HERBICIDE RESISTANCE IN WEEDS: EARLY DETECTION, MECHANISMS, DISPERSAL, NEW INSIGHTS AND MANAGEMENT ISSUES

EDITED BY: Ilias Travlos, Rafael De Prado, Demosthenis Chachalis and Dimitrios J. Bilalis

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HERBICIDE RESISTANCE IN WEEDS: EARLY DETECTION, MECHANISMS, DISPERSAL, NEW INSIGHTS AND MANAGEMENT ISSUES

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EDITORIAL
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Editorial: Herbicide Resistance in Weeds: Early Detection, Mechanisms, Dispersal, New Insights and Management Issues

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Editorial on the Research Topic

Herbicide Resistance in Weeds: Early Detection, Mechanisms, Dispersal, New Insights and Management Issues

Herbicides are considered to be among the most widely used plant protection products. Weed management is mainly based on herbicide applications, partially due to their rapid action, high efficacy, and low cost (De Prado et al., 2004; Travlos et al., 2017). Chemical weed control by means of the use of herbicides was broadly adopted in many agricultural areas, reducing the labor costs and the mechanical means dependence. For instance, in USA, herbicide usage in 1950 accounted only for 5–10% of the total area in cotton, maize, and wheat fields, whereas in 1980 the corresponding value was 90–99% (Fernandez-Cornejo et al., 2014).

However, this tremendous over-reliance on herbicides along with the absence of any preventive or other cultural practices resulted in the evolution and spread of herbicide-resistant weeds (De Prado et al., 2000; Travlos and Chachalis, 2010, 2013; Travlos et al., 2018). However, any solution should first be based on the accurate identification of the problem, the current status and the deep knowledge on several key aspects. Therefore, the main objectives of this Research Topic was not only to present the most advanced research dealing with the management of resistant weeds, but also to attract new insights on biology, ecology, physiology, genetics and epigenetics, population dynamics, mechanisms, and dispersal of the resistant weeds.

HERBICIDE RESISTANCE: STATUS, NEW CASES, AND MECHANISMS

Unfortunately, the number of resistant weed biotypes keeps increasing at a worrying rate, while climate change along with new cropping systems pose new challenges (Heap, 2020). New cases of herbicide resistant weeds are recorded and the involvement of several mechanisms is systematically studied. Tahmasebi et al. characterized the resistance to diquat/paraquat (bipyridiliums) in an *Epilobium ciliatum* biotype collected in an olive orchard from Chile. Physiological and biochemical studies determined the resistance to diquat of the R1 biotype, which was due to impaired translocation. Oliveira et al. investigated the genetic control of mesotrione resistance in an *Amaranthus tuberculatus* population from USA and the results of this study aid to understand the genetics and inheritance of a non-target-site resistance to HPPD inhibitor herbicides. Panozzo et al. studied the cross-resistance pattern, the resistance mechanism and the fitness costs of

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an ALS inhibiting *Echinochloa crus-galli* population and reported for the first time the Ala-122-Asn amino-acid change in the ALS gene that confers high levels of cross-resistance to several ALS inhibitors. Bracamonte, Fernández-Moreno, Bastida, et al. reported the first case of herbicide resistance in Cuba. Their results revealed a glyphosate resistant (GR) population of *Chloris elata*, with its resistance involving both non-target site and target-site mechanisms. This pattern of two different resistance mechanisms (both target-site and non-target site) was also reported for GR populations of *Lolium perenne* and *L. multiflorum* by Fernandez-Moreno, Alcántara-de la Cruz et al. Additionally, Torra et al. also reported two 2,4-D non-target site resistance mechanisms (reduced translocation and enhanced metabolism) being present in two *Papaver rhoeas* populations. The involvement of other mechanisms is also common in several herbicide resistant weed species. Brunharo and Hanson found that vacuolar sequestration is involved in the resistance to paraquat in a population of *L. perenne* spp. *multiflorum*. Yanniccari et al. found that the over-expression of EPSPS appears to be the main mechanism responsible for resistance to glyphosate in a *L. perenne* population from Argentina. This over-expression was something previously reported by Tani et al. (2016). Fernandez-Moreno, Bastida et al. confirmed a reduced glyphosate uptake and translocation as being the main mechanism involved in glyphosate resistance in a GR biotype of *L. rigidum*. The objective of the study conducted by Nakka et al. was to investigate the mechanism of HPPD-inhibitor (mesotrione) resistance in *A. palmeri*. Their findings revealed that mesotrione resistance in *A. palmeri* is conferred primarily by rapid detoxification (non-target-site based) of mesotrione; additionally, increased HPPD gene expression (target-site based) also contributes to the resistance mechanism in the evolution of herbicide resistance in this naturally occurring weed species. Furthermore, Iwakami et al. found that seven of the nine accessions of *Alopecurus aequalis* grown in a single wheat field survived application of standard field rates of thifensulfuron-methyl, indicating that severe infestations likely result from herbicide resistance. Resistant plants carry a mutation in either the *ALS1* or *ALS2* gene, with all mutations causing an amino acid substitution at the Pro197 residue, which is known to confer SU resistance. Transcription of each *ALS* gene copy was confirmed by reverse transcription PCR, supporting involvement of these mutations in SU resistance. The information on the copy number and full-length sequences of *ALS* genes in *A. aequalis* will aid future analysis of the mechanism of resistance.

Among the herbicides, glyphosate is the most widely used globally for the control of weeds in both agricultural and non-agricultural areas for more than three decades (Andert et al., 2019). The worldwide market of this active ingredient is continuously increasing (annually higher than 5 billion *e*), with the overall consumption reaching more than 0.8 million tons per year and the annual production accounting for more than 1.1 million tons per year. It has to be noted that glyphosate represents 12% of the overall pesticide market globally by 2012 (Székács and Darvas, 2018). It is also noticeable that in countries like Germany, glyphosate market is large and the sales of glyphosate-based products covers more than one third of the total herbicide amount (Steinmann et al., 2012). The overreliance on this herbicide has resulted in the development of several cases of weeds resistant to glyphosate (Feng et al., 2004; Vidal et al., 2007; Travlos and Chachalis, 2010; Gonzalez-Torralva et al., 2012). Currently, there are 48 glyphosate-resistant species worldwide (Heap, 2020). The evolution of resistance to glyphosate is evolving at a steady pace (Heap and Duke, 2018) and results in low efficacy, with the weed management costs significantly increased. Therefore, it is rather anticipated that more and more researchers from different countries study the potential resistance of several weeds to glyphosate and the mechanisms involved. For instance, Karn and Jaseniuk investigated the frequency of target-site mutations conferring resistance to glyphosate combined with the frequency of resistant individuals in 14 *L. perenne* populations. Seven unique alleles were detected at codon site 106, four of which have been previously shown to confer target-site-based resistance to glyphosate. Four different resistance alleles were detected, indicating that resistance to glyphosate has evolved multiple times in the region. In two populations, no EPSPS mutations were detected despite the presence of resistant plants, strongly suggesting that non-target-site-based mechanisms confer resistance to glyphosate in these populations. It is likely that resistance to glyphosate in these 14 California populations of *L. perenne* derives from at least five evolutionary origins, indicating that adaptive traits can evolve repeatedly over agricultural landscapes.

Bracamonte, Fernández-Moreno, Barro et al. studied the resistance level and mechanisms of different *Parthenium hysterophorus* accessions (three collected in Cuba and four collected in the Dominican Republic) under greenhouse and laboratory conditions. Results have shown that high glyphosate resistance values were determined by the number of defense mechanisms (target-site and non-target-site resistance) possessed by the different *P. hysterophorus* accessions. Alcántara-de la Cruz, Rojano-Delgado et al. studied three glyphosate-resistant populations of *Lepthochloa virgate* collected in citrus orchards from Mexico were studied in terms of their resistance mechanisms. It was shown that the three resistant *L. virgata* populations show reduced absorption and translocation of ^{14}C-glyphosate. Moreover, a mutation and an enhanced EPSPS basal activity at target-site level confers higher resistance to glyphosate. In the first report of *Bidens pilosa* populations with glyphosate resistance, Alcantara-de la Cruz, Fernández-Moreno et al. revealed that target-site mutations associated with a reduced translocation were responsible for the higher glyphosate resistance in the one resistant population. The low-intermediate resistance of the other resistant population was mediated by reduced translocation.

Moreover, studies on interactions and effects of soil parameters, climatic conditions and cultural practices on herbicide resistance of weeds are becoming more common (Kleinman et al., 2015; Robinson et al., 2015; Matzrafi et al., 2016). In another study, Matzrafi, Shaar-Moshe et al. studied the potential role of temperature on chemical control and particularly pinoxaden resistance in *Brachypodium hybridum*. It was suggested that elevated activity of enzymatic processes at high-temperature may induce rapid and efficient

pinoxaden metabolism leading to temperature-dependent herbicide resistance.

POTENTIAL DISPERSAL, EARLY DETECTION AND FITNESS COST OF HERBICIDE RESISTANT WEED BIOTYPES

Regarding the potential dispersal of herbicide resistant biotypes, special attention should be paid to the early detection of herbicide-resistance which could mitigate the spread of the problem (Schulz et al., 2014). The first step to preventing herbicide resistance and efficiently managing is early detection. Patterson et al. proposed a KASP genotyping method to identify northern watermilfoil (*Myriophyllum sibiricum* Kom.), Eurasian watermilfoil (*M. spicatum* L.), and their interspecific hybrids (*M. spicatum* × *M. sibiricum*). Accurate and rapid identification of hybrid individuals within populations is very important due to their usually higher fitness and reduced sensitivity to some commonly used herbicides. Lakes with complex species distribution dynamics, such as a low proportion of hybrids, are where herbicide application must be carefully chosen so as not to select for the more vigorous and less herbicide-sensitive hybrid individuals. Matzrafi, Herrmann et al. proposed a toolbox based on hyperspectral technologies and data analyses aimed to predict *A. palmeri* seed germination and response to the herbicide trifloxysulfuron-methyl. Sensitive and resistant plants were identified with high degrees of accuracy from leaf hyperspectral reflectance profiles acquired prior to herbicide application and thus more targeted control methods can be developed.

Herbicide resistance may or may not constitute a competition penalty in the resistant populations (Vila-Aiub et al., 2009; Travlos, 2013; Yanniccari et al., 2016). Development of integrated management strategies for herbicide-resistant weeds clearly requires an understanding of population dynamics and potential impacts of the resistant biotypes. Therefore, research on the relative competitiveness of herbicide-resistant and -susceptible biotypes is crucial. Fernández-Escalada et al. studied the genetics in *Amaranthus palmeri* and particularly the pleiotropic effects of *EPSPS* transcript abundance and of glyphosate application on the aromatic amino acid and branched chain amino acid synthesis pathways. Their findings suggest that the high copy number variation of *EPSPS* in *A. palmeri* GR populations has no major pleiotropic effect on the expression of amino acid biosynthetic genes. This finding is probably related to the limited fitness cost associated with *EPSPS* copy number variation in *A. palmeri*. Glyphosate resistant populations of *L. perenne* and *L. multiflorum* studied by Fernández-Moreno, Alcántara-de la Cruz et al. probably had a fitness cost and consequently a competitive disadvantage.

Frenkel et al. studied the effects of environmental conditions on the fitness penalty in herbicide resistant *Brachypodium hybridum*. The application of PSII inhibitors may have created selective pressure toward TSR dominancy; termination of herbicide application gave an ecological advantage to S plants, creating changes in the composition of the seed bank. Alternatively, the high radiation intensities found in the Mediterranean-like climate may reduce the fitness penalty associated with TSR. Our results may suggest that by integrating non-herbicidal approaches into weed-management programs, we can reduce the agricultural costs associated with herbicide resistance. Zangeneh et al. evaluated germination and seedling emergence characteristics of three rigid ryegrass biotypes (one susceptible and two resistant populations with different mutations, namely Ile 1781 Leu and Ile 2041 Asn) and of competition between this weed and wheat. There was no apparent fitness penalty associated to ACCase-inhibitor resistance, while different mutations may impose different competitive ability and therefore require case-specific management strategies.

Osipitan and Dille assessed the fitness cost of glyphosate resistance compared to susceptibility in six kochia populations at different life history stages, namely rate of seed germination, increase in plant height, days to flowering, biomass accumulation at maturity, and fecundity. Of the life history stages measured, fitness differences between the GR and GS kochia populations were consistently found in their germination characteristics. The GR kochia showed reduced seed longevity, slower germination rate, and less total germination than the GS kochia. In the field, increases in plant height, biomass accumulation, and fecundity were not clearly different between GR and GS kochia populations (irrespective of neighbor density).

Matzrafi, Gerson et al. showed that individuals of *L. rigidum* resistant to ACCase inhibitors had significantly lower grain weight and early vigor compared with the sensitive ones. The study conducted by Keshtkar et al. was the first evaluation of potential fecundity and vegetative fitness cost of a non-target site resistant black-grass (*Alopecurus myosuroides*) population. According to the results, the resistant black-grass has no fitness cost and probably it will persist in the field even in the cessation of fenoxaprop-P-ethyl. The different fitness between resistant and susceptible phenotypes of *Echinochloa crus-galli* reported by Panozzo et al. suggests that keeping the infestation density as low as possible can increase the reproduction success of the susceptible phenotype and therefore contribute to lowering the ratio between resistant and susceptible alleles.

WHAT ABOUT MANAGEMENT?

Studies like the above-mentioned ones on fitness cost evaluation could provide some valuable information that will be useful for predicting the evolutionary dynamics of resistant populations and consequently for developing appropriate resistance management strategies. In fact, the confirmed presence of resistant biotypes with a fitness penalty may provide an opportunity to minimize or reverse herbicide resistance evolution through the implementation of effective and integrated weed management practices.

Küpper et al. studied eight different populations of *Amaranthus palmeri* by analyzing patterns of phylogeography and diversity to ascertain whether resistance evolved independently or spread from outside to an Arizona locality. Their data confirmed that both scenarios were possible,

highlighting the complicated nature of the problem and underlying the need of integrated weed management and seed dispersal prevention. Ganie and Jhala evaluated the interaction of glufosinate plus 2,4-D and/or dicamba for control of GR giant ragweed, and determined their effect on GR giant ragweed density, biomass, maize injury, and yield. Their results revealed that tank-mixing glufosinate with 2,4-D or dicamba showed an additive effect and will be an additional tool with two effective modes of action for the management of GR giant ragweed in maize. Fernández-Moreno, Bastida et al. indicated that herbicide applications at the later growth stage tended to be less effective in terms of immediate effects on population size than earlier applications, and that only in some cases, the removal of at least 85% of the GR biotype of *L. rigidum* was achieved. However, with few exceptions, the alternative treatments tested appeared to be highly effective in reducing the seed bank irrespective of the growth stage. Chahal et al. showed that the effective control of multiple herbicide-resistant *A. palmeri* can be achieved with PRE followed by POST programs that include herbicides with overlapping residual activity to maintain season-long control. Barnes et al. evaluated and proposed several preplant and post-emergence applications of various herbicides for the efficient control of GR *Ambrosia artemisiifolia* in glufosinate-resistant soybean.

Consequently, alternative herbicides can be used (alone, in mixtures or in sequence) in order to control the herbicide resistant weeds. Unfortunately, we can't rely on many new herbicide discoveries since developing and registering a new herbicide could cost more than 300 million dollars (Beckie et al., 2019). Globally, there is also an increasing demand for a reduction in herbicides use and dependence (Böcker et al., 2018) and therefore, the role of non-chemical approaches is crucial. Such practices could favor the herbicide sensitive weed biotypes and significantly reduce the selection pressure needed for herbicide resistance evolution. Crop rotation, mulches and cover crops, biological and natural herbicides are often very effective tools for both the pro- or the re-active resistance management (Radhakrishnan et al., 2018; Alonso-Ayuso et al., 2020; Bottrill et al., 2020). Cultural practices like false seedbed can be also included in integrated weed management programs in several annual crops (Travlos et al., 2020), while decision support systems (DSS) can provide information regarding weed seedbank dynamics in the soil in order to suggest management options (both chemical and non-chemical) not only within a single period but also in a rotational view (Kanatas et al., 2020). Under this concept, the emergence and integration of new weed management tools is imperative (Dayan, 2019). In conclusion, it could be said that herbicide resistance is a reality; however, the systematic research (part of which was presented in this Research Topic) allows us to remain optimist for an effective integrated and sustainable weed management. Such a sustainable weed management and prevention of herbicide resistance should be based on both agronomy and weed science and certainly on a sound knowledge of biology, ecology, physiology, genetics and epigenetics, population dynamics, mechanisms and dispersal of the resistant weeds.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Target and Non-target Site Mechanisms Developed by Glyphosate-Resistant Hairy beggarticks (*Bidens pilosa* L.) Populations from Mexico

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In 2014 hairy beggarticks (*Bidens pilosa* L.) has been identified as being glyphosate-resistant in citrus orchards from Mexico. The target and non-target site mechanisms involved in the response to glyphosate of two resistant populations (R1 and R2) and one susceptible (S) were studied. Experiments of dose-response, shikimic acid accumulation, uptake-translocation, enzyme activity and 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) gene sequencing were carried out in each population. The R1 and R2 populations were 20.4 and 2.8-fold less glyphosate sensitive, respectively, than the S population. The resistant populations showed a lesser shikimic acid accumulation than the S population. In the latter one, 24.9% of ^{14}C-glyphosate was translocated to the roots at 96 h after treatment; in the R1 and R2 populations only 12.9 and 15.5%, respectively, was translocated. Qualitative results confirmed the reduced ^{14}C-glyphosate translocation in the resistant populations. The EPSPS enzyme activity of the S population was 128.4 and 8.5-fold higher than the R1 and R2 populations of glyphosate-treated plants, respectively. A single (Pro-106-Ser), and a double (Thr-102-Ile followed by Pro-106-Ser) mutations were identified in the EPSPS2 gene conferred high resistance in R1 population. Target-site mutations associated with a reduced translocation were responsible for the higher glyphosate resistance in the R1 population. The low-intermediate resistance of the R2 population was mediated by reduced translocation. This is the first glyphosate resistance case confirmed in hairy beggarticks in the world.

Keywords: 5-enolpyruvyl shikimate-3-phosphate synthase, *Bidens pilosa*, EPSPS2, resistance mechanisms, glyphosate translocation, TIPS mutation

INTRODUCTION

Mexico is the top producer and exporter of limes and lemons worldwide (U.S. Department of Agriculture [USDA], 2016). Persian lime (*Citrus latifolia* Tan.) is the most economically important crop (Servicio de Información Agropecuaria y Pesquera [SIAP], 2016), because of its large volume of exports. Weeds are the main limiting factor in lime production, and the use of herbicides has

been adopted as the main tool for weed control in this crop, mainly glyphosate, which is applied up to 4 treatments per year (Pérez-López et al., 2014). Glyphosate use has induced great changes in weed flora in Persian lime (*C. latifolia* Tan.) groves, where two cases of glyphosate resistance have been reported in Mexico: tropical sprangletop [*Leptochloa virgata* (L.) P. Beauv.], and hairy beggarticks (*Bidens pilosa* L.) (Pérez-López et al., 2014; Heap, 2016).

Hairy beggarticks is a native asteraceae from Mexico, widely spread over the country's tropical and subtropical regions and in the world (Vibrans, 1995). It is an annual weed that reproduces itself by seeds, affecting annual and perennial crops (Rzedowski and Rzedowski, 2008). In 1991 it was reported as being resistant to paraquat in coffee plantations from Kenya, and in 1993 to ALS-inhibiting herbicides in soybean crops in Brazil (Heap, 2016). Hairy beggarticks is susceptible to glyphosate, but field prospections made by this research group in citrus orchard areas of Mexico have allowed the identification of glyphosate-resistant populations of this weed (Heap, 2016).

Glyphosate is a systemic non-selective herbicide that has been used globally for over 40 years in weed management (Duke and Powles, 2008; Szeìkaìcs and Darvas, 2012). When it is properly used, i.e., following label recommendations, it does not have any adverse effects on wildlife (Giesy et al., 2000). Glyphosate acts rapidly in reducing photosynthesis activity (Duke et al., 2003), and it is translocated with photosynthates from the leaves to the meristematic tissue to reach the target-site, achieving maximum uptake at 96 h after treatment (Cruz-Hipolito et al., 2011; González-Torralva et al., 2012b). Glyphosate is a phosphonomethyl derivative of the amino acid glycine (Szeìkaìcs and Darvas, 2012), and kills plants by preventing the synthesis of three essential amino acids (phenylalanine, tyrosine, and tryptophan; Franz et al., 1997), inhibiting the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) (Duke and Powles, 2008; Szeìkaìcs and Darvas, 2012). Its effect is broader, the biosynthesis of chorismate, an intermediate in the shikimate pathway, is blocked causing the accumulation of high levels of shikimate-3-phosphate (Amrhein et al., 1980; Franz et al., 1997). Thereby, aromatic substances are disturbed in sensitive plants treated with this herbicide (Pline et al., 2002), affecting the production of flavonoids, phenolic compounds, monolignol polymerization, lignin synthesis, other secondary metabolites (Boerjan et al., 2003). This compounds can account for as much as 35% of a plant's biomass (Franz et al., 1997). In addition, glyphosate causes a deregulation of the carbon flow to other essential pathways (Orcaray et al., 2012).

Depending almost exclusively on the use of glyphosate for weed management has led to the evolution of resistant populations (Alcántara-de la Cruz et al., 2016). During 20 years there was no evidence of any glyphosate-resistant weed populations (Duke and Powles, 2008). The first case reported was *Lolium rigidum* in 1996 (Powles et al., 1998). Since then, 258 glyphosate resistance cases have been reported in 35 weed species (Heap, 2016), mainly, but not only, influenced by adoption of transgenic glyphosate-resistant crops (Duke and Powles, 2008).

Glyphosate resistance in weeds is due to different mechanisms (Salas et al., 2015), grouped and commonly known as non-target site resistance (NTSR) and target site resistance (TSR) mechanisms (Sammons and Gaines, 2014). The NTSR mechanisms limit glyphosate reaching its site of action (EPSPS; Alcántara-de la Cruz et al., 2016). This group includes: reduced uptake (Michitte et al., 2007; de Carvalho et al., 2012), altered translocation (Perez-Jones et al., 2007), increased vacuolar sequestration (Ge et al., 2012), and metabolism to non-toxic compounds (de Carvalho et al., 2012; González-Torralva et al., 2012b), causing less glyphosate transport via phloem to the EPSPS. These mechanisms are influenced by enhanced physiological and biochemical characteristics (Alcántara-de la Cruz et al., 2016), and generally, each of these mechanisms confers moderate levels of glyphosate resistance (Yu et al., 2015).

The TSR mechanisms are those related to the EPSPS, either by a loss of affinity between the linking protein and glyphosate caused by mutations, or by the EPSPS overexpression (Sammons and Gaines, 2014). Different single mutations in the Pro-106 position (to Ala, Thr, and Leu) of EPSPS gene have been identified as conferring low-intermediate glyphosate resistance in weeds (de Carvalho et al., 2012; González-Torralva et al., 2012a, 2014; Alarcón-Reverte et al., 2015; Salas et al., 2015). Moreover, a double mutation was found in the Thr-102-Ile position followed by Pro-106-Ser conferring higher resistance in *Eleusine indica* (Chen et al., 2015; Yu et al., 2015). This double mutation is used in transgenic crops (Sammons and Gaines, 2014). Multiple EPSPS copy numbers and/or increased EPSPS expression are also involved in glyphosate resistance. These mechanisms have been described in mono and dicotyledonous weed species (Alarcón-Reverte et al., 2015; Chatham et al., 2015; Salas et al., 2015; Wiersma et al., 2015; Malone et al., 2016). In this paper, the target and non-target site mechanisms involved in glyphosate resistance of two resistant populations (R1 and R2) of hairy beggarticks in comparison to one susceptible (S) (as control), were studied by physiological, biochemical and molecular methods.

MATERIALS AND METHODS

Biological Material and Experiment Conditions

Seeds of resistant populations (R1 and R2) were harvested directly in two Persian lime groves of the San Manuel farm, Puebla, Mexico, (20° 06′ 28″ N, 97° 09′ 34″ W) from at least 20 plants that had been survived to the last glyphosate treatment at the recommended field rate [720 g acid equivalent (ae) ha^{-1}]. Persian lemon groves had a history of 6 (R2) and 13 (R1) years of continuous use of glyphosate (3–4 application per year). Seeds of a susceptible population (S) never treated were collected near the Persian lime groves. Seeds collected from a grove were bulked and constitute a sample from a single population.

Seeds were seeded on trays (15 cm × 15 cm × 8 cm) with peat saturated at a field capacity. The trays were covered with plastic layer until germination and placed in a growth chamber under controlled conditions (day/night temperature of 26/18°C, photoperiod of 16 h at 850 $\mu mol^{-2}s^{-1}$ of light intensity, and 60% relative humidity).

The seedlings were transplanted individually into 250 mL pots containing a mixture of sand/peat (1:1 v/v) + 0.4 g of fertilizer (NPK 17-09-11 + 2% MgO). The pots were placed in the growth chamber under the conditions described above and watered daily.

The glyphosate applications (Roundup Energy 45% w/v, Monsanto, Madrid, Spain) for the dose-response, foliar retention and shikimic acid assays were made with a Generation III Research Track Sprayer (DeVries Manufacturing Inc., Hollandale, MN, USA) equipped with an 8002EVS nozzle (TeeJet, Spraying System Spain, S.L., Madrid, Spain) delivering 200 L ha^{-1}. The plants were treated with four true leaves counted from the bottom.

Dose-Response Assays

Plants from the S, R1 and R2 populations were treated with the following doses of glyphosate: 0, 31.25, 62.5, 125, 250, 500, 1000, and 2000 g ae ha^{-1}. At 21 days after treatment (DAT), the plants were cut off at ground level and wrapped in filter paper envelopes. Later, the plants were dried in a stove (JP Selecta S.A., Barcelona, Spain) at 60°C for 1 week and weighed to determine their dry weight. Data were expressed as a percentage of dry weight, compared to untreated control plants (Cruz-Hipolito et al., 2011). The experiment was arranged in a completely random design with 10 replications per dose. The assays were repeated twice.

Foliar Retention Assays

The methodology adapted by González-Torralva et al. (2010) was employed. Na-fluorescein was used as labeling reagent for determination of herbicide solution amount was retained. Seven plants from each population, in a completely random design, were treated with a solution containing 360 g ae ha^{-1} of glyphosate (0.5 of field rate) + 100 mg L^{-1} Na-fluorescein. When the solution applied on the plant's foliage dried (20–25 min after application), the treated plants were cut off at ground level and washed with 50 mL of NaOH 5 mM in a test tube shaking it vigorously for 30 s. The washing solution was recovered in glass flasks and the absorbance of fluorescein was immediately measured at $490_{exc}/510_{em}$ nm (Hitachi F-2500 spectrofluorimeter). The plants were wrapped in filter paper envelopes and dried in a stove at 60°C for 1 week, and weighed. The retention was expressed in μL of glyphosate solution g^{-1} dry matter.

Shikimic Acid Accumulation

An assay at different intervals of time was carried out. Plants from S, R1, and R2 populations, were treated with glyphosate at 360 g ae ha^{-1}. Samples of 50 mg of tissue corresponding to the first and second leaf of treated and untreated plants (the latter used as a control) were cut at 24, 48, 72, and 96 hour after treatment (HAT). The samples were placed in an Eppendorf with 1 mL of HCl 1 M, immediately frozen in liquid nitrogen and stored at −40°C up to their analysis. The shikimic acid accumulation was determined by the methodology described by Cromartie and Polge (2002). Sample absorbance was measured with a spectrophotometer (Beckman DU-640, Fullerton, CA, USA) at 380 nm. The shikimic acid accumulation was obtained from the difference between treated and untreated plants, its rate was measured at between 24 and 96 HAT and the results were expressed in mg of shikimic acid g^{-1} fresh tissue. Five treated and untreated plants from each population at each time evaluated were used in a completely random design.

In leaf segment bioassay, young leaf disks 4 mm in diameter were taken until completing 50 mg of plant tissue from plants of hairy beggarticks populations S, R1, and R2 with four true leaves (Dayan et al., 2015). The disks were placed in Eppendorfs containing 999 μL of monoammonium phosphate ($NH_4H_2PO_4$ 10 mM, pH 4.4). Next, 1 μL of glyphosate at different concentrations were added (0, 1, 10, 50, 100, 200, 400, 600, 1000, and 10000 μM). The samples were incubated for 24 h in the growth chamber under controlled conditions described above. Then the samples were frozen at −20°C until their analysis. After thawing the samples at room temperature, they were incubated at 60°C for 30 min. Volumes of 250 μL of HCl 1.25 N were added and incubated again at 60°C for 15 min. Aliquots of 250 μL were transferred to new Eppendorfs adding 500 μL of periodic acid (0.25% w/v) and sodium metaperiodate (0.25% w/v) at a ratio of 1:1. The samples were incubated at room temperature (25°C) for 90 min, and next, 500 μL of a mix of sodium hydroxide (NaOH 0.6 N) + sodium sulfite (Na_2SO_3 0.22 N) was added at a ratio of 1:1, and mixed. Absorbance was measured at 380 nm in a spectrophotometer (Beckman DU-640). The experiment was arranged in a completely random design with three replications for each glyphosate concentration. The absorbance values were converted into mg of shikimic acid g^{-1} fresh weight.

Uptake and Translocation of ^{14}C-glyphosate

Plants from S, R1, and R2 populations were treated with a solution of ^{14}C-glyphosate [glycine-2-^{14}C] (specific activity 273.8 MBq $mmol^{-1}$, American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) + commercial glyphosate. The solution applied contained a specific activity of 0.834 kBq^{-1} μL and a glyphosate concentration of 1.8 g ea L^{-1} (360 g ea ha^{-1} in 200 L). One drop of 1 μL $plant^{-1}$ of solution was applied with a micropipette (Lab Mate HTL, Matosinhos, Portugal) on the adaxial surface of the first–second leaf. The treated leaf was washed three times separately with 1 mL of water-acetone (1:1 v/v) to recover the non-absorbed ^{14}C-glyphosate at 24, 48, 72, and 96 HAT. The washing solution was mixed with 2 mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard), and analyzed by liquid scintillation spectrometry (LSS) in a scintillation counter (LS 6500, Beckman Coulter Inc., Brea, Fullerton, USA). Complete plants were carefully removed from the pot and washed. They were divided into treated leaf, remainder of the plant and root, and stored individually in flexible combustion cones (Perkin-Elmer, BV BioScience Packard). The samples were dried in a stove at 60°C for 1 week and combusted in a biological oxidizer (Packard Tri Carb 307, Packard Instrument Co., Downers Grove, IL, USA). The CO_2 released from the combustion was captured in 18 mL of a mix of Carbo-Sorb E and Permafluor (9:9 v/v) (Perkin-Elmer, BV BioScience Packard). The radioactivity of individual sample was

quantified by LSS. The experiment was arranged in a completely random design with five replications per population at each time evaluated. The radioactive values were used to calculate recovery as: (kBq in treated leaf + kBq in plant + kBq in roots + kBq from washes/kBq total applied) × 100. The average total recovery of ^{14}C-glyphosate applied was >94%.

The glyphosate translocation was visualized in plants from S, R1, and R2 populations. At 24, 48, 72, and 96 HAT, whole plants were washed, fixed on filter paper (25 cm × 12.5 cm) and dried at room temperature for 1 week. The samples were placed for 6 h beside a phosphor storage film (Storage Phosphor System: Cyclone, Perkin–Elmer Packard BioScience BV). A phosphor imager (Cyclon, Perkin-Elmer, Packard BioScience BV) was used to reveal the translocation. The experiment was carried out using three plants per population at each evaluation time.

Glyphosate Metabolism

Randomized plants of the three hairy beggarticks populations were treated with 100 g ae ha^{-1}. Untreated plants were used as controls. The methodology described by Rojano-Delgado et al. (2010) was used to determinate the percentage of glyphosate and its metabolites (aminomethyl phosphonate (AMPA), glyoxylate, sarcosine and formaldehyde) at 4, 8, and 12 DAT. Standard compounds used were provided by Sigma–Aldrich, Spain.

Basal and Enzyme Activity of the EPSPS

Plants S, R1 and R2 populations were grown in pots (25 cm in diameter × 15 cm high: 4 plants per pot) under greenhouse conditions, in temperatures ranging from 17 to 31°C, and a photoperiod of 16 h. The natural light was complemented by 900 μmol^{-2}s^{-1} photosynthetic photon flux density delivered by incandescent and fluorescent lights. The two youngest totally expanded leaves of plants with four true leaves were harvested until completing 5 g of foliar tissue for each population. Samples were frozen and stored at −40°C up to the protein extraction. The EPSPS extraction assays were conducted following the methodology described by Sammons et al. (2007). The total content of proteins in the raw extract was measured using the colorimetric method of Bradford (1976) following the manufacturer's instructions with a Modified Lowry Kit for Protein Determination (Sigma–Aldrich, Madrid, Spain) following the manufacturer's instructions.

The specific EPSPS activity in plants from S, R1, and R2 populations was studied in the presence and absence of glyphosate. In order to determine the EPSPS activity, a continuous assay of the release of inorganic phosphate was made with EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The glyphosate concentrations used were: 0, 1, 10, 100, 1000, and 10000 μM. Three replicates at each glyphosate concentration were analyzed. The release of phosphate above background level was measured during 10 min at 360 nm in a spectrophotometer (Beckman DU-640). The EPSPS activity was calculated to determine the amount of phosphate (μmol) released μg of total soluble protein (TSP)$^{-1}$ min^{-1}.

Amplification and Sequencing of the EPSPS Gene

Samples (100–200 mg) of young leaf tissue were collected of plants from S, R1, and R2 populations and stored at −80°C for RNA extraction. The frozen samples were milled with liquid nitrogen in a STAR-BEATER 412-0167 mill (VWR International Eurolab S.L., Barcelona, Spain). Total RNA was isolated following the methodology described by Pistón (2013). Integrity of RNA was verified in 0.8% agarose gel and it was quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Walthman, MA, USA). First strand complementary DNA (cDNA) synthesis was carried out using 1 μg from the total RNA in all the samples. An iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was employed following the manufacturer's instructions.

The PCR reactions were carried out with cDNA samples from each populations (R1, R2, S) using the following primers: Bidens-F13 (5′-TTGCCYGGRTCMAAGTCTTT-3′) and Bidens-R11 (5′-GTCCCAASTATCACTRTGTTC-3′) designed with software Primers3Plus[1] based on EPSPS gene sequences of *Amaranthus tuberculatus* (Accession FJ869880.1, FJ869881.1), *A. palmeri* (FJ861242.1), *A. spinosus* (KF569213.1), *Conyza bonariensis* (EF200074.1), *C. canadensis* (AY545666.1, AY545667.1, FR872821.1), *C. sumatrensis* (AY834207.1), *Helianthus salicifolius* (AY545662.1) from the GenBank. A total volume of 25 μL which contained 50 ng of cDNA, 0.2 μM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Madrid, Spain), 2 mM $MgCl_2$, 1X buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain) per reaction using a thermocycler (Gene Amp PCR System 9700; Applied Biosystems, CA, USA). The PCR conditions were: 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were checked by 1% agarose gel. The amplified fragments of 639 bp in length included the Thr-102 and Pro-106 positions, which corresponds to the sequence of the EPSPS gene of *Arabidopsis thaliana* (GenBank: CAA29828.1), point mutations associated with glyphosate resistance in weeds (Sammons and Gaines, 2014; Chen et al., 2015; Yu et al., 2015).

The PCR products were ligated using the pGEM-T Easy Vector System (Promega Biotech Ibérica, SL, Madrid, Spain) following the manufacturer's instructions, and cloned into competent cells of *Escherichia coli* DH5α. Positive transformants were selected. The fragment insertion was confirmed through a PCR using the M13F (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and M13R (5′-TCACACAGGAAACAGCTATGAC-3′) primers at a total volume of 15 μL containing 0.2 μM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Madrid, Spain), 2 mM $MgCl_2$, 1X buffer, and 0.625 units of non-proofreading (*Thermus thermophilus*) polymerase (BIOTOOLS, Madrid, Spain) per reaction. The PCR conditions were as

[1]http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

follows: 94°C for 5 min, 28 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. The plasmids were purified with the illustra plasmidPrep Mini Spin kit (GE Healthcare, Buckinghamshire, UK), following the manufacturer's instructions. Sanger sequencing was carried out by the STABVIDA sequencing service (Caparica, Portugal). Five biological samples were used per population. A total of 15 clones from each population were sequenced. The assembly of the sequences was carried out by SeqMan Pro (Version 11, DNASTAR; Wisconsin, USA) and Geneious (Version 8.1.8, Biomatters Ltd, Auckland, New Zealand) software's.

A second EPSPS sequencing with 15 new individuals from R1 population to confirm mutations was carried out. A total of 45 clones were sequenced.

The hairy beggarticks EPSPS cDNA sequences information can be found in GenBank with accession numbers KU984452–KU984458.

Statistical Analysis

The dry weight and survival percentage data were submitted to a non-linear regression analysis. The dose needed to reduce the growth of a population by 50% (ED_{50}), the mortality by 50% (LD_{50}), and to inhibit EPSPS activity by 50% (I_{50}) were calculated. The log-logistic model was conducted using SigmaPlot (Version 11.0, Systat Software, Inc., USA) software. The statistical model is:

$$Y = c + \{(d - c)/[1 + (x/g)^b]\}$$

Where Y is the dry weight, survival and/or EPSPS inhibiting percentage with respect to the untreated control, c and d are coefficients corresponding to the upper (maximum growth) and lower (minimum growth) asymptotic limits), b is the Hill slope, g is the herbicide dose (ED_{50}, LD_{50} or I_{50}) at the mean point of inflection between the upper and lower asymptote and x (independent variable) corresponds to the herbicide dose.

Statistical analyses between the hairy beggarticks populations were performed using Statistix version 8.0 Analytical Software. The experimental results were subjected to analysis of variance, and means were compared using Tukey's or LSD test's at the 95% probability level.

RESULTS

Dose-Response

This experiment confirmed the resistance of R1 and R2 hairy beggarticks populations to glyphosate. A large reduction of biomass in population S was observed at low glyphosate doses in comparison to that of the resistant populations (**Figure 1A**). The ED_{50} value for the S population was 51.7 g ae ha^{-1}, whereas the R1 and R2 populations exhibited a higher ED_{50}, with resistance index (RI) values ($ED_{50}R/ED_{50}S$) of 20.4- and 2.7-fold more resistant, respectively (**Table 1**). The R1 population showed an ED_{50} value 1.46-fold higher than the glyphosate field rate recommended (720 g ae ha^{-1}).

According to LD_{50} values, R1 and R2 populations were 9.5- and 3.4-fold more resistant than the S population (**Figure 1B**; **Table 1**). A field rate of glyphosate showed total control for the S population. A glyphosate field rate > 2.7-fold was needed to kill 50% R1 population plants. Even though the R2 population presented fivefold less ED_{50} than the field rate, just 50% of the population was eradicated (LD_{50} = 774.4 g ae ha^{-1}) with this rate. The chlorosis symptoms caused by glyphosate application in resistant populations became evident as the glyphosate doses increased, although they were not sufficient to control the R1 population, in which plants survived treatment at 21 DAT, and continued growing up to the reproductive phase.

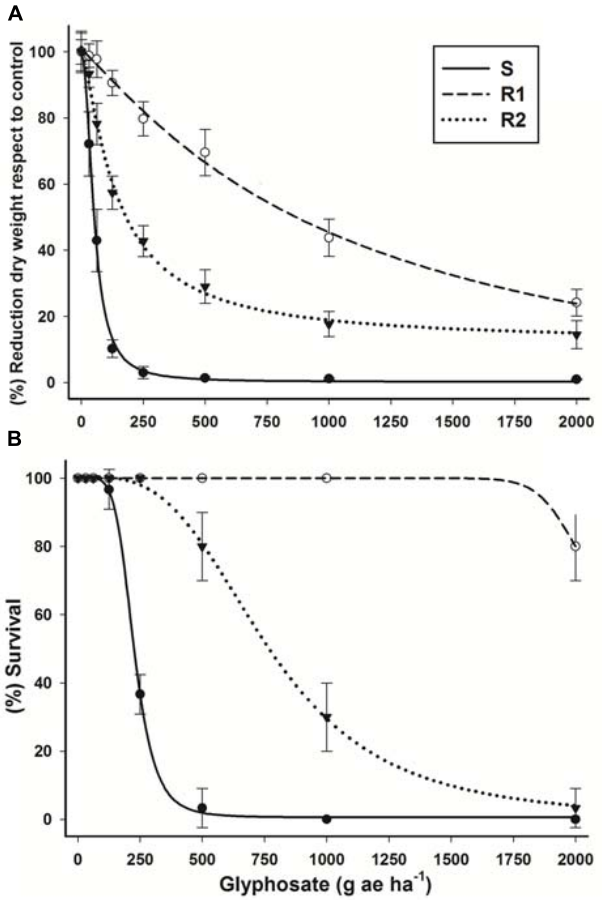

FIGURE 1 | Log–logistic curves of glyphosate-susceptible and -resistant *Bidens pilosa* populations evaluated at 21 days after treatment (DAT). (A) Dose-response curve with respect to percentage of dry mass reduction. The equations of log–logistic curves to estimates the ED_{50} values are: S: $Y = 0.178 + \{(99.354 - 0.178)/[1 + (dose/ED_{50})^{2.134}]\}$; R1: $Y = -3.295 + \{(100.56 + 3.295)/[1 + (dose/ED_{50})^{1.140}]\}$; R2: $Y = 12.121 + \{(101.39 - 12.121)/[1 + (dose/ED_{50})^{1.288}]\}$. **(B)** Dose-response curve with respect to percentage of survival. The equations of log–logistic curves to estimates the LD_{50} values are: S: $Y = 0.623 + \{(100.17 - 0.623)/[1 + (dose/LD_{50})^{5.396}]\}$; R1: rates used did not permit to estimate LD_{50} value; R2: $Y = -1.156 + \{(101.47 + 1.156)/[1 + (dose/LD_{50})^{3.238}]\}$. Vertical bars represent the standard error of the mean ($n = 10$).

TABLE 1 | ED_{50}, LD_{50}, and ID_{50} values of glyphosate-susceptible and -resistant *Bidens pilosa* populations.

Population	ED_{50} (g ae ha^{-1})	RI[a]	LD_{50} (g ae ha^{-1})	RI[a]	I_{50} (μM)	RI[a]
S	51.7 ± 2.3		225.4 ± 4.4		0.96 ± 0.0	
R1	1055.8 ± 34.9	20.4	>2000	>8.7	122.7 ± 2.1	128.4
R2	142.7 ± 10.8	2.8	774.4 ± 79.5	3.4	8.2 ± 1.1	8.5

[a]*RI, Resistance indexes (R/S) calculated using the ED_{50}, LD_{50}, or I_{50} values of the resistant populations respect to the susceptible population. ±Standard error of the mean.*

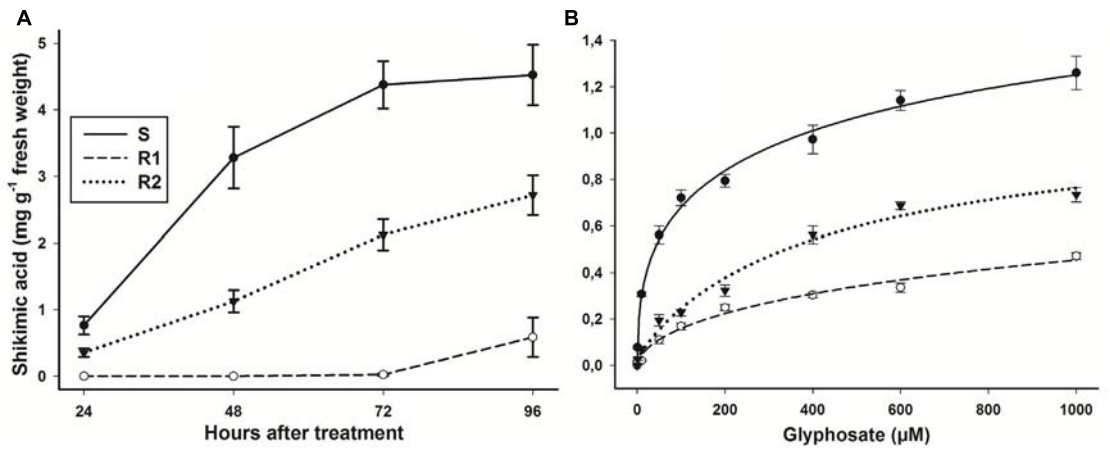

FIGURE 2 | Shikimic acid accumulation of glyphosate-susceptible and -resistant *B. pilosa* populations. (A) Shikimic acid accumulation after a glyphosate application at 360 g ae ha^{-1} at different intervals of time. **(B)** Shikimic acid accumulation at different glyphosate concentrations. Vertical bars represent the standard error of the mean ($n = 6$ technical replicates).

Foliar Retention

There were significant differences in foliar retention between hairy beggarticks populations ($P = 0.0045$; DF = 2; $n = 21$). The R2 (A) population retained the highest amount of glyphosate solution (392 ± 39 μL g^{-1} of dry weight), followed by the S (B) population with a mean value of 343 ± 34 μL g^{-1} of dry weight, whereas the R1 (B) population reached a mean of 328 ± 32 μL.

Shikimic Acid Accumulation

In the assay at different time intervals with whole plants, the S population presented an accumulation of 0.76 ± 0.13 mg shikimic acid g^{-1} of fresh weight at 24 HAT, reaching up to 4.5 ± 0.52 mg g^{-1} of fresh weight at 96 HAT (**Figure 2A**). The S and R2 populations showed an accumulation of shikimic acid since 24 HAT, while R1 population alone presented a considerable accumulation as from 72 HAT. Thus, S population was 7.7-fold more susceptible than population R1, and 1.6-fold in comparison to R2 population. The leaf segment bioassay results obtained from different glyphosate concentrations were consistent with the results obtained in the assays with the whole plants. The hairy beggarticks populations accumulated shikimic acid as the glyphosate concentrations increased (**Figure 2B**). The greater accumulation of shikimic acid exhibited by population S was consistent with the greater reduction in growth observed in plants of these populations at these low rates (**Figure 1A**). Populations R1 and R2 were 3.3- and 1.9-fold more resistant, respectively, than S population.

^{14}C-glyphosate Uptake and Translocation Assays

The differences in foliar uptake of ^{14}C-glyphosate between the resistant hairy beggarticks populations compared to the S population were highly significant ($P < 0.0001$; DF = 2; $n = 60$) (**Figure 3A**). The amount of ^{14}C-glyphosate absorbed ranged between 29.7 and 47.8%, 13.9 and 38.5%, 15.2 and 41.6%, for populations S, R1, and R2, respectively, between 24 and 96 HAT. At 24 and 96 HAT, the S population showed a greater uptake compared to R1 and R2 populations. However, after 48 and 72 HAT, the values were similar in the three populations.

With respect to the ^{14}C-glyphosate translocation, the initial amount quantified from 68.2% at 24 HAT in the treated leaf diminished to 42.6% at 96 HAT in S population. Conversely, the larger amount of herbicide applied was retained in the leaf treated in the resistant populations, dropping from 79.6 to 64.6% in R1 population, and from 73.3 to 59.7% in R2 population at 24 and 96 HAT, respectively. In the S population, an average of 24.9% of the glyphosate translocated reached the root at 96 HAT, whereas in R1 and R2 populations it was only of 12.9 and 15.5%, respectively. **Table 2** shows the results of the percentage of ^{14}C-glyphosate translocated to the remainder of the plant and root in hairy beggarticks plants.

The images obtained in the Phosphor Imager confirmed the previous results obtained for translocation. At 96 HAT it was seen how, in the plants of resistant populations, the glyphosate was retained mainly in the treated leaf, and only small amounts were

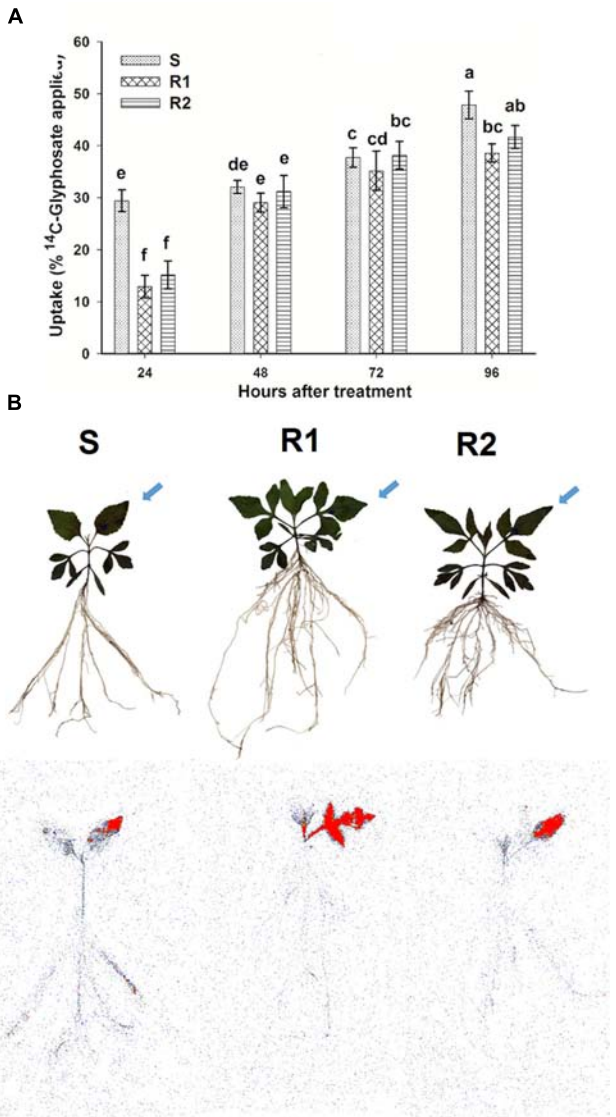

FIGURE 3 | ^{14}C-Glyphosate uptake and translocation in glyphosate-susceptible and -resistant plants of *B. pilosa* populations. **(A)** ^{14}C-glyphosate uptake in glyphosate-susceptible and -resistant *B. pilosa* plants. Different letters are statistically different at 95% probability determined by LSD test. Vertical bars represent the standard error of the mean ($n = 5$). **(B)** Digital images (upper plants) and autoradiograph images (lower plants) that show the distribution of ^{14}C-glyphosate translocation in glyphosate-susceptible and -resistant *B. pilosa* plants at 96 HAT. The highest concentration of ^{14}C-glyphosate is highlighted in red. Arrows indicate the treated leaf.

translocated across the remainder of the plant in comparison to the S population (**Figure 3B**).

Metabolism

Glyphosate and its metabolites were quantified by reversed-polarity capillary-electrophoresis. There were no significant differences between hairy beggarticks populations. The amount of glyphosate quantified was of 100% at 4 DAT, and higher than 95% at 8 and 12 DAT from total applied. Only small amounts of AMPA and glyoxylate were detected at this time in all populations.

TABLE 2 | Translocation percentage of ^{14}C-glyphosate in plants of glyphosate-susceptible and -resistant *Bidens pilosa* populations.

Population	HAT	Traslocation (% from uptake)[a]		
		Treated leaf	Remainder of plant	Root
S	24	68.2 ± 2.0 c	18.3 ± 1.1 ef	13.5 ± 1.0 cde
	48	59.7 ± 1.6 d	23.0 ± 1.6 c	18.5 ± 1.2 b
	72	52.3 ± 2.2 e	29.1 ± 1.7 b	19.6 ± 0.6 b
	96	42.6 ± 2.0 f	33.5 ± 0.6 a	24.9 ± 1.5 a
R1	24	79.6 ± 2.1 a	13.0 ± 0.5 g	7.4 ± 2.3 g
	48	73.6 ± 2.3 b	16.9 ± 3.2 ef	10.5 ± 0.9 f
	72	67.6 ± 3.3 c	19.1 ± 2.4 e	13.3 ± 1.3 cde
	96	64.6 ± 2.0 c	22.5 ± 2.5 cd	12.9 ± 1.0 de
R2	24	73.3 ± 2.3 b	15.2 ± 0.8 fg	11.5 ± 1.3 ef
	48	68.5 ± 1.9 b	17.8 ± 2.2 ef	13.7 ± 1.6 cde
	72	66.2 ± 4.1 c	19.4 ± 1.9 de	14.4 ± 1.2 cd
	96	59.7 ± 2.8 d	24.8 ± 2.3 c	15.5 ± 1.3 c

[a]*Means with different letter within a column are statistically different at 95% probability determined by the Tukey test. ±Standard error of the mean (n = 5).*

Enzyme Activity

There were no significant differences ($P = 0.34$; DF = 2; $n = 9$) in the basal EPSPS activity (average = 0.39 μmol μg TSP^{-1} min^{-1}) in plants of glyphosate-susceptible and -resistant hairy beggarticks populations in the absence of glyphosate (**Figure 4A**). The EPSPS enzyme was inhibited by glyphosate in plants of susceptible and resistant populations. For the S population, only 0.95 μM of glyphosate was necessary to inhibit EPSPS activity by 50% (I_{50}). The resistant plants of R2 and R1 populations, on average, were 8.5- and 128.4-fold, respectively, less sensitive to glyphosate than the susceptible plants (**Figure 4B**).

Sequencing of the EPSPS Gene

The sequencing from cDNA revealing the presence of two different EPSPS genes that are expressed in the three hairy beggarticks populations (**Table 3**; **Figure 5**), showed one homology above 92% between EPSPS1 and EPSPS2 genes based on their predicted proteins, and above 80% with respect to *Arabidopsis thaliana* (GenBank: CAA29828.1) (**Figure 5**). In the three populations, some individuals only showed the EPSPS1 gene, others the EPSPS2 gene, and others showed both genes.

Some R1 population plants were identified with a single mutation in Pro-106 position alone, and other plants presented a double mutation in the Thr-102 and Pro-106 positions in the EPSPS2 gene (**Figure 5**). The amino acid substitutions consisted of Threonine (ACC) to Isoleucine (ATC) in Thr-102 position, and from Proline (CCA) to Serine (TCA) in Pro-106 positon (**Figure 5**). Mutations were not found in the EPSPS1, and the R2 population did not show any mutation. Of the 20 individuals sequenced from R1 population, only 2 had the I102-S106 (TIPS)

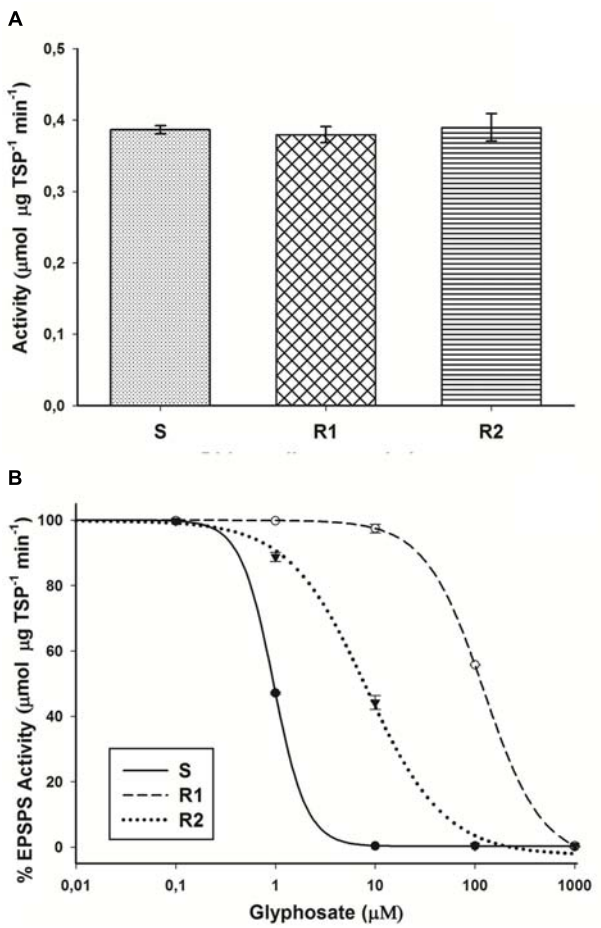

FIGURE 4 | 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) activity in glyphosate-susceptible and -resistant plants of *B. pilosa* populations. (A) Basal EPSPS activity for glyphosate-susceptible and -resistant *B. pilosa* plants. Histograms represent treatment means and vertical bars SE of the mean ($n = 3$). **(B)** EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from glyphosate-susceptible and resistant *B. pilosa* populations. The equations of log–logistic curves to estimates the I_{50} values are: S: $Y = 0.227 + \{(99.98 - 0.227)/[1 + (\text{dose}/I_{50})^{2.619}]\}$; R1: $Y = -4.065 + \{(99.92 + 4.065)/[1 + (\text{dose}/I_{50})^{1.477}]\}$; R2: $Y = -2.471 + \{(99.79 + 2.471)/[1 + (\text{dose}/I_{50})^{1.108}]\}$. Vertical bars represent the standard error of the mean ($n = 3$).

allele; 11 individuals the T102-S106 allele, and 7 had the wild type allele T102-P106 corresponding to 10, 55 and 35% of the sample size analyzed. Percentage frequency of EPSPS genes and alleles implicated in glyphosate resistance are shown in the **Table 3**.

DISCUSSION

The ED_{50}, LD_{50} and I_{50} parameters (**Table 1**) showed the highest level of resistance developed by resistant hairy beggarticks populations. Our results of dose-response results are according with other glyphosate-resistant species. For example, the resistance index (RI) in resistant *C. bonariensis, C. canadensis,* and *C. sumatrensis* populations, asteraceae species like hairy beggarticks, ranged between 7- and 17-fold more compared to their respective susceptible population (Koger et al., 2004; González-Torralva et al., 2010, 2012b). Other dicotyledonous species such as *A. palmeri* and *Kochia scoparia* presented similar variation of resistance level showed RI between 3- and 18-fold (Whitaker et al., 2013; Ribeiro et al., 2014; Wiersma et al., 2015). A resistant *A. palmeri* population showed an RI 18-fold higher than its S population with an ED_{50} of 2565 g ae ha^{-1} (Whitaker et al., 2013).

On the basis of LD_{50}, the RI ranged between 3- and 15-fold in weed species, such as *Lolium perenne* spp. *multiflorum* and *K. scoparia* (Salas et al., 2015; Wiersma et al., 2015). To achieve a total control in resistant hairy beggarticks populations, one needs to apply at least double the rate of glyphosate of that of their corresponding LD_{50}. However, higher doses increase selection pressure and will accelerate the evolution of resistant populations (Salas et al., 2015).

Shikimic acid and enzyme EPSPS activity tests are accepted as appropriate parameters to determine susceptibility level to glyphosate (Dayan et al., 2015). With respect to EPSPS activity, some resistant *L. perenne* spp. *multiflorum* and *Echinochloa colona* populations showed differences in basal EPSPS activity compared to their respective susceptible populations (Salas et al., 2012; Alarcón-Reverte et al., 2015), but these differences were associated with a greater number of copies of the EPSPS gene. Therefore, the similar basal EPSPS activity between hairy beggarticks populations suggests that resistant populations could not have any differences in the number of copies of the EPSPS gene respect to susceptible populations. Multiple EPSPS copy numbers and/or increased EPSPS expression have been described as glyphosate resistance mechanisms in dicotyledonous species such as *A. palmeri, A. tuberculatus*, *K. scoparia* (Ribeiro et al., 2014; Chatham et al., 2015; Wiersma et al., 2015), among others species. However, similar gene copy numbers may not necessarily show the same level of resistance to glyphosate (Salas et al., 2012). As in resistant hairy beggarticks populations, higher glyphosate concentrations were necessary in resistant *L. perenne* spp. *multiflorum* and *E. colona* populations to inhibit EPSPS activity by 50% (I_{50}) (Salas et al., 2012; Alarcón-Reverte et al., 2015).

In both assays of shikimic acid, the resistant hairy beggarticks populations showed a lesser shikimic acid accumulation than the S population (**Figure 2**). This evidenced the high susceptibility to glyphosate of the S population, and a different resistance level between R1 and R2 populations. Similar results have been reported in other species of glyphosate-resistant weeds, for instance, resistant *L. rigidum*, *E. colona,* and *Poa annua*) populations (Perez-Jones et al., 2007; Alarcón-Reverte et al., 2013; Cross et al., 2015). Any species with a low accumulation of shikimic acid requires a larger amount of glyphosate in order for it to be lethal (Cruz-Hipolito et al., 2011; Alcántara-de la Cruz et al., 2016). This can happen when, in the differential accumulation of shikimic acid, glyphosate does not reach the target site in sufficient amounts due to altered translocation patterns (Alarcón-Reverte et al., 2015; Cross et al., 2015). In this work, both susceptible and resistant populations accumulated

TABLE 3 | Frequency percentage of 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) genes, and polymorphisms at 102 and 106 positions in glyphosate-susceptible and -resistant plants of *Bidens pilosa* populations.

Population	Number of individuals/clones	Gene	Gene Frequency (%)	Alleles[a]	Allele frequency (%)
S	5/15	EPSPS1	60.0	T102-P106	60.0
		EPSPS2	40.0	T102-P106	40.0
R1	20/60	EPSPS1	56.7	T102-P106	56.7
		EPSPS2	43.3	T102-P106	14.3
				T102-S106	23.3
				I102-S106	6.6
R2	5/15	EPSPS1	53.3	T102-P106	53.3
		EPSPS2	46.7	T102-P106	46.7

[a] *T102-P106 = wild type or glyphosate susceptible; T102-S106 = low-intermediate glyphosate resistance; and I102-S106 = high glyphosate resistance.*

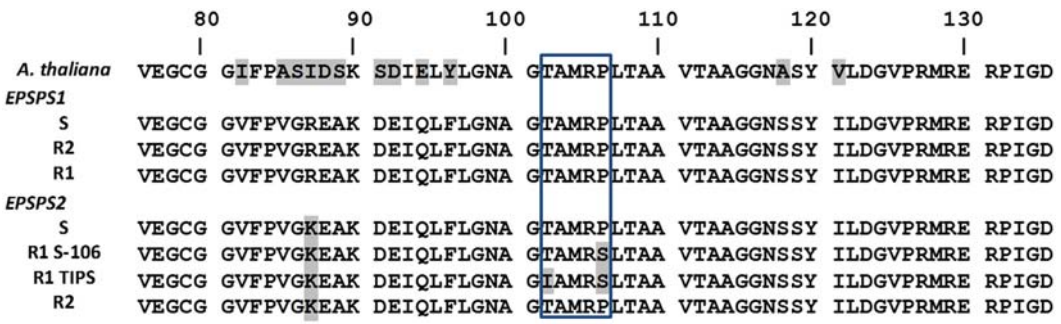

FIGURE 5 | Partial alignment of protein sequences of EPSPS1 and EPSPS2 genes in glyphosate-susceptible and -resistant *B. pilosa* populations. The highlighted color indicates changes to codons from the consensus nucleotide sequence. Box includes from the 102 to 106 positions (amino acid number based on the start codon (ATG) of *A. thaliana* [GenBank: CAA29828.1] EPSPS sequence). The *B. pilosa* EPSPS cDNA sequences information can be found in GenBank with accession numbers KU984452–KU984458.

shikimic acid. This indicated that the glyphosate arrived at the target site inhibiting the EPSPS, but that that inhibition was at different levels (Duke and Powles, 2008; Alarcón-Reverte et al., 2015), this being significantly greater in the S population.

Herbicide foliar retention and uptake are influenced by physiological and morphological traits (Cruz-Hipolito et al., 2011; Alcántara-de la Cruz et al., 2016), and are not major mechanisms conferring glyphosate resistance. Foliar retention capacity depends on the phenology of the plants. Studies on *Conyza* spp. showed that foliar retention was greater during the elongation of the stem than during flowering (González-Torralva et al., 2010). Only few weed species have presented differences in reduced glyphosate uptake and foliar retention as mechanisms involved in their resistance. For instance, resistant *A. tuberculatus*, *L. multiflorum,* and *Digitaria insularis* populations showed a reduced uptake (Michitte et al., 2007; de Carvalho et al., 2012; Nandula et al., 2013); and only *L. multiflorum* presented a lower foliar retention. These traits play an important role in innate glyphosate-tolerant species (Cruz-Hipolito et al., 2011; Alcántara-de la Cruz et al., 2016). In hairy beggarticks populations neither foliar retention nor ^{14}C-glyphosate uptake were not mechanisms involved in the resistance. However, the ^{14}C-glyphosate translocation results suggest that a reduced translocation could be the main mechanism involved in resistance (**Table 2**; **Figure 3B**), but the different resistance levels and shikimic acid accumulation between resistant hairy beggarticks populations suggests that their resistance mechanisms may differ between each other. Sammons and Gaines (2014) pointed out several cases with at least two resistance mechanisms. These cases involved TSR and NTSR mechanisms.

Shikimic acid pathway consists at least of two separate pathways, the presence of multiple EPSPS genes or isoforms is common in higher plants (Jensen, 1986). EPSPS isozymes have been identified differentially localized in plastids and cytosol (Mousdale and Coggings, 1985). This justifies the presence of the two EPSPS genes identified in hairy beggarticks populations. Weed species such as *C. sumatrensis* and *E. colona* also expressed two genes of EPSPS, one of them showing a mutation in Pro-106 position conferring glyphosate resistance (González-Torralva et al., 2014; Alarcón-Reverte et al., 2015).

A single Pro-106 mutation has been widely described in several resistant weed species to glyphosate (González-Torralva et al., 2012a, 2014; Alarcón-Reverte et al., 2015; Cross et al., 2015; Salas et al., 2015; between others). The levels of resistance conferred by single target-site mutation to date tend to vary at between 2- and 10-fold, depending on whether a target site mutation or reduced translocation is the mechanism (Bostamam et al., 2012), and up to 4 to 15-fold when two different mechanisms are involved (Sammons and Gaines, 2014),

for instance, resistant *A. tuberculatus* (Nandula et al., 2013), *L. rigidum* (Yu et al., 2007; Bostamam et al., 2012) and *L. multiflorum* (González-Torralva et al., 2012a) populations, exhibited a mutation in Pro-106 position, and a reduced translocation with an RI fivefold for the resistant *A. tuberculatus* and *L. multiflorum* populations, whereas for those of *L. rigidum*, the RI was six–eightfold (Bostamam et al., 2012), and 14-fold (Yu et al., 2007) higher than their susceptible population.

The Thr-102-Ile mutation would be unlikely to occur first or independently (Weinreich et al., 2006), it has usually been associated with the Pro-106-Ser mutation, commonly known as TIPS. Such mutation has been reported to provide high resistance/tolerance to glyphosate in studies with *E. coli* (Healy-Fried et al., 2007), and it is used in transgenic glyphosate-resistant crops (see **Table 3**, review by Sammons and Gaines, 2014). Recently, the TIPS mutation was identified in two resistant *E. indica* populations. The latter was one population from Malaysia, as the first TIPS mutation naturally identified in weed species (Yu et al., 2015), and one from China (Chen et al., 2015). In both cases, the frequency of I102-S106 (high resistance) allele corresponding to TIPS mutation was lower (26%) of frequency in the population from the Malaysia (3 from 193 individuals), and 16.7% in the population from China (8 from 30 individuals) than T102-S106 (low-intermediate resistance) and/or T102-P106 (wild type or susceptible) alleles. The hairy beggarticks R1 population also presented a low frequency (6.6%) of I102-S106 allele in 2 from 20 individuals studied. This is another example of naturally evolved of TIPS mutation. Due to the insect pollination that shows hairy beggarticks, the allele of TIPS mutation could be easily spread to other Persian lime groves by insects. For this reason, future studies will focus on characterizing glyphosate-resistant genotypes using dCAPS markers, as well as the fitness cost of glyphosate resistance in this species.

Glyphosate-resistant species such as *D. insularis* and *C. canadensis* (de Carvalho et al., 2012; González-Torralva et al., 2012b) presented metabolism as a mechanism of resistance. However, the metabolism was not involved in the resistance to glyphosate in hairy beggarticks populations of this study. These results were consistent with other studies in *C. canadensis* and *E. colona* (Dinelli et al., 2006; Alarcón-Reverte et al., 2015), which demonstrated no contribution to resistance.

Additionally, EPSPS isozymes may have different response to glyphosate (Ream et al., 1988). Due to each isozyme was not isolated individually, we do not know which isozyme (EPSPS1- or EPSPS2-isozyme) is more sensitive to glyphosate. This can explain the differences observed in the EPSPS activity between the S and R2 populations, were no mutation was identified.

CONCLUSION

These results revealed the first case of a double mutation (TIPS) evolved on the target site in a wild dicotyledonous weed, due to high selection pressure exerted by repeated glyphosate applications (3–4 times per year) in citrus groves from Mexico (Pérez-López et al., 2014).

The reduced translocation and the relationship between the parameters of ED_{50}, LD_{50}, and I_{50} confirmed glyphosate resistance of hairy-beggarticks. The R2 population used reduced translocation of ^{14}C-glyphosate, to its target site (EPSPS) as major mechanism, to resist against glyphosate presenting a low-intermediate resistance. Although Ser-106 and TIPS mutations found in the EPSPS2 gene presented a low frequency, in association with a reduced glyphosate translocation, those were responsible for conferring high resistance in the R1 population.

The confirmation of this resistance suggests the need to include other pre-emergent and post-emergent herbicides to manage hairy beggarticks in citrus groves. In addition, practices that contributing to the germination and presence of susceptible hairy beggarticks plants, allowing their reproduction with resistant plants in order to reduce the resistance level.

AUTHOR CONTRIBUTIONS

JD-V, Provided the seeds used in this work. RA, HC-H, FB, JD-V, and RP, Idea and designed the experiments. RA, AR-D, CO, and PF-M, Performed the research. RA, AR-D, CO, and PF-M. Interpretation and analysis of results (of raw data). RA, AR-D, CO, HC-H, FB, JD-V, and RP: Wrote and approved the manuscript.

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First Resistance Mechanisms Characterization in Glyphosate-Resistant *Leptochloa virgata*

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Leptochloa virgata (L.) P. Beauv. is an annual weed common in citrus groves in the states of Puebla and Veracruz, Mexico limiting their production. Since 2010, several *L. virgata* populations were identified as being resistant to glyphosate, but studies of their resistance mechanisms developed by this species have been conducted. In this work, three glyphosate-resistant populations (R8, R14, and R15) collected in citrus orchards from Mexico, were used to study their resistance mechanisms comparing them to one susceptible population (S). Dose-response and shikimic acid accumulation assays confirmed the glyphosate resistance of the three resistant populations. Higher doses of up to 720 g ae ha^{-1} (field dose) were needed to control by 50% plants of resistant populations. The S population absorbed between 7 and 13% more ^{14}C-glyphosate than resistant ones, and translocated up to 32.2% of ^{14}C-glyphosate to the roots at 96 h after treatment (HAT). The R8, R14, and R15 populations translocated only 24.5, 26.5, and 21.9%, respectively. The enzyme activity of 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) was not different in the S, R8 and R14 populations. The R15 Population exhibited 165.9 times greater EPSPS activity. Additionally, this population showed a higher EPSPS basal activity and a substitution in the codon 106 from Proline to Serine in the EPSPS protein sequence. EPSPS gene expression in the R15 population was similar to that of S population. In conclusion, the three resistant *L. virgata* populations show reduced absorption and translocation of ^{14}C-glyphosate. Moreover, a mutation and an enhanced EPSPS basal activity at target-site level confers higher resistance to glyphosate. These results describe for the first time the glyphosate resistance mechanisms developed by resistant *L. virgata* populations of citrus orchards from Mexico.

Keywords: 5-enolpyruvyl shikimate-3-phosphate synthase, EPSPS gene, Pro-106 substitution, reduced absorption and translocation, tropical sprangletop.

INTRODUCTION

Glyphosate is the most extensively used systemic foliar herbicide of broad spectrum used for over 40 years (early 1970s) to control of annual and perennial weeds (Gomes et al., 2014; Armendáriz et al., 2016). The target-site of glyphosate is the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), an intermediate in the shikimate pathway (Gomes et al., 2014). EPSPS inhibition by glyphosate does not allow the synthesis of phenylalanine, tyrosine, and tryptophan resulting in accumulation of shikimic acid and depletion of aromatic amino acid pools (Gomes et al., 2014; Maroli et al., 2016). Therefore, glyphosate acts rapidly in reducing photosynthesis activity, carbon metabolism, mineral nutrition, and oxidative events, and to disturb plant–microorganism interactions (Gomes et al., 2014). To date 36 weed species have been reported as being resistant to glyphosate with more than 266 cases (Heap, 2016).

Resistance to glyphosate could be caused by different mechanisms either in the target or non-target site (Salas et al., 2015). Both groups limiting the amount of glyphosate which reaches the EPSPS at toxic levels, or causing a loss of affinity between the EPSPS and the glyphosate (Powles, 2010). Target-site resistance (TSR) mechanisms includes amino acid substitutions in DNA sequence, represented by nucleotide changes in Thr-102 and Pro-106 positions, that synthesizes the EPSPS enzyme (Sammons and Gaines, 2014; Chen et al., 2015; Yu et al., 2015; Alcántara-de la Cruz et al., 2016b); multiple EPSPS gene copy numbers resulting in a higher amplification, expression and activity of the EPSPS (Gaines et al., 2013; Vila-Aiub et al., 2014). To date, glyphosate is the only herbicide known to evolve amplification of the target-site (EPSPS) conferring resistance with field applications (Ribeiro et al., 2014).

Non-target-site resistance (NTSR) mechanisms can confer unpredictable resistance to herbicides (Déyle, 2013). Some are: limited glyphosate leaf absorption (Vila-Aiub et al., 2012); reduced glyphosate translocation to meristematic zones, mainly restricted within the treated leaves (Shaner, 2009; Yanniccari et al., 2012); sequestration of glyphosate in the vacuole (Ge et al., 2012); and glyphosate degradation to non-toxic compounds (sarcosine, glyoxylate, formaldehyde and amino methyl phosphonate) (Duke, 2011) resulting in significantly less glyphosate throughout the whole plants.

Weeds can evolve several herbicides resistance mechanisms, and the individuals of the same species can express different mechanism(s) (Alarcón-Reverte et al., 2015). In *Leptochloa virgata* (L.) P. Beauv., studies about of glyphosate efficacy by dose-response assay, genetic diversity characterization using inter simple sequence repeat (ISSR) markers of different glyphosate-resistant *L. virgata* populations (Alcántara-de la Cruz et al., 2016c), and field trials to provide alternative herbicides for their control have been carried out (Pérez-López et al., 2014). However, no studies on the glyphosate resistance mechanisms involved in their resistance have been conducted, and currently there are no reports of others resistant populations of this species elsewhere in the world.

Based on previous reports, and the lack of any knowledge on the glyphosate resistance mechanisms of *L. virgata*, we hypothesized that the resistance mechanisms developed by *L. virgata* plants could be similar to the mechanisms evolved by others glyphosate resistant species. Therefore, the objective of this work was characterize for the first time whether the glyphosate resistance developed by three resistant *L. virgata* populations was due either target or non-target site mechanisms, comparing them to one susceptible population (S). These populations were used as being representatives of the species in citrus cropping systems from Mexico.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Seeds of four *L. virgata* populations (S, R8, R14, and R15) collected in Persian lime groves from Veracruz, previously characterized with different levels of susceptibility to glyphosate by Alcántara-de la Cruz et al. (2016c), were used. Individuals of the *L. virgata* populations studied had a similar glyphosate response glyphosate within them. Glyphosate resistant R14 and R15 populations from Cuitláhuac municipality, were selected for being geographically close (18.75° N, 96.53° W), but genetically different. Also, the most glyphosate resistant population (R8) from Martínez de la Torre municipality (20.16° N, 97.07° W) was included in this study. The S population was used as control (18.79° N, 96.69° W).

The seeds were sown on trays with peat saturated at field capacity. The trays were covered with a layer of plastic until emergence and placed in a growth chamber at 26/18°C (day/night) with a photoperiod of 16 h at 850 mmol m^{-2} s^{-1} of light density and 60% relative humidity. Germinated seedlings were individually transplanted into plastic pots in 250 mL of substrate (sand and peat 1:1). Subsequently, the pots were placed in a growth chamber under the conditions described above and watered daily.

Dose-Response Assays

Plants of the four populations with 3–4 true leaves were treated with the following glyphosate rates: 0, 45, 90, 180, 360, 720, 1040, 1440, and 1800 g ae ha^{-1}. Glyphosate (Roundup Energy 45% w/v, Monsanto, Spain) applications were carried out in a treatment chamber (Devries Manufacturing, Hollandale, MN, USA) equipped with TeeJet 8002EVS flat fan nozzle calibrated at 200 kPa, a height of 50 cm and 200 L ha^{-1} of application volume. The plants were harvested at ground level 21 days after treatment (DAT) and stored separately in paper envelopes. The samples were dried in an oven (JP Selecta S.A., Barcelona, Spain) at 60°C for 4 days and weighed to determine the dry weight. Data were expressed as percentage of dry weight compared to the untreated control plants. The experiment was repeated twice in a completely randomized design with 10 replicates per dose.

Shikimic Acid Accumulation

Samples of 50 mg (leaf disks 4 mm in diameter) of plant tissue were taken from young leaves of three plants with 3–4 true leaves of each *L. virgata* population according to Dayan et al. (2015). The disks were placed in 2 mL-Eppendorf tubes containing

999 μL of monoammonium phosphate ($NH_4H_2PO_4$ 10 mM, pH 4.4). Volumes of 1 μL of glyphosate solutions at different concentrations were added (0, 0.1, 1, 10, 50, 100, 200, 400, 600, and 1000 μM). The samples were incubated for 24 h in the growth chamber under growing conditions described above. Next, they were incubated at 60°C for 30 min. Volumes of 250 μL of HCl 1.25 N were added and incubated at 60°C for 15 min. Aliquots of 250 μL were transferred to new tubes adding 500 μL of periodic acid (0.25% w/v) and sodium metaperiodate (0.25 % w/v) in a 1:1 ratio. The samples were incubated at room temperature (25°C) during 90 min, and next, 500 μL of a mix of sodium hydroxide (NaOH 0.6 N) plus sodium sulfite (Na_2SO_3 0.22 N) in a 1:1 ratio was added and mixed.

The experiment was arranged in a completely randomized design with three technical replications by sample of each population for each glyphosate concentration, and the study was repeated twice. A standard curve was done using known concentrations of shikimate. The absorbance of samples was measured at 382 nm in a spectrophotometer (DU-640, Beckman Coulter Inc., Fullerton, CA, USA). The absorbance values were converted into mg of shikimic acid per g of fresh weight.

Absorption and Translocation

Plants with 3–4 true leaves from the four *L. virgata* populations were treated with a solution of ^{14}C-glyphosate [glycine-2-^{14}C] (specific activity 273.8 MBq mmol^{-1}, American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) + commercial glyphosate. The solution applied contained a specific activity of 0.834 kBq μL^{-1} and a glyphosate concentration of 1.8 g ea L^{-1} (360 g ea ha^{-1} in 200 L). One drop of 1 μL plant^{-1} of solution was applied with a micropipette (Lab Mate HTL, Matosinhos, Portugal) on the adaxial surface of the first-second leaf. After treatment, the plants were maintained in the growth chamber at the growing conditions described above. At 24, 48, 72, and 96 HAT, the treated leaves were washed three times separately with 1 mL of water-acetone (1:1 v/v) to recover the non-absorbed ^{14}C-glyphosate. The washing solution was mixed with 2 mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard), and analyzed by liquid scintillation spectrometry (LSS) in a scintillation counter (LS 6500, Beckman Coulter Inc., Fullerton, CA, USA) during 10 min per sample. The whole plants were carefully removed from the pot and washed, mainly the roots. The plants were individually divided into treated leaf, remainder of the plant and root. The samples were stored in flexible combustion cones (Perkin-Elmer, BV BioScience Packard), dried in an oven at 60°C for 4 days. Next, the samples were combusted in a biological oxidizer (Packard Tri Carb 307, Packard Instrument Co., Downers Grove, IL, USA). The CO_2 released from the combustion was captured in 18 mL of a mix of Carbo-Sorb E and Permafluor (1:1 v/v) (Perkin-Elmer, BV BioScience Packard). The radioactivity of each individual sample was quantified by LSS during 10 min per sample. The experiment was arranged in a completely random design with five replicates per population at each time evaluated. The radioactive values were used to calculate recovery percentage as: [(kBq in treated leaf + kBq in plant + kBq in roots + kBq from washes)/kBq total applied] × 100. The average total recovery of ^{14}C-glyphosate applied was >96% to the S, R8, and R14 populations, and <93% from the R15 population.

To visualize the ^{14}C-glyphosate translocation, three whole plants per population at each visualization time (24, 48, 72, and 96 HAT) were treated under the same conditions as in the previous assay. The plants were washed individually, fixed on filter paper (25 cm × 12.5 cm) and dried at room temperature for 1 week. The samples were placed for 4 h beside a phosphor storage film (Storage Phosphor System: Cyclone, Perkin–Elmer Packard BioScience BV). A phosphor imager (Cyclon, Perkin-Elmer, Packard BioScience BV) was used to reveal the translocation.

Enzyme Activity of the EPSPS

Plants of the four *L. virgata* populations were grown in pots (25 cm in diameter × 15 cm high: four plants per pot) under greenhouse conditions, in temperatures ranging from 17 to 31°C, and a photoperiod of 16 h. The natural light was complemented by 900 μmol^{-2} s^{-1} photosynthetic photon flux density delivered by incandescent and fluorescent lights. Samples of 5 g of foliar tissue from each population were obtained from the second and third youngest totally expanded leaves.

The methodology described by Sammons et al. (2007) was used for EPSPS extraction. The total soluble protein (TPS) in the extract was measured using a Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain) following the manufacturer's instructions. The specific EPSPS activity in plants from *L. virgata* populations was studied in the presence and absence (basal activity) of glyphosate. The EPSPS activity was determined using a EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The glyphosate concentrations used were: 0, 1, 10, 100, 1000, 10000 μM. Three replicates at each glyphosate concentration were analyzed. The release of phosphate on the bottom level was measured during 10 min at 360 nm in a spectrophotometer (DU-640, Beckman Coulter Inc., Fullerton, CA, USA).

EPSPS Gene Sequencing

Samples of young leaf tissue (≈100 mg) from 5 glyphosate-susceptible (S) and 15-resistant (R8, R14, and R15) *L. virgata* individuals were taken, and stored at −80°C for RNA extraction. Total mRNA was isolated following the methodology described by Pistón (2013). Integrity of RNA was verified in 0.8% agarose gel and quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Walthman, MA, USA). First strand complementary DNA (cDNA) synthesis was carried out using 1 μg of RNA in all the samples. An iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. Hercules, CA, USA) was employed following the manufacturer's instructions. Lv-F3 and Lv-R2 primers (**Table 1**) were designed using the software Primers3Plus[1], based on conserved regions of the EPSPS gene sequences from *Eleusine indica* (GenBank Accession: AY157642.1, HQ403647.1) and *Lolium multiflorum* (GenBank

[1]http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

TABLE 1 | Names and sequences of primers used in EPSPS gene sequencing and expression analysis of *L. virgata* populations.

Name	Sequence (5′ to 3′)
EPSPS gene sequencing and cloning	
Lv-F3[a]	AAGAGCTGTWGTYGTTGGCTG
Lv-R2[a]	AATAGCACCTCGCACTTGAG
M13F	CGCCAGGGTTTTCCCAGTCACGAC
M13R	TCACACAGGAAACAGCTATGAC
qPCR	
qLv-For1[a]	GGCAGGTTCCCGATTGARAA
qLv-Rev1[a]	YGCATTTCCACCAGCAGCTA
ADP-RF(a) For	TCTCATGGTTGGTCTCGATG
ADP-RF(a) Rev	GGATGGTGGTGACGATCTCT
β-Act F1[a]	ATGGTAGGGATGGGACAGAA
β-Act R1[a]	TCCATGTCATCCCAGTTGCT

[a]*The primers were designed with the software Primers3Plus online.*

Accession: DQ153168.2). Individual PCR reactions were carried out using cDNA from each sample of the S and R15 populations. Each PCR reaction was performed using 50 ng of cDNA, 0.2 μM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Madrid, Spain), 2 mM $MgCl_2$, 1X buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain) in a final volume of 25 μL. The PCR conditions were: 1 cycle of 94°C for 5 min; followed by35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products (10 μL) were checked by 1% agarose gel to corroborate amplification. The PCR products were ligated using the pGEM-T Easy Vector System (Promega Biotech Ibérica, SL, Madrid, Spain) following the manufacturer's instructions, and cloned into competent cells of *E. coli* DH5α. Positive transformants were selected and fragment insertion confirmed through PCR using the M13F and M13R primers (**Table 1**). A total volume of 15 μL per sample containing 0.2 μM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Madrid, Spain), 2 mM $MgCl_2$, 1X buffer, and 0.625 units of non-proofreading (*Thermus thermophilus*) polymerase (BIOTOOLS, Madrid, Spain). The PCR conditions were: 1 cycle of 94°C for 5 min; followed by 28 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. The plasmids were purified with the ilustra plasmidPrep Mini Spin kit (GE Healthcare, Buckinghamshire, UK), following the manufacturer's instructions. Sanger sequencing was carried out by the STABVIDA sequencing service (Caparica, Portugal). A total of 30 clones from each population were sequenced. The assembly of the sequences was carried out by SeqMan Pro (Version 11, DNASTAR; Madison, WI, USA) and Geneious (Version 8.1.8, Biomatters Ltd, Auckland, New Zealand) software's.

The *L. virgata* EPSPS cDNA sequences information can be found in GenBank with accession numbers KX425854 and KX425855.

EPSPS Gene Expression

Young leaf samples (≈100 mg) from six untreated plants of both S and R15 populations were taken before treatment. Plants were then treated with 360 g ae ha^{-1} of glyphosate in the conditions used in the dose-response assays, and new samples were collected at 24 HAT. In both cases, the samples were stored at −80°C for RNA extraction and cDNA synthesis in the same conditions described in the previous section. The qLvFor1 and qLv-Rev1 primers (**Table 1**) to amplify a fragment of 114 bp were designed from the EPSPS gene sequences obtained in the previous section. The β-Actin and ADP-ribosylation factor genes were used as reference genes. Pairs of β-Actin primers (**Table 1**) were designed on the basis of the *Agrostis stolonifera* (JX644005.1), *Avena sativa* (KP257585.1), *Lolium multiflorum* (AJ585201.1), *Triticum monococcum* (AF326781.1) and *Zea mays* (U60510.1) sequences from GenBank. β-Actin and qLv primers were designed using Primers3Plus. The ADP-RF(a) primers designed by Giménez et al. (2011) were used to amplify the ADP-ribosylation factor gene (**Table 1**). For each quantitative RT-PCR reaction 40 ng of cDNA, 10 μL PerfeCTa SYBR Green FastMix ROX (Quanta Bioscience), and 0.2 μM of both forward and reverse primers in a 15 μL final reaction volume were used. The PCR conditions were: initial cycle at 94°C for 5 min; 40 cycles of 94°C 30 s and 62°C 1 min. The PCR reactions were carried out using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Three to four technical replications per plant were carried out in a factorial design of two glyphosate treatments and two populations.

The PCR efficiency for each pair of primer and sample was determined by the program qPCR data analysis LinRegPCR (version 11) according to Ruijter et al. (2009) using raw fluorescence as input data. Expression level of both the reference and target genes for each sample was determined with the follow equation:

$$N_0 = 0.2/E^{Cq} \quad (1)$$

Where N_0 is expression of the gene, E is the PCR efficiency for each primer, Cq is the number of cycles needed to reach 0.2 arbitrary units of fluorescence. The mean PCR efficiency for each gene, population and treatment was determined according to Giménez et al. (2011). The stability of the expression of the reference genes (β-Actin and ADP-ribosylation factor) and Normalization Factor (NF) were determined using geNorm software for each sample according to Vandesompele et al. (2002).

Statistical Analysis

The percentages data of dry weight reduction, survival and EPSPS enzyme activity were submitted to a non-linear regression analysis. The dose of glyphosate needed to reduce the weight of a population (ED_{50}), mortality (LD_{50}), and to inhibit EPSPS activity (I_{50}) by 50% were calculated. A log-logistic model of four parameters was conducted using the *drc* statistical package (Ritz et al., 2015) in the program R version 3.2.5. The statistical model is:

$$Y = c + \{(d - c)/[1 + (x/g)^b]\} \quad (2)$$

Where Y is the percentage of dry weight, survival and/or EPSPS-inhibiting with respect to the control, c and d are coefficients

corresponding to the upper and lower asymptotic limits, *b* is the Hill slope, *g* is the glyphosate dose (ED_{50}, LD_{50}, or I_{50}) at the mean point of inflection between the upper and lower asymptote and *x* (independent variable) corresponds to the glyphosate dose. The data were plotted using SigmaPlot 11.0 (Systat Software, Inc., USA).

EPSPS normalized expression level was calculated for each qPCR reaction, the average and standard error from technical replicates were recorded for each plant and population.

Absorption, translocation and EPSPS expression results were subjected to ANOVA using Statistix version 9.0 from Analytical Software (Tallahassee, FL, USA). When necessary, the means were compared using Tukey's test's at the 95% probability level.

RESULTS

Glyphosate Dose–Response

Glyphosate resistance was confirmed in the three *L. virgata* populations (R8, R14, and R15) collected in Persian lime groves from Veracruz. These glyphosate resistant populations were less sensitive to glyphosate than the S population (**Figure 1**). R8 and R14 populations had similar response to glyphosate (**Figure 1A**). The resistant index (RI) of the glyphosate resistant populations ranged between 2.9 to 5.2 with respect to S population (**Table 2**).

Based on 50% mortality (LD_{50}), the R8, R14 and R15 populations were, respectively, 2.5, 2.3, and 4.6 times more resistant than the S population (**Figure 1B**). A field dose of glyphosate (720 g ae ha^{-1}), used in Persian lime groves of Veracruz, was enough to achieve full control in the S population. However, with this dose of glyphosate, only 50% mortality was observed in R8 and R14 populations, and to the R15 for population, a dose 1.8 times more glyphosate than the field dose was required to obtain the same level of control. The ED_{50} and LD_{50} parameters showed the different resistance levels to glyphosate acquired by resistant *L. virgata* populations (**Table 2**).

Shikimic Acid Accumulation

The amounts of shikimic acid accumulated after glyphosate treatment is highly variable between species and populations. In this work, the shikimic accumulation was different in each *L. virgata* population. The S population presented the highest level of shikimic acid accumulation (**Figure 2**). This accumulation was exhibited at lower glyphosate concentrations (between 0.1 to 100 μM) reaching an average of 12.35 mg shikimic g^{-1} fresh weight from 200 μM of glyphosate. The resistant populations only presented an appreciable accumulation from 100 μM of glyphosate. The averages of shikimic acid accumulated were 8.4, 7.9, and 2.6 mg shikimic g^{-1} fresh weight for the R8, R14, and R15 populations, respectively, at the glyphosate concentration of 1000 μM.

The response patterns to glyphosate in shikimate accumulation assays were similar to that observed in the dose-response study. The lowest shikimate accumulation observed in the R15 population compared to the R8 and R14 populations was consistent with lower growth reduction and mortality observed in the plants (**Figures 1** and **2**).

Absorption and Translocation

The four *L. virgata* populations presented a high absorption index of ^{14}C-glyphosate, absorbing amounts of over 50% from recovered herbicide at 96 HAT (**Figure 3**). At 24 HAT, the S population presented the highest ^{14}C-glyphosate absorption rate. Between 48 to 96 HAT, glyphosate absorption was similar

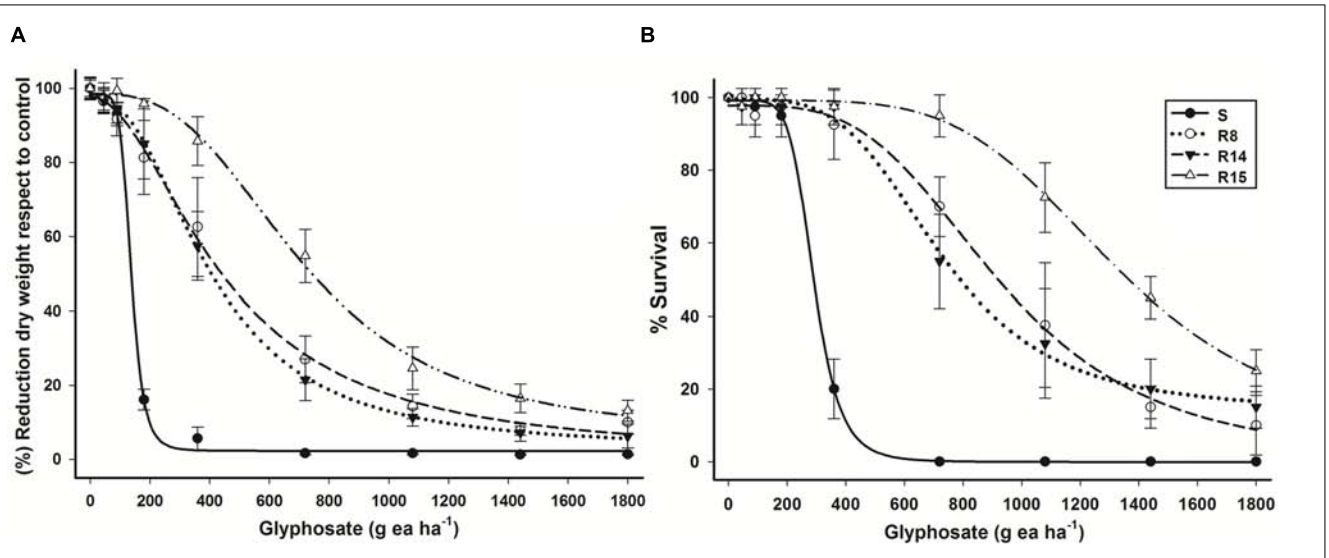

FIGURE 1 | Log–logistic curves of glyphosate-susceptible and -resistant *L. virgata* populations evaluated at 21 DAT. (A) Dose–response curve with respect to percentage of dry mass reduction. **(B)** Dose–response curve with respect to percentage of survival. Vertical bars represent the standard error of the mean ($n = 10$).

TABLE 2 | Parameters of the sigmoidal equation used to estimate ED_{50}, LD_{50} and I_{50} values of the glyphosate-susceptible and resistant *L. virgata* populations.

Population	c	d	b	R^2aj	Mean dose[a] (CI 95%)	RI[b]
Parameters of ED_{50} value (g ae ha^{-1})[c]						
S	2.28	98.34	6.62	0.99	137.7 (129.2, 146.1)	
R8	0.70	97.69	1.94	0.97	447.6 (403.5, 491.8)	3.3
R14	2.80	98.17	2.33	0.98	402.0 (371.2, 433.0)	2.9
R15	5.67	98.49	2.90	0.98	719.8 (668.2, 771.4)	5.2
Parameters of LD_{50} value (g ae ha^{-1})[c]						
S	−0.07	99.19	6.47	0.99	291.3 (263.7, 318.7)	
R8	0.10	97.80	3.52	0.95	932.3 (777.0, 1087.6)	3.2
R14	13.94	99.51	3.79	0.95	724.4 (624.9, 823.0)	2.5
R15	6.97	99.18	4.57	0.96	1330.3 (1084.2, 1576.4)	4.6
Parameters of I_{50} value (μM)[d]						
S	0.12	100.0	2.9	0.99	0.32 (0.04, 0.59)	
R8	0.10	100.0	11.0	0.99	0.67 (0.12, 1.22)	2.1
R14	0.11	100.0	13.0	0.99	0.50 (0.16, 0.84)	1.6
R15	1.64	100.2	1.2	0.99	53.09 (30.39, 75.78)	165.9

c = lower limit, d = upper limit, b = Hill's slope, R^2aj = 1 - (sums of squares of the regression/corrected total sums of squares). [a]*Effective mean dose required: ED_{50} = to reduce the dry biomass by 50%; LD_{50} = to kill 50% plants of a population; and I_{50} = to reduce the EPSPS enzyme activity by 50%.* [b]*RI = Resistance index (R/S) calculated using the corresponding ED_{50}, LD_{50} or I_{50} values of the resistant populations respect to the susceptible one.* [c]*CI values are the 95% confidence intervals (n = 10).* [d]*CI values are the 95% confidence intervals (n = 3).*

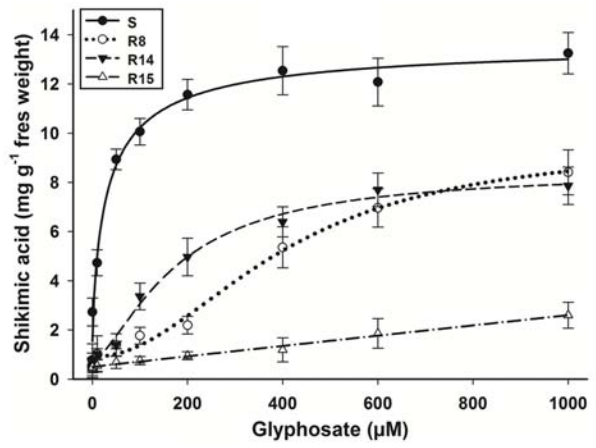

FIGURE 2 | Shikimic acid accumulation of glyphosate-susceptible and -resistant *L. virgata* populations at different glyphosate concentrations. Vertical bars represent the standard error of the mean ($n = 3$).

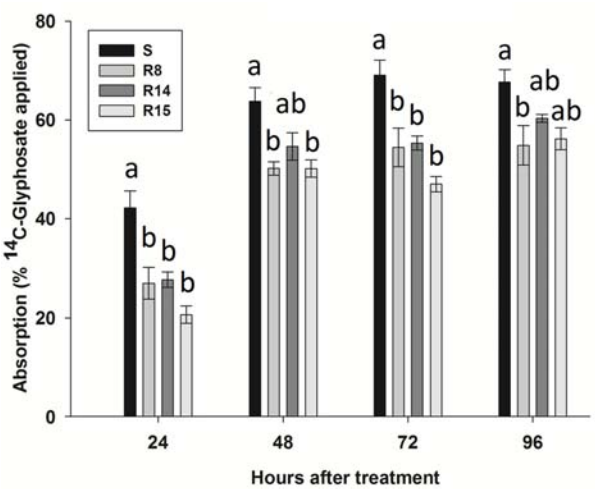

FIGURE 3 | ^{14}C-glyphosate absorption in glyphosate-susceptible and -resistant plants of the *L. virgata* populations. Different letter at each evaluation time is statistically different at 95% probability determined by the Tukey's test. Vertical bars represent the standard error of the mean (n = 5).

among populations, ranging from 47.7 to 67.7%. However, R15 population showed a clear reduced ^{14}C-glyphosate absorption in comparison to the S population (**Figure 3**).

L. virgata populations showed similar ^{14}C-glyphosate translocation patterns. In the aerial part of the plant (treated leaves and rest of plant). However, the greatest differences of ^{14}C-glyphosate translocation were found in the roots. At 96 HAT, R15 population had the lowest rate of translocation to the root (21.9% of the absorbed herbicide), translocating ±10% less herbicide than the S population. The R8 and R14 populations translocated an average of 24.5 and 26.5%, respectively, of herbicide translocated to the roots at 96 HAT (**Table 3**). At this time, the S population had already reached a balanced distribution of herbicide between treated leaf, rest of plant and roots. The Phosphor Imager images confirmed the previous results of ^{14}C-glyphosate translocation. At 96 HAT, the plants of resistant populations, mainly the R15 population ones, translocated smaller amounts of ^{14}C-glyphosate from treated leaf to the root than the S population plants (**Figure 4**).

Enzyme Activity

In the absence of glyphosate, significant differences ($P = 0.035$; $DF = 3$; $n = 12$) in the basal EPSPS activity between plants of *L. virgata* populations were found. R15 population presented an average of 0.41 μmol μg TSP^{-1} min^{-1}, while the S, R8, and R14 populations an average of 0.29 μmol μg TPS^{-1} min^{-1}. The EPSPS enzyme activity was inhibited by glyphosate in plants from susceptible and resistant populations while the concentrations increased. To inhibit EPSPS activity by 50% (I_{50}) for the S population was required 0.32 μM of glyphosate (**Table 2**). Plants from the R15 population showed an RI of 165.9 higher with respect to the S population plants (**Figure 5**). According to the confidence intervals (CI95%), R8, R14, and S population plants showed no significant differences in their EPSPS enzyme activity.

EPSPS Sequencing and Gene Expression

A fragment of 559 bp in length were amplified included the Thr-102 and Pro-106 positions in the protein sequence. The predicted amino acid sequence of S population presented as the same consensus of *Lolium rigidum* (GenBank: AF349754), and others grass glyphosate susceptible species. In the Thr-102 position no mutation was found. Only the R15 population showed a codon change from CCA to TCA resulting in an amino acid substitution from Proline to Serine at 106 position (**Figure 6**).

No significant differences ($P = 0.6924$; $DF = 3$; $n = 12$) were found in the EPSPS expression in untreated (0 HAT) plants used as a control between populations. The S and R15 populations showed an increased in the EPSPS expression level after glyphosate application at 24 HAT, but it was similar in both populations, with an average of 3.84 times more expression of the EPSPS (**Table 4**).

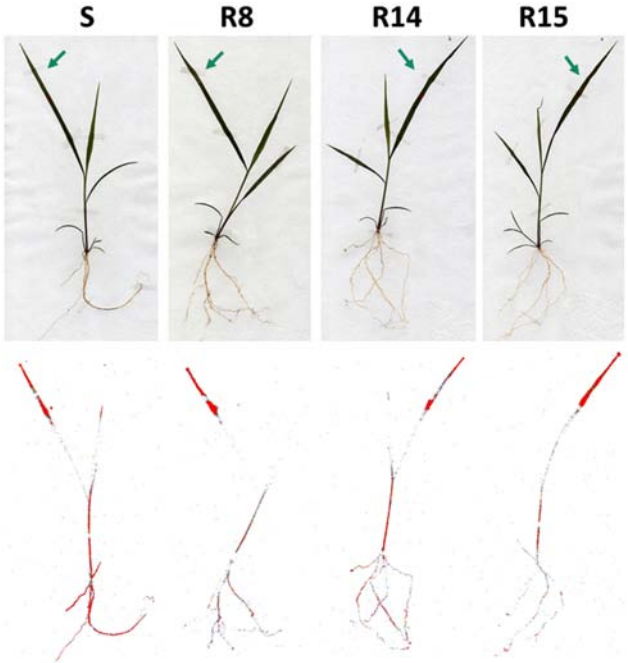

FIGURE 4 | Digital images (top row) and autoradiograph images (bottom row) of ^{14}C-glyphosate translocation in glyphosate-susceptible and -resistant plants of *L. virgata* populations. The autoradiograph images were obtained from treated plants at 96 HAT. The highest concentration of ^{14}C-glyphosate is highlighted in red. Arrows indicate the treated leaf.

TABLE 3 | Translocation percentage of ^{14}C-glyphosate in plants of glyphosate-susceptible and -resistant *L. virgata* populations.

Population	HAT	Translocation (% from absorbed)[a]		
		Treated leaf	Rest of plant	Root
S	24	75.1 ± 2.0 ab	16.0 ± 0.8 h	8.9 ± 2.6 j
	48	55.7 ± 2.6 c	24.2 ± 2.0 ef	20.2 ± 1.3 defg
	72	44.7 ± 1.0 de	30.3 ± 1.4 bcd	25.0 ± 1.1 bc
	96	33.9 ± 1.9 g	33.9 ± 1.0 ab	32.2 ± 1.2 a
R8	24	74.5 ± 2.1 ab	16.8 ± 1.5 gh	8.7 ± 1.2 j
	48	55.1 ± 1.9 c	27.9 ± 1.8 cde	17.0 ± 2.0 fgh
	72	45.3 ± 2.4 de	33.8 ± 2.6 ab	20.9 ± 1.5 cdef
	96	38.2 ± 1.1 fg	37.3 ± 3.8 a	24.5 ± 2.6 bcd
R14	24	70.3 ± 0.9 b	18.8 ± 1.1 fg	10.9 ± 0.4 ij
	48	56.4 ± 1.9 c	27.5 ± 1.0 cde	16.0 ± 2.0 gh
	72	43.5 ± 2.3 def	36.2 ± 1.2 a	20.3 ± 2.1 cdefg
	96	39.8 ± 2.8 ef	33.7 ± 3.5 ab	26.5 ± 1.0 b
R15	24	78.0 ± 2.2 a	12.3 ± 1.1 gh	9.6 ± 1.3 j
	48	58.7 ± 1.7 c	26.4 ± 1.7 de	14.9 ± 0.8 hi
	72	48.7 ± 2.2 d	32.3 ± 1.0 abc	19.0 ± 1.6 efgh
	96	43.2 ± 1.5 def	34.9 ± 1.5 ab	21.9 ± 2.2 cdef

[a]*Means with different letter within a column are statistically different at 95% probability determined by the Tukey's test. ±SEM (n = 5).*

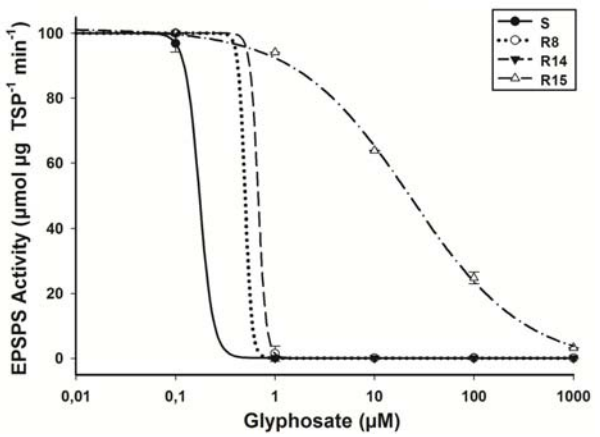

FIGURE 5 | EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from glyphosate-susceptible and resistant *L. virgata* populations. Vertical bars represent the standard error of the mean ($n = 3$).

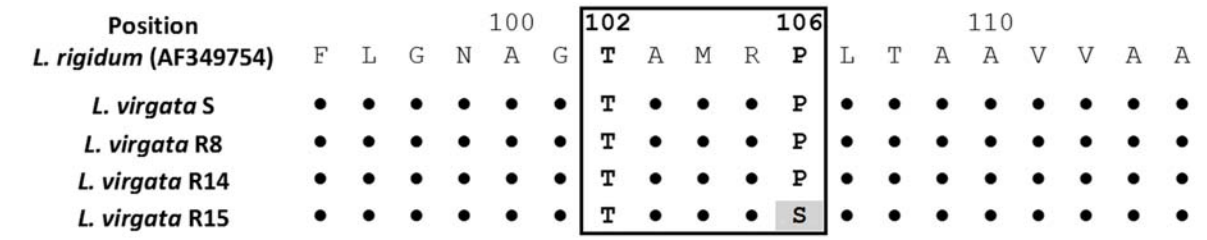

FIGURE 6 | Partial alignment of protein sequences of EPSPS gene in glyphosate-susceptible and -resistant *L. virgata* populations. The highlighted color indicates a change at 106 position from CCA (Proline) to TCA (Serine) in the consensus nucleotide sequence. Box includes from the 102 to 106 positions (amino acid number based on the start codon of *L. rigidum* [GenBank: AF349754] EPSPS sequence), corresponding to point mutations associated for conferring glyphosate resistance. The *L. virgata* EPSPS cDNA sequences information can be found in GenBank with accession numbers KX425854 and KX425855.

TABLE 4 | EPSPS expression level in treated and untreated plants of the glyphosate-susceptible and resistant *L. virgata* populations.

Population	Expression level[a] (EPSPS:NF[b])	EI[c]	EPSPS expression level (0 HAT:24 HAT)[d]	EI[c]
S	4.67 ± 0.50		4.02 ± 0.61	
R15	4.50 ± 0.53	0.96	3.67 ± 0.44	0.91
Mean	4.58 ± 0.49		3.84 ± 0.54	

[a]*Values ($\times 10^3$) obtained from untreated (0 HAT) plants.* [b]*NF = Normalization Factor.* [c]*Expression index EI = Expression level R/Expression level S.* [c]*The coefficients of expression were estimated as: Expression level at 24 HAT/Expression level at 0 HAT of each population. ±SEM (n = 6).*

DISCUSSION

The index resistance (IR) of resistant *L. virgata* populations ranged from 2.9 to 5.2 times more than the S population. Previously, *L. virgata* was effectively controlled with glyphosate in citrus orchards of Veracruz with 720 g ae ha^{-1} (field dose), until the identification of the first resistant populations (Pérez-López et al., 2014). The LD_{50} values estimated in the resistant populations studied in this work were higher than those at the field glyphosate dose. Other glyphosate-resistant grass weeds such as: *Bromus diandrus, Echinochloa colona, Eleusine indica, Lolium perenne* spp. *multiflorum, L. rigidum, Poa annua,* among others (González-Torralva et al., 2012; Alarcón-Reverte et al., 2015; Chen et al., 2015; Cross et al., 2015; Fernandez et al., 2015; Salas et al., 2015; Yu et al., 2015; Malone et al., 2016), exhibited RI values that ranged between 3 to 19, and between 4 to <182 based on in their ED_{50} or LD_{50}, respectively. Differences in the level of glyphosate resistance between these species were due to various resistance mechanisms.

Both shikimic acid accumulation and dose-response results indicated that the R8 and R14 populations of *L. virgata* possessed similar levels of glyphosate resistance, and the R15 population was the most resistant one. R8 and R14 populations had similar glyphosate application history (3–4 times per year for 8–10 years), despite coming from different geographical places. R15 population was subjected to 3–4 application per year for over 15 years (Pérez-López et al., 2014), in a citrus grove geographically close to the grove where R14 population was collected (Alcántara-de la Cruz et al., 2016c). It is evident that the selection pressure exerted by glyphosate in the R15 population caused low sensitivity to the herbicide.

Glyphosate absorption is a biphasic process. First the glyphosate should penetrate rapidly through the cuticle, and then is slowly absorbed through the phloem (Gomes et al., 2014). Reduced absorption is a mechanism not usually involved in the glyphosate resistance and has been described in a few species such as *D. insularis*, *L. multiflorum,* and *S. halepense* (Michitte et al., 2007; de Carvalho et al., 2012; Vila-Aiub et al., 2012). Yanniccari et al. (2012) suggested that there is an unidentified barrier that prevents glyphosate to load into the phloem. In the resistant *L. virgata* populations, the reduced glyphosate absorption played an important role, mainly in the first 24 HAT, due to a clear reduction compared to the S population. The absorption process time, depends on the treated species, age of the plant, herbicide concentration and environmental conditions (Gomes et al., 2014). Considering that the populations were studied in similar controlled conditions, it is clear that resistant *L. virgata* populations have limited the foliar absorption of glyphosate, mainly in the first hours after treatment, where the S population absorbs up to twice the herbicide that the most resistant population (R15). Thus, the tropical rainy conditions of the citrus region of Veracruz end up favoring resistant *L. virgata* populations, reducing the amount of glyphosate that could be absorbed by the treated plants and reach the target-site. Similar patterns of reduced absorption were observed in *B. pilosa*, where the S population absorbs at least twice more herbicide that the resistant populations in the first 24 HAT (Alcántara-de la Cruz et al., 2016b).

Different glyphosate concentrations in the tissue are related to differences in glyphosate efficacy (Alarcón-Reverte et al., 2015). Glyphosate will reach active metabolic sites, such as root and shoot meristems to act (Gomes et al., 2014). Greater movement of glyphosate to the meristematic tissues is crucial for plant mortality (Adu-Yeboah et al., 2014). However, reduced glyphosate translocation in resistant plants is mostly retained within the treated leaves (Yanniccari et al., 2012). Plants of the resistant *L. virgata* populations showed low glyphosate translocation to the meristematic tissues of the roots. The lowest one being exhibited by the R15 population. Reduced

glyphosate translocation is due to a trait (unknown barrier) that restricts glyphosate movement within resistant plants (Powles, 2010), or an altered subcellular distribution of glyphosate, keeping it away from the target-site (Kleinman and Rubin, 2016). The barrier may exist either in the phloem system or in the mesophyll cells, where glyphosate must enter to be translocated (Yanniccari et al., 2012). Glyphosate resistance by this mechanism confer by a single nuclear gene with complete- or semi-dominance (Shaner, 2009). R15 population was selected by glyphosate during a longer time period, this explains why it has the lowest translocation rate. However, the genetic determinants of NTSR continues to be poorly know (Déyle, 2013). Reduced translocation has been reported as being a mechanism responsible for endow glyphosate resistance in different grass weed species such as *D. insularis, L. multiflorum, L. perenne*, *L. rigidum, Sorghum halepense* (Bostamam et al., 2012; de Carvalho et al., 2012; González-Torralva et al., 2012; Vila-Aiub et al., 2012; Adu-Yeboah et al., 2014; Fernandez et al., 2015; Ghanizadeh et al., 2015), among others. In some cases, it was reported as a major resistance mechanism (Adu-Yeboah et al., 2014). Glyphosate resistant biotypes of *C. bonariensis* showed an altered subcellular distribution of glyphosate (Kleinman and Rubin, 2016). We suggest that *L. virgata* developed reduced absorption and translocation as resistance mechanisms against glyphosate first, independently of their geographic or genetic relationship.

The different shikimic acid accumulation, reduced absorption and translocation patterns presented by the R15 population, suggest that their glyphosate resistance mechanism may differ from that of the R8 and R14 populations. In addition, reduced glyphosate absorption by plants and higher EPSPS activity, have been associated with a decreased sensitivity to glyphosate (Salas et al., 2012).

On the other hand, EPSPS enzyme activity tests, in addition to any other appropriate parameter to determine glyphosate resistance (Dayan et al., 2015), also allows us to suspect the possible mechanisms that may be involved in the target-site. A greater basal activity is often associated with a larger number of copies or overexpression of the EPSPS gene (Salas et al., 2012). Also, a greater content EPSPS protein, allows that some molecules act as sponge to absorb glyphosate, while others molecules continue their essential function in the shikimic pathway (Powles, 2010). The R15 population presented the highest EPSPS content among the *L. virgata* populations. Gene amplification is a well-characterized phenomenon in plant evolution (Powles, 2010). However, the R15 population of *L. virgata* evolved under anthropogenic selection pressures, showing an enhanced EPSPS basal activity that allows to cope with the presence of glyphosate. The EPSPS of glyphosate-resistant plants could be equally sensitive to glyphosate (Powles, 2010). This happens when the EPSPS gene does have not mutations in the Thr-102 and Pro-106 positions yet. The R8 and R14 populations of *L. virgata*, showed no significant differences in their inhibition of the EPSPS enzyme by glyphosate with respect to the S population. Moreover, these populations showed no mutation in the EPSPS gene. This suggests that their resistance mechanism did not involve the target-site. Similar results were reported in resistant populations of *E. indica* (population R3) and *E. colona* (population RLB2) (Alarcón-Reverte et al., 2015; Chen et al., 2015), in which no important differences in their inhibition of the EPSPS activity with respect to their sensitive populations were reported.

Therefore, the scant inhibition of EPSPS enzyme activity by glyphosate in the R15 population, also presented a mutation found in the Pro-106 position of EPSPS gene, that provides of a higher resistance to glyphosate. Amino acid substitution at Pro-106 position in the EPSPS gene from Proline to Serine, Alanine, Threonine and/or Leucine have been widely reported to partially confer resistance to glyphosate, and often are accompanied by another mechanism (Sammons and Gaines, 2014). Some species which presented a mutation in combination with other resistance mechanisms (either reduced absorption, reduced translocation, multiple EPSPS gene copy number, overexpression of the EPSPS gene, and even metabolism) are: *A. palmeri. A. tuberculatus, B. diandrus, B. pilosa, D. insularis, E. colona, E. indica, L. perenne* sp. *multiflorum, L. rigidum, K. scoparia* (Bostamam et al., 2012; de Carvalho et al., 2012; González-Torralva et al., 2012; Alarcón-Reverte et al., 2015; Chatham et al., 2015; Chen et al., 2015; Fernandez et al., 2015; Salas et al., 2015; Wiersma et al., 2015; Alcántara-de la Cruz et al., 2016b; Malone et al., 2016).

Due to only the R15 population showed a greater basal activity, the EPSPS expression was studied in this resistant population in comparison to the S population. However, our qPCR analysis from cDNA (**Table 4**), indicated that the greater EPSPS basal activity of the R15 population, cannot be ascribed to an overexpression of the EPSPS gene. Alarcón-Reverte et al. (2015), suggested that it could have been an enhanced basal EPSPS activity as an additional TSR against glyphosate, possibly due to post-transcriptional regulation mechanisms increased mRNA stability and/or reduced enzyme degradation, as a consequence of the selection pressure exerted by glyphosate. A higher number of EPSPS gene copies correlate positively with higher EPSPS transcription (Gaines et al., 2013; Vila-Aiub et al., 2014). However, the manifestation of more EPSPS gene copies does not always result in differences in the amount of protein content (Salas et al., 2015). It is possible that the EPSPS copy number content of the R15 population could be greater than of the S population. However, we did not study this parameter among S and R15 populations, because no difference in the expression of the EPSPS gene was observed. Resistant populations of *A. palmeri, A. tuberculatus. B. diandrus, E. colona, E. indica, K. skoparia, L. perenne* spp. *multiflorum* (Salas et al., 2012; Gaines et al., 2013; Ribeiro et al., 2014; Alarcón-Reverte et al., 2015; Chatham et al., 2015; Chen et al., 2015; Wiersma et al., 2015; Malone et al., 2016), are examples of species which presented differences in the EPSPS copy numbers and/or overexpression of the EPSPS gene as glyphosate resistance mechanisms, in some cases as major ones. This results indicates that the R15 population presented NTSR and TRS mechanisms against glyphosate: reduced absorption, reduced translocation, enhanced EPSPS basal activity, and target-site mutation.

Glyphosate metabolism could contribute to natural tolerance. However, there is no evidence that metabolism plays a significant role in resistance to glyphosate. It could be developed due to an extreme selection pressure, but only when treated plants did not evolve glyphosate resistance via other mechanisms (Duke, 2011). We analyzed *L. virgata* aerial and roots tissue of plants randomly of the four populations treated with 360 ae ha^{-1} collected at 4 and 8 DAT, following the methodology used by de Carvalho et al. (2012). Resistant *L. virgata* plants analyzed did not show glyphosate metabolism as resistance mechanism.

According with the results of this work, the glyphosate resistance of *L. virgata* is a consequence of the selection pressure exerted by herbicide, and it did not present innate glyphosate tolerance. In addition, *L. virgata* was effectively controlled previously with glyphosate (Pérez-López et al., 2014; Alcántara-de la Cruz et al., 2016c). According to bibliography, innate tolerance to glyphosate not involve target site tolerance mechanisms within the same species, and the tolerant populations generally are compared with sensitive populations of other species (Yuan et al., 2002; Alcántara-de la Cruz et al., 2016a; Fernández-Moreno et al., 2016; Mao et al., 2016). To demonstrate the innate glyphosate tolerance in species such as: *Avena sterilis, Cologania broussonetii, Dicliptera chinensis, Liriope platyphylla, Liriope spicata, Ophiopogon japonicas*, among others, it has been necessary compare them with glyphosate sensitive species.

CONCLUSION

The reduced absorption and translocation are the main mechanisms of resistance of *L. virgata* to glyphosate. In addition, an enhanced EPSPS basal activity and a mutation in the glyphosate target-site endow a high level of resistance in this species. These results demonstrate that *L. virgata* evolves similar glyphosate resistance mechanisms like many other weeds, independently of agronomic system.

To prevent the resistance spread of *L. virgata* across citrus groves, the growers must properly clean agricultural implements. Given the current global scale of glyphosate use, current field trials are being carried out by this research group, to find chemical and non-chemical alternatives, which are economically and environmentally viable, to integrate *L. virgata* management where this weed is common.

AUTHOR CONTRIBUTIONS

RA-C, HC-H, FB, JD-V, and RDP: Idea and designed the experiments; RA-C, AR-D, and MG: Performed the research; RA-C, AR-D, and MG: Interpretation and analysis of results (of raw data); RA-C, AR-D, MG, HC-H, FB, JD-V, and RDP: Wrote and approved the manuscript.

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Glyphosate-Resistant *Parthenium hysterophorus* in the Caribbean Islands: Non Target Site Resistance and Target Site Resistance in Relation to Resistance Levels

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Glyphosate has been the most intensely herbicide used worldwide for decades, and continues to be a single tool for controlling weeds in woody crops. However, the adoption of this herbicide in a wide range of culture systems has led to the emergence of resistant weeds. Glyphosate has been widely used primarily on citrus in the Caribbean area, but a study of resistance in the Caribbean islands of Cuba and the Dominican Republic has never been carried out. Unfortunately, *Parthenium hysterophorus* has developed glyphosate-resistance in both islands, independently. The resistance level and mechanisms of different *P. hysterophorus* accessions (three collected in Cuba (Cu-R) and four collected in the Dominican Republic (Do-R) have been studied under greenhouse and laboratory conditions. In *in vivo* assays (glyphosate dose causing 50% reduction in above-ground vegetative biomass and survival), the resistance factor levels showed susceptible accessions (Cu-S $\geq$ Do-S), low-resistance accessions (Cu-R3 $<$ Do-R4), medium-resistance accessions (Do-R3 $<$ Cu-R2 $<$ Do-R2) and high-resistance accessions (Do-R1 $<$ Cu-R1). In addition, the resistance factor levels were similar to those found in the shikimic acid accumulation at 1000 μM of glyphosate (Cu-R1 $\geq$ Do-R1 $>$ Do-R2 $>$ Cu-R2 $>$ Do-R3 $>$ Do-R4 $>$ Cu-R3 $>>$ Cu-S $\geq$ Do-S). Glyphosate was degraded to aminomethylphosphonic acid, glyoxylate and sarcosine by $>$88% in resistant accessions except in Cu-R3 and Do-R4 resistant accessions (51.12 and 44.21, respectively), whereas a little glyphosate ($<$9.32%) was degraded in both susceptible accessions at 96 h after treatment. There were significant differences between *P. hysterophorus* accessions in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity enzyme with and without different glyphosate rates. The R accessions showed values of between 0.026 and 0.21 μmol μg^{-1} TSP protein min^{-1} basal EPSPS activity values with respect to the S (0.024 and 0.025) accessions. The same trend was found in the EPSPS enzyme activity treated with glyphosate, where a higher enzyme activity inhibition (glyphosate μM) corresponded to greater resistance levels in *P. hysterophorus* accessions. One amino acid substitution was found at position

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106 in EPSPS, consisting of a proline to serine change in Cu-R1, Do-R1 Do-R2. The above-mentioned results indicate that high resistance values are determined by the number of defense mechanisms (target-site and non-target-site resistance) possessed by the different *P. hysterophorus* accessions, concurrently.

Keywords: *P. hysterophorus*, target-site and non-target-site mechanisms, resistance levels, glyphosate

INTRODUCTION

Herbicide resistance is an evolutionary phenomenon that allows resistant weed biotypes to be exposed to the normal dose of a herbicide undergoing any suffering growth alterations (Fernández et al., 2016). This biological phenomenon is favored by intensive herbicide applications with the same active ingredient or with the same mode of action (Neve et al., 2014; Evans et al., 2016). Glyphosate weed resistance is one of the world's most interesting cases, 35 glyphosate-resistant species have been detected and characterized (mainly using test dose response curves and shikimic acid accumulation) up to date (Heap, 2016).

Glyphosate ((N-phosphonomethyl)-glycine) is a post-emergent herbicide that is non-selective, highly systemic and widely used for weed control around the world (Franz et al., 1997; Székács and Darvas, 2012). It is well metabolized in plants and slow-acting with visible phytotoxic symptoms in sensitive plants at 10–20 days after application (Amrhein et al., 1980; Shingh and Shaner, 1998; Monquero et al., 2004). It inhibits the shikimate pathway by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the synthesis reactions of aromatic amino acids involved in the formation of essential proteins in plants (Sammons and Gaines, 2014).

Glyphosate resistance selection is due to two different mechanisms known as non-target site resistance (NTSR) and target site resistance (TSR) (Shaner et al., 2012; Sammons and Gaines, 2014). NTSR involves a reduced rate of herbicide in the meristem tissues due to limited absorption/translocation, and/or sequestration of the herbicide into compartments such as vacuoles (Michitte et al., 2007; Ge et al., 2012; Vila-Aiub et al., 2012). Metabolic pathways capable of degrading the herbicide to non-toxic compounds in plants also belong to these group mechanisms (De Prado and Franco, 2004; Cruz-Hipólito et al., 2009, 2011; Busi et al., 2011; de Carvalho et al., 2012; González-Torralva et al., 2012; Alcántara-de la Cruz et al., 2016a). TSR has been produced by one or more mutations in the DNA sequence (González-Torralva et al., 2014; Sammons and Gaines, 2014; Fernández et al., 2015; Yu et al., 2015), or by the overexpression of the EPSPS protein by gene amplification (Gaines et al., 2010; Salas et al., 2012, 2015).

When growers reported noticing any deficiency in their weed control, they usually increased the glyphosate doses, which increased the pressure selection as well as triggering the acquisition of a second resistance mechanism (Jasieniuk et al., 1996; González-Torralva et al., 2012). Then, the level of weed resistance to glyphosate increased (Bostamam et al., 2012).

Ragweed parthenium (*Parthenium hysterophorus* L.) is a troublesome annual weed of the *Asteraceae* family that is native to the Gulf of Mexico and other Latin American countries (Rosario et al., 2013). Its prolific seed production (130,000–200,000 seeds m^{-2}), as well as the seeds's ability to persist in the soil and germinate over a wide range of temperatures, have contributed to the widespread distribution of ragweed parthenium in perennial and annual crops (orchards, citrus, soybean, corn) as well as in surrounding areas (Joshi, 1991; Pandey et al., 2003; Navie et al., 2004; Adkins and Shabbir, 2013). In addition, the subtropical environment of the Caribbean Islands (Cuba and Dominican Republic) allows year-round germination, growth, and reproduction of ragweed parthenium, which also contributes to its widespread distribution in the region. Glyphosate has been used repeatedly in perennial crop areas and fallow fields in the Caribbean Islands for many years to manage ragweed parthenium and other troublesome weeds. However, growers have recently observed reduced ragweed parthenium control with single or multiple glyphosate applications. Previous reports have documented glyphosate-resistant ragweed parthenium in Colombia (Rosario et al., 2013), Florida (southeast US) (Fernandez, 2013) and Dominican Republic (Jimenez et al., 2014), but in these three cases the causes of resistance to glyphosate have been inconclusive.

The main objective of this work is a survey of *P. hysterophorus* in Cuba and the Dominican Republic that had never been done before. The specific objectives were to determine (1) the level of glyphosate resistance of different accessions; (2) the possible NTSR and TSR mechanisms involved; and (3) to find out if the resistance genes may also increase the multiplicative or additive resistance levels in *P. hysterophorus*.

MATERIALS AND METHODS

Plant Material

In 2013, mature *P. hysterophorus* seeds were collected from plants not controlled with glyphosate at doses normally used (2 L ha^{-1}; 720 g ae ha^{-1}) in areas with perennial crops in two Caribbean Islands. Seeds from Cu-S and Do-S accessions never exposed to glyphosate were collected from adjacent areas and used as a reference control (**Table 1**). Seeds collected from 25 mature plants were stored under laboratory conditions (25°C) for 2 weeks and then placed in paper bags at 4°C. Approximately 300 seeds of these accessions were sown directly into trays (40 × 60 × 15 cm), containing a mixture of sand and peat (2:1, v/v) and placed in a greenhouse at 28/20°C day/night under a 16 h photoperiod with 850 μmol m^{-2} s^{-1} photon flux density, and 80% relative humidity. At the four leaf stage plants of all accessions were treated with glyphosate at 720 g ae ha^{-1} using a laboratory spray chamber equipped with a flat fan nozzle (TeeJet 8002 EVS) with a total output volume of 200 L ha^{-1} water at

TABLE 1 | History of different *P. hysterophorus* accessions used in this study.

Accessions[a]	Location	Crop	Glyphosate[b] (time of applications per year), number of application years
Cu-R1	Ceiba	Orchards[c]	720 (2 or 3 times), >10
Cu-R2	Ceiba	Citrus[c]	720 (1 time), >10
Cu-R3	Arimao	Citrus	720 (2 times), unknown
Cu-S	Arimao	Road trails	No herbicide treatment
Do-R1	Villa Altagracia	Citrus[c]	900 (2 times), >15
Do-R2	San Cristobal	Citrus	900 (2 times), >15
Do-R3	Monseñor Nouel	Citrus	720 (2 times), >10
Do-R4	Maria T. Sánchez	Orchards	720 (1 time), >10
Do-S	Maria T. Sanchez	Road trails	No herbicide treatment

[a]*Cu, P. hysterophorus harvested in Cuba; Do, P. hysterophorus harvested in Dominican Republic;* [b]*glyphosate g ae ha*$^{-1}$*;* [c]*the last application was performed manually for every plant.*

a pressure of 200 kPa. Four weeks after glyphosate treatment plant survival of the resistant accessions was estimated, and seed produced from surviving plants was collected and stored in paper bags for all subsequent trials. In the case of susceptible accessions (Cu-S and Do-S), no plant survival was observed 4 weeks after glyphosate treatment.

Dose-Response Assay

Seeds of putative resistant (Cu-R1, Cu-R2, Cu-R3, Do-R1, Do-R2, Do-R3, and Do-R4) and susceptible (Cu-S and Do-S) of the *P. hysterophorus* accessions were germinated in trays (12 × 12 × 6 cm) containing the same substrate as described before and placed in a growth chamber of similar environmental conditions controlled as before. One week after germination, individual seedlings were transplanted into pots (6 × 6 × 8 cm) and grown under fluctuating 30/20°C day/night with a 14 h photoperiod and 850 μmol m^{-2} s^{-1} photon flux density, and 80% relative humidity. As glyphosate (EPSPS inhibitor) is used in early post-emergence, at the four leaf stage, resistant and susceptible *P. hysterophorus* seedlings were treated with increasing glyphosate doses: 0, 31.25, 62.5, 125, 250, 500, 1000, 2000, 4000, and 8000 g ae ha^{-1} (Roundup Energy 45% w/v, SL, Monsanto Spain). The experiment were conducted with 10 replications (one plant pot^{-1}) of each accession per herbicide dose, and the experiments were repeated twice. Thirty days after herbicide treatment, herbicide effects on plant survival (LD) and above-ground vegetative biomass (GR) were assessed.

Leaf Segment Shikimate Accumulation Assay

Leaf segments (50 mm diameter) were harvested from the youngest fully expanded leaf from a batch of 15 plants per *P. hysterophorus* accessions at the 4–6 leaf stage (Hanson et al., 2009). Approximately 50 mg of fresh tissue was transferred to 2 mL Eppendorf tubes containing 1 mL of 1 mM $NH_4H_2PO_4$ (pH 4.4). Glyphosate was added to the tubes at the following concentrations: 0, 0.1, 0.5, 1, 5, 10, 50, 100, 200, 400, 500, 600, and 1000 μM. The Eppendorf tubes were incubated in a growth chamber during 24 h under the previously described conditions. After 24 h, the tubes were stored at −20°C until analysis. Eppendorf tubes were removed from the freezer and thawed at 60°C for 30 min. Two hundred and fifty micro liters of 1.25 N HCL was added to each tube, and placed at 60°C for 15 min. A 125 μL aliquot from each tube was pipetted into a new 2 mL Eppendorf tube, and 500 μL of periodic acid and sodium metaperiodate (0.25% [wt/v] each) was added. They were incubated at room temperature for 90 min, after which 500 μL of 0.6 N sodium hydroxide and 0.22 M sodium sulfite was added. The contents of all tubes were transferred to glass vials. Samples were measured in a spectrophotometer at 380 nm within 30 min. For each glyphosate concentration and accession, three replications were stablished and repeated twice.

^{14}C Glyphosate Absorption and Translocation

Absorption and translocation study was carried out following the methodology proposed by Cruz-Hipólito et al. (2011) The ^{14}C-glyphosate was mixed with commercially formulated glyphosate to prepare a solution with a specific activity of 0.834 kBq μL^{-1} and a glyphosate concentration of 1.8 g ae L^{-1} (360 g ae ha^{-1} in 200 L). *P. hysterophorus* plants at 4-leaf stage were treated with the radiolabeled herbicide by applying one droplet of 1 μL of glyphosate solution (0.834 kBq μL^{-1}) on the adaxial surface of the second leaf in each plant using a micropipette (LabMate). The ^{14}C-glyphosate unabsorbed in the treated leaf was removed with 3 mL of water: acetone solution (1:1, v/v) 96 h after droplet application. Preliminary assays with two accessions (Cu-R1 and Cu-S) studied had revealed that the glyphosate absorption leveled-off at 96 h after the droplet applications. The rinsate was mixed with 2 mL of scintillation liquid and analyzed by liquid scintillation spectrometry (LSS) (Scintillation Counter, Beckman LS 6500, Fullerton CA). The plants were separated into the treated leaf, rest of the shoot and root after being placed in cellulose cones. The plant tissue was dried at 60°C over 96 h and combusted in a biological sample oxidizer (Packard Tri Carb 307, Perkin-Elmer, Waltham, MA). The $^{14}CO_2$ evolved was trapped and counted in 18 mL of a mixture of Carbo-Sarb E and Permafluor (9:9, v/v) (Perkin-Elmer). Thus, over 95% of the total radioactivity applied was recovered. There were five replications and the experiment was arranged in a completely randomized design, and repeated twice. The proportion of absorbed herbicide was expressed as:

$$[\%\ \text{absorbed} = (\text{kBq in combusted tissue}/(\text{kBq in combusted tissue} + \text{kBq in leaf washes})) \times 100].$$

Glyphosate Metabolism

P. hysterophorus plants were treated with a glyphosate rate of 360 g ae ha^{-1} at 4–6 leaf stage. At 96 h after treatment (HAT), glyphosate and its metabolites, i.e., AMPA (aminomethylphosphonic acid), glyoxylate and sarcosine, were determined by reversed-polarity capillary electrophoresis following the methodology described by Rojano-Delgado

et al. (2010). The calibration equations were established using non-treated plants and known concentrations of glyphosate and its metabolites, which were determined from their enclosed areas under the peaks in the electropherogram. The average value for the amount of glyoxylate naturally produced by the plant was subtracted from the average of the produced or reduced amount after treatment of each accession (Rojano-Delgado et al., 2010). The experiment was arranged in a completely randomized design with four replications per accession and repeated three times.

EPSPS Enzyme Activity Assays

The enzyme extraction was conducted according to the protocol described by Dayan et al. (2015). Five gram of the leaf tissue of all *P. hysterophorus* accessions **(Table 1)** were ground to fine powder in a chilled mortar. Immediately after that, the powdered tissue was transferred to tubes containing 100 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mMKCl and 0.5 mM benzamidine) containing 70 μL of β-mercaptoethanol and 1% in polyvinylpolypyrrolidone (PVPP). Samples were stirred and subsequently centrifuged for 40 min (18,000 g) at 4°C. The supernatant was decanted into a beaker using a cheesecloth. $(NH_4)_2SO_4$ was added to the solution to obtain 45% (w/v) concentration, with stirring during 30 min. After that, the mix was centrifuged at 20,000 g for 30 min at 4°C. The previous step was repeated to precipitate the protein in the extracts but in that case with a $(NH_4)_2SO_4$ concentration of 80% (w/v) stirring for 30 min. Finally, they were centrifuged at 20,000 × g for 30 min at 4°C.

All the pellets were dissolved in 3 mL of extraction buffer and dialyzed in 2 L of dialysis buffer (30 mm, 1000-MWC dialysis tubing at 4°C on a stir plate) over 12 h. The protein concentrations were determined by Bradford assay (Bradford, 1976).

The assay for the determination of EPSPS activity followed the methodology described by Dayan et al. (2015) using the EnzCheck phosphate assay Kit (Invitrogen, Carlsbad, CA) to determine the inorganic phosphate release. The EPSPS activity from the nine accessions was determined in the presence and absence of glyphosate. The glyphosate concentrations used were: 0, 0.1, 1, 10, 100, and 1000 μM to determine the enzyme activity inhibition (I_{50}). The assay buffer was composed of 1 mM $MgCl_2$, 10% glycerol, and 100 mM MOPS, 2 mM sodiummolybdate and 200 mM NaF. The experiments were conducted with three replications of each accession per glyphosate concentration and repeated three times. EPSPS enzyme activity was expressed as percentage of enzyme activity in presence of glyphosate respect to the control (without glyphosate).

EPSP Synthase Gene Sequencing

For RNA extraction 100–200 mg of young leaves were taken from plants of each *P. hysterophorus* accession, and stored at −80°C for the extraction of RNA. Their tissue was ground in liquid nitrogen in a STAR-BEATER 412–0167 mill (VWR International Eurolab S.L., Barcelona, Spain). Total RNA was isolated from leaves as described by Pistón (2013), and the amount and quality were determined in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Walthman, MA, USA). The synthesis to cDNA was from total RNA being adjusted to the same concentration in all the samples (50 ng μL^{-1}). An iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. CA, USA) at 40 μL reaction volume was used following the manufacturer's instructions.

The PCR reactions were carried out with cDNA samples from each of the accession using the primers *Bidens*-F10 (5′- GGTTGTGGYGGTVTRTTTCC-3′) and *Bidens*-R11 (5′-GTCCCAASTATCACTRTGTTC-3′) based on EPSPS gene sequences described previously (Alcántara-de la Cruz et al., 2016b). PCR conditions were also as described (Alcántara-de la Cruz et al., 2016b). The PCR on cDNA amplified fragments of 462 bp in length, comprising the region of Thr-102 and Pro-106, which corresponds to the sequence of the EPSPS gene of *Arabidopsis* Klee et al. (1987), in which point mutations conferring resistance to glyphosate have been associated (Sammons and Gaines, 2014; Yu et al., 2015).

The PCR fragments were cloned in the pGEM®-T Easy Vector System (Promega Biotech Ibérica, SL, Madrid, Spain) and transformed into competent cells of *E. coli* DH5α (Promega). Transformation was confirmed through PCR using the M13F and M13R primers as described (Alcántara-de la Cruz et al., 2016b). The colonies containing the length of the fragment were sequenced by the STABVIDA sequencing service (Caparica, Portugal). Five biological samples were used per accession providing 15 clones in all for each one. The quality and assembly of cDNA sequences and consensuses were determined employing the programs of SeqMan Pro™ versión 11(DNASTAR; Wisconsin, USA) and Geneious® versión 8.1.8 (Biomatters Ltd, Auckland, New Zealand). The multiple sequences were aligned by means of the Muscle algorithm incorporated into SeqMan Pro versión 11.

Data Analysis

Dose-Response and EPSPS enzyme activity data were subjected to non-linear regression analysis (Seefeldt et al., 1995; Burgos et al., 2013) using a three-parameter log-logistic equation (Equation 1) to determine the glyphosate dose causing 50% reduction in above-ground vegetative biomass (GR_{50}), 50% mortality (LD_{50}), and inhibition of EPSPS activity by 50% (I_{50}).

$$Y = \{[(d) / (1 + (x/g)^b)]\} \quad (1)$$

Where Y is the EPSPS activity, survival or above-ground biomass at herbicide x dose, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve, and g is the herbicide rate at the point of inflection halfway (i.e., LD_{50}, GR_{50}, I_{50}).

Regression analyses were conducted using the *drc* package (Ritz et al., 2015) for the statistical environment R (R 3.2.4; R Core Team, 2015). Resistance indices were computed as R-to-S GR_{50} LD_{50}, or I_{50} ratios. To test for a common GR_{50}, LD_{50}, or I_{50} for R and S accessions, i.e., Resistance Index equals to 1, a lack-of-fit test was used to compare the model consisting of curves with accessions-specific g values with a reduced model with common g (Ritz et al., 2015).

Analysis of variance (ANOVA) was conducted using Statistix 9.0 (Analytical Software, USA) to test for differences between

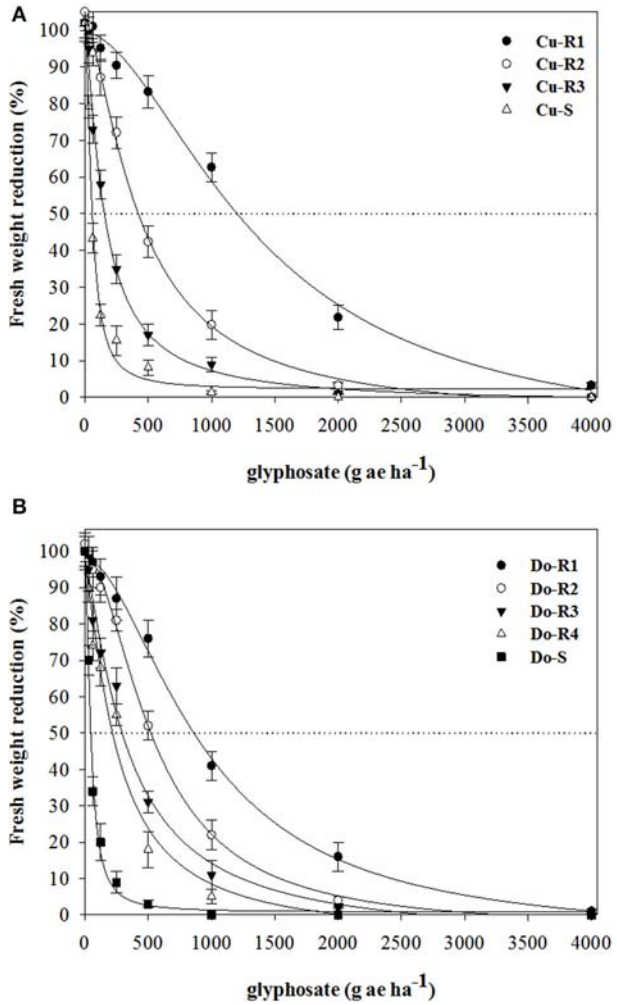

FIGURE 1 | Shoot biomass in glyphosate-resistant and susceptible *P. hystherophorus* accessions from Cuba (A) and Dominican Republic **(B)** 30 days after treatment. Symbols denoted mean ($n = 10$) ± standard errors of the mean.

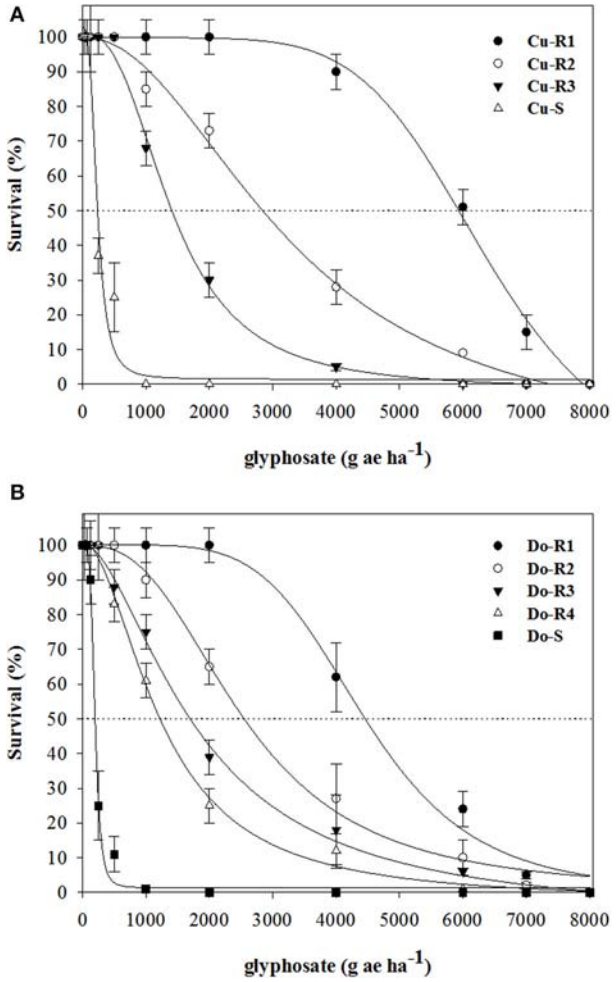

FIGURE 2 | Survival plants in glyphosate-resistant and susceptible *P. hystherophorus* accessions from Cuba (A) and Dominican Republic **(B)** 30 days after treatment. Symbols denoted mean ($n = 10$) ± standard errors of the mean.

R and S accessions in shikimate accumulation at 1000 μM glyphosate in the leaf segment; and proportion of the different glyphosate metabolites; proportion of applied ^{14}C-glyphosate taken up by leaves, and proportions of absorbed ^{14}C-glyphosate remaining in the treated leaf, translocated to roots and to the rest of the plant at 96 HAT; and basal enzyme activity. Percentage data were previously transformed (arcsine of the square root) to meet model assumptions. Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected. When needed, differences between means were separated using the Tukey HSD test.

RESULTS

Physiological Studies

Dose-response assays showed the existence of the first case of glyphosate-resistant weeds in the Caribbean (Cuba and Dominican Republic). The two susceptible weeds (Cu-S and Do-S) had similar susceptibility levels (**Figures 1**, **2**; **Table 2**). The *P. hyterophorus* accessions from Cuba island had resistance index (RI) values (based on the GR_{50} and LD_{50} values) that ranged from 2.7 to 24.6, and 6.1 to 27.5 fold resistance, respectively, while on Dominican Republic island values were between 5.4 to 20, and 6.3 to 22.7 fold resistance, respectively (**Table 2**).

The fact that plants treated with glyphosate increase shikimic acid accumulation in leaf disks due to the inhibition of EPSPS activity led us to carry out the experiment depicted in **Figures 3A,B**. Considering the values obtained *in vivo* (GR_{50} and LD_{50}) and the shikimic acid accumulation in leaf disks at 1000 μM of glyphosate, the resistance order of the *P. hystherophorus* accessions was Cu-R1 ≥ Do-R1 > Do-R2 > Cu-R2 > Do-R3 > Do-R4 > Cu-R3 >> Cu-S ≥ Do-S. There were significant differences at 1000 μM glyphosate between R and S accessions of Cuba ($p = 0.0013$, $DF = 3$, $n = 12$) and Dominican Republic ($p = 0.0008$, $DF = 4$, $n = 15$).

TABLE 2 | Parameters of the log-logistic equations used to calculate the glyphosate rates required for 50% survival (LD_{50}) and reduction fresh weight (GR_{50}) of the different accessions of *P. hyterophorus* from Cuba and Dominican Republic.

Accessions	Survival[a] (%)						Fresh weight reduction[b] (%)					
	d	*b*	R^2	LD_{50} (g ae ha^{-1})	RI	*p*	*d*	*b*	R^2	GR_{50} (g ae ha^{-1})	RI	*p*
Cu-R1	99.8	6.1	0.98	6364 ± 122	27.5	<0.0001	99.4	1.8	0.99	1370 ± 191	24.5	<0.0001
Cu-R2	98.9	2.9	0.99	2794 ± 90	12.0	<0.0001	103.0	1.5	0.95	437 ± 28	7.8	<0.0001
Cu-R3	100.9	2.6	0.99	1415 ± 55	6.1	<0.0001	103.3	1.3	0.96	151 ± 13	2.7	0.003
Cu-S	102.7	3.1	0.97	232 ± 23	–	–	103.2	1.7	0.98	56 ± 6	–	–
Do-R1	100.1	5.1	0.96	4456 ± 76	22.7	<0.0001	98.2	1.8	0.98	939 ± 25	20.0	<0.0001
Do-R2	99.9	2.7	0.98	2550 ± 92	13.0	<0.0001	99.6	1.8	0.99	547 ± 30	11.6	<0.0001
Do-R3	100.7	1.7	0.99	1821 ± 63	9.3	<0.0001	97.9	1.3	0.99	339 ± 27	7.2	<0.0001
Do-R4	100.9	1.9	0.99	1242 ± 65	6.3	<0.0001	96.4	1.3	0.96	255 ± 33	5.4	<0.0001
Do-S	100.5	4.5	0.97	196 ± 8	–	–	100.6	1.7	0.98	47 ± 4	–	–

[a] *For $Y = \{(d) / [1 + (x/ LD_{50}) \exp b]\}$ Where Y is the survival expressed as a percentage of the untreated control, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve in LD_{50}, LD_{50} is the herbicide rate at the point of inflection halfway, and x is the herbicide dose.*

[b] *For $Y = (d) / [1 + (x/ GR_{50}) \exp b]$ Where Y is the above-ground weight expressed as a percentage of the untreated control, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve in GR_{50}, GR_{50} is the herbicide rate at the point of inflection halfway, and x is the herbicide dose.*

TABLE 3 | ^{14}C-glyphosate absorption (% of recovered radioactivity) and translocation (% of absorbed radioactivity) in the different *P. hysterophorus* accessions at 96 h after treatment (HAT).

Accessions	Absorption[a] ($p = 0.0001, DF = 8, n = 45$)	Translocation		
		Treated leaf ($p = 0.0003, DF = 8, n = 45$)	Rest of shoot ($p = 0.0001, DF = 8, n = 45$)	Root ($p = 0.0004, DF = 8, n = 45$)
Cu-R1	59.3 ± 4.9 BC	77.9 ± 5.7 AB	12.1 ± 2.1 BCD	10.0 ± 2.3 BC
Cu-R2	60.2 ± 2.1 BC	82.4 ± 4.2 A	9.3 ± 1.9 D	8.3 ± 3.4 BCD
Cu-R3	56.8 ± 3.9 C	80.1 ± 3.9 AB	15.7 ± 3.4 B	4.2 ± 1.2 D
Cu-S	82.2 ± 6.7 A	35.5 ± 2.3 C	41.6 ± 6.2 A	22.9 ± 4.8 A
Do-R1	63.1 ± 6.8 B	78.3 ± 6.7 AB	10.5 ± 2.7 CD	11.2 ± 2.1 B
Do-R2	55.9 ± 7.8 C	79.3 ± 3.4 AB	16.2 ± 4.9 B	4.5 ± 1.4 D
Do-R3	60.4 ± 3.7 BC	75.6 ± 5.1 B	14.1 ± 3.8 BC	10.3 ± 3.8 B
Do-R4	58.4 ± 2.3 BC	81.4 ± 6.3 A	12.7 ± 4.3 BCD	5.9 ± 2.7 CD
Do-S	78.8 ± 5.6 A	39.1 ± 1.9 C	37.8 ± 2.3 A	23.1 ± 5.6 A

[a] *Over 95% of the total radioactivity applied was recovered.*

Mean value ($n = 5$) ± standard error. Means on a same column followed by the same letter were not significantly different at $\alpha = 0.05$.

There were marked differences in glyphosate absorption between the resistant and susceptible glyphosate *P. hysterophorus* accessions at 96 h after treatment (HAT) ($p = 0.0001$, $DF = 8$, $n = 45$) (**Table 3**). All accessions obtain maximum absorption at 96 HAT, and the two susceptible accessions absorbed an average of 80.5%, while the resistance accessions absorbed an average of 59.2% of ^{14}C-glyphosate which was recovered.

Translocation assays suggest marked differences at 96 HAT between the Cu-S and Do-S accessions compared to the Cu-R1, Cu-R2, Cu-R3, Do-R1, Do-R2, Do-R3, and Do-R4 ones in treated leaf ($p = 0.0003$, $DF = 8$, $n = 45$), rest of the shoots ($p = 0.0001$, $DF = 8$, $n = 45$), and root ($p = 0.0004$, $DF = 8$, $n = 45$) (**Table 3**). There were no significant differences in translocation between the two susceptible accessions (Cu-S and Do-S) from Caribbean Islands. But there were small significant differences in the resistant accessions (Cu-R1, Cu-R2, Cu-R3, Do-R1, Do-R2, Do-R3, and Do-R4). Nonetheless, the high amount of ^{14}C-glyphosate in each resistant accession remained in the treated leaf. Due to differences in levels of glyphosate resistance between the *P. hysterophous* resistant accessions, we suspect that other mechanisms could be involved (**Tables 2**, **3**, **Figure 3**).

Biochemical Studies

Previous tests demonstrated that the highest glyphosate translocation and metabolism was reached at 96 HAT in the *P. hysterophorus* accessions (unpublished data). There were significant differences at 96 HAT in glyphosate metabolism levels between accessions ($p = 0.0014$, $DF = 8$, $n = 36$). Glyphosate levels decreased, whereas glyphosate metabolites (AMPA, glyoxylate and sarcosine) increased at 96 HAT in the Cu-R1, Do-R1, Do-R2, Cu-R2, and Do-R3 accessions. Higher glyphosate levels remained in the Cu-R3 and Do-R4 (low resistance), and

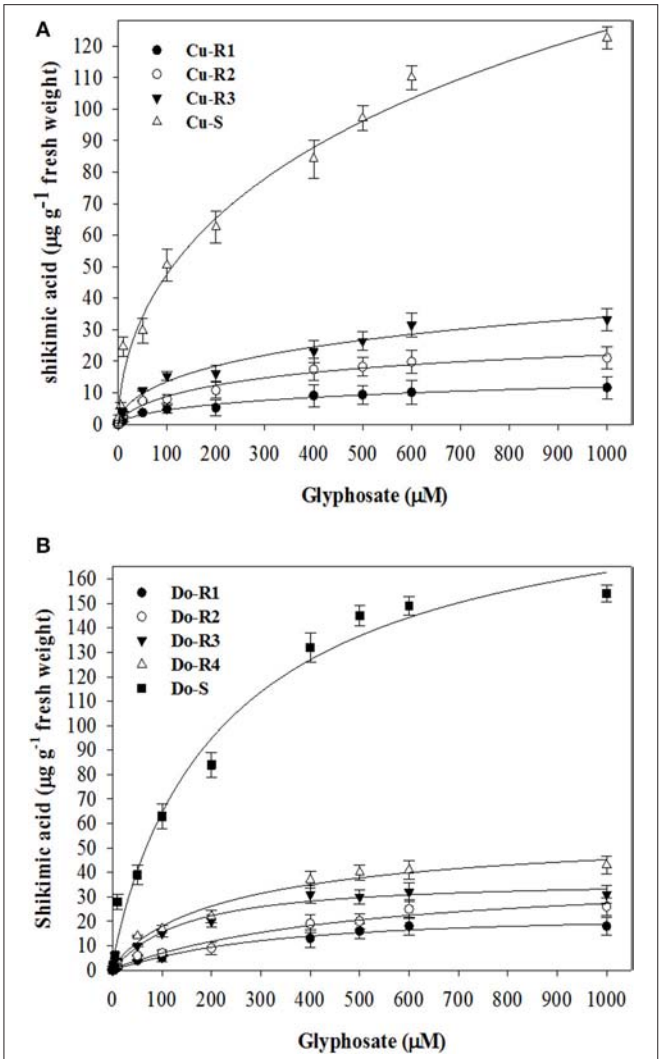

FIGURE 3 | Shikimic acid accumulation in leaf segments of plants from Cuba (A) and Dominican Republic **(B)** accessions of *P. hysterophorus*. Symbols denoted mean ($n = 3$) ± standard errors of the mean.

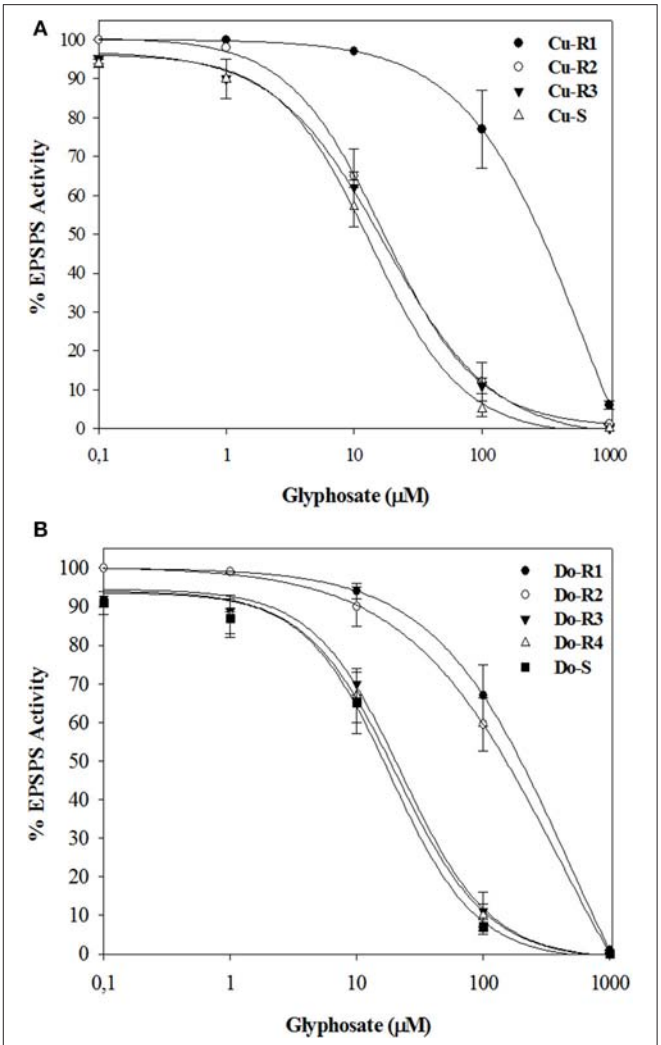

FIGURE 4 | EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from Cuba (A) and Dominican Republic **(B)** accessions of *P. hysterophorus*. Symbols denoted mean ($n = 3$) ± standard errors of the mean.

very high one in the Cu-S and Do-S (susceptible) accessions. In these last accessions, sarcosine was not detected (**Table 4**). These results can also explain the low level of resistance of the accession (Cu-R3 and Do-R4) with a single resistance mechanism, while the other glyphosate resistant accessions have at least two mechanisms (**Tables 3**, **4**).

The EPSPS enzymes of all the accession plants were inhibited by glyphosate. The I_{50} (herbicide dose which reduces the enzyme activity to 50%) values were different in all accessions, ranging between approximately 47.65 in Cu-R1, 25.2 in Do-R1, 22.1 in Do-R2, 1.4 in Cu-R2, 1.2 in Do-R3, 1.2 in the Cu-R3, and 1.1-fold resistance in Do-R4 accessions relative to their susceptible accession, respectively (**Figure 4**, **Table 5**). These results were in accordance with the *in vivo* resistance level shown for the different accessions, and suggest that multiple mechanisms in the target-site could be expressed in these accessions.

The basal activity of EPSPS enzyme (without glyphosate) in the resistant accessions was between 0.026 and 0.21 µmol μg^{-1} protein min^{-1}, while the susceptible accessions (Cu-S and Do-S) were lower with 0.024 and 0.025 µmol μg^{-1} protein min^{-1}, respectively (**Figure 5**). There were market differences between accessions in both Cuba ($p = 0.0001$, $DF = 3$, $n = 12$), and Dominican Republic ($p = 0.0002$, $DF = 4$, $n = 15$). The Cu-R1, Do-R1, and Do-R2 exhibited 8.8, 7.2, and 4.8-times higher basal enzyme activities than their susceptible accessions, respectively. For Cu-R2, Do-R3, Do-R4 ,and Cu-R3 accessions the values were similar to those found for their susceptible accessions, respectively.

Molecular Studies

A total of 462 bp of the EPSPS gene of *P. hysterophorus* plants of resistant and susceptible accessions were sequenced. The fragments were aligned and numbered based on a published

TABLE 4 | Glyphosate metabolism expressed as a percentage of total glyphosate and its metabolites in *P. hystherophorus* susceptible and resistant-glyphosate accessions at 96 HAT.

Accessions	Glyphosate ($p = 0.0014, DF = 8, n = 36$)	Metabolites		
		AMPA ($p = 0.0003, DF = 8, n = 36$)	Glyoxylate ($p = 0.0001, DF = 8, n = 36$)	Sarcosine ($p = 0.0002, DF = 8, n = 36$)
Cu-R1	9.80 ± 1.70 D	60.54 ± 1.32 B	18.14 ± 0.32 C	11.52 ± 0.96 A
Cu-R2	21.12 ± 0.93 C	55.31 ± 1.57 B	20.80 ± 0.51 AB	2.77 ± 0.31 E
Cu-R3	73.42 ± 3.63 B	26.14 ± 0.26 C	0.44 ± 0.02 E	ND
Cu-S	91.82 ± 4.81 A	7.68 ± 0.33 E	0.50 ± 0.02 E	ND
Do-R1	11.83 ± 0.74 D	58.94 ± 2.79 B	21.74 ± 0.97 A	7.49 ± 0.27 C
Do-R2	11.37 ± 0.80 D	64.70 ± 2.93 A	18.54 ± 0.83 C	5.39 ± 0.15 D
Do-R3	9.56 ± 0.72 D	60.95 ± 2.71 B	20.36 ± 0.94 B	9.13 ± 0.53 B
Do-R4	71.21 ± 1.06 B	20.05 ± 2.20 D	7.28 ± 0.93 D	1.01 ± 0.71 F
Do-S	90.68 ± 4.39 A	8.86 ± 1.06 E	0.46 ± 0.03 E	ND

Mean value (n = 4) ± standard error. Means on a same column followed by the same letter were not significantly different at α = 0.05.
ND, non-detected; AMPA, aminomethylphosphonic acid.

EPSPS sequence of *Arabidopsis thaliana* (L.) Heynh. (GenBank: CAA29828.1). The resistant accessions of *P. hysterophorus* Cu-R1 from Cuba, and Do-R1 and Do-R2 from Dominican Republic, showed an amino acid substitution at position 106 consisting of a Proline to Serine (**Figure 6**).

TABLE 5 | Parameter estimates of the equation used to calculate the sensitivity of EPSPS enzyme activity to glyphosate in extracts from leaf tissue of the different accessions of *P. hyterophorus* from Cuba and Dominican Republic.

Accessions	*d*	*b*	R^2	I_{50} (μM)[a]	RI	*P*
Cu-R1	100.1	0.9	0.97	646.2 ± 35.8	47.6	<0.0001
Cu-R2	99.8	0.8	0.96	18.9 ± 1.4	1.4	0.1902
Cu-R3	97.0	1.0	0.99	17.4 ± 2.8	1.2	0.2186
Cu-S	96.2	1.2	0.98	13.6 ± 2.2	–	–
Do-R1	100.0	0.8	0.99	468.1 ± 22.0	25.2	<0.0001
Do-R2	100.4	0.7	0.99	410.7 ± 26.1	22.1	<0.0001
Do-R3	94.5	1.2	0.98	22.6 ± 1.5	1.2	0.3714
Do-R4	94.0	1.2	0.96	20.8 ± 6.1	1.1	0.6042
Do-S	93.6	1.2	0.99	18.5 ± 5.7	–	–

[a]*For $Y = \{(d) / [1 + (x/ I_{50}) \exp b]\}$ Where Y is the EPSPS activity, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve in I_{50}, I_{50} is the herbicide rate at the point of inflection halfway, and x is the herbicide dose.*

DISCUSSION

P. hysterophorus is universally recognized for its widespread distribution and high seed production, commonly known as the parthenium weed. Parker (1989) identified two biotypes with different flowering patterns in Mexico (Caribbean area), and they were genetically distinct biotypes (Clermont and Toogoolawah). Moreover, Hanif et al. (2011) found that these two biotypes differed in their morphology and reproductive behavior; in particular, the Toogoolawah biotype shows a greater tendency toward self-pollination, but these biotypes can also present out-crossing. It makes sense that it would reproduce prolifically and that higher resistance levels due to accumulation of multiple mechanisms, by multiple crossings, would proliferate within populations (**Table 6**).

Glyphosate has been used repeatedly in perennial crop areas and fallow fields in the Caribbean Islands for many years to manage *P. hysterophorus* and other troublesome weeds. However, using glyphosate alone without any additional alternative and/or IWM (Integrated Weed Management) led to the emergence of glyphosate-resistant weeds early in the second decade of the 21st century (**Tables 1**, **2**). Herbicide response between different locations depends on local ecological factors, such as a variation in soil type, tillage practices, types of crops, fertilizers, etc., (Shaner and Beckie, 2014; Jussaume and Ervin, 2016). Our results showed different glyphosate resistance levels between the *P. hysterophorus* accessions. This differences could be addressed to the use of different glyphosate formulations and dose rate, the application technique (manual or mechanical) employed by farmers, and the agro environment conditions (Neve et al., 2014; Renton et al., 2014; Jussaume and Ervin, 2016; Matzrafi et al., 2016; Owen, 2016). It has been shown that an increase in the relative humidity and temperature increases the glyphosate absorption, translocation, and toxicity in many weed species (Ge et al., 2011; Hatterman-Valenti et al., 2011; Vila-Aiub et al., 2012; Santos et al., 2016). This research also revealed that the low GR_{50} and LD_{50} values for the susceptible accessions showed that glyphosate has been a very effective tool for farmer for over 15 years, as has been shown in *P. hysterophorus* from Colombia, Dominican Republic, and Florida (Fernandez, 2013; Rosario et al., 2013; Jimenez et al., 2014).

Plants with low levels of GR_{50} and LD_{50} are related to an increased inhibition of EPSPS activity and a greater accumulation of shikimic acid (Shaner et al., 2005; Gaines et al., 2010; Fernández et al., 2015). High levels of resistance (RI) and low shikimic acid accumulation observed in the different *P. hystherophorus* accessions were consistent with those of plants

which have acquired resistance to the addition of more than one NTSR and/or TSR mechanisms, as has been shown in dicotyledonous weed species such as *Amaranthus tuberculatus* (Nandula et al., 2013), *Conyza sumatrensis* (González-Torralva et al., 2014), and several grass weed species (Michitte et al., 2007; de Carvalho et al., 2012; Fernández et al., 2015).

According to Shepherd and Griffiths (2006), a cuticular wax layer provides a protective barrier for a wide range of abiotic stresses (pesticide). Resistant and tolerant plants have displayed a cuticle containing a massive amount of epicuticular wax which forms a nonuniform 3D cover as has been revealed by scanning electron micrographs (De Prado et al., 2005; Wang and Liu, 2007; Rojano-Delgado et al., 2012; Alcántara-de la Cruz et al., 2016a). The limited glyphosate absorption by the resistant *P. hysterophorus* accessions was likely to have been due to differences in outer leaf surfaces. Different translocation can be explained by ^{14}C-glyphosate and/or its metabolite accumulation in the tips of the resistant treated leaves, while ^{14}C was removed from the susceptible treated leaves (**Table 3**). Since the first case of glyphosate resistance was detected in a population of *Lolium rigidum* in Australia (Powles et al., 1998), both previously mentioned mechanisms were considered responsible for this resistance (Wakelin et al., 2004; Michitte et al., 2007; Preston and Wakelin, 2008; de Carvalho et al., 2012; González-Torralva et al., 2012, 2014; Nandula et al., 2013; Fernández et al., 2015). Subsequent studies in the main dicot and monocotyledonous glyphosate-resistant weeds seem to have demonstrated that the main NTSR mechanism involved in their resistance is due to a lesser glyphosate absorption and/or -translocation (Feng et al., 2004; Michitte et al., 2007; de Carvalho et al., 2012; González-Torralva et al., 2012, 2014; Vila-Aiub et al., 2012; Nandula et al., 2013; Adu-Yeboah et al., 2014).

In some plants, the glyphosate degradation to glyoxylate and AMPA is carried out by a glyphosate oxidoreductase (GOX), and the glyphosate degradation to sarcosine and inorganic phosphate by a C–P lyase. These steps have been reported by some authors such as Liu et al. (1991); Komoba et al. (1992); Saroha et al. (1998); Al-Rajab and Schiavon (2010), and Duke (2012) among others. However, only a few works unify these two degradation pathways to explain the glyphosate metabolism in leguminous plants and weeds (de Carvalho et al., 2012; Rojano-Delgado et al., 2012). Some authors consider that metabolism has a low contribution to the resistance or, even more, that it is nonexistent (Saroha et al., 1998; Feng et al., 2004; Duke, 2012; Sammons and Gaines, 2014). However, the fact is that this mechanism involves a decrease in the concentration of the herbicide glyphosate around the target-site, diminishing the EPSPS inhibition rate (Duke, 2012; Sammons and Gaines, 2014; Alcántara-de la Cruz et al., 2016a). The GOX gene that encodes the glyphosate metabolizing enzyme glyphosate oxidoreductase was cloned from *Achromobacter* sp. *strain* LBAA (Barry et al., 1994). Neither plant GOX nor the gene(s) encoding it have been isolated or elucidated. A plant gene encoding GOX might be useful in genetically engineering crops and weed resistance development (Duke, 2012; Rojano-Delgado et al., 2012). Some researchers have proposed additive effects of concurrent glyphosate resistance mechanisms in the same weed species (Gaines et al., 2010; Yu et al., 2010; Bostamam et al., 2012; Rojano-Delgado et al., 2012), which would explain the difference in the resistance between accessions keeping the same percentage of metabolic degradation (**Table 6**). However, genetic basic controlling absorption/translocation and/or metabolism including genes involved have not been identified so far (Yuan et al., 2006; Delye, 2013; Délye et al., 2013). This could be a highly promising research area in the future.

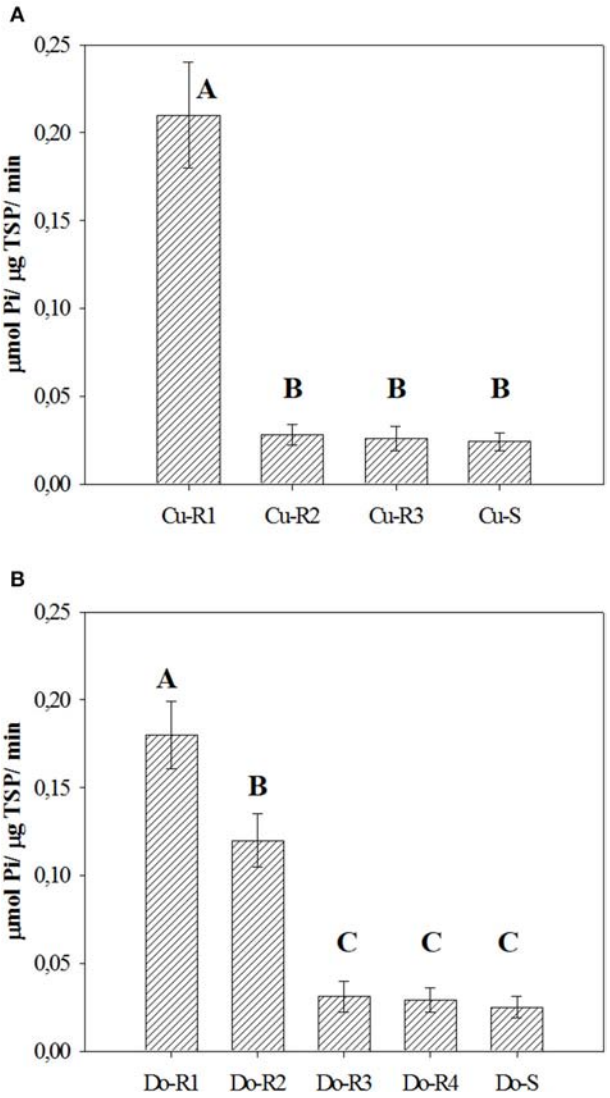

FIGURE 5 | Basal EPSPS activity for glyphosate-susceptible and resistant from Cuba (A) and Dominican Republic **(B)** accessions of *P. hysterophorus*. Vertical bars are ± standard errors of the mean. Means by the same letter were not significantly different at $\alpha = 0.05$.

Taking into account these results, resistance could be associated with target enzyme overexpression. Some species as ryegrass (Yu et al., 2007; Dayan et al., 2012) have shown differences in the basal EPSPS enzyme activity as a consequence of the EPSPS gene overexpression. However, in the *L. perenne* spp. *multiflorum* population from Arkansas, no differences were

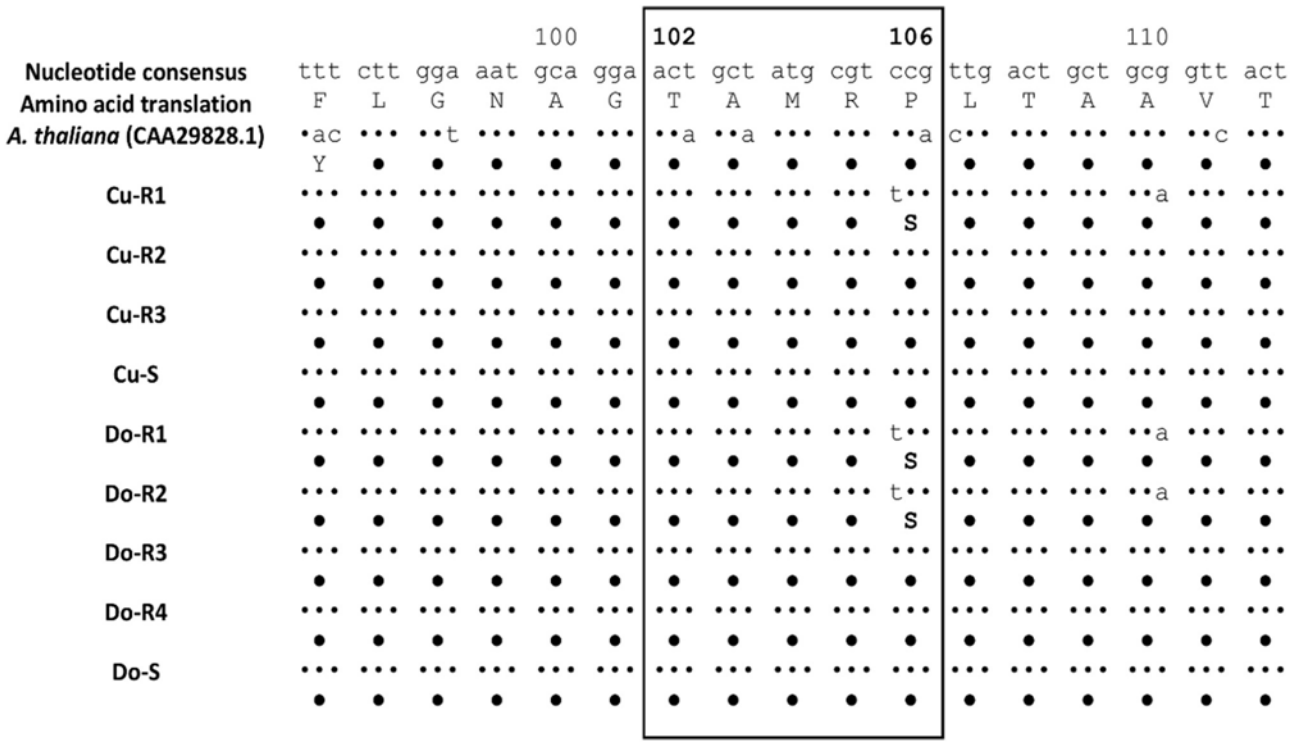

FIGURE 6 | Partial protein sequence alignment of the EPSPS gene of resistant and susceptible *P. hysterophorus* plants. The box comprising the region of Thr-102 and Pro-106 point mutations associated to confer glyphosate resistance. The points indicate homology between the different sequences.

TABLE 6 | Summary of glyphosate resistance mechanisms accumulated by *P. hysterophorus* accessions studied in this work.

Accessions	GR_{50}[a]	LD_{50}[a]	Absorption and translocation	Glyphosate metabolism	Enhanced EPSPS basal activity[b]	EPSPS (I_{50}[b])	Pro106Ser
Cu-R1	1370	6364	Low	High	Yes	High	Yes
Cu-R2	437	2794	Low	High	No	Low	No
Cu-R3	151	1415	Low	Medium	No	Low	No
Cu-S	56	232	High	Low	No	Low	No
Do-R1	939	4456	Low	High	Yes	High	Yes
Do-R2	547	2550	Low	High	Yes	High	Yes
Do-R3	339	1821	Low	High	No	Low	No
Do-R4	255	1242	Low	Medium	No	Low	No
Do-S	47	196	High	Low	No	Low	No

[a]*glyphosate g ae ha*$^{-1}$; [b]*glyphosate* μM.

observed in the I_{50} values, which could be explained as a lack of effective mutations in the binding site of the enzyme (Salas et al., 2015). In our case, some accessions are candidates to possessing an effective mutation (**Figure 6**, **Table 6**) or a possible EPSPS overexpression, explaining their high resistance to glyphosate compared to other accessions. We are aware of that fact, and effective research is currently in progress to characterize the EPSPS overexpression resistance mechanism involving these accessions.

Results reported here are in agreement with previous works, in which the Proline to Serine substitution was found to confer glyphosate resistance in other weed species such as *A. tuberculatus*, *C. sumatrensis*, *Echinochloa colona*; *L. perenne* spp. *multiflorum* and *L. rigidum* (Bostamam et al., 2012; González-Torralva et al., 2012, 2014; Nandula et al., 2013; Fernández et al., 2015; Han et al., 2016). However, mutations in the Pro-106 position generally provide only a low level (2–4-fold) of glyphosate resistance (Kaundun et al., 2011). Here, *P. hysterophorus* accessions that presented Pro-106 mutation had a resistance factor of >12. These three accessions (Cu-R1, Do-R1, and Do-R2) were more highly resistant to glyphosate as a result of showing different concurrent resistance mechanisms, including reduced absorption and translocation, glyphosate metabolism, and EPSPS gene mutation.

In some species, at least more than one glyphosate resistance mechanism have been reported, such as *A. tuberculatus* (Nandula et al., 2013), *L. rigidum* (Bostamam et al., 2012), *L. perenne* spp. *multiflorum* (González-Torralva et al., 2012), and *L. perenne* (Ghanizadeh et al., 2015) populations which exhibited a mutation in Pro-106 position, and a reduced translocation. Besides, other species such as *Digitaria insularis* presented a pool of mechanisms (absorption, translocation, metabolism, and EPSPS gene mutation; de Carvalho et al., 2012). The involvement of several resistance mechanisms is evident when looking at the resistance levels of accessions Cu-R2, Cu-R3, Cu-R4, Do-R3, Do-R4, and Do-R5 of *P. hysterophorus*, which did not show any mutation in the Pro-106 position. This is the first time that a mutation in the target-site has been reported in glyphosate-resistant *P. hysterophorus*.

In summary, we have confirmed resistance to glyphosate in different *P. hysterophorus* accessions harvested in the Caribbean Islands. Their resistance levels depend on the different resistance mechanisms (NTSR and TSR) that are accumulated by these accessions (**Table 6**), due to increasing selection pressure and out-crossing. The evolution of multiple mechanisms found in this resistance species is worrying. The farmers should implement manage practices such as the use of cover crops, which prevent soil erosion and allow the use of grazing, as well as the use of other non-selective herbicides in an integrated weed management (IWM) to facilitate the reduction and suppression of herbicide-resistant accessions.

AUTHOR CONTRIBUTIONS

EB, PF, and RD performed the glyphosate plant dose-response and shikimic acid accumulation. EB, PF, FB, and RD carried out the EPSPS activity assays. EB, PF, and RD did the ^{14}C-glyphosate absorption/translocation, and metabolism study. FB performed the EPSP synthase gene sequencing.

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Study of Fitness Cost in Three Rigid Ryegrass Populations Susceptible and Resistant to Acetyl-CoA Carboxylase Inhibiting Herbicides

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Evaluation of fitness differences between herbicide-resistant and susceptible weed biotypes, allows a better prediction of further dispersal of herbicide-resistance populations and the design of a management strategy in order to achieve a mitigation of the problem in the absence of herbicide. In this study, an evaluation of germination and seedling emergence characteristics of three rigid ryegrass biotypes (one susceptible and two resistant populations with different mutations, namely Ile 1781 Leu and Ile 2041 Asn) and of competition between this weed and wheat using replacement series experiments was conducted. The results showed that when seeds were on the soil surface (0 cm) to depth of 2 cm and again for the depth of 6 cm, there were not any significant differences between the biotypes regarding seed germination percentage. On the contrary, when seeds were sown in 2–4 cm depth, R-1781 consistently displayed lower emergence than the S and R-2041 biotypes. Moreover, when seeds were sown at 8 cm depth, final proportions of emerged seedlings were similar for R-2041 and R-1781, while both populations had significantly higher emergence than the S population. The competitive ability of the three biotypes was similar, as determined by a replacement series experiment with wheat. Our results under competitive conditions revealed that *Triticum aestivum* was more competitive than *Lolium rigidum*. Overall, there was no apparent fitness penalty associated to ACCase-inhibitor resistance, while different mutations may impose different competitive ability and therefore require case-specific management strategies.

Keywords: 1781-Leu, 2041-Asn, ACCase mutation, competition, germination, soil burial

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INTRODUCTION

Acetyl-coenzyme A carboxylase (ACCase) inhibitors consist a group of commercially important, very effective and, selective graminicides which are applied postemergence and introduced since 1970s. These herbicides are members of the Group A (1) of herbicides (Mallory-Smith and Retzinger, 2003; Anonymous, 2004). By inhibiting ACCase activity, fatty acid synthesis is also

inhibited, resulting in growth cessation in meristematic tissues and finally plant death (Délye, 2005; Powles and Yu, 2010; Ahmad-Hamdani et al., 2012).

The extended and worldwide use of ACCase inhibitors has resulted in resistance evolution in about in 48 different weed species (Heap, 2016). It has to be noted that the first reports of resistance to these herbicides have been reported more than 30 years ago (Heap and Knight, 1982). An important mechanism that induces ACCase herbicide resistance in grasses is target site mutation (Délye, 2005; Powles and Yu, 2010). Nine distinguished aminoacid replacements in the CT domain of plastidic Acetyl-COA carboxylase genes have been identified to cause target-site resistance to certain ACCase inhibiting herbicides in grass weeds (Délye et al., 2005; Powles and Yu, 2010; Collavo et al., 2011). Target site resistance is probably due to critical changes in the optimal binding of the herbicide. Six mutations in the gene encoding ACCase in rigid ryegrass (*Lolium rigidum*) were shown to confer resistance to herbicides inhibiting this enzyme. In particular, they cause amino-acid substitutions at codon positions 1781 (Ile-to-Leu), 2027 (Trp-to-Cys), 2041 (Ile-to-Asn), 2078 (Asp-to-Gly), 2096 (Gly-to-Ala), and 1756 (Gln-Glu; Zhang and Powles, 2006). Powles and Yu (2010) declared that resistance level depends on the herbicides, rate, weed species, and number of resistant alleles in individual plants. Menchari et al. (2008) reported that diversity fitness costs could be connected to mutant ACCase alleles.

Fitness can be defined as the ability to reproduce to the next generation. Regarding herbicide resistance (HR), fitness costs can help illustrate the relative abundance of resistance alleles (Menchari et al., 2008; Vila-Aiub et al., 2009). Fitness costs observed in weeds resistant to herbicides because of some mutations have been reported to be lower (Beversdorf et al., 1988; Darmency and Pernes, 1989) or even higher that 25% (Tardif et al., 2006). Some resistant biotypes of rigid ryegrass were also shown to be more competitive in terms of seed size compared to the susceptible population (Pedersen et al., 2007). Travlos (2013) showed that there were not any significant fitness differences between ACCase-inhibitor

TABLE 1 | List of potentially resistant and susceptible (S) ryegrass populations to ACCase inhibitor herbicides obtained from Khuzestan area, Iran.

Population code	Collection area	UTM coordinate (zone 38)		Area (ha)	Status of resistance
		X	Y		
AH1	Ahvaz	240857.70	3456064.44	20	Potentially resistant
AH2	Ahvaz	272347.02	3500817.47	8	Potentially resistant
AH3	Ahvaz (CA)	245382.12	3445968.63	4	Potentially resistant
AH4	Ahvaz (CA)	246576.01	3455925.23	2	Potentially resistant
AH5	Ahvaz (CA)	264054.28	3469940.39	10	Potentially resistant
AH6	Ahvaz (CA)	241021.63	3462717.69	20	Potentially resistant
AH7	Ahvaz	258677.41	3484485.27	9	Potentially resistant
AH8	Ahvaz	272347.02	3500817.48	10	Potentially resistant
AH9	Ahvaz	272371.39	3501926.28	4	Potentially resistant
BOS1	Bostan	215786.07	3481127.57	20	Potentially resistant
BOS2	Bostan	216676.82	3478886.90	22	Potentially resistant
BOS3	Bostan	217657.95	3479966.94	10	Potentially resistant
BOS4	Bostan	215755.87	3480018.56	10	Potentially resistant
BOS5	Bostan	223393.50	3480923.15	10	Potentially resistant
DA1	Dashteazadegan (Sus)	225926.71	3468649.34	30	Potentially resistant
DA2	Dashteazadegan	235334.77	3463969.08	25	Potentially resistant
DA3	Dashteazadegan	226907.63	3469733.44	10	Potentially resistant
DA4	Dashteazadega	236259.29	3462836.21	17	Potentially resistant
HAM1	Hamidiyeh	265205.19	3478789.33	25	Potentially resistant
HAM2	Hamidiyeh	256724.91	3482311.79	20	Potentially resistant
HAM3	Hamidiyeh	250443.55	3497993.38	25	Potentially resistant
HAM4	Hamidiyeh	258677.41	3484485.27	12	Potentially resistant
HAM5	Hamidiyeh	252870.02	3480183.48	10	Potentially resistant
HAM6	Hamidiyeh	252975.11	3484618.89	10	Potentially resistant
HAM7	Hamidiyeh	258677.41	3484485.27	7	Potentially resistant
HAM8	Hamidiyeh	250443.55	3497993.38	15	Potentially resistant
GOT	Gotvand	265973.30	3554212.50	8	Potentially resistant
KHO	Khorramshahr	231038.35	3368655.53	21	Potentially resistant
RAM	Ramshir	347069.78	3418507.70	10	Potentially resistant
HF (S)	Haftkel	342131.32	3471799.10	8	Susceptible

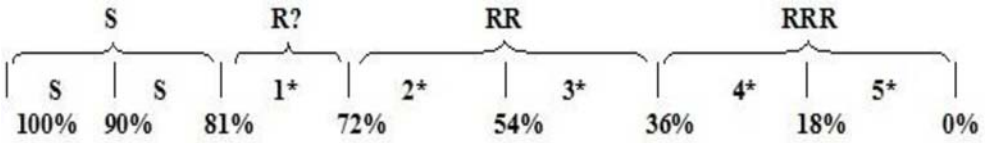

FIGURE 1 | Star (*) and R rating system to distinguish the degree of resistance, according to the response to a single dose of herbicide in resistance screening (Moss et al., 1999).

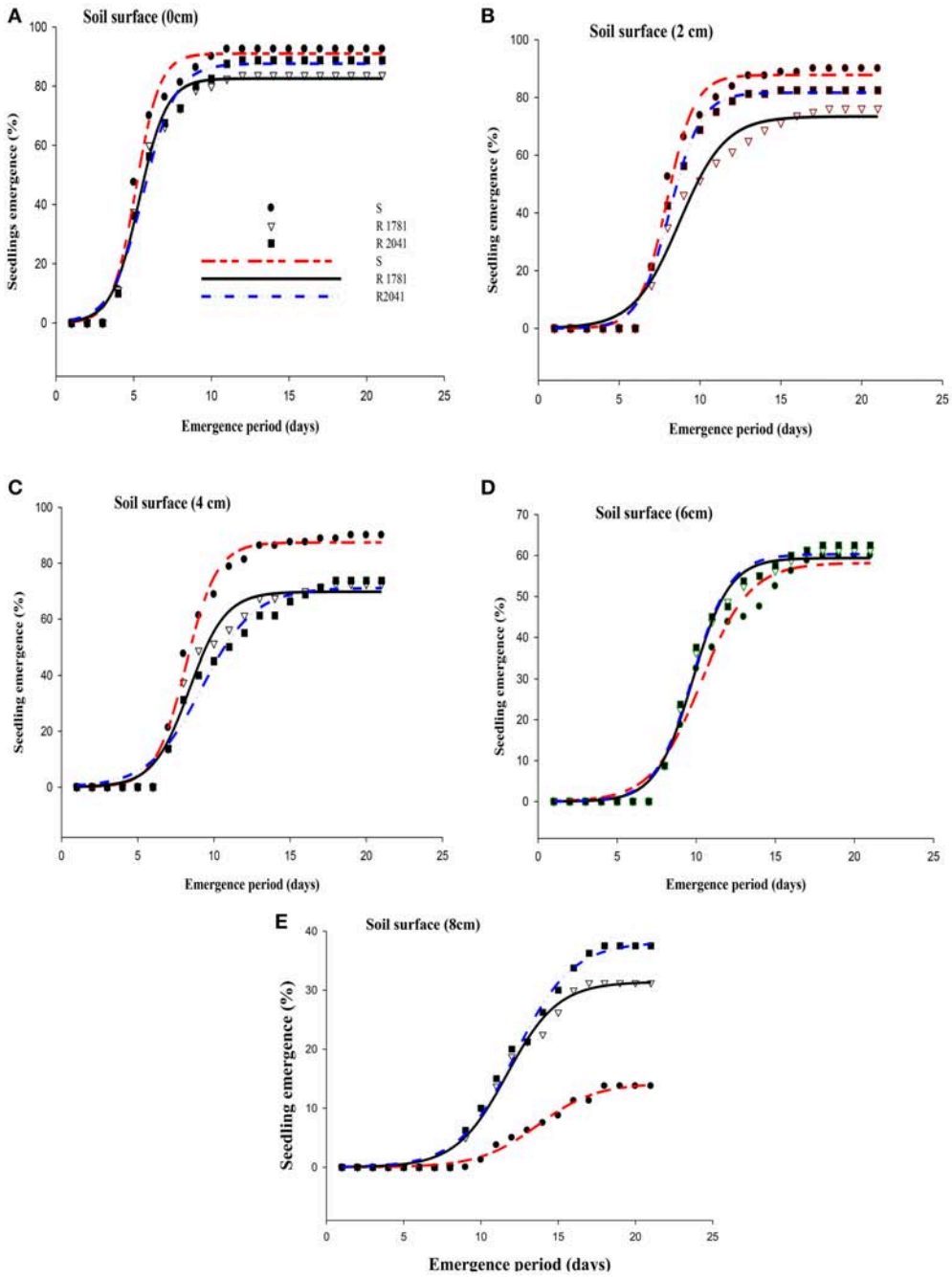

FIGURE 2 | Effects of sowing depth on the percentage of seedling emergence of the S (circle), R-2041 (square), and ACCase (triangle) populations. (A) Soil surface (0 cm), **(B)** Soil surface (2 cm), **(C)** Soil surface (4 cm), **(D)** Soil surface (6 cm), and **(E)** Soil surface (8 cm).

TABLE 2 | Parameter evaluation from sigmoidal model that explains the effect of different sowing depth on seedling emergence percentage for the population S, R-1781, and R-2041.

Depth (cm)	Population *a*			t_{E50}			b		
	S	R-1781	R-2041	S	R-1781	R-2041	S	R-781	R-2041
0	91.02 (1.02)	87.62 (1.02)	82.59 (0.86)	5.15 (0.09)	5.63 (0.11)	5.34 (0.09)	0.77 (0.081)	1.08 (0.09)	0.89 (0.08)
2	87.80 (1.14)	73.45 (1.56)	81.69 (0.84)	7.95 (0.10)	8.72 (0.19)	8.15 (0.08)	0.86 (0.09)	1.41 (0.17)	0.96 (0.07)
4	87.41 (1.21)	69.76 (1.41)	71.13 (1.81)	8.18 (0.11)	8.38 (0.18)	9.22 (0.24)	1.02 (0.10)	1.19 (0.15)	1.73 (0.20)
6	58.20 (1.36)	59.39 (0.89)	60.33 (1.03)	10.30 (0.20)	9.77 (0.12)	9.76 (0.14)	1.55 (0.17)	1.17 (0.10)	1.20 (0.12)
8	14.24 (0.45)	31.40 (0.67)	38.13 (0.85)	13.67 (0.22)	11.68 (0.17)	12.22 (0.17)	1.90 (0.17)	1.65 (0.14)	1.84 (0.14)

The numbers in parentheses represent the standard errors of the means.

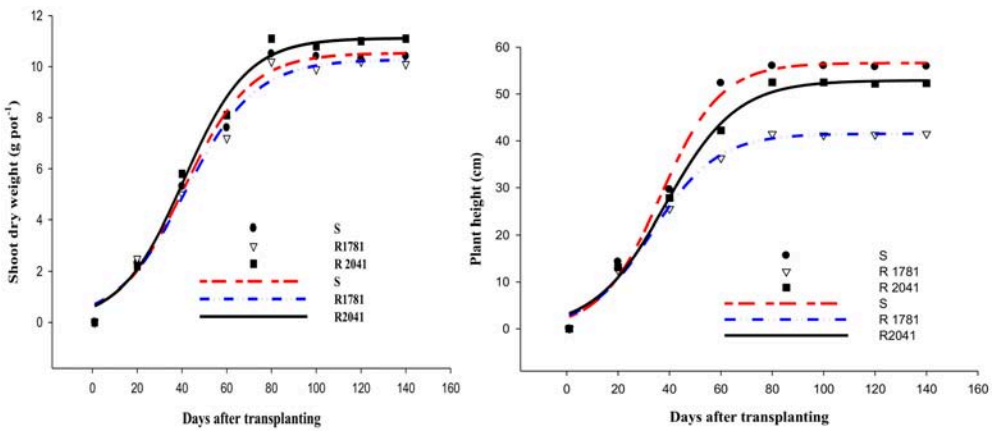

FIGURE 3 | Shoot dry weight (SDW) and plant height of R-1781, R-2041, and S rigid ryegrass populations under noncompetitive conditions.

resistant and susceptible biotypes of sterile wild oat (*Avena sterilis*).

Fitness costs play a significant evolutionary role contributing among the others to the maintenance of genetic polymorphism within populations (Yanniccari et al., 2016). However, even if fitness costs associated with plant defensive traits are widely anticipated, they are not commonly detected (Van Etten et al., 2016). Hence, the main purpose of the present study was to compare the competitiveness of a susceptible (S) rigid ryegrass biotype, compared with two resistant (R) biotypes, in terms of several growth parameters. Moreover, 30 biotypes were exerted to determine the possible pleiotropic effects of R-1781 and R-2041 ACCase alleles in greenhouse conditions, in competition with *Triticum aestivum*. Such data could be useful in order to understand fitness cost's sides and implement the optimal resistance management strategies.

MATERIALS AND METHODS

Seed Collection

Seeds used in the present study were collected in the spring of 2013 from 29 wheat fields infected with rigid ryegrass and located in the southwestern part of Iran (Khuzestan province) (**Table 1**). In the specific area, there were often complains on the low efficacy of ACCase herbicides, while there were several reports of herbicide resistance development. Seeds were mainly collected at the beginning of maturity from herbicide-treated wheat fields along with some fields that had never been treated with herbicides (in order to use them as susceptible). Each surveyed field was walked through by the two diagonals seeds were separated, air-dried, and stored at room temperature (22–26°C) until their use. Rigid ryegrass seeds were treated with 10 ppm of gibberellic acid (GA) at 5 days before sowing, in order to relieve any seed dormancy.

Assessment of Herbicide Resistance

Ten germinated seeds of the potentially resistant and susceptible populations were sowed in pots filled with 500 ml of soil. An herbicide-free soil mixed with a common peat substrate (1:1, V/V) was used. Throughout the experiments the pots were uniformly watered as needed. Shortly after emergence, plants were thinned to a final density of seven seedlings per pot. The pots were fertilized as required and kept under conditions set at 20–30/10–15°C day/night with a 14 h photoperiod and a relative humidity of about 65%. The required amount of light was provided by a combination of fluorescent and incandescent lamps. At 21 DAE[1] (at the 2–3 leaf stage), seedlings were treated with the recommended dose of clodinafop-propargyl (CLD) and pinoxaden (PIN). Particularly, CLD and PIN were applied at

[1]Days after emergence.

rates of 64 and 45 g ai ha^{-1}, respectively. Plants were harvested at 28 DAT[2] and the fresh weight of foliage was recorded, with the number of dead and surviving plants also being measured.

CAPS and dCAPS Methods

After the above-mentioned evaluation of efficacy and potential resistance, the populations were used for leaf sampling and subsequent DNA extraction. Particularly, in a CAPS (cleaved amplified polymorphic sequences) analysis, gene-specific primers were applied to reproduce DNA pattern, and SNPs (single nucleotide polymorphisms) were identified. In the dCAPS (Derived cleaved amplified polymorphic sequences) technique, a limitation enzyme identification site, which contains the SNP, was introduced into the PCR amplicon by a primer containing one or more inconformities to the DNA template. The PCR amplicon is then digested by a limitation enzyme, and the presence or absence of the SNP is defined by the resulting digestion restriction template as previously described by Yu et al. (2008).

Evaluation of the Effect of Burial Depth on Emergence of Resistant and Susceptible Populations

In this study, the effect of sowing depth on emergence of three rigid ryegrass populations (a susceptible population and two resistant populations) was evaluated. Mean seed weight (MSW) was similar for the three populations (MSW of 1.85 ± 0.25 mg, $n = 100$) in order to eliminate any side effects. Twenty seeds of each population were sown in 12-cm diameter pots at five depths (0, 2, 4, 6, and 8 cm). Pots were placed in a controlled environment greenhouse with the same conditions as above. The experiment was set up under a completely randomized experimental design with four replicates. Seedling emergence was measured daily for 21 days, while in the case of 0 cm, seedlings were considered emerged when the coleoptile length was 3 mm long (Steadman et al., 2003).

Growth of Resistant and Susceptible Populations under Non-competitive Conditions (Monoculture)

Fifteen seeds of each population were sown in a potting soil compound in 12-cm diameter pots. Pots were placed in a controlled environment greenhouse with the same conditions as above and watered uniformly as needed. Shortly after emergence, plants were thinned to a final density of eight seedlings per pot. The experiment was set up under a completely randomized experimental design with four replicates. The experiments were established in October 2014 and 2015. Four wheat and rigid ryegrass plants were harvested at 20-days intervals, from 20 to 160 DAE. Plants were cut in the soil surface and height was also measured. The plants were dried at 74°C for 72 h and dry weight was determined.

[2]Days after treatment.

TABLE 3 | Seed production (number and weight) for ACCase inhibitor-resistant (R) and -susceptible (S) rigid ryegrass populations under noncompetitive conditions.

Population	Total seed number	Seed weight	
	No. $plant^{-1}$	g 1000 $seeds^{-1}$ (TSW)	g $plant^{-1}$
S	296 (5.64)	1.6 (0.09)	0.48 (0.011)
R-1781	267 (9.33)	1.8 (0.07)	0.47 (0.009)
R-2041	365 (12.75)	1.7 (0.08)	0.63 (0.013)

The numbers in parentheses indicate the standard errors of the means.

Evaluation of Competitive Ability, Growth, and Seed Production of S and R Populations under Competitive and Non-competitive Conditions

These tests were conducted in order to evaluate the competitive ability, growth, and seed production of S and R rigid ryegrass populations and wheat under competitive and non-competitive conditions. For that purpose, pot experiments were conducted in the greenhouse of Agricultural Research Center of Khuzestan province, Iran. Seedlings (after seed placement in Petri dishes and their germination) were transplanted into 12-cm diameter pots filled with 500 ml of a manure-loam-sand mixture in 1:1:1 ratio. Competition between rigid ryegrass populations and wheat was studied by means of a replacement series experiment at five proportions (100:0, 75:25, 50:50, 25:75, and 0:100) at a fixed density of 8 plants (ryegrass/wheat) per pot. The pots were kept under conditions set at 20–30/10–15°C day/night with a 14-h photoperiod and watered uniformly as needed. The required amount of light was provided by a combination of fluorescent and incandescent lamps. The experiment was set up in a completely randomized experimental design with four replicates. The replacement series experiment was established on October 2014 and repeated on April 2015. The plants were dried at 74°C for 70 h and weighed. Plants from each species were separated into shoots and spikes, while plant height was also measured at harvest.

Statistical Analyses

Differences between seed populations were evaluated after data expression as percentage of the untreated control. The percentage of surviving plants was also recorded at 28 DAT.

In order to evaluate the effect of burial depth on the emergence of resistant and susceptible populations regression and curve fitting is considered as a recommended type of analysis. A functional three parameter sigmoid (S) model using nonlinear least squares regression (Curve Fitter) was performed by means of the software SigmaPlot 12 (Systat Software, Inc.) and shown below:

$$y = \frac{a}{1 + e^{-\left[\frac{x - t_{E50}}{b}\right]}} \quad (1)$$

In Equation (1), y is whole appearance (emergence) on time x, a is the highest emergence (percentage), t_{E50} is the time to attain

50% of ultimate seedling emergence (day) and *b* represent the slope around t_{E50}. Emergence values were angular transformed. Parameter estimates were compared by one-way ANOVA and means were separated using least significant difference (LSD) ($\alpha = 5\%$).

A similar procedure as the one mentioned above was also followed for the evaluation of competitive ability, growth, and seed production of S and R populations under competitive and non-competitive conditions. Data on plant height and dry weight were subjected to ANOVA. Total shoot dry weight (SDW) was compared with the theoretical yields for equal competitive ability using 95% confidence interval (CI). For this statistical analysis SAS software was used (SAS Institute, 1987).

RESULTS

Assessment of Herbicide Resistance

Assessment of herbicide resistance was conducted by means of the rating system given in **Figure 1** as described by Moss et al. (1999). The results showed that *L. rigidum* fresh weight and survival were significantly different between populations at 28 DAT with CLD. Populations AH3, AH4, BOS1, BOS2, BOS5, DA1, DA2, HAM1, HAM2, HAM3, HAM4, HAM5, HAM6, HAM7, and KHO showed the lower fresh weight reduction, while other populations were adequately controlled by CLD.

These differences between the several potentially R populations indicate that inadequate control of ryegrass in other locations may be attributed to other reasons like an improper application time or method and not *per se* to herbicide resistance. The results of our screening tests showed that 10 populations of *L. rigidum* have resistance to CLD (34.5% of the total). After confirming the resistance of the populations to this herbicide, they have been used for leaf sampling for DNA extraction as described above.

CAPS and dCAPS Methods

Leaves of these plants were analyzed by CAPS and dCAPS methods for identification of possible mutations occurring in 2088, 2078, , 2041, and 1781 locations of ACCase enzyme coding gene which is involved in the resistance of *L. rigidum* to ACCase inhibitors. Finally, there have been recognized two mutations (Ile 1781Leu and Ile 2041Asn) and it was found that the mutation 2041-Asn confers sufficient level of resistance to clodinafop-propargyl. Molecular analyses of resistance confirmed that the Ile 1781 Leu and Ile 2041 Asn mutations in the resistant biotypes endowed resistance to FOPs, but not to DIMs and DEN.

Evaluation of the Effect of Burial Depth on Emergence of R and S Populations

Our results showed that when seeds were placed on the soil surface (0 cm) and in a depth of 2 cm, the S biotype had slightly higher seed germination percentage than the two R populations (2041-Asn and 1781-Leu), but this difference was not statistically significant (**Figure 2A** and **Table 2**). In the case of seeds placed on soil surface, seedlings of the R-2041 population had the lowest appearance (83%) compared with populations R-1781 and S (88 and 91%, respectively). When the seeds were sown in a depth of 2–4 cm, R-1781 biotype systematically exhibited lower emergence than S and R-2041 biotypes (**Figures 2B,C** and **Table 2**). On the contrary, seeds sown in a depth of 6 cm had a germination percentage of 60, 61.25, and 62% for R-2041, R-1781, and S biotypes, respectively. This finding indicates no-significant difference in the percentage of germination (parameter *a*) between the three biotypes at this depth (**Figure 2D** and **Table 2**). However, when seeds were sown in a depth of 8 cm, final percentage of emerged seedlings was similar for R-2041 and R-1781, while both biotypes had significantly higher emergence than the S biotype (**Figure 2E** and **Table 2**). A functional three parameter sigmoid (S) model (equal) presented a significant fit ($P < 0.001$, $R^2 = 0.98$–0.99) to emergence data for all combinations of population and sowing depth. Values of t_{E50} were increased with sowing depth (**Table 2**). R-1781 population exhibited significantly higher t_{E50} values than the S and R-2041 populations at 0 and 2 cm depths, while R-2041 population had the highest values at depths of 4, 6, and 8 cm (**Table 2**).

In all three populations, increasing depth resulted to an emergence reduction. It has also to be noted that at the depth of 0 cm, the highest germination rate was that of S population (**Table 2**). Some of these findings are in accordance with similar results obtained in the study of Vila-Aiub et al. (2015b).

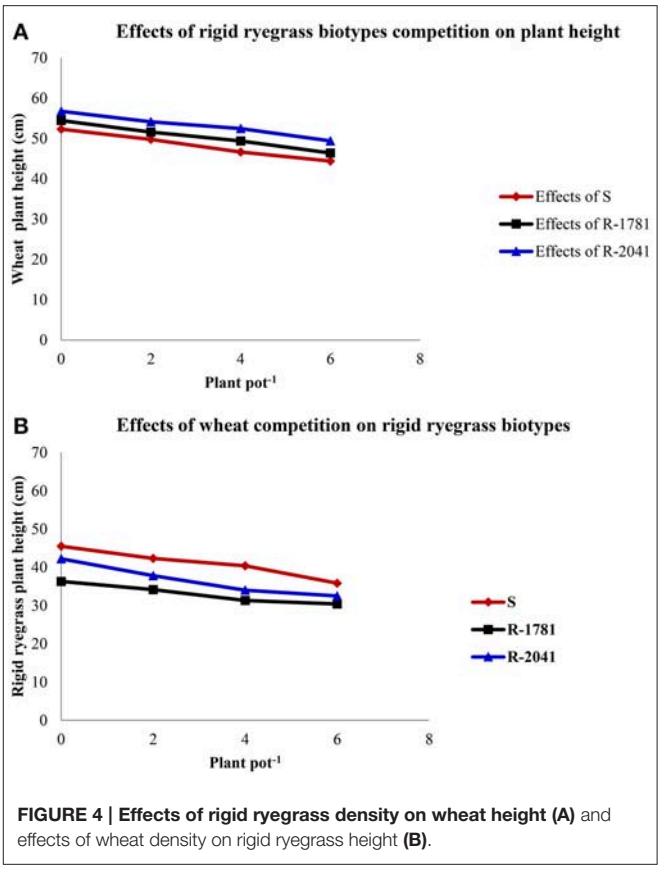

FIGURE 4 | Effects of rigid ryegrass density on wheat height (A) and effects of wheat density on rigid ryegrass height **(B)**.

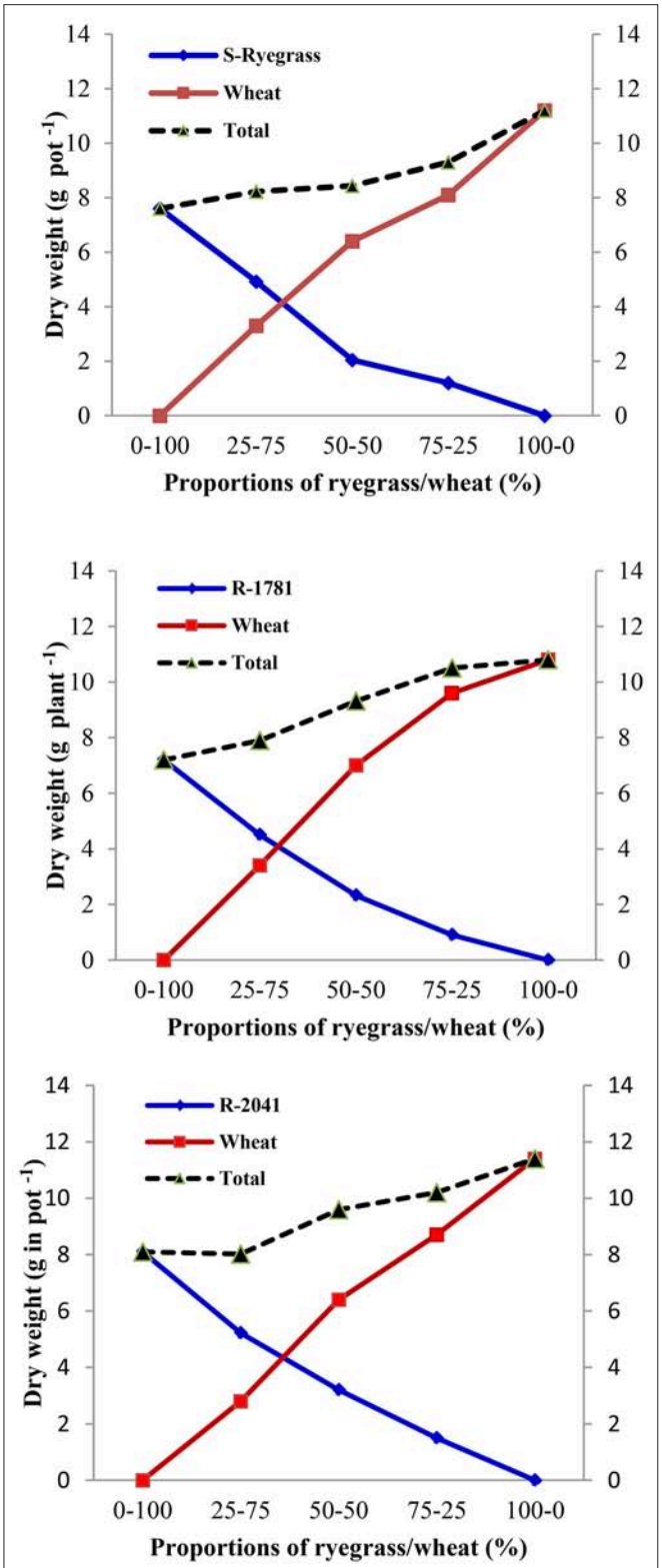

FIGURE 5 | Replacement series diagrams for shoot dry weight of ACCase inhibitor-resistant (R-2041 and R-1781) and -susceptible (S) rigid ryegrass biotypes grown under competitive conditions at different proportions with wheat.

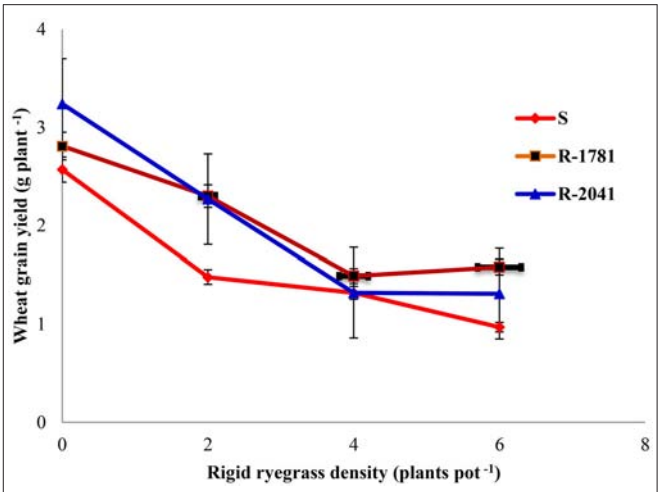

FIGURE 6 | Effect of wheat and rigid ryegrass density on wheat grain yield. Vertical bars indicate standard errors of the mean (data from both years were combined, since there were not significant differences between the years).

Growth of R and S Populations under Non-competitive Conditions (Monoculture)

The results of biomass and plant height data fit to a functional three parameter sigmoid curve for the populations R-781, R-2041, and S under non-competitive conditions as given in **Figure 3**. The highest biomass and plant height were reached at 80 day after sowing (DAS). Our results showed that biomass of the R-2041 and R-1781 populations was similar to that of the S population. Regarding the maximum dry weight, there were no significant differences ($p > 0.05$) between the resistant and susceptible populations. As anticipated, the data followed a typical sigmoid growth pattern. Similar results have been found by other researchers on resistant and susceptible populations of weeds like kochia and downy brome (Thompson et al., 1994; Christoffoleti et al., 1997; Park et al., 2004). Concerning plant height, our results revealed some significant differences between the rigid ryegrass populations with S population being significantly higher than R-1781 population (**Figure 3**). These findings are not in full agreement with results of previous studies (Thompson et al., 1994; Christoffoleti et al., 1997; Park et al., 2004; Menchari et al., 2008).

Seed production of the three studied populations was also within a wide range. As shown in **Table 3**, R-2041 was the population with highest seed production, 19 and 27% higher than the corresponding value for S and R-1781 population, respectively. However, regarding thousand seeds' weight (TSW) there were not significant differences between the several populations.

Previous studies on several weeds have shown either higher seed production for R compared with the S population (Purrington and Bergelson, 1997; Park et al., 2004), or no differences at all between the several biotypes (Alcocer-Ruthling et al., 1992; Thompson et al., 1994; Travlos, 2013).

Evaluation of Competitive Ability, Growth, and Seed Production of S and R Populations under Competitive and Non-competitive Conditions

Evaluation of the competitive ability of R and S *L. rigidum* populations showed that increasing rigid ryegrass density from 2 to 6 plants pot^{-1} reduced wheat height compared to the non-competitive conditions (**Figures 4A,B**). The results showed that interspecific competition (rigid ryegrass-wheat) was more intense than the intraspecific competition (rigid ryegrass-rigid ryegrass or wheat-wheat).

It has to be noted, that in our study wheat emerged 2–5 days earlier than rigid ryegrass. Consequently, this early emergence of *Triticum aestivum* allowed its high biomass accumulation, and therefore its competitiveness was high. In Australia, Rerkasem et al. (1980) found that the competitive ability of rigid ryegrass under field conditions was low when rigid ryegrass emerged later or simultaneously with wheat. Early wheat emergence in comparison with weeds is considered to increase wheat competitive ability as previously reported by Kropff et al. (1992) and O'Donovan et al. (1985).

Our experiments also showed that a density of 2–6 wheat plants pot^{-1} decreased SDW per plant of rigid ryegrass at different densities (data not shown). As shown in **Figures 4**, **5**, increased wheat density seems to increase the competition against rigid ryegrass populations which may be due to the dense wheat canopy and reduction of the availability of light, nutrients, and moisture for rigid ryegrass (Tanji et al., 1997). In another study and by using different rigid ryegrass densities in a field in Australia, Medd et al. (1981) found that SDW of rigid ryegrass in the presence of 40 or 74 wheat plants m^{-2} was twice that obtained in the presence of 200 wheat plants m^{-2}. These findings agree with the results of our study showing that all densities of rigid ryegrass (2, 4, and 6 plant per pot) reduced SDW of wheat and grain yield per pot^{-1} (**Figures 5**, **6**).

When populations R-1781, R-2041, and S were sown under competitive situations with wheat, their dry weight and height were significantly affected (**Figure 5**). SDW of the S biotype confirmed the theoretically anticipated response showing the fitness cost of R-1781, R-2041, biotypes. Dry weight and plant height of R-1781, R-2041, and S biotypes were different from non-competitive situation, being various in all studied proportions (**Figures 4A**, **5** and **Table 4**).

DISCUSSION

Gill et al. (1996) stated that there was not any significant fitness cost of rigid ryegrass resistant to ACCase inhibitor herbicides. A similar result was found in foxtail millet, *Setaria faberi* populations (Wiederholt and Stoltenberg, 1996), but in these two studies mechanism of resistance of these grass weeds to herbicides was not identified and this absence of fitness cost can be due to the involvement of several

TABLE 4 | Shoot dry weight (SDW), number of tillers, and plant height for ACCase inhibitor-resistant (R) and -susceptible (S) rigid ryegrass biotypes in a replacement series experiment at five proportions (100:0, 75:25, 50:50, 25:75, 0:100) of (ryegrass:wheat).

	Biotype	Proportions of mixture (%)				
		100 0	75 25	50 50	25 75	0 100
SDW (gr per pot)	S	7.60 (0.16)	4.92 (0.22)	2.04 (0.11)	1.20 (0.13)	
	W		3.30 (0.15)	6.40 (0.15)	8.10 (0.36)	11.20 (0.30)
No. of tillers	S	2.70 (0.12)	2.08 (0.13)	1.62 (0.11)	1.51 (0.14)	
	W		1.00 (0.09)	1.14 (0.09)	1.15 (0.11)	1.70 (0.09)
Plant height(cm)	S	45.50 (1.50)	42.22 (1.57)	40.38 (1.15)	35.85 (1.09)	
	W		44.40 (1.34)	46.62 (1.62)	49.75 (1.49)	52.31 (1.03)
SDW (gr per pot)	R 1781	7.20 (0.14)	4.50 (0.18)	2.32 (0.17)	0.90 (0.07)	
	W		3.40 (0.15)	7.00 (0.16)	9.60 (0.23)	10.80 (0.24)
No. of tillers	R 1781	2.45 (0.14)	2.60 (0.13)	1.49 (0.12)	0.98 (0.07)	
	W		1.48 (0.13)	1.35 (0.09)	1.62 (0.12)	1.80 (0.13)
Plant height (cm)	R 1781	36.31 (1.34)	34.15 (1.32)	31.30 (1.21)	30.40 (1.04)	
	W		46.40 (1.41)	49.38 (1.21)	51.60 (1.32)	54.45 (1.43)
SDW (gr per pot)	R 2041	8.1 (0.16)	5.22 (0.16)	3.2 (0.20)	1.5 (0.16)	
	W		2.8 (0.16)	6.4 (0.16)	8.7 (0.18)	11.4 (0.16)
No. of tillers	R 1781	2.88 (0.16)	3.11 (0.17)	3.20 (0.23)	2.30 (0.11)	
	W		1.34 (0.13)	1.18 (0.09)	1.68 (0.16)	2.03 (0.12)
Plant height (cm)	R 1781	42.22 (1.30)	37.83 (0.99)	34.00 (0.96)	32.50 (0.98)	
	W		49.40 (1.68)	52.44 (1.90)	54.13 (1.34)	56.75 (1.18)

The numbers in parentheses represent the standard errors of the means.

resistance mechanisms. Earlier studies on ACCase herbicide-resistant rigid ryegrass and blackgrass indicated lack of significant difference on the competitiveness between resistant populations with the ACCase 1781-Leu (R-1781) mutation and populations without this mutation (Menchari et al., 2008; Vila-Aiub et al., 2015b). Such studies highlight the need of the detailed knowledge of resistance mechanisms and of any mutations in order to correlate them with fitness cost data.

Our results revealed a significant fitness cost in terms of plant growth and seed production of a R-1781 rigid ryegrass population and this finding is not in agreement with results of previous studies (Vila-Aiub et al., 2005; Yu et al., 2010). Furthermore, Vila-Aiub et al. (2005) correlated fitness cost with increased herbicide metabolism (HM), while there was any further fitness cost in plants with both increased HM and R-1781 mutation.

In our study, S plants grew faster than R-1781 seedlings, matured, and flowered earlier and eventually produced more tillers and more seeds than the R-1781 plants. There was a tradeoff between tiller and seed production on the one hand and seed weight and seedling emergence on the other hand. Moreover, this is the first study with herbicide-resistant plants showing that different mutation can be responsible for the different competitive ability of R plants (2041-Asn plants exhibit better fitness ingredients than S and R-1781-Leu ACCase counterparts in some traits).

So far, only a few studies have been conducted on the evaluation of fitness costs associated with 2041-Asn ACCase. A R-2041 biotype of *Alopecurus myosuroides* showed a fairly decreased enzyme activity (Délye et al., 2003). R-2041 has been observed in two grasses species: ryegrass (Délye et al., 2003; Zhang and Powles, 2006; Yu et al., 2007) and wild oat (Liu et al., 2007). The results of the present study also confirmed that there was any fitness cost on plant growth or seed production connected with R-2041 in *L. rigidum* plants grown in competition with *Triticum aestivum*.

According to our results, seed size in the susceptible biotype was smaller than that of the resistant biotypes and this can result to lower seed survival and lower emergence rate especially for seeds sown at the 6–8 cm depth. This observation can be of high ecological importance, since it seems that R biotypes in some cases do not have any fitness penalty but also can germinate and emerge even from higher soil depths. On the contrary, the lower emergence potential of seeds of S populations is counterbalanced by their higher seed production. In previous studies on the identified R-1781 ACCase mutation, it was shown that the R biotype had slower germination than S plants (Vila-Aiub et al., 2015b).

The present study provides some valuable information that will be useful for predicting the evolutionary dynamics of resistant populations and also for devising appropriate resistance management strategies. More studies on additional R and S rigid ryegrass biotypes need to be conducted, since previous studies on *L. rigidum* have found that high variability occurred in R and S biotypes regarding their relative growth and phenological development (Gill et al., 1996). Furthermore, since these experiments were conducted under well-watered conditions in a greenhouse, extrapolation of the results under field conditions may be limited and should be clearly investigated. In all cases, evaluation of fitness costs associated with herbicide- resistance genes is useful to parameterize modeling herbicide resistance (Gressel and Segel, 1990; Maxwell et al., 1990; Yanniccari et al., 2016) and provide an evolutionary frame for answering basic questions about the fitness cost in plants (Vila-Aiub et al., 2009, 2015a,b). Eventually, evaluation of fitness cost status associated with ACCase resistance in *Lolium rigidum* may be exploited in terms of the development weed management strategies.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Effects of Environmental Conditions on the Fitness Penalty in Herbicide Resistant *Brachypodium hybridum*

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Herbicide-resistance mutations may impose a fitness penalty in herbicide-free environments. Moreover, the fitness penalty associated with herbicide resistance is not a stable parameter and can be influenced by ecological factors. Here, we used two *Brachypodium hybridum* accessions collected from the same planted forest, sensitive (S) and target-site resistance (TSR) to photosystem II (PSII) inhibitors, to study the effect of agro-ecological parameters on fitness penalty. Both accessions were collected in the same habitat, thus, we can assume that the genetic variance between them is relatively low. This allow us to focus on the effect of PSII TSR on plant fitness. S plants grains were significantly larger than those of the TSR plants and this was associated with a higher rate of germination. Under low radiation, the TSR plants showed a significant fitness penalty relative to S plants. S plants exhibiting dominance when both types of plants were grown together in a low-light environment. In contrast to previous documented studies, under high-light environment our TSR accession didn't show any significant difference in fitness compared to the S accession. Nitrogen deficiency had significant effect on the R compared to the S accession and was demonstrated in significant yield reduction. TSR plants also expressed a high fitness penalty, relative to the S plants, when grown in competition with wheat plants. Two evolutionary scenarios can be suggested to explain the coexistence of both TSR and S plants in the same habitat. The application of PSII inhibitors may have created selective pressure toward TSR dominancy; termination of herbicide application gave an ecological advantage to S plants, creating changes in the composition of the seed bank. Alternatively, the high radiation intensities found in the Mediterranean-like climate may reduce the fitness penalty associated with TSR. Our results may suggest that by integrating non-herbicidal approaches into weed-management programs, we can reduce the agricultural costs associated with herbicide resistance.

Keywords: fitness cost, herbicide resistance, photosystem II inhibitors, *psb*A gene, seed bank, weed control

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INTRODUCTION

Since plants were first domesticated ~10,000 years ago, crop plants have been exposed to recurrent infestations by weed plants. To date, weeds are the most important biotic factor affecting agriculture production and causing yield losses in various crops worldwide [e.g., *Corylus avellana* (Kaya-Altop et al., 2016), *Zea mays* (Soltani et al., 2016) and *Oryza sativa* (Chauhan and Johnson, 2011; Chauhan and Opena, 2012)]. Chemical control using herbicides is considered the most

cost-effective and efficient method of weed management. However, the use of routine herbicide applications to reduce weed infestations also imposes continuous selective pressure on diverse weed populations and can lead to the evolution of herbicide resistance (Jasieniuk et al., 1996; Kaundun et al., 2012; Busi and Powles, 2013). Herbicide resistance had been reported in more than 240 weed species, including resistance to almost every known herbicidal mode of action (MOA; Heap, 2016).

Photosystem II (PSII) inhibitors include several herbicide chemistries (e.g., triazine, triazinone, and substituted urea). Herbicides that work via this MOA compete with the plastoquinone B (PQ_B) at the PQ_B binding site located on the D_1 protein of the PSII complex enzyme (Arntzen et al., 1982), causing the formation of free radicals, which lead to plant death (Fuerst and Norman, 1991). Due to their effectiveness, PSII inhibitors are routinely used for weed control in agro-systems, forests, and roadsides. The first reported case of resistance to a PSII inhibitor in 1970 involved resistance to simazine in common groundsel (*Senecio vulgaris*; Ryan, 1970). Since then, resistance to PSII inhibitors has become widespread all over the world; 231 cases of resistance have been reported for atrazine alone (Heap, 2016). Mechanisms of resistance to PSII inhibitors in weeds typically involve an altered target site (TS; Heap, 2014). Several different point mutations in the *psb*A gene have been showed to confer TSR to PSII inhibitors (e.g., Hirschberg and McIntosh, 1983; Mengistu et al., 2005; Park and Mallory-Smith, 2006; Mechant et al., 2008; Perez-Jones et al., 2009; Thiel and Varrelmann, 2014). TS resistance is related to chloroplastic gene (*psb*A), which lead to maternally inheritance resistance (e.g., Plowman et al., 1999). Non-target site resistance to PSII inhibitors is less abundant, but there are some cases that have been reported over the years. Both chlorotoluron and isoproturon resistance was found to be related to enhanced metabolic activity [e.g., *Alopecurus myosuroides* (Hyde et al., 1996) and *Phalaris minor* (Singh et al., 1998), respectively].

Plants' adaptation to environmental conditions is characterized by the selection of a natural population toward a phenotype that best suits the prevailing environmental conditions (Fisher, 1930). Moreover, alleles that confer higher adaptive value in one environment may have a detrimental impact on fitness in another environment (Orr, 2005). Fitness penalty under herbicide-free environments as result of gerbicide-resistance mutations was reported for several MOA's such as acetyl-CoA carboxylase (Vila-Aiub et al., 2005), 5-enolpyruvylshikimate-3-phosphate synthase (Yanniccari et al., 2016) and photosystem II (Benyamini et al., 1991; Matzrafi et al., 2014) inhibitors. This can be associated with the fact that it alters the natural function of important biological processes in the cell (Ahrens and Stoller, 1983; Vermaas and Arntzen, 1983). The fitness penalty associated with resistance to PSII inhibitors is not a fixed parameter and its magnitude is influenced by ecological factors such as radiation (Holt and Radosevich, 1983), temperature (Vencill et al., 1987), inter-accession competition (Conard and Radosevich, 1979) and inter-species competition (Williams et al., 1995). It has also been suggested that fitness penalties may be more evident under stressful environmental conditions (Vila-Aiub et al., 2009).

The fitness penalty associated with TSR to PSII inhibitors has been reported to involve different physiological and biochemical aspects, such as significantly reduced photosynthetic potential (Holt et al., 1981; Ahrens and Stoller, 1983), reduced vegetative growth (e.g., Holt, 1988), delayed flowering (e.g., Beversdorf et al., 1988), reduced reproductive potential (e.g., Weaver and Warwick, 1982), decreased competitive ability (e.g., Conard and Radosevich, 1979) and more damage from photo-inhibition (e.g., Sundby et al., 1993). The magnitude of the fitness penalty associated with resistance to PSII inhibitors implies that in an herbicide-free environment, there will be strong selective pressure against mutations in the *psb*A gene. This subject had been modeled (Gressel and Segel, 1990) and validated in several different studies (Benyamini et al., 1991; Sibony and Rubin, 2003b).

Brachypodium, a Mediterranean temperate winter wild grass, has emerged as an attractive experimental model species for biotic and abiotic stress (Fursova et al., 2012; Tripathi et al., 2012; Benavente et al., 2013; Matzrafi et al., 2014; Shaar-Moshe et al., 2015). Recently, we identified a *Brachypodium hybridum* population in a planted forest that includes both individuals that are sensitive (S) to PSII inhibitors and individuals that exhibit TSR to those herbicides (Matzrafi et al., 2014). Here, we used two accessions, each exhibiting one of these phenotypes, to study the effect of ecological parameters on the fitness penalty associated with this TSR. By using accessions from the same habitat, we were able to minimize the genetic variation within the experiment (Cousens et al., 1997) and emphasize the fitness penalty associated with PSII TSR.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Sensitive (BrI-638, **S**) and resistant (BrI-637, **TSR**) *Brachypodium hybridum* accessions from the BrI Collection (Matzrafi et al., 2014) were used in this work. Both accessions were collected in the same habitat (planted forest), were atrazine was applied as a conventional practice to assist in the young trees establishment. Seeds from each accession were germinated in plastic trays (50 cm × 25 cm × 5 cm) filled with growth mixture (Tuff Marom Golan, Israel). The trays were placed in a dark, cold room (18°C) to break the seeds' dormancy until germination. After emergence, young seedlings were transplanted as specified for each experiment below.

Herbicide Dose Response

Seedlings of the S and R accessions were transplanted into 0.2-L pots (6 cm × 6 cm × 6 cm), one per pot, filled with growth mixture (Tuff Marom Golan, Israel) and grown in an environmentally controlled chamber (16/10°C, day/night). Thirty-day-old plants (3–4 leaves) were exposed to increased rates (0, 1/8, 1/4, 1/2, 1, 2, 4, 8, 16, 32 and 64 kg ha^{-1}) of atrazine (Atranex® 50% SC, ADAMA-Agan, 50% active ingredient) $-X = 1000$ g ha^{-1}, using a chain-driven sprayer delivering 300 L ha^{-1}, with four replicates. Twenty-one days after treatment (DAT), all of the aboveground tissue was harvested,

oven-dried (80°C, 48°h) and weighed to obtain shoot dry weight (DW) data. Relative values were calculated by dividing the DW of the treated shoots by that of the control shoots.

Phenotypic Measurements

Seedlings of the S and R accessions were transplanted into 0.2-L pots filled with growth mixture (one per pot) and grown in a net house under approximately 90% radiation (100% = 1000–1100 μmol m^{-2} s^{-1}), a day/night temperature of 20/9°C and short-day (10 h of light) conditions. For the analysis of chlorophyll content, the flag leaf (10 mg) was sampled 31 days after transplanting and immersed in 2 mL of N,N-dimethylformamide in the dark for 48 h at 4°C. The absorbance of the supernatant at 647 and 664 nm was measured using a spectrophotometer (ST-VS-723, LAB-KITS, Hong Kong) and chlorophyll *a* and *b* concentrations were calculated as described in Moran (1982). Plants were harvested 79 days after transplanting. At that point, all aboveground tissue was harvested, oven-dried (80°C, 48 h) and weighed to obtain shoot DW data.

Grain Shape and Emergence Rate

Subsamples of the S and TSR accessions (50 seeds per subsample) were weighed using an analytic scale (ED124S, Sartorius Weighing Technology GmbH, Germany), to obtain grain weight (GW) data. Grains were individually scanned using a flatbed scanner (HP Scanjet G2710, HP, USA) and grain length (GL), width (Gwid) and area (GA) were measured using the SmartGrain software (Tanabata et al., 2012). Emergence rates were then analyzed by planting 10 uniform seeds of each accession (S and TSR) 1 cm deep in 0.4-L pots (12 cm × 7 cm × 4 cm) filled with growth mixture. Ten pots of each accession were placed in a dark, cold room (18°C). Seedling emergence was recorded daily over 14 days.

Effects of Radiation and Competition on Plant Productivity

Seedlings of the S and TSR accessions were transplanted into 4.5-L pots (26 cm × 16 cm × 11 cm), one per pot, filled with a mixture of 80% field brown-red degrading sandy soil (Rhodoxeralf; 76% sand, 8% silt and 16% clay) and 20% growth mixture at high density level that mimics non-agricultural natural conditions (480 plants/m^2, 20 plants per pot). The pots were placed in a phytotron under short-day conditions (10 h of light) and temperatures of 16/10°C (day/night) for 48 days and then subjected to long-day conditions (14 h of light) at 22/16°C, to mimic the natural *Brachypodium* growing conditions in the Mediterranean climate. Experiments were organized in a two-factorial completely randomized design with four replicates for each treatment. Different radiation levels; 100% (control) and 40% (low), were used in this experiment. The low radiation level was achieved using a black shading net. Competition ability was examined either intra-accession (by transplanting plants from each accession separately) or inter-accession (by growing them in a mixture in the same pot). Plants were harvested 113 days after transplanting and tillers and spikes number were counted. All aboveground biomass was harvested and oven-dried (80°C for 48 h) and shoot DW was determined.

Effect of Nitrogen Conditions on Plant Productivity

S and TSR seedlings were hydroponically grown in 15-L plastic tanks (35 cm × 29 cm × 15 cm) containing nutrient solutions (Supplementary Table S1) with two levels of nitrogen: 100% (control) and 6% (The lowest level that can be accurately measured in the system). The hydroponic solutions were continuously aerated and replaced every 7 days. Ten replicates of each treatment, with 16 plants in each plastic tank, were placed in a climate-controlled greenhouse (22/15°C day/night) under short-day conditions (10 h of light), in a completely randomized design. The plants were harvested at 56 days after transplanting, tillers and spikes were counted and all aboveground biomass was harvested, oven-dried (80°C for 48 h) and weighed to obtain shoot DW data.

Effect of Competition with Wheat Plants on *B. hybridum* Productivity

S and TSR *B. hybridum* seedlings and bread wheat (*Triticum aestivum*, cv. Zahir) seedlings were transplanted into 15-L boxes (35 cm × 30 cm × 15 cm) filled with a mixture of 80% soil and 20% growth mixture. Each *B. hybridum* accession was arranged either alone (intra-accession competition) or together with wheat with equal number of plants (inter-species competition). A total of 28 plants were grown per box (to mimic normal field density of 266 plants/m^2) in four replicates for each treatment, when both species were placed in the same box they were transplanted in a mixture. Plants were grown in a climate-controlled greenhouse (22/15°C day/night) under long-day (14 h of light) conditions, in a completely randomized design. At 60 days after transplanting, the numbers of tillers and spikes were recorded and all aboveground biomass was harvested, oven-dried (80°C for 48 h) and then weighed.

Statistical Analyses

JMP Pro ver. 12 software (SAS Institute Inc., Cary, NC, USA), was used for all statistical analyses. Differences between two treatments were examined using Student's *t*-test at a significance level of $P \leq 0.05$. Analysis of variance (ANOVA) was performed to examine the effect of each single variable and interaction term. Dose-response curves were constructed by plotting the shoot DW data (21 DAT) for the different accessions as a percentage of that of the untreated control. These data were analyzed using SigmaPlot (ver. 10) software (Systat Software Inc., San Jose, CA, USA) and ED_{50} (herbicide rate reducing shoot FW by 50%) values were extracted. A non-linear curve model (sigmoidal logistic, three parameters; Seefeldt et al., 1995) was adjusted to analyze the effects of the tested herbicides in the different experiments.

$$Y = \frac{a}{1 + \left(\frac{x}{X_0}\right) b}$$

In the model, if $b > 0$, then a describes the upper limit of Y. $X_0 = ED_{50}$ and b describes the slope of the curve in ED_{50}. The resistance index (RI) was calculated as the ratio of the ED_{50} value of the resistant accession to the ED_{50} of the sensitive accession.

RESULTS

Plant Fitness Following the Application of Atrazine

Brachypodium hybridum accession BrI-637, previously shown to carry a mutation ($A_{790}\rightarrow$ G) in the chloroplast gene *psb*A (**TSR,** resistant), and BrI-638, which carries the WT gene (**S**, sensitive), (Matzrafi et al., 2014) were characterized for their response to PSII inhibitors. While the S accession was fully controlled by a 1/2X dose of atrazine, the TSR accession survived up to 64X of the recommended dose (**Figure 1A**; Supplementary Table S2; Supplementary Figure S1). Following herbicide application, the shoot DW of the R accession was significantly greater than that of the S accession, as assessed in terms of ED_{50} values (8.124 kg ha^{-1} vs. 0.161 kg ha^{-1}, respectively).

Plant Fitness under Natural Conditions in an Herbicide-Free Environment

The two *B. hybridum* accessions were grown in a nethouse under Mediterranean-winter conditions (i.e., vegetative growth under short-day conditions, followed by long-day conditions to induce flowering). The TSR accession began to flower 7 days later than the S accession (85 days vs. 92 days, respectively; **Figure 1B**; Supplementary Table S3). The shoot DW of the TSR accession was significantly lower than that of the S accession (0.82 g $plant^{-1}$ vs. 1.05 g $plant^{-1}$, respectively; **Figure 1C**). An analysis of flag-leaf chlorophyll content revealed significantly ($P < 0.0001$) higher chlorophyll *a* and *b* content in the S accession, as compared to the TSR accession (2.52 mg g^{-1} vs. 1.74 mg g^{-1} and 0.78 mg g^{-1} vs. 0.53 mg g^{-1}, respectively; Supplementary Table S4). Notably, the chlorophyll *a/b* ratio of both accessions was similar (3.26 vs. 3.34, S and TSR accessions, respectively).

Grain Shape and Emergence Rate

A time course of seed emergence revealed the significantly faster and greater emergence rate of the S accession, as compared with the TSR accession (**Figure 1D**). At the end of the experiment (14 days after transplanting), 84% of the S seeds had emerged, with only 20% emergence observed among the TSR seeds (**Figure 1E**).

Previous studies involving various plant species have reported a positive correlation between fitness penalties and reproductive biomass (Ahrens and Stoller, 1983; Holt, 1988). In this study, the grains of the S accession were larger than those of the TSR accession (**Figures 2A–D**). S grains showed a significant advantage over the R grains in terms of total weight (4.01 mg vs. 3.53 mg), area (6.96 mm^2 vs. 5.96 mm^2), length (7.07 mm vs. 6.67 mm) and width (1.26 mm vs. 1.15 mm; **Figures 2C,D**; Supplementary Table S5).

Effect of Environmental Conditions on the Ecological Fitness Penalty

Solar radiation is a key element in the plant's photosynthetic productivity. When both accessions were grown in a controlled environment (in the phytotron) under natural level of light intensity (i.e., 100%), both accessions showed similar shoot DW (**Table 1**). When grown under low-light (i.e., 40%) conditions that mimic a cloudy environment, the TSR accession exhibited significantly ($P = 0.015$) less shoot DW, as compared with the S accession (0.56 g vs. 0.79 g, respectively; **Table 1**).

Nitrogen deficiency has a crucial effect on photosynthetic capacity and carbon fixation in plants (Khamis et al., 1990). Significant differences in shoot DW were observed between S and TSR plants grown hydroponically in nitrogen-rich (100%) and nitrogen-poor (6%) solutions. In the presence of an adequate (100%) nitrogen supply, TSR plants developed less shoot DW than S plants. The same trend was observed under deficient nitrogen conditions (6%). Another parameter of plant productivity is the number of spikes produced by the plant. Relative to the adequate (100%) nitrogen concentration, it appears that nitrogen deficiency (6%) had a greater effect on the production of spikes among the R plants (2 vs. 0.6, 100% vs. 6%, respectively) than among the S plants (9.9 vs. 6.6, 100% vs. 6%, respectively) plants (**Table 2**).

Competition between Weed Species

Competition among plants of a single species can be divided into two types: intra-accession competition and inter-accession competition (Wiederholt and Stoltenberg, 1996). We examined both types of competition to gain a better understanding of the interaction between S and TSR plants under field conditions. Under intra-accession competition, plants from both S and TSR accessions had similar shoot DW levels (1.06 g vs. 0.91 g, respectively). In contrast, under inter-accession competition, TSR plants showed significantly less shoot DW than S plants (0.88 vs. 1.35, respectively; **Figure 3**; **Table 1**). Similar trends were noted for other parameters such as number of tillers and number of spikes (Supplementary Table S4).

Competition between Wild Weeds and a Domesticated Crop

Competition between two different species in the same habitat can significantly affect plant fitness. We investigated the competition of a wild weed (*B. hybridum*) with a domesticated cereal crop (*T. aestivum*) bred for uniformity and productivity (Borlaug, 1983). Comparisons of the S and TSR accessions grown in competition with wheat showed that all growth parameters of both accessions were affected by that competition (**Figure 4**). Examination of the agro-ecological performance of the S and TSR accessions revealed that the TSR plants were more negatively affected by competition than the S plants. Significantly less biomass (0.19 g vs. 0.33 g), shorter plants (30.7 cm vs. 44.6 cm) and fewer tillers (2.46 vs. 2.94) were observed among

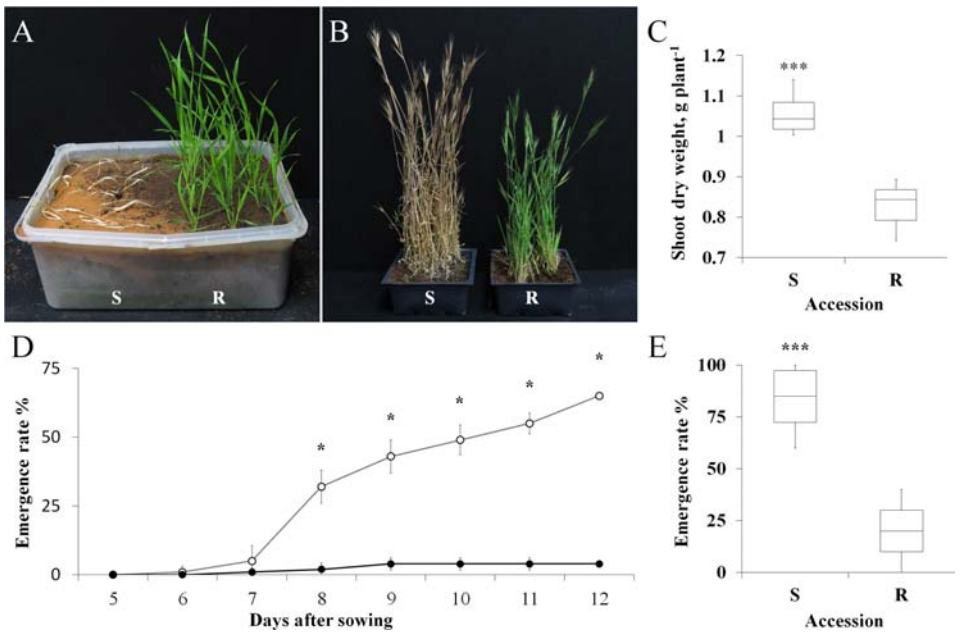

FIGURE 1 | (A) A representative picture of sensitive (S, BrI-638) and resistant (TSR, BrI-637) *Brachypodium hybridum* accessions, 14 days after the application of atrazine (1 kg ha^{-1}). **(B)** A representative picture of S and TSR plants grown in an herbicide-free environment, 130 days after transplanting. **(C)** Shoot dry weights of S and TSR accessions grown in an herbicide-free environment. **(D)** Time course showing the emergence rates of the S (open circles) and TSR (close circles) accessions. **(E)** Boxplot of the emergence rate of S and TSR accessions 60 days after sowing. Data are 1st, 2nd and 3rd quartiles and the minimum and maximum values among all of the data ($n = 10$). * and *** indicate significant differences between accessions as determined by Student's *t*-test at $P \leq 0.05$ and $P < 0.001$, respectively.

the TSR plants, as compared to the S plants, when each accession was grown in competition with wheat (**Figure 4**; Supplementary Table S6). The wheat plants showed similar levels of growth and productivity when grown in competition with the S and TSR weeds (**Figures 4C–E**; Supplementary Table S6).

DISCUSSION

Photosystem II inhibitors have been used as primary herbicides to control weeds in agro-ecological systems since the 1950s'. Over-use and misapplication have resulted in the evolution of increasing numbers of resistant weeds (Heap, 2016). Most cases of TSR to PSII inhibitors involves a single substitution of Gly_{264} (Heap, 2014). The fitness penalty caused by this mutation has been demonstrated in various resistant biotypes (Sundby et al., 1993; Arntz et al., 2000), as in the current study (**Figure 1B**). Reductions in photosynthetic efficiency (Ireland et al., 1988; Arntz et al., 2000), reproductive ability (Conard and Radosevich, 1979) and seed production (Park and Mallory-Smith, 2006) have all been reported to be correlated with PSII TSR.

Light intensity is a major factor limiting photosynthesis, affecting carbohydrate production and eventually growth (Mauseth, 2014). It has been suggested that under both low (Schonfeld et al., 1987) and high-light intensities (Hart and Stemler, 1990; Alfonso et al., 1996), the fitness of the mutated plants (TSR) is reduced relative to S plants. Accordingly, under lower radiation (400–450 μmol m^{-2} s^{-1}), the S accession exhibited a significant advantage in biomass (74% vs. 61%, respectively; **Table 1**). The chlorophyll content of the R plants was less than that of the S plants, corresponding to the observed differences in biomass. However, in our high-radiation (∼1000 μmol m^{-2} s^{-1}) treatment, there were no significant differences between the two accessions in terms of biomass. This experiment was repeated four times to validate these results.

Nitrogen is important for the production of chlorophyll and photosynthesis (Vos et al., 2005). While the S accession exhibited an advantage in terms of biomass production under control and low N conditions, the two accessions reacted similarly to the low-N treatment (82% of the control biomass vs. 79% of the control biomass for the S and TSR accessions, respectively; **Table 2**). These results suggest that the fitness penalty of the TSR accession can be associated with the efficiency of the photosynthetic apparatus (due to structural modification of the D1 protein), and less with chlorophyll density.

In natural environment, plants compete for resources such as water, light, and space. Vigorous plants that use resources more efficiently will eventually produce more seeds and dominate the population. When TSR and S accessions were grown together (inter-accession competition), the S biomass increased and the TSR biomass decreased, as compared to the biomass levels observed when the accessions were grown apart from one another (intra-accession competition; 1.35 g vs. 1.06 g and 0.88 g vs. 0.91 g, for the S and R, respectively; **Table 1**). Our results, as well as theoretical models (Gressel and Segel, 1990), indicate

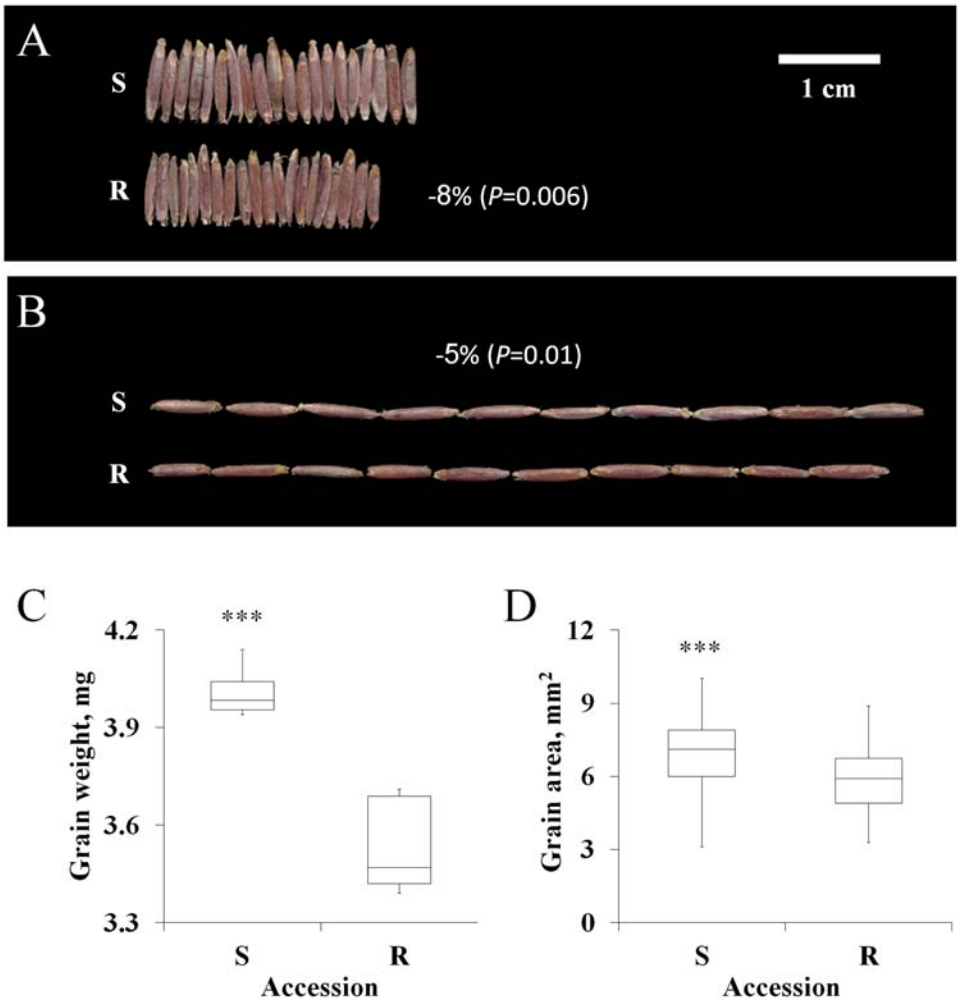

FIGURE 2 | Characteristics of the grain of the sensitive (S, BrI-638) and resistance (TSR, BrI-637) *Brachypodium hybridum* accessions. **(A)** A representative picture showing grain width ($n = 20$) and **(B)** grain length ($n = 10$). **(C)** Boxplot of the grain weight and **(D)** grain area of the S and TSR accessions. Data are 1st, 2nd and 3rd quartiles and the minimum and maximum values among all of the data ($n = 10$ and 50, respectively). *** indicates significant differences, as determined by Student's *t*-test at $P < 0.001$.

that in herbicide-free environments, the proportion of R plants in a population will decrease due to their low productivity in a highly competitive environment. Likewise, reductions of more than 30% in the reproductive ability of PSII TSR *Senecio vulgaris* and *Bromus tectorum* (Holt, 1988; Park and Mallory-Smith, 2005) were observed when those plants were grown in competition with S plants of the same species.

Following the examination of the competition between the TSR and S accessions, we also examined their relative fitness in a situation involving inter-species competition (*B. hybridum* / *T. aestivum*), to model an actual agricultural system. When these plants were grown in competition with wheat, reduced fitness penalties was observed for all of the examined growth parameters (biomass: −33% S vs. −55% TSR, height: −9% S vs. −25% TSR, number of tillers: −51% S vs. −56% TSR, respectively; **Figure 4**; Supplementary Table S6). Herbicide applications are usually carried out during the periods in which crop plants are most sensitive to the damage caused by weeds (Knezevic et al., 2002). In the current study, we observed a substantial reduction in the biomass and height of TSR plants under competitive conditions, which may indicate a lower competitive ability. By using non-herbicidal techniques such as crop competition, we can reduce the frequency of TSR seeds to a negligible level in the seed bank. These results are in accordance with those of other studies that have suggested the rapid extinction of TSR individuals under high field densities (reviewed by Vila-Aiub et al., 2015). One can assume that due to the high fitness penalty found in the current study, the TSR plants would be eliminated from this habitat. The presence of both TSR and S plants in the same habitat can be explained by two scenarios. The first scenario is as follows: The strong selection pressure of repeated PSII application resulted in a population shift that enriched the seed bank with R seeds, giving the R seeds/plants an advantage

TABLE 1 | Analysis of variance of the effect of *Brachypodium hybridum* accessions [sensitive (BrI-638) and resistant (BrI-637)], competition (intra- and inter-accession), and radiation level [high (100%) and low (40%)] on dry weight biomass production, number of tillers and number of spikes, under an herbicide-free environment.

Accession	Competition	Radiation	Dry weight, g	No. of tillers	No. of spikes
BrI-638	Intra-accession	High	1.06 ± 0.03	5.3 ± 0.21	5.8 ± 0.34
BrI-637	Intra-accession	High	0.91 ± 0.09	4.6 ± 0.48	5.0 ± 0.46
BrI-638	Intra-accession	Low	0.79 ± 0.03*	3.8 ± 0.29	3.9 ± 0.28*
BrI-637	Intra-accession	Low	0.56 ± 0.08	3.1 ± 0.21	3.5 ± 0.13
BrI-638	Inter-accession	High	1.35 ± 0.11**	6.7 ± 0.36***	6.9 ± 0.34**
BrI-637	Inter-accession	High	0.88 ± 0.05	4.8 ± 0.17	5.0 ± 0.14
Source	**d.f.[1]**			**Sum of square**	
Accession (A)	1		0.668***	7.031***	8.168***
Competition (C)	1		0.088	0.587	1.918*
Radiation (R)	1		0.890***	30.681***	26.584***
A × C	1		0.078	1.253	0.834
A × R	1		0.004	1.125	0.459
C × R	1		0.005	0.681	0.945
A × C × R	1		0.031	0.347	0.543
Error	24		0.693	7.431	7.715
Total	31		2.457***	49.135***	47.166***

[1]*d.f., degrees of freedom. *, **, and ***indicate significance levels of $P \leq 0.05$, $P < 0.01$ and $P < 0.001$, respectively.*

TABLE 2 | Analysis of variance of the effect of *Brachypodium hybridum* accession [sensitive (BrI-638) and resistant (BrI-637)] and level of nitrogen [high (100%) and low (6%)] on dry weight biomass production, number of tillers and number of spikes, in an herbicide-free environment.

Accession	Nitrogen	Dry weight, g	No. of tillers	No. of spikes
BrI-638	High	0.93 ± 0.03***	10.4 ± 0.54	9.9 ± 0.41***
BrI-637	High	0.58 ± 0.03	9.5 ± 0.53	2.0 ± 0.49
BrI-638	Low	0.71 ± 0.02***	8.0 ± 0.19	6.6 ± 0.53***
BrI-637	Low	0.46 ± 0.03	8.1 ± 0.35	0.6 ± 0.24
Source	**d.f.[1]**		**Sum of square**	
Accession (A)	1	0.774***	1.074	400.973***
Nitrogen (N)	1	0.252***	31.112***	44.338***
A × N	1	0.017	1.843	7.871*
Error	30	0.182	49.003	44.775
Total	33	1.355***	85.882***	537.882***

[1]*d.f., degrees of freedom. ***indicate significance differences at levels of $P < 0.001$.*

over the S seeds/plants (Rubin et al., 2004). The herbicide concentration required to control the R accession (BrI-637) was 50-fold higher than that needed to control the S accession (BrI-638; Supplementary Figure S1). As a consequence, under repeated herbicide applications, the TSR accession exhibited greater fitness (**Figure 1A**). Exclusion of these herbicides from the ecosystem (i.e., an herbicide-free environment created a shift toward S seed, due to the strong agro-ecological fitness penalty of the TSR accession. Due to seed bank enrichment with TSR seeds, this transition is still in progress and we are now witnessing the decline of TSR individuals in the population.

The second scenario is as follows: In Mediterranean environments, light intensities are very high (1000–1500 μmol m^{-2} s^{-1}), compensating for the deficiency in the photosynthetic activity of TSR plants. These high levels of radiation can explain the abundance of TSR plants, due to the lower fitness penalty seen under high-light intensities. This can also explain the relative abundance of other PSII TSR mutant plants (e.g., *Conyza canadensis*; Matzrafi et al., 2015), as well as PSII TSR mutants of C_4 plant species such as *Amaranthus retroflexus* (Van Oorschot and Van Leeuwen, 1984) and *Amaranthus blitoides* (Sibony and Rubin, 2003a), whose photosynthetic apparatus is more efficient than that of C_3 plants.

CONCLUSION

Both accessions examined in this study were collected from a planted forest in which multiple applications of PSII

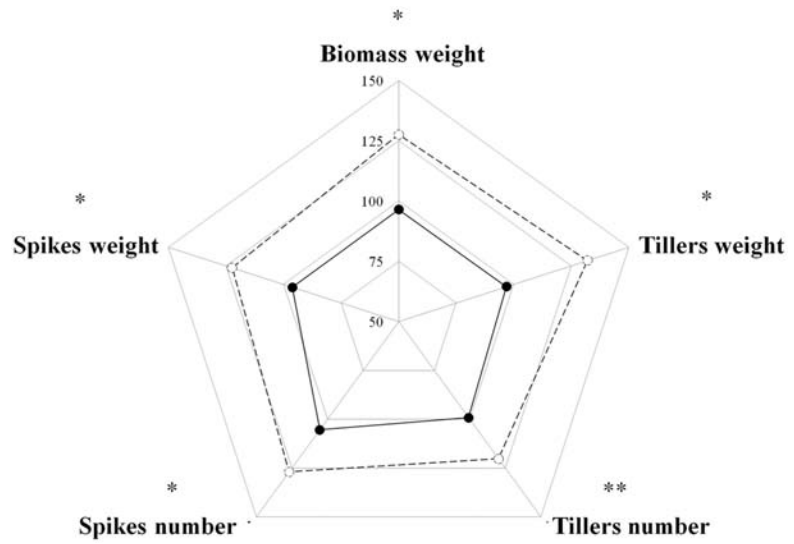

FIGURE 3 | Radar chart comparing the productivity parameters of the sensitive (S, BrI-638, dashed line) and resistant (TSR, BrI-637, full line) *Brachypodium hybridum* accessions ($n = 4$). The data was analyzed using one-way ANOVA followed by Student's *t*-test. * and ** indicate significant differences between accessions; $P \leq 0.05$ and $P < 0.01$, respectively.

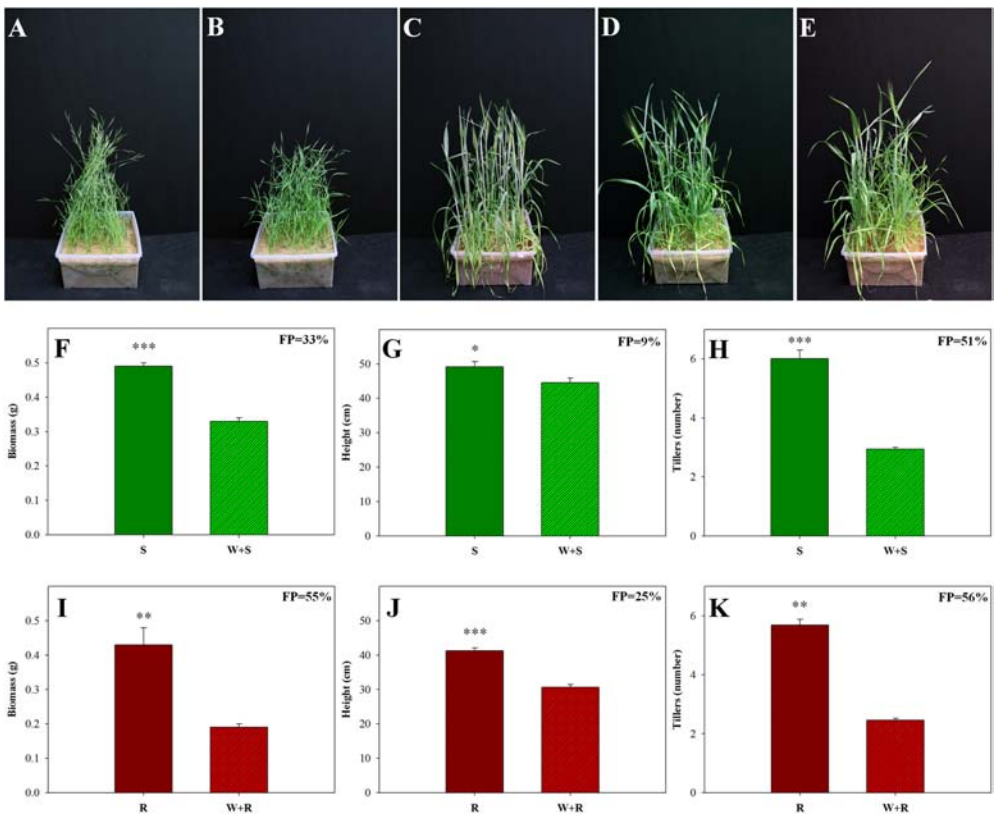

FIGURE 4 | Effect of intra- and inter-species competition between sensitive (S, BrI-638) and resistant (TSR, BrI-637) *Brachypodium hybridum* accessions and bread wheat (W, cv. Zahir) on plant phenology and productivity. Representative pictures of **(A)** S plants, **(B)** TSR plants, **(C)** wheat plants, **(D)** competition between S and wheat plants (S + W) and **(E)** competition between TSR and wheat plants (R + W). Pictures were taken 60 days after transplanting. Comparison between S **(F–H)** and TSR **(I–K)** plants grown in competition with each other and in competition with wheat. **(F,I)** Biomass production, **(G,J)** plant height and **(H,K)** number of tillers. *, **, and *** indicate significant differences between accessions at $P \leq 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

inhibitors were made in the past (to help the young trees establishment), but discontinued at least 20 years ago. The fitness penalty exhibited by TSR plants under competitive conditions can be exploited in efforts to overcome herbicide resistance with non-herbicidal techniques. The development of integrated weed management practices that increase competition such as the use of vigorous cultivars, shading crops and controlled mineral deficiency could help control resistant weeds and contribute to further reductions in their seed production.

AUTHOR CONTRIBUTIONS

EF, MM, BR, and ZP designed the experiments. EF and MM conducted the experiment. EF, MM, BR, and ZP analyzed data and wrote the paper. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00094/full#supplementary-material

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Copy Number Variation in Acetolactate Synthase Genes of Thifensulfuron-Methyl Resistant *Alopecurus aequalis* (Shortawn Foxtail) Accessions in Japan

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Severe infestations of *Alopecurus aequalis* (shortawn foxtail), a noxious weed in wheat and barley cropping systems in Japan, can occur even after application of thifensulfuron-methyl, a sulfonylurea (SU) herbicide. In the present study, nine accessions of *A. aequalis* growing in a single wheat field were tested for sensitivity to thifensulfuron-methyl. Seven of the nine accessions survived application of standard field rates of thifensulfuron-methyl, indicating that severe infestations likely result from herbicide resistance. Acetolactate synthase (ALS) is the target enzyme of SU herbicides. Full-length genes encoding ALS were therefore isolated to determine the mechanism of SU resistance. As a result, differences in *ALS* gene copy numbers among accessions were revealed. Two copies, *ALS1* and *ALS2*, were conserved in all accessions, while some carried two additional copies, *ALS3* and *ALS4*. A single-base deletion in *ALS3* and *ALS4* further indicated that they represent pseudogenes. No differences in ploidy level were observed between accessions with two or four copies of the *ALS* gene, suggesting that copy number varies. Resistant plants were found to carry a mutation in either the *ALS1* or *ALS2* gene, with all mutations causing an amino acid substitution at the Pro197 residue, which is known to confer SU resistance. Transcription of each *ALS* gene copy was confirmed by reverse transcription PCR, supporting involvement of these mutations in SU resistance. The information on the copy number and full-length sequences of *ALS* genes in *A. aequalis* will aid future analysis of the mechanism of resistance.

Keywords: shortawn foxtail, ALS inhibitor, herbicide resistance, target-site resistance, copy number variation

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INTRODUCTION

Herbicides that inhibit acetolactate synthase (ALS) cause depletion of branched chain amino acids such as valine, leucine and isoleucine, leading to plant death (Duggleby et al., 2008; Yu and Powles, 2014). However, recurrent use of these herbicides has resulted in the rapid evolution of herbicide resistance in weeds. Globally, resistance to ALS inhibitors has been reported in 159 weed species

(Heap, 2016). ALS inhibitors are categorized into five groups based on their chemical structure: sulfonylurea (SU), triazolopyrimidine (TP), pyrimidinylthiobenzoate (PTB), imidazolinone (IMI), and sulfonylaminocarbonyltriazolinone (SCT). In Japan, SUs are predominantly used due to their excellent crop safety, broad spectrum of weed control and low toxicity to animals. Thus, most cases of resistance to ALS-inhibiting herbicides reported in Japan, such as *Monochoria vaginalis* (Ohsako and Tominaga, 2007), *Schoenoplectus juncoides* (Uchino et al., 2007), and *Sagittaria trifolia* (Iwakami et al., 2014b), have evolved under SU selection (Uchino et al., 2016).

Resistance of weeds to herbicides is caused by target-site and /or non-target-site resistance mechanisms (Powles and Yu, 2010). Target-site resistance involves alterations to the target site such as overproduction and amino acid substitution of the target protein, while non-target-site resistance includes all other mechanisms such as enhanced herbicide metabolism, restricted herbicide translocation and reduced herbicide uptake. In the case of ALS inhibitor resistance, target-site resistance resulting from amino acid substitution frequently occurs (Yu and Powles, 2014). A substitution at one of eight amino acid residues in the ALS protein sequence of *Arabidopsis thaliana*, Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, or Gly654, was found to cause resistance to ALS inhibitors (Yu and Powles, 2014).

Accumulating evidence further suggests that copy numbers of genes encoding herbicide targets also has an effect on the evolution of herbicide resistance. For example, in a polyploid, a single mutation in one homoeologous copy encoding a target enzyme tends to confer lower levels of herbicide resistance compared to diploid plants carrying the same mutation in a single-copy gene (Yu et al., 2013). The locus of a mutated copy among multiple homoeologous copies of genes encoding herbicide targets could also influence the degree of herbicide resistance (Ostlie et al., 2015). In addition, within-species copy number variation (CNV) was previously observed in the gene encoding the glyphosate target enzyme, 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS), of which a higher copy number was associated with glyphosate resistance (Sammons and Gaines, 2014). It is therefore important to determine copy numbers of genes encoding target enzymes and identify the particular copy carrying a resistance-conferring mutation.

Alopecurus aequalis Sobol. (shortawn foxtail), family Poaceae, is a diploid species distributed throughout Europe, temperate Asia and North America (Cope, 1982). Its strong tillering capacity allows it to out-compete wheat seedlings, causing yield losses of more than 50 % (Guo et al., 2015a). In Japan, *A. aequalis* is a major weed in barley and wheat fields. Since the early 1990s, management has relied on postemergence application of thifensulfuron-methyl, a SU herbicide. However, resistance to thifensulfuron-methyl was confirmed in 2004, after seven consecutive years of herbicide treatment (Uchikawa et al., 2005). Resistance of *A. aequalis* to ALS inhibitors was also recently reported in China (Guo et al., 2015b, 2016; Xia et al., 2015). Although mutations in *ALS* gene causing Pro197Arg, Pro197Thr, or Trp574Leu have been reported, it remains unknown whether these populations are homozygous or heterozygous at the *ALS* loci and which copies of the *ALS* gene carry a mutation. In this study, we therefore determined the full-length sequences and copy numbers of *ALS* genes in *A. aequalis*, and identified mutations in resistant plants thought to be responsible for resistance to thifensulfuron-methyl. CNV in *ALS* genes in *A. aequalis* was also examined.

MATERIALS AND METHODS

Plant Materials

Seeds of nine *A. aequalis* plants (hereafter referred to as accessions) were collected in May 2012 from a ∼1-ha wheat field in Kumamoto City, Kumamoto Prefecture, Japan (**Table 1**). The field was under a rice–wheat cropping system and was severely infested with *A. aequalis* after thifensulfuron-methyl treatment. Single plants of each accession were grown in a greenhouse and self-pollinated once and the seeds assayed for thifensulfuron-methyl sensitivity. Nucleic acids were also extracted from the seedlings for gene cloning and Southern blot analysis. Individual seedlings from each accession were self-pollinated and used for gene expression and genotyping analyses. An *A. japonicus* accession was also used in this study, and was collected in May 2012 from a wheat field in Mifune, Kumamoto Prefecture, Japan.

Thifensulfuron-Methyl Dose-Response Assay

Seeds were germinated on 0.6% agar plates in a growth chamber at 25/15°C (day / night) with a 12 h photoperiod. After germination, six seedlings per accession were transplanted in a cell tray filled with soil and kept at an ambient temperature in a vinyl greenhouse at Kyoto University during winter 2016 (January to March). At the 3–4 leaf stage, plants were treated with a commercial formulation of thifensulfuron-methyl (Harmony, DuPont, Tokyo, Japan) at 0, 1/3, 1 and 3× the recommended rate (75 g a.i. ha^{-1}), respectively. Three weeks after thifensulfuron-methyl application, the dry weights of shoots were measured to compare relative growth among the accessions. The experiment was repeated twice with three replications. Results of a single experiment are shown since they were similar between experiments.

Statistical analyses were performed using square root transformed data. One-way ANOVA with Dunnett's post-test was performed using R version 3.3.1 (R core team, 2016) to determine differences in sensitivity of the Sugi-1 accession with all other accessions.

Isolation and Sequencing of *ALS* Genes

Isolation and sequencing analysis of *ALS* genes was carried out using plants of all nine accessions grown in a greenhouse. Green leaves were harvested at the heading stage, snap-frozen and stored at −80°C until use. RNA was isolated using the RNeasy

TABLE 1 | *ALS* gene copy numbers and mutations in *Alopecurus aequalis* accessions.

Accession	Resistance status*	Copy number status		Substitution at Pro197	
		Copy number	Identified gene	Gene	Substituted amino acid
Sugi-1	S	2	*ALS1, ALS2*	–	–
Sugi-2	R	2	*ALS1, ALS2*	*ALS1*	Ser
Sugi-3	R	2	*ALS1, ALS2*	*ALS2*	Leu
Sugi-5	R	4	*ALS1, ALS2, ALS3, ALS4*	*ALS1*	Leu
Sugi-11	R	4	*ALS1, ALS2, ALS3, ALS4*	*ALS1*	Leu
Sugi-14	R	4	*ALS1, ALS2, ALS3, ALS4*	*ALS1*	Leu
Sugi-18	R	2	*ALS1, ALS2*	*ALS1*	Thr
Sugi-24	S	2	*ALS1, ALS2*	–	–
Sugi-29	R	4	*ALS1, ALS2, ALS3, ALS4*	*ALS1*	Thr

**R, resistant; S, sensitive.*

TABLE 2 | Primers used for 5′- and 3′-RACE cloning of *ALS* genes from *A. aequalis*.

Gene	Primer sequence		
	1st PCR	2nd PCR	3rd PCR
5′-RACE			
ALS1	5′-AGGCTTCCTGAATGACGCGGGGAATG-3′	5′-GGGTGACCTCGACTATGGGCGTCTCT-3′	–
ALS2	5′-AGGCTTCCTGAATGACGCGGGGGATA-3′	5′-GTGACCTCGACTATGGGCGTCTCC-3′	–
3′-RACE			
ALS1	5′-CAATGGAGATCCACCAGGCGCTCACA-3′	5′-CGTCTCCGCGCTCGCCGATGCTCTT-3′	–
ALS2	5′-CAATGGAGATCCACCAGGCGCTCACG-3′	5′-GATCGGCACGGACGCCTTCCAG-3′	–
FPIN PCR (5′ Isolation)			
ALS3, ALS4	5′-GTGATGGAGCGGGTGACCTCCA-3′	5′-TCCACTATGGGCGTCTCCTGGAAAG-3′	5′-GCGTATCCGGACGCCGCGAAG-3′
TAIL PCR (5′ Isolation)			
ALS1	5′-AGGCTTCCTGAATGACGCGGGGAATG-3′	5′-GGGTGACCTCGACTATGGGCGTCTCT-3′	5′-ACCATGGGGATGGAGTCGAGA-3′
TAIL PCR (3′ Isolation)			
ALS3, ALS4	5′-CTCACGCCGCGCTCTCAACTCT-3′	5′-GATCGGCACGGACGCTTTCCAG-3′	5′-GGAGGACATTCCCCGCGTCATC-3′

Plant Mini Kit (Qiagen, CA, USA) and genomic DNA removed using the TURBO DNA-*free* Kit (Life Technologies, CA, USA). Complementary DNA (cDNA) was synthesized from the RNA using the SMART RACE cDNA Amplification Kit (TaKaRa, Otsu, Japan), and extracted using the DNeasy Plant Mini Kit (Qiagen).

Partial *ALS* genes of all accessions were amplified from genomic DNA samples using KOD FX (Toyobo, Osaka, Japan) with primers designed based on *ALS* genes from other grass species: forward primer, 5′-AAGGGCGCSGACATCCT-3′; reverse primer, 5′-ATCTGCTGYTGGATGTCCTT-3′. Amplicons were subjected to direct sequencing using BigDye Terminator V3.1 on a 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). Fragments of *ALS* genes amplified from accessions Sugi-5 and Sugi-24 were cloned into pGEM-T Easy (Promega, Madison, WI, USA) and the inserts sequenced.

The 5′ and 3′ regions of each *ALS* gene fragment from Sugi-5 were determined by Rapid Amplification of cDNA Ends (RACE) according to either the manufacturer's instructions for the SMART RACE cDNA Amplification Kit, Thermal Asymmetric Interlaced (TAIL) PCR (Liu and Whittier, 1995) or fusion primer and nested integrated (FPNI) PCR (Wang et al., 2011). The gene-specific primers used for PCR are listed in **Table 2**. The resulting amplicons were then subjected to direct sequencing or cloned and sequenced as described above. Full-length *ALS* gene sequences were amplified using KOD FX with the primers listed in **Table 3** and the sequences confirmed by direct sequencing.

Phylogenetic Analysis of ALS Protein Sequences

Amino acid sequences of plant *ALS* genes were obtained from GenBank, the MSU Rice Genome Annotation Project[1] or Phytozome v11.0[2], and a phylogenetic tree generated using MEGA6 (Tamura et al., 2013). Sequences were aligned using ClustalW, and the JTT matrix-based method used to compute evolutionary distances.

Analysis of Genetic Inheritance of the *ALS3* and *ALS 4* Genes

Seedlings of accession Sugi-5 were self-pollinated twice using the single-seed descent method in the greenhouse at Kyoto

[1]http://rice.plantbiology.msu.edu/index.shtml
[2]https://phytozome.jgi.doe.gov/pz/portal.html

TABLE 3 | Primers used for amplification of full-length *ALS* genes from *A. aequalis*.

Gene	Forward	Reverse
ALS1	5′-CAATAAAAATCTCATGCCCGT-3′	5′-CATGGTTCACAGTTGACCACA-3′
ALS2	5′-ACGCTCGCATAAAAAGCCA-3′	5′-GTCCTCTAGGTCGAGCTCTTGATT-3′
ALS3 and *ALS4*	5′-CACACACTCAGATAAAAAGCCA-3′	5′-AGGTCGAGCTCTTGCTGAAG-3′

TABLE 4 | Primers used for analysis of *ALS* gene expression.

Gene	Forward	Reverse	Expected band size (bp)
ALS1	5′-GCCAACCCAGGGGTCACA-3′	5′-CATGGTTCACAGTTGACCACA-3′	514
ALS2	5′-CGTTGCAGGGCTTGAACA-3′	5′-GTCCTCTAGGTCGAGCTCTTGATT-3′	786
ALS3 and *ALS4*	5′-GCCAACCCAGGTGTAACC-3′	5′-AGGTCGAGCTCTTGCTGAAG-3′	464

University. DNA was then extracted from shoot tissues using the One Step Method according to instructions provided by the manufacturer of KOD FX polymerase. PCR was conducted using KOD FX with primers designed from conserved regions of the *ALS3* and *ALS4* genes (**Table 3**). The amplicons were then subjected to direct sequencing to determine which sequence is carried in the progeny.

Southern Blot Analysis

Genomic DNA was extracted from shoots of individual plants of single-self-pollinated Sugi-5 and Sugi-24 seedlings using a Plant Genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Ten microgram of DNA was digested with *Eco*RV and *Hind*III (New England BioLabs Japan, Tokyo, Japan), then separated electrophoretically on 1% agarose gels and transferred to positively charged nylon membranes (Roche, Basel, Switzerland). A probe for the *ALS* gene was prepared by PCR using the PCR DIG Probe Synthesis Kit (Roche) with primers (forward, 5′-CTTTTGCAAGCAGGTCCAAG-3′; reverse, 5′-ATCCCCATCAATGTCAACAAC-3′) and the *ALS1* PCR product as a template. Hybridization was conducted according to the DIG Application Manual (Roche) and the signals detected using CDP-Star (Roche) on ChemiDoc Touch (Bio-Rad, Irvine, CA, USA).

Flow Cytometric Analysis

Ploidy levels were analyzed using an Attune Flow Cytometer (Applied Biosystems). *A. aequalis* leaves were chopped using a razor blade in extraction buffer (Galbraith et al., 1983). After filtration using a CellTrics® 20-μm filter (Sysmex, Kobe, Japan), propidium iodide (25 mg/l) was added to stain the DNA. The nuclei suspension was then analyzed by laser excitation at 488 nm.

Transcription of *ALS* Genes

Plants were grown in a growth chamber at 25°C with a 12 h photoperiod for 20 days after germination. Shoot and root RNA was extracted as described above from three Sugi-5 and Sugi-24 plants, respectively. RNA samples (1 μg) were reverse-transcribed using ReverTra Ace (Toyobo) and PCR amplification conducted using rTaq DNA polymerase (Toyobo) with primers listed in **Table 4**. PCR conditions were as follows: 94 °C for 2 min followed by 35 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 30 s, and ending with 72°C for 5 min. The PCR products were run on 1% agarose gel and stained with ethidium bromide. Experiments were conducted twice using RNA samples from distinct individuals. Similar results were obtained in each experiment.

RESULTS

Thifensulfuron-Methyl Sensitivity

Figure 1 shows the results of the thifensulfuron-methyl sensitivity assay in nine *A. aequalis* accessions collected from a severely infested wheat field. Growth of two accessions, Sugi-1 and Sugi-24, was severely affected by application of 1/3× the recommended field rate, with dry weights reaching only 35% that of the untreated control. Moreover, plants of both accessions died under treatment with 1 and 3× the recommended field rate. These accessions were therefore considered thifensulfuron-methyl susceptible. In comparison, the remaining seven accessions survived application of 1× the recommended field rate, achieving dry weights of >60% that of the untreated control. Even under 3× the field rate, these accessions exhibited dry weights ≥50% that of the untreated control, indicating thifensulfuron-methyl resistance. All plants from each accession responded to each dose of thifensulfuron-methyl in a similar manner, with no segregation of resistance.

Isolation and Analysis of *ALS* Genes

Partial fragments of *ALS* genes were amplified from genomic DNA of each accession and subjected to direct sequencing. More than one peak was observed at some nucleotide positions in chromatograms of each accession, suggesting the existence of multiple copies of the *ALS* gene. Interestingly, the chromatogram patterns differed among accessions, with double peaks observed in some accessions, and very sharp single peaks in others (**Figure 2A**), suggesting CNV of the *ALS* genes.

PCR amplification of the 3′ and 5′ regions of the partial sequences resulted in the isolation of two copies of the *ALS* gene (*ALS1* and *ALS2*) from accession Sugi-24 and four

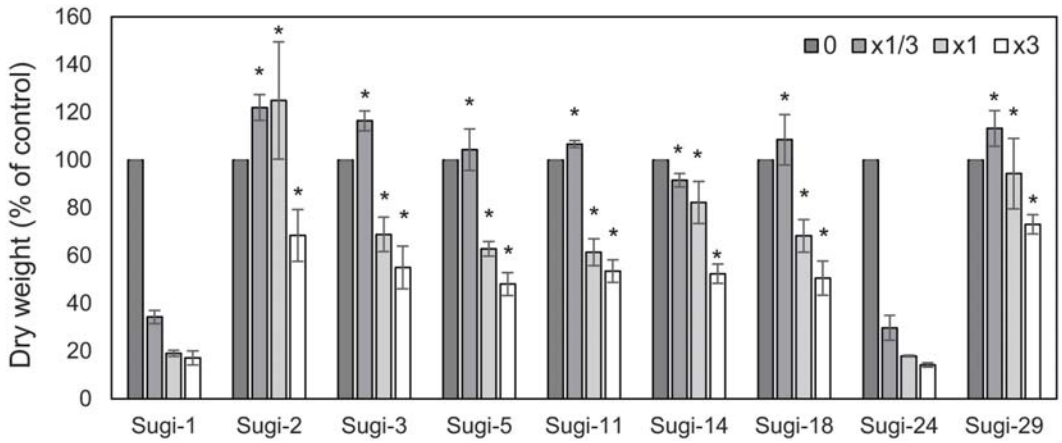

FIGURE 1 | Thifensulfuron-methyl responses of the *Alopecurus aequalis* accessions. Each accession was treated with 0, 1/3, 1, or 3× the recommended field application rate of thifensulfuron-methyl. Shoot dry weights are shown as percentages of untreated controls. Bars indicate the standard error ($n = 3$). Dry weights of accession Sugi-1 were compared with remaining accessions using Dunnett's test. *$p < 0.01$.

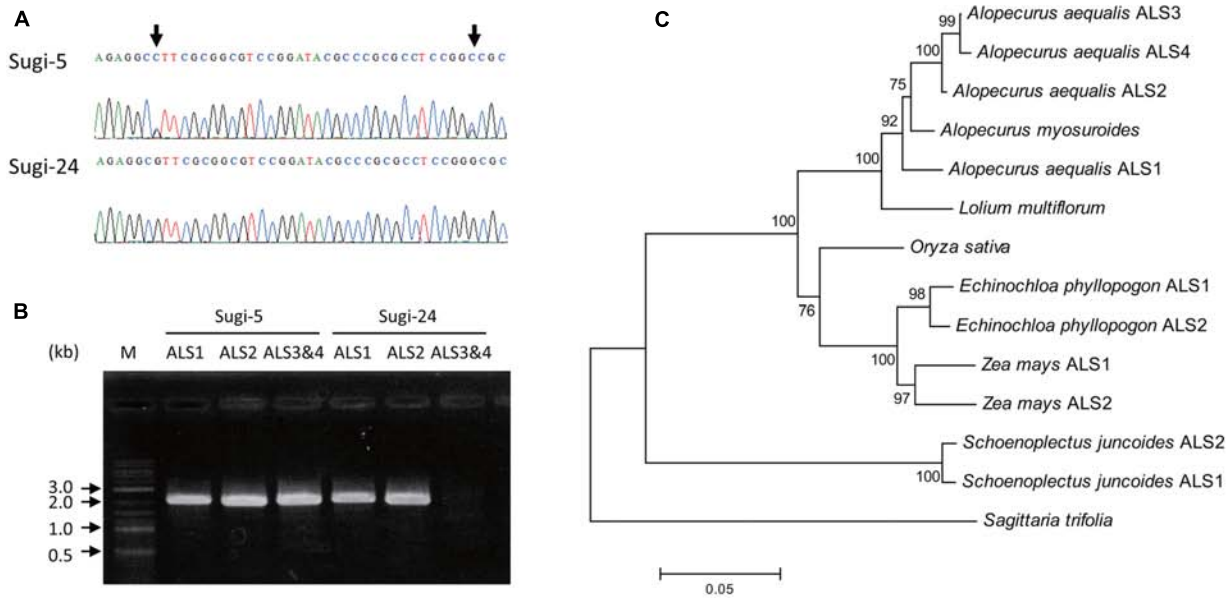

FIGURE 2 | *ALS* genes in *A. aequalis*. (A) Chromatograms of DNA sequences of an *ALS* gene fragment. Arrows indicate double peaks. **(B)** Amplification of full-length *ALS* genes from accessions Sugi-5 and Sugi-24. M, DNA size standard. **(C)** Phylogenetic tree of the ALS. Numbers at each node indicate the reliability of branches estimated by bootstrap analysis with 1,000 replicates. Accession numbers for each sequence are as follows: *Alopecurus myosuroides*, AJ437300; *Lolium multiflorum*, AAG30931.1; *Oryza sativa*, LOC_Os02g30630.2; *Echinochloa phyllopogon* ALS1, AB636580.1; *Echinochloa phyllopogon* ALS2, AB636581.1; *Zea mays* ALS1, GRMZM2G143008_T01; *Zea mays* ALS2, GRMZM2G143357_T01; *Schoenoplectus juncoides* ALS1, BAE97675.1; *Schoenoplectus juncoides* ALS2, BAE97677.1; *Sagittaria trifolia*, AB301496.1. In the case of rice ALS, an additional ALS-like sequence, LOC_Os04g32010.1, was found in the MUS database; however, this sequence was excluded due to its low level of expression and the large number of unconserved residues among grass ALSs.

copies (*ALS1*, *ALS2*, *ALS3*, and *ALS4*) from accession Sugi-5 (accession numbers: LC200800 to LC200803) (**Figures 2, 3**). The double peaks observed in direct sequencing of the Sugi-5 PCR product (**Figure 2A**) therefore represented *ALS3* and *ALS4*. The *ALS2* gene sequence was identical to that of the *A. aequalis ALS* gene sequence previously deposited in GenBank (JQ743908.1). The *ALS3* and *ALS4* genes each contained a single base deletion in a region containing four consecutive Cs at positions +146 to +149 (**Figure 3**), respectively, which would result in aberrant protein sequences. Thus, *ALS3* and *ALS4* are thought to be pseudogenes that share 99.5% identity with only three single nucleotide polymorphisms (SNPs) in the coding sequence (**Figures 2C, 3**). Polymorphisms between *ALS3* and *ALS4* were limited even in untranslated

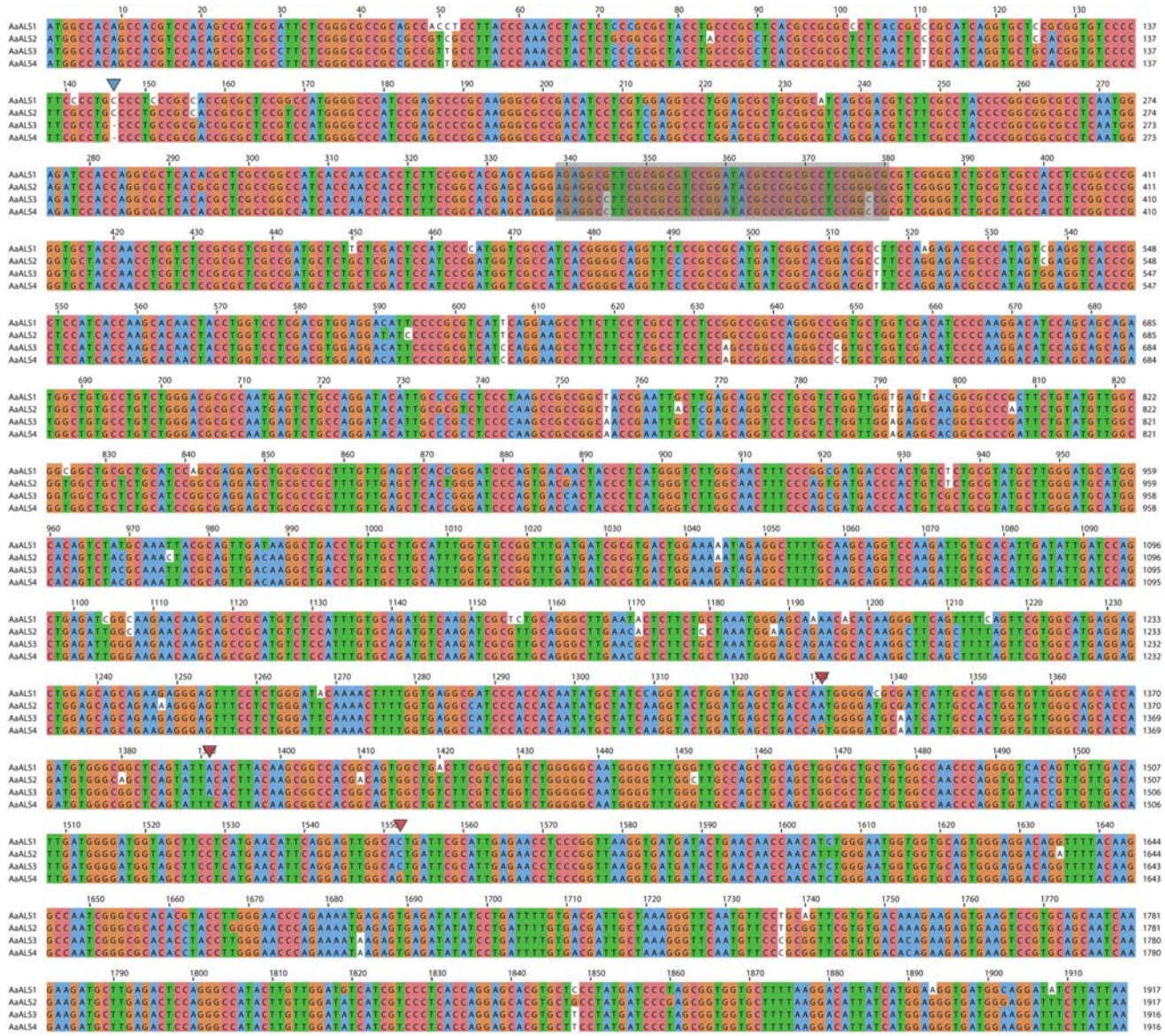

FIGURE 3 | Sequence alignment of coding sequences of *ALS* genes in *A. aequalis*. The gray box (+339 to +380) represents the region shown in the chromatogram in **Figure 2A**. Red arrows indicate the positions of SNPs between *ALS3* and *ALS4*, and the blue arrow indicates the deletion that caused a frameshift in *ALS3* and *ALS4*.

regions (UTRs) and the promoter region; no polymorphisms were observed in the 3′-UTR (157 bp), while four SNPs and three bp deletions were revealed in the promoter and 5′-UTR, respectively (603 and 600 bp for *ALS3* and *ALS4*, respectively) (Supplementary Figure S1). This discovery of a limited number of polymorphisms between *ALS3* and *ALS4* precluded the design of gene-specific primers. None of the *A. aequalis ALS* genes contained introns, as previously observed in *ALS* genes in Poaceae (Iwakami et al., 2012). The predicted protein sequences of ALS1 and ALS4 exhibited high homology with those of ALS proteins from *A. myosuroides* (AJ437300) at 96.2 to 97.5% identity (**Figure 2C**). Furthermore, ALS3, which shared 99.5 % identity with ALS4, exhibited 95.8 and 98.9% identity with ALS1 and ALS2, respectively.

The possibility that *ALS3* and *ALS4* represent alleles of a single gene, rather than two different genes, was subsequently investigated since the two were found to be highly homologous. To do so, sequences were investigated in the self-pollinated progeny of next-generation plants carrying both sequences. Full-length sequences of *ALS3* and *ALS4* were amplified and the DNA fragments directly sequenced. A chromatogram of all the progeny (more than 30 individuals) showed a double peak at the three polymorphic sites between the *ALS3* and *ALS4* sequences

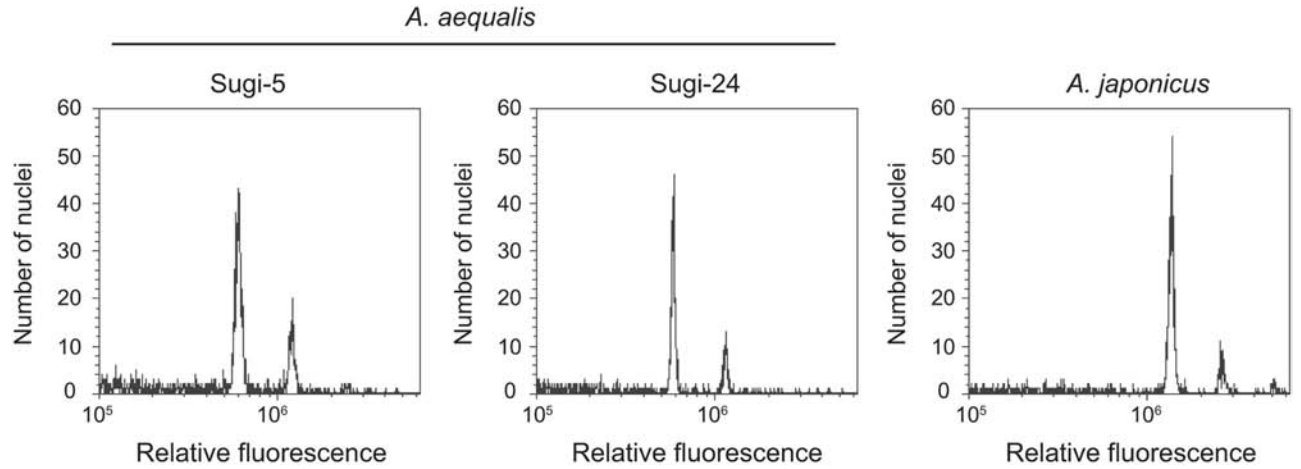

FIGURE 4 | Relative fluorescence intensities from flow cytometry analysis.

only. This finding confirms that all the progeny carried both the *ALS3* and *ALS4* sequences, contradicting the suggestion that they represent alleles and providing strong evidence that they represent two different genes.

The results of Southern blot analysis supported the differences in copy number among accessions. In Sugi-24, which carried two copies (*ALS1* and *ALS2*), two signals were detected both in the *Eco*RV and *Hind*III digests (Supplementary Figure S2). In contrast, three signals were detected in the *Hind*III digest from Sugi-5, in which an additional two copies were found, although no differences in hybridization patterns of the *Eco*RV digests were observed. In both accessions, lower molecular weight signals in the *Eco*RV digest were thought to represent *ALS2*, considering the restriction sites of *Eco*RV (Supplementary Figure S2C).

The full-length sequences of *ALS* genes amplified from all nine accessions were subsequently sequenced. *ALS1* and *ALS2* were observed in all accessions analyzed so far (48 accessions from eight fields, data not shown), while some were found to carry additional *ALS3* and *ALS4* copies (**Table 1**). All sequences of each gene were identical, except at the codon corresponding to Pro197, mutations of which are known to confer resistance to several SU herbicides. Here, all of the thifensulfuron-methyl resistant accessions carried mutations at Pro197, with substitutions of Ser, Leu or Thr identified in *ALS1* (**Table 1**). However, in Sugi-3, a Leu substitution was observed in the Pro197 of *ALS2*.

Ploidy Levels

Two nuclei peaks were detected in flow cytometric analyses of two accessions of *A. aequalis* and one accession of *A. japonicus* (**Figure 4**). The fluorescence intensities of the first and second peaks were the same in the *A. aequalis* accessions, with two and four copies of the *ALS* genes. In contrast, peak intensities of the closely related tetraploid *A. japonicus* (Xu et al., 2014) were double those of the *A. aequalis* accessions, suggesting that DNA contents of single nuclei from two- and four-copy *A. aequalis* accessions are equal and approximately half that of *A. japonicus*.

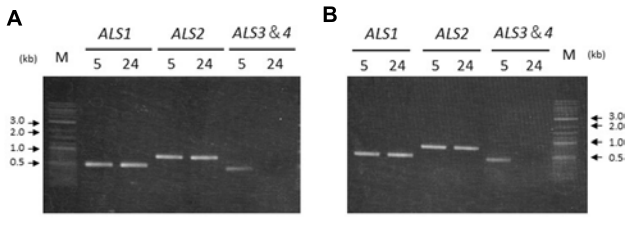

FIGURE 5 | Transcription of *ALS* genes in two *A. aequalis* accessions. Labels 5 and 24 represent the Sugi-5 and Sugi-24 accessions, respectively. The primers do not discriminate between the *ALS3* and *ALS4* genes. M, DNA size standard. **(A)** Seedling shoots. **(B)** Seedling roots.

Transcription of the *ALS* Genes

To confirm transcription of the *ALS* genes in seedlings from accessions carrying two or four copies, reverse transcription (RT)-PCR was conducted. PCR amplification could not distinguish *ALS3* and *ALS4* due to the lack of nucleotide polymorphisms. Thus, primers targeting the conserved region between the two genes were designed in order to amplify the area containing the polymorphisms. The resulting PCR product was subjected to direct sequencing to confirm which genes were transcribed.

ALS1 and *ALS2* were transcribed both in the shoots and roots in accessions carrying two or four copies of the *ALS* genes; namely, Sugi-24 and Sugi-5, respectively (**Figure 5**). *ALS3* and *ALS4* DNA fragments were subsequently amplified from Sugi-5 shoot and root template cDNA. Sequencing of the PCR products revealed transcription of both the *ALS3* and *ALS4* genes. No DNA fragments of any of the *ALS* genes were detected in controls without reverse transcriptase (data not shown).

DISCUSSION

In the present study, thifensulfuron-methyl resistant *A. aequalis* accessions were found to carry a mutation at the Pro197 residue in either the *ALS1* or *ALS2* gene. All amino acid substitutions identified in this study are known to confer SU resistance (Yu and Powles, 2014). Moreover, both the *ALS1* and *ALS2* genes were found to be transcribed in seedlings of *A. aequalis*, supporting involvement of this mutation in thifensulfuron-methyl resistance. However, it remains to be determined whether the mutation site or number of mutated loci influences the degree of resistance. More detailed characterization of these accessions will therefore be carried out in the future.

The number of *ALS* gene sequences differed among the nine accessions. Differences in the copy number of genes can result from CNV or from different ploidy levels within a species. In the present study, analysis of ploidy level revealed no differences in the amount of nuclear DNA among accessions. It is therefore unlikely that the higher-copy number accessions are tetraploid, although ploidy level variation within a single species has been reported in *Alopecurus* spp. including *A. aequalis* (Sieber and Murray, 1980; Koul and Gohil, 1990). The two highly similar DNA sequences of the higher–copy number accessions (*ALS3* and *ALS4* sequences) suggested that they might represent alleles of a single gene rather than two different genes. However, analysis of self-pollinated progeny revealed that all offspring carried both sequences, confirming that they are not alleles. Meanwhile, Southern blot analysis indicated two or three bands rather than four, which is consistent with different restriction enzymes (data not shown). This discrepancy can be explained by the hypothesis that *ALS3* and *ALS4* are tandemly duplicated and tightly linked on the same chromosome, supporting the observation that no three-copy individuals were present among 48 accessions in eight fields (data not shown). The occurrence of CNV in *ALS* genes has also been suggested in the tetraploid *A. japonicus* (Feng et al., 2016), with three copies identified in one population and an additional copy in another. This additional copy might represent an ortholog of *ALS3* and *ALS4* identified in this study, since one of the subgenomes of the hexaploid *A. japonicus* is thought to have been derived from *A. aequalis* (Matumura, 1968).

The CNV revealed in this study is unlikely to influence the evolution or level of resistance since both additional copies were thought to be pseudogenes without functions. However, copy number amplification of functional *ALS* genes could have resulted in ALS inhibitor resistance as previously observed in cultured cells (Odell et al., 1990), although resistance levels would be lower than that of target-site resistance resulting from amino acid substitution. Activity of plant ALS is subject to feedback inhibition from branched chain amino acids (Duggleby and Pang, 2000) and is not necessarily positively correlated with protein levels. In line with this, overproduction of ALS was found to result in only a slight decrease in ALS inhibitor sensitivity (Odell et al., 1990; Whaley et al., 2007).

The discovery of CNV in the *ALS* gene warrants further analysis of copy numbers in genes encoding herbicide targets, especially *EPSPS*. *ALS* genes are frequently used as an internal control in real-time PCR analysis of copy numbers of *EPSPS* genes, since *ALS* copy number was previously thought to be stable among individuals. However, this study revealed that the *ALS* copy number is, in fact, unstable. Selection of genes for use as an internal control should therefore be made carefully, and copy number amplification of genes of interest validated by other methods such as Southern blotting or fluorescence *in situ* hybridization as in Gaines et al. (2010), Jugulam et al. (2014), and Dillon et al. (2016). Alternatively, additional real-time PCR using a second internal control gene such as the cinnamoyl-CoA reductase gene (Salas et al., 2012, 2015) should also be performed for validation.

One interesting finding of this study was the presence of various mutation patterns in accessions randomly collected from a small field. Since *A. aequalis* is a self-fertile species, one might expect resistant plants in a single field to be derived from a single progenitor, as previously seen in resistant populations of *M. vaginalis* in Japan (Imaizumi et al., 2008). However, the discovery of four resistant genotypes suggests that the evolution of resistance occurred multiple independent times. Further research is now needed to determine how these resistance alleles evolved.

Despite the findings, the present results do not exclude the possible involvement of a non-target-site resistance mechanism. Non-target-site resistance is a major mechanism in *A. myosuroides*, which is a close outcrossing relative of *A. aequalis* (Délye et al., 2011). Non-target-site resistance to ALS inhibitors is often associated with enhanced activity of cytochrome P450 (Yuan et al., 2007). Recently, we discovered that overexpression of herbicide-metabolizing P450 genes is associated with resistance to ALS inhibitors in *Echinochloa phyllopogon*, a self-pollinating Poaceae species (Iwakami et al., 2014a). It is therefore possible that resistance evolved via a similar mechanism in *A. aequalis*.

AUTHOR CONTRIBUTIONS

SI, YS, YM, AU, and TT designed the experiments; SI, YS, YM, ME, and HS performed the experiments and analyzed the results; and SI, YS, AU, and TT wrote and approved the manuscript.

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Evidence, Mechanism and Alternative Chemical Seedbank-Level Control of Glyphosate Resistance of a Rigid Ryegrass (*Lolium rigidum*) Biotype from Southern Spain

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Rigid ryegrass (*Lolium rigidum*) is one of the most troublesome weeds in different crops in the Mediterranean region. A rigid ryegrass biotype from an olive grove in Jaén province (Andalusía, southern Spain), potentially resistant to glyphosate (RG), was tested for its resistance level through dose-response assays using a susceptible biotype (SG). To test the hypothesis of a non-target-site-based resistance, as point mutations are far less common mechanisms of glyphosate resistance, studies were also conducted to elucidate whether resistance was associated with biochemical, metabolism, molecular and/or physiological mechanisms. Alternative herbicide-based control options, including single-herbicide or herbicide mixtures with glyphosate, applied at seedling, tillering or full heading stages, were tested in field experiments for 2 years for their efficacy against rigid ryegrass plants and their effects on the soil seed bank. Resistance levels of the RG biotype were 23- (LD_{50}) and 7-fold (GR_{50}) higher compared to the SG biotype. The SG biotype exhibited a significantly greater shikimic acid accumulation than the RG one. At 96 HAT, 58 and 89% of applied ^{14}C-glyphosate was up taken by leaves of RG and SG biotype plants, respectively, and, at this time, a significantly higher proportion of the glyphosate taken up by the treated leaf remained in its tissue in RG plants compared to the SG ones. The RG biotype did not reveal any point mutation in the glyphosate target site EPSP synthase. Overall, results confirmed reduced glyphosate uptake and translocation as being the mechanism involved in glyphosate resistance in the RG biotype. RG biotype responses to the alternative treatments tested *in situ* indicated that herbicide applications at the later growth stage tended to be less effective in terms of immediate effects on population size than earlier applications, and that only in some cases, the removal of at least 85% of the RG biotype was achieved. However, with few exceptions, the alternative treatments tested appeared to be highly effective in reducing the seed bank irrespective of the growth stage. The frequency of the resistant phenotype in the progeny of surviving plants of the RG biotype was dependent on treatment. Results suggest that a potential exists for effective management of glyphosate-resistant rigid ryegrass in olive groves in southern Spain.

Keywords: rigid ryegrass, resistance, glyphosate, seedbank, target-site and non-target-site mechanisms

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INTRODUCTION

Rigid ryegrass (*Lolium rigidum* Gaud.) is one of the most relevant weed problems in cereal and other grain crops, both in its Mediterranean area of origin (Izquierdo et al., 2003) and in south-western Australia (Holtum and Powles, 1991; Powles et al., 1998), where it was purposely introduced as a pasture plant (Kloot, 1983). Widespread and large populations of this weed are also characteristic of other crop types in the Mediterranean region, including fruit tree orchards, olive groves, and vineyards. Apart from being extensively cultivated in southern Australia, this plant species is also managed as a cover crop for reducing soil losses in erosion-prone crops, particularly olive groves in southern Spain (Alcántara-de la Cruz et al., 2016b). Rigid ryegrass is ranked among the weeds exhibiting most reported cases of herbicide resistance (Heap, 2017) and, currently, resistant populations appear to be highly frequent in surveyed countries, including Australia (Owen et al., 2007) and Spain (Loureiro et al., 2010), among others (Kaundun et al., 2011; Bostamam et al., 2012). First reports of herbicide resistance in this weed species date back to the early 1980s with ACCase- and ALS-inhibiting herbicides being involved (Heap and Knight, 1982). Thereafter, additional cases of resistance across different herbicide modes of action, and also multiple and cross resistance (Heap and Knight, 1986; Fernandez et al., 2016), have been repeatedly reported across 12 countries (Heap, 2017), including resistance to glyphosate. In fact, the first case of weed resistance to glyphosate was reported in 1996 for *L. rigidum*, in Australia (Powles et al., 1998).

Because of its broad control spectrum and fast degradation, glyphosate has become the most widely used herbicide since its introduction in 1974 (Duke and Powles, 2008; Powles and Yu, 2010). Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) thus preventing biosynthesis of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, and of many secondary aromatic compounds (Amrhein et al., 1980). Inhibition of EPSPS leads to a rapid accumulation of shikimate and, eventually, to plant death (Shaner et al., 2012).

Resistance to glyphosate, currently identified in populations of 37 species worldwide (Heap, 2017), results from different mechanisms, generally classified as target-site and non-target-site resistance (NTSR) (Sammons and Gaines, 2014). Target-site resistance (TSR) can involve an EPSPS gene mutation or an over expression of the EPSPS enzyme. In the former case, the molecular basis for TSR was revealed to be a point mutation in the EPSPS gene consisting of a substitution at amino acid Pro-106 position by Ser, Thr, Ala, or Leu (Ng et al., 2005). As a result of this substitution, a decrease in the affinity of EPSPS for glyphosate binding is observed. This mutation has been reported in rigid ryegrass (Fernandez et al., 2015) and also in other weed species, including Italian ryegrass (Perez-Jones et al., 2007) and goosegrass (Baerson et al., 2002a). Recently, Yu et al. (2015) and Alcántara-de la Cruz et al. (2016a) reported glyphosate-resistant populations of goosegrass and hairy beggarticks, respectively, showing simultaneous Pro-106-Ser and Thr-102-Ile mutations. These are the first reports of a naturally evolving double mutation of the EPSPS gene in weeds, although it has been purposely used in transgenic maize (GA21) (Lebrun et al., 2003). While a single target-site mutation in the EPSPS gene seems to confer low levels of resistance to glyphosate in the order of two- to fourfold, the double mutation greatly increases resistance levels (Sammons and Gaines, 2014; Chen et al., 2015).

Amplified basal expression of EPSPS, also a TSR mechanism, has been found in glyphosate-resistant lines derived from the rigid ryegrass population in which glyphosate resistance was first described (Baerson et al., 2002b), and also in glyphosate-resistant horseweed (Dinelli et al., 2006) and hairy fleabane (Dinelli et al., 2008). Similarly, incremented basal EPSPS enzyme activity, associated with EPSPS gene amplification, has been reported as being a glyphosate-resistance mechanism in Italian ryegrass (Salas et al., 2012) and Palmer amaranth (Gaines et al., 2010). Amplified EPSPS expression provides additional active sites for PEP and S3P to bind normally and continue to move carbon flux through the shikimate pathway. For instance, resistant individual plants of Palmer amaranth had, on average, 77-fold more copies of the EPSPS gene, a 35-fold higher expression of EPSPS mRNA and an approximately 20-fold higher expression of EPSPS protein (Gaines et al., 2010).

Non-target-site resistance, which results from reduced glyphosate absorption and/or translocation, is far more common (Délye, 2013). As herbicide effects of glyphosate result from interference with the shikimate pathway, which is most active in meristematic tissues, translocation of the herbicide to these growing points must occur to a great extent. Glyphosate translocation takes place via phloem from treated leaves to sink meristematic tissues following sucrose movement. Phloem mobility of the glyphosate molecule is due to its unique combination of three acidity functions and one basic one. Any change in the structure of glyphosate that affects its zwitterionic characteristics reduces its ability to move through the plant (Shaner et al., 2012).

Unlike TSR, that only confers resistance to herbicides targeting the protein concerned, NTSR results in unpredictable resistance levels to different herbicides largely varying in their mode of action (Petit et al., 2010). Several studies have described NTSR in rigid ryegrass for up to 16 herbicide molecules with nine different action modes (Burnet et al., 1994). NTSR has also been described as being the most common mechanism of resistance to glyphosate (Powles and Yu, 2010), providing a 3- to 12-fold increase in resistance levels. Apart from rigid ryegrass (Burnet et al., 1994; Adu-Yeboah et al., 2014), NTSR mechanisms have also been reported in other *Lolium* species such as Italian ryegrass (Michitte et al., 2007; Nandula et al., 2008) and perennial ryegrass (Ghanizadeh et al., 2015), and in several other weed species, including johnsongrass (Vila-Aiub et al., 2012), sourgrass (de Carvalho et al., 2012), and horseweed (Koger and Reddy, 2005). On the other hand, rapid sequestration of glyphosate into vacuoles, leading to reduced amounts in the target-site, has been found in populations of *Conyza* and *Lolium* species. This mechanism has proved to confer a 14-fold increase in resistance to glyphosate (Ge et al., 2011, 2012). Enhanced metabolism to non-toxic, or less toxic, compounds including aminomethylphosphonic acid

(AMPA), glyoxylate, sarcosine and formaldehyde has also been described as an underlying mechanism of glyphosate-NTSR (Gonzalez-Torralva et al., 2014). However, metabolism is not a frequent mechanism in weed resistance to glyphosate (Duke, 2011).

Jaén province (Andalusía, southern Spain) is the largest olive oil producer worldwide, contributing to approximately 20% of global annual production. Over recent years, farmers in this area have been experiencing increased difficulties in obtaining acceptable control levels of rigid ryegrass populations in olive groves under long-lasting glyphosate-based management schemes.

The ultimate success and sustainability of management practices is usually more determined by the long-term fate they impose on a weed population rather than by their effects on current population size (Mortensen et al., 2000).

On the basis of reported continuous lack of severe injuries following glyphosate applications at the labeled rate, we selected a population of rigid ryegrass from an olive orchard in Jaén province as a putative resistant biotype (RG). Experiments were conducted to (1) characterize response to glyphosate of the RG biotype relative to a susceptible biotype of rigid ryegrass, (2) determine the mechanisms involved in the lack of response, and (3) evaluate chemical control alternatives according to both immediate effectiveness in removing the standing RG biotype and longer-term effects through an ability to reduce its soil seed bank.

MATERIALS AND METHODS

Plant Material

Mature seeds of a putative glyphosate-resistant (RG) rigid ryegrass biotype were collected in July 2013 from an olive grove, "El Álamo," located in Beas de Segura, Jaén province, southern Spain. This olive grove had been treated with larger field doses than 1800 g ae ha^{-1} glyphosate for at least 11 consecutive years (Roundup®, 360 g ae L^{-1} as isopropylamine salt). Seeds of a susceptible (SG) ryegrass biotype were collected from a nearby olive grove that had never received glyphosate treatments. Seeds were stored for 3 months under laboratory conditions and thereafter germinated in Petri dishes with filter paper moistened with distilled water, placed in a growth chamber at 28/18°C (day/night), i.e., near optimal temperature conditions (Steadman et al., 2003), under a photoperiod of 16 h, 850 μmol m^{-2} s^{-1} photosynthetic photon flux, and 80% relative humidity. Resulting seedlings of RG and SG biotypes were transplanted into pots containing sand/peat in a 1:1 (*v/v*) ratio and placed in a growth chamber under the environmental conditions described.

Glyphosate Whole Plant Dose-Response Assays

Herbicide treatments were applied at the 3–4 leaf growth stage. Glyphosate was applied in a laboratory chamber (SBS-060 DeVries Manufactering, Hollandale, MN, USA) equipped with 8002 flat fan nozzles delivering 200 L ha^{-1} at 250 KPa at the height of 50 cm. The following glyphosate (Roundup® Energy SL, 450 g ae L^{-1} as isopropylamine salt, Monsanto) rates were used: 0, 31.25, 62.50, 125, 250, 500, 1000, 2000, and 4000 g ae ha^{-1}. The experiment was designed using five replicates per rate. Plant mortality and dry mass were evaluated 21 days after the application (DAT). Dry mass was measured for aboveground parts of RG and SG plants after drying at 60°C for 72 h in a heater (J.P. Selecta S.A., Barcelona, Spain).

Shikimate Accumulation in Leaves

The time patterns and extent of shikimate accumulation in glyphosate-exposed leaves of SG and RG rigid ryegrass plants were studied following two different spectrophotometric analyses. In the first analysis, 50 4-mm leaf disks were harvested from the youngest fully expanded leaf at the 3–4 tiller stage from 15 plants per biotype (Hanson et al., 2009). Five disks of fresh tissue were transferred to 2 mL eppendorfs containing 1 mL of 1 mM $NH_4H_2PO_4$ (pH 4.4). One microliter of glyphosate was added to eppendorfs at the following concentrations: 0, 0.1, 0.5, 1, 5, 10, 50, 100, 200, 400, 500, 600, and 1000 μM. The eppendorfs were incubated in a growth chamber during 24 h under the above conditions. After 24 h, the eppendorfs were stored at −20°C until further analysis. Eppendorfs were removed from the freezer and thawed at 60°C for 30 min. Thereafter, 250 μL of 1.25 N HCL was added to each eppendorf. Again, they were introduced at 60°C for 15 min. A 125 μL aliquot from each eppendorf was pipetted into a new 2 mL eppendorf, and 500 μL of periodic acid and sodium metaperiodate (0.25% [wt/v] each) was added. After incubation at room temperature for 90 min, 500 μL of 0.6 N sodium hydroxide and 0.22 M sodium sulfite were added. Finally, the eppendorf's content was transferred to glass vials. Samples were measured in a spectrophotometer at 380 nm within 30 min. For each glyphosate concentration and biotype, five replications were established and repeated twice.

In the second analysis, RG and SG plants at the 3- to 4-leaf stage were treated with glyphosate at 300 g ae ha^{-1} using the laboratory spray chamber and treatment conditions described above. At 24, 48, 72, and 96 h after treatment (HAT), 50 mg of plant tissue was harvested and placed in a vial containing 1 mL of 1 M HCl and then immediately frozen in liquid nitrogen. Shikimic acid accumulation was determined according to Singh and Shaner (1998). Sample absorbance was measured with a Beckman DU-640 spectrophotometer at 380 nm. Net shikimic acid accumulation was deduced from the difference between treated and non-treated plants in each biotype. The test was performed in triplicate on five treated and five non-treated plants per biotype.

^{14}C-Glyphosate Uptake and Translocation

The assays were carried out according to Cruz-Hipolito et al. (2011). ^{14}C-glyphosate (American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) was added to a commercial glyphosate to achieve a specific activity of 0.834 kBq μL^{-1}. The final glyphosate concentration corresponded to 300 g ae ha^{-1} applied in 200 L ha^{-1}. Plants of the RG and SG biotypes at the 3- to 4-leaf growth stage were treated with a drop of 1 μL (0.834 kBq $plant^{-1}$)

deposited with a micropipette (LabMate) onto the adaxial surface of the second leaf.

The treated leaf was washed with 3 mL of water: acetone (1:1 v/v) solution to remove non-absorbed ^{14}C-glyphosate at 12, 24, 48, 72, and 96 h after drop application. The rinsate was mixed with 2 mL of scintillation cocktail and analyzed by liquid scintillation spectrometry (LSS) on a scintillation counter (Beckman LS 6500, Fullerton, CA, USA). The remainder of the plant was removed from the pot, and its roots were carefully washed with distilled water. The plant was divided into treated leaf, remaining shoot tissue, and roots. The plant parts thus obtained were dried at 60°C for 96 h and combusted in a Packard Tri Carb 307 biological sample oxidizer. Evolved $^{14}CO_2$ was trapped and counted by LSS in a 18 mL mixture of Carbo-Sorb E and Permafluor E+ (1:1v/v) (PerkinElmer, Packard Bioscience BV). The amount of radiolabel deposited was checked by washing a treated leaf excised immediately after deposition. The experiment was arranged in a completely randomized design with five replicates and repeated twice. The mean radioactivity recoveries were 93.17 ± 2.48% and 95.86 ± 3.39% for RG and SG biotypes, respectively. The proportion of absorbed herbicide was expressed as [kBq in combusted tissue/(kBq in combusted tissue + kBq in leaf washes)] × 100.

^{14}C-Glyphosate Visualization

Translocation of ^{14}C-glyphosate in plants of RG and SG biotypes of rigid ryegrass was also visualized using a phosphor imager (Cyclone, PerkinElmer). Plants were treated and collected in the same way as described in the uptake and translocation assays. The whole plants were gently rinsed, pressed, and then left to dry at room temperature during 4 days. The dried plants were placed adjacent to a 25 cm × 12.5 cm phosphor storage film for 13 h and scanned for radiolabel distribution on a phosphor imager. Three plants were analyzed per biotype.

Glyphosate Metabolism

Plants of RG and SG biotypes at the 3- to 4-leaf growth stage were treated with glyphosate at a rate of 300 g ae ha^{-1} as described in the dose-response assays section, while other plants were kept without treatment as non-treated controls. At 96 HAT, following the methodology described by Rojano-Delgado et al. (2010), glyphosate and its metabolites, i.e., aminomethylphosphonic acid (AMPA), glyoxylate, sarcosine, and formaldehyde, were determined by reversed polarity capillary electrophoresis using a G1600A Capillary Electrophoresis System (Agilent, Santa Clara, CA, USA) instrument equipped with a diode array detector (DAD, wavelength range 190–600 nm). Glyphosate, AMPA, sarcosine, formaldehyde, and glyoxylate were used as standards. Leaf tissues were washed with distilled water, flash-frozen in liquid nitrogen, and stored at −40°C until use. The aqueous background electrolyte consisted of 10 mM potassium phthalate, 0.5 mM hexadecyltrimethylammonium bromide, and 10% acetonitrile at pH 7.5. Calibration equations were established from non-treated plants and known concentrations of glyphosate and its metabolites, which were determined from their peak areas in the electropherogram. The average value for the content of glyoxylate naturally produced by the plant was subtracted from the average content of each biotype. The experiment was arranged in a completely randomized design with five replications per biotype and repeated twice.

EPSPS Enzyme Activity Assays

The enzyme extraction was conducted according to the protocol described by Sammons et al. (2007). Five gram of the leaf tissue of RG and SG biotypes of rigid ryegrass plants were ground to fine powder in a chilled mortar. Immediately after that, the powdered tissues were transferred to tubes containing 100 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5 mM benzamidine) containing 70 μL of β-mercaptoethanol and 1% in polyvinylpolypyrrolidone (PVPP). Samples were previously stirred and subsequently centrifuged for 40 min (18,000 × *g*) at 4°C. The supernatant was decanted into a beaker through a cheesecloth. $(NH_4)_2SO_4$ was added to the solution to obtain 45% (w/v) concentration, with stirring during 30 min. After that, the mix was centrifuged at 20,000 × *g* for 30 min at 4°C. The previous step was repeated to precipitate the protein in the extracts but in that case with a $(NH_4)_2SO_4$ concentration of 80% (w/v) stirring for 30 min. Finally, they were centrifuged at 20000 × *g* for 30 min at 4°C. All the pellets were dissolved in 3 mL of extraction buffer and dialyzed in 2 L of dialysis buffer (30 mm, 1000-MWC dialysis tubing at 4°C on a stir plate) over 12 h. The protein concentrations were determined by Bradford assay.

The assay for the determination of EPSPS activity followed the methodology described by Dayan et al. (2015) using the EnzCheck® phosphate assay Kit (Invitrogen, Carlsbad, CA, USA) to determine the inorganic phosphate release. The EPSPS activity from biotypes was determined in the presence and absence of glyphosate. The glyphosate concentrations used were 0, 0.1, 1, 10, 100, and 1000 μM to determine the enzyme activity inhibition. The assay buffer used was composed of 1 mM $MgCl_2$, 10% glycerol, and 100 mM MOPS, 2 mM sodium molybdate and 200 mM NaF. The experiment was repeated three times with three replications at each glyphosate concentration. EPSPS enzyme activity was expressed as percentage of enzyme activity in presence of glyphosate with respect to the control (without glyphosate). The EPSPS activity was calculated to determine the amount of phosphate (μmol) released μg of total soluble protein (TSP)$^{-1}$ min^{-1}.

EPSP Synthase Sequencing

Total RNA was isolated from leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was then treated with TURBO DNase (RNase-Free; Ambion, Warrington, UK) to eliminate any DNA contamination and stored at −80°C. RNA integrity was verified in 0.8% agarose gel. The amount and quality of the RNA was measured by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Walthman, MA, USA). First strand complementary DNA (cDNA) synthesis was started from the totality of the RNA adjusted to the same concentration in all the samples (50 ng μL^{-1}). An iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a total reaction volume of 40 μL was employed following the manufacturer's instructions.

Primers (forward: 5′ AGCTGTAGTCGTTGGCTGTG 3′; reverse: 5′ GCCAAGAAATAGCTCGCACT 3′) were used and expanded a 543-bp fragment of the EPSPS gene that contains the mutation site described as conferring resistance to glyphosate in *Lolium* species. The PCR reactions were carried out using cDNA from 50 ng of total RNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 1 × buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain) in a final volume of 25 μL. All PCR reactions were in duplicate and cycling conditions were: 94°C for 3 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with a final extension cycle of 72°C for 10 min. An aliquot of the PCR product was loaded in a 1% agarose gel to check the correct band amplification. The rest of the PCR product was then purified using ExoSAP-IT® for PCR Product Clean-Up (USB, Cleveland, OH, USA) as indicated by the manufacturer. Fifteen purified PCR products per biotype were sequenced (STAB VIDA, Caparica, Portugal). The resulting fragments were aligned and numbered based on published EPSPS sequences for rigid ryegrass [R rigid ryegrass from Fernandez et al. (2015); S rigid ryegrass from GenBank: AF349754].

Chemical Alternatives to Glyphosate: Effects on the Standing Population and on the Soil Seed Bank

Field and laboratory/greenhouse experiments were conducted to evaluate the effectiveness of different alternative herbicide treatments in terms of immediate "*in situ*" control of the standing RG biotype of rigid ryegrass and of longer-term effects on soil seed bank size.

Three field trials were carried out during two consecutive seasons, 2013–2014 and 2014–2015. Single-herbicide or herbicide mixtures with glyphosate were applied either at early post-emergence (3- to 8-leaf stage, trial 1), at tillering (trial 2) or at full heading, immediately before flowering (trial 3) (**Table 1**). Three completely randomized blocks were established each study year within the cultivation row. Blocks were located within the cultivation row. The experimental unit was a plot of 2 m × 15 m. The herbicides were applied using a Pulverex backpack sprayer with a T coupling for the wand equipped with four flat fan nozzles, at a spraying pressure of 200 kPa, and calibrated to deliver a volume of 200 L ha^{-1}. A strip of 1 m was established between plots within a block to prevent treatment overlap. Direct treatment effects on target plants were evaluated 60 days after application (DAT) in terms of percentage reduction of rigid ryegrass soil cover with respect to untreated plots, and treatment effectiveness was finally expressed as the complementary percentage.

Apart from immediate effects on the abundance of the standing biotype, selected treatments were also evaluated according to their ability to reduce the soil seed bank on which future infestations would be dependent. To quantify treatment effects on seed bank size, we first estimated for surviving plants their mean seed set, i.e., the proportion of flowers produced by an individual plant transformed into single-seeded fruits (referred to as seeds throughout the paper). Rigid ryegrass plants of the RG biotype surviving the different herbicide treatments in the field trials were let to flower and fruiting. At maturity, inflorescences were bulk collected for each treatment within each block and stored in the laboratory for 3 months. As long as after-ripened rigid ryegrass seeds readily germinate under favorable laboratory conditions (Steadman et al., 2003), the seed set was measured as the germination fraction of floret units sampled from mature inflorescences. In each of the two study years, seed germination tests were carried out using 50 randomly chosen florets from each bulk collection. Floret samples were placed in plastic Petri dishes sealed with parafilm inside a growth chamber under the environmental conditions described above. Percentage of germination was recorded after 14 days. Seed set

TABLE 1 | Herbicide treatments in rigid ryegrass in three different phenologic stages.

Early post-emergence, 3–8 leaves 07/03/2014 – 01/03/2015[a]			Tillering stage 08/04/2014 – 03/04/2015			Full heading stage 23/04/2014 – 29/04/2015		
Active ingredient[b]	**HRAC group[c]**	**g ai ha^{-1}**	**Active ingredient**	**HRAC group**	**g ai ha^{-1}**	**Active ingredient**	**HRAC group**	**g ai ha^{-1}**
Control			control			control		
Glyphosate	G	1800[d]	Clethodim + glyphosate	A + G	100 + 1800	Glyphosate	G	1800
Clethodim	A	100	Cycloxydim + glyphosate	A + G	250 + 1800	Glufosinate	H	750
Cycloxydim	A	250	Quizalofop-*p*-ethyl + glyphosate	A + G	125 + 1800	Clethodim	A	100
Flazasulfuron	B	50				Cycloxydim	A	250
Flazasulfuron + glyphosate	B + G	50 + 1800				Quizalofop-*p*-ethyl	A	125
Quizalofop-*p*-ethyl	A	125				Diquat	D	800

g ai ha^{-1} = grams of active ingredient per hectare. [a] *year 1 – 2;* [b] *glyphosate: Roundup Energy®, 45% p/v. SL, Monsanto, Spain; clethodim: Centurion Plus®, 12% p/v. EC, Bayer CropScience, Spain; cycloxydim: Focus Ultra®, 10% p/v. EC, BASF, Spain; flazasulfuron: Terafit®, 25% WG, Syngenta, Spain; quizalofop-p-ethyl: Master D®, 5% p/v. EC, Dow AgroSciences, Spain; glufosinate: Finale®, 15% p/v. SL, Bayer CropScience, Spain; diquat: Reglone®, 20% p/v. SL, Syngenta, Spain;* [c] *Abbreviations: HRAC, Herbicide-Resistance Action Committee; G, EPSPS inhibitors; A, ACCase inhibitors; B, ALS inhibitors; H, glutamine synthase inhibitors; D, PSI electron diverter;* [d] *g ae ha^{-1} = grams of acid equivalent per hectare.*

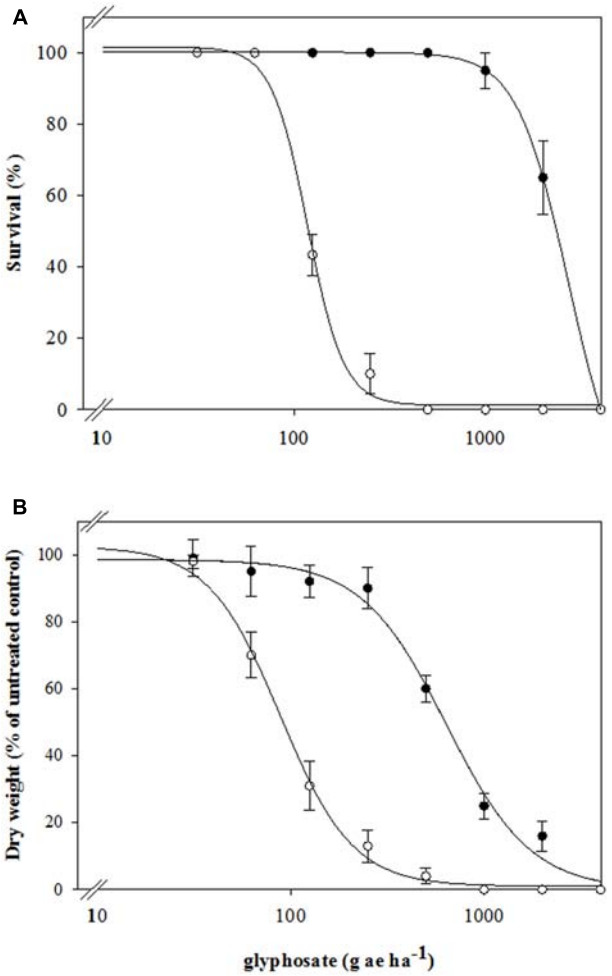

FIGURE 1 | Glyphosate dose-response on (A) survival and **(B)** above-ground dry weight expressed as percentage of the mean untreated control of the R (•) and S (○) biotypes of rigid ryegrass. Symbols denoted mean (n = 5) ± standard errors of the mean.

of surviving plants was established for the different treatments relative to seed set of untreated plants, and the resulting fractions were multiplied by the corresponding mean values of relative soil cover at 60 DAT to obtain relative measurements of the contribution of the standing population to the soil seed bank following exposure to the different herbicide treatments. The same as above, effectiveness in reducing the seed bank was finally expressed as the complementary percentages.

The frequency of the glyphosate-resistant phenotype among the progeny of plants of the RG biotype surviving the different "*in situ*" treatments was also estimated as follows. Seedlings resulting from germination tests were individually transferred to pots and placed in the greenhouse. At the 3- to 4-leaf stage, seedlings were treated with glyphosate at the labeled field rate (720 g ae ha^{-1}) using the laboratory spray chamber described previously. Due to space limitations, the seedlings were divided into three lots per maternal treatment, and seedlings from each lot were simultaneously treated in the chamber. The number of plants surviving glyphosate treatment, i.e., exhibiting a resistant phenotype, was counted 21 days after treatments.

TABLE 2 | Parameters of the log-logistic equations used to calculate the glyphosate rates required for 50% survival (LD_{50}), reduction dry weight (GR_{50}), or inhibit EPSPS activity (I_{50}) of R and S biotypes of rigid ryegrass.

Survival (%)						
	c	***d***	***b***	**LD_{50} (g ae ha^{-1})**	**RI**	***p***
R	−2.82	100.08	3.22	2712.05 ± 45.41	23.04	<0.0001
S	1.51	101.50	4.69	117.74 ± 4.54		
Above-ground dry weight (%)						
	c	***d***	***b***	**GR_{50} (g ae ha^{-1})**	**RI**	***p***
R	1.25	98.10	1.98	637.83 ± 32.90	7.17	<0.0001
S	1.41	102.41	2.38	88.87 ± 5.14		
EPSPS activity (%)						
	c	***d***	***b***	**I_{50} (μM)**	**RI**	***p***
R	1.14	100.59	1.65	8.23 ± 2.34	1.18	0.6034
S	1.29	101.36	1.96	6.94 ± 1.98		

g ae ha^{-1} = grams of acid equivalent per hectare.

Data Analysis

Whole plant dose-response and EPSPS enzyme activity data were subjected to non-linear regression analysis using a four-parameter log-logistic equation (Eq. 1) to determine the glyphosate dose causing 50% reduction in growth (GR_{50}), 50% mortality (LD_{50}), and inhibition of EPSPS activity by 50% (I_{50}).

$$Y = [(d-c)/(1 + (x/g)^b)] + c \quad (1)$$

where Y is above-ground weight or survival expressed as a percentage of the non-treated control, c and d are the parameters corresponding to the lower and upper asymptotes, b is the slope of the curve at the inflection point, g the herbicide rate at the inflection point (i.e., GR_{50}, LD_{50}, I_{50}), and x (independent variable) is the herbicide rate.

Regression analyses were conducted using the *drc* package (Ritz et al., 2015) for the statistical environment R (R 3.2.4; R Core Team, 2015). Resistance indices were computed as RG-to-SG GR_{50}, LD_{50}, or I_{50} ratios. To test for a common GR_{50}, LD_{50}, or I_{50} for RG and SG biotypes, i.e., Resistance Index equal to 1, a lack-of-fit test was used to compare the model consisting of curves with biotype-specific g values with a reduced model with common g (Ritz et al., 2015).

Analysis of variance (ANOVA) was conducted using Statistix 9.0 (Analytical Software, USA) to test for differences between RG and SG biotypes in shikimate accumulation in leaves, either at 1000 μM glyphosate in the leaf segment or at 96 HAT in the whole plant analysis; proportion of the different glyphosate metabolites; proportion of applied ^{14}C-glyphosate taken up by leaves, and proportions of absorbed ^{14}C-glyphosate remaining in the treated leaf, translocated to roots and to the rest of the plant at 96 HAT; and basal enzyme activity. Percentage data were

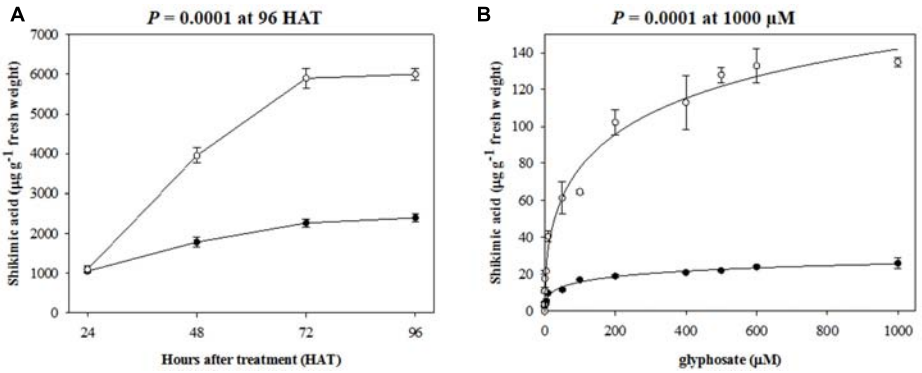

FIGURE 2 | Shikimic acid accumulation in (A) plant leaves, and **(B)** leaf segments of plants from R (•) and S (○) biotypes of rigid ryegrass. Symbols denoted mean (n = 5) ± standard errors of the mean.

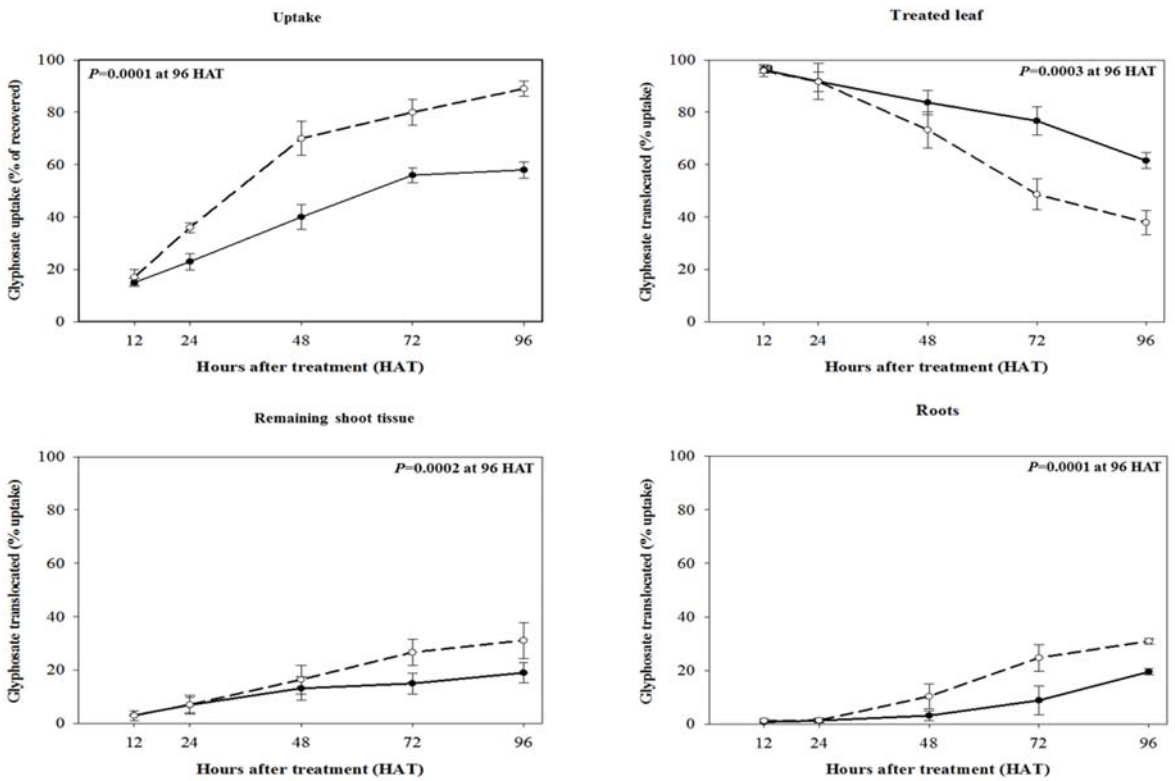

FIGURE 3 | ^{14}C-glyphosate foliar uptake and translocation to plant sections in R (•) and S (○) biotypes of rigid ryegrass at 12, 24, 48, 72, and 96 h after glyphosate application. Symbols denoted mean (n = 5) ± standard errors of the mean.

previously transformed (arcsine of the square root) to meet model assumptions of normality of error distribution and homogeneity of variance. Model assumptions were graphically inspected. When needed, differences between means were separated using the Tukey HSD test.

ANOVA was conducted in R accounting for the experimental design to test for the effects of herbicide treatment and year, and their interaction, on effectiveness in reducing soil cover of the R biotype in the field trials, and on seed set of plants surviving the treatments to fruiting. As above, response variables were previously transformed and model assumptions of homogeneity of variance and normality of errors were graphically inspected. Generalized linear mixed models (GLMM) with binomial error distribution and logit link function were used to test for treatment and year effects on the survival rate of the progeny from glyphosate in laboratory/greenhouse experiments. GLMMs allow for a proper treatment of hierarchical designs and, in the case of proportions, also they correct for varying initial

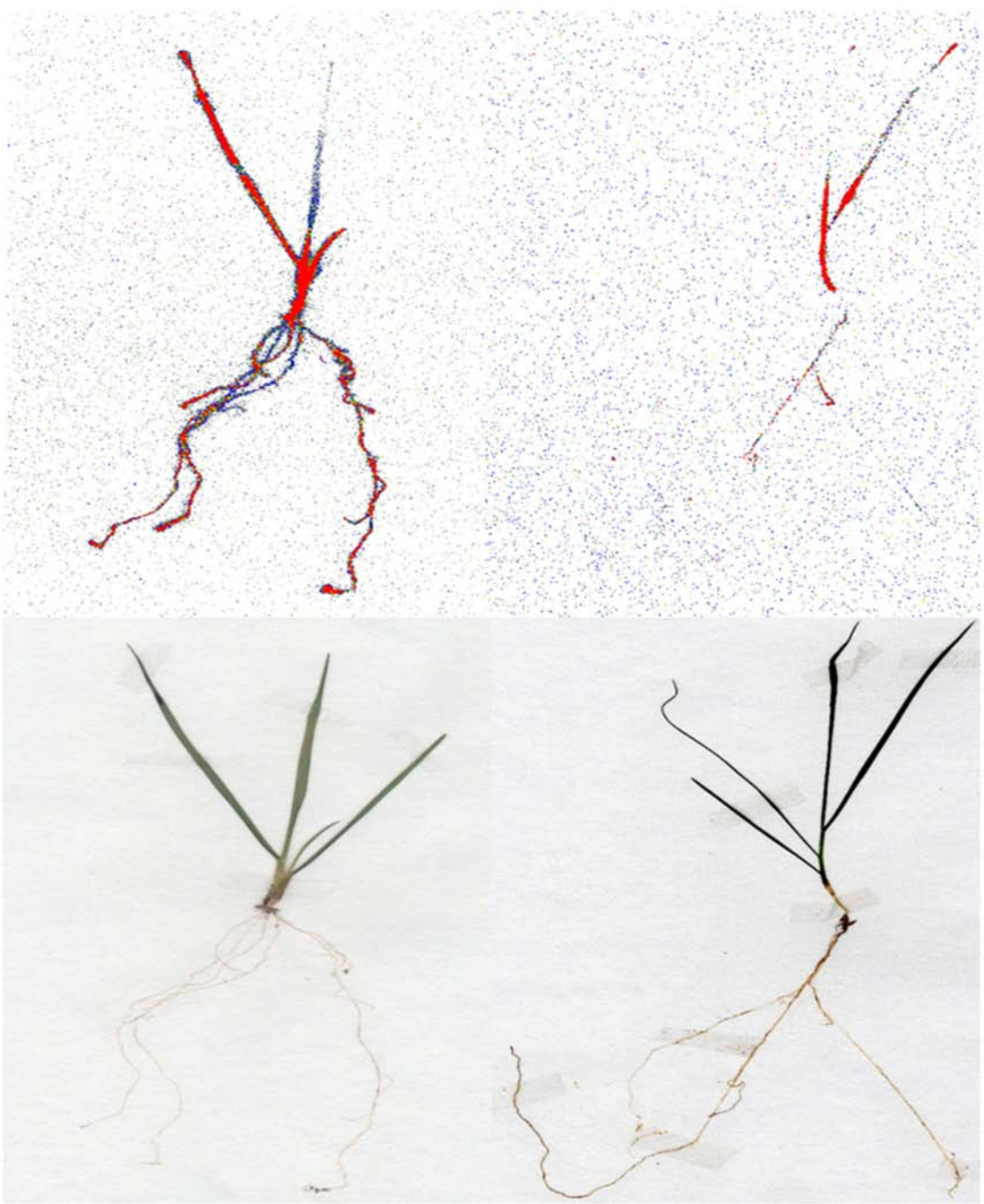

FIGURE 4 | Phosphorimaging visualization of ^{14}C-glyphosate in plants of rigid ryegrass of susceptible (left) and resistant **(right)** biotypes at 96 h after treatment.

sample sizes. GLMMs were conducted in R using the package lme4 (Bates et al., 2015). Model assumptions were graphically checked. For each maternal growth stage, we compared survival rates resulting from each maternal treatment to survival rates of untreated plants (the reference class), judging the effect as significant when the parameter of a specific treatment in the linear component of the model was significantly different from 0. Non-significant terms, starting with the interaction, were dropped one at a time and, in every step, the reduced model was compared with the previous model using likelihood ratio tests. We continued this process up to when no additional terms could be dropped.

RESULTS

Glyphosate Whole Plant Dose-Response

Dose-response assays clearly established differential susceptibility to glyphosate between the SG and the putative resistant RG biotypes of rigid ryegrass, both in terms of survival and aboveground dry biomass (**Figure 1** and **Table 2**). As expected, the SG biotype was highly susceptible to glyphosate and at the labeled field rate in Spain (720 g ae ha^{-1}) it showed a very low survival and biomass (**Figure 1**). At this rate, however, 97% of the plants of the RG biotype survived the treatment, and aboveground dry biomass was only reduced to 48% of untreated plant biomass (**Figure 1**). The test for lack of fit, comparing a reduced model with common *g* parameter for SG and RG curves to a model with biotype-specific *g* values, was significant ($p < 0.01$) for both survival (LD_{50}) and dry biomass (GR_{50}), indicating that both rates do differ between biotypes and thus confirming resistance to glyphosate of the studied RG biotype from southern Spain (**Table 2**).

Shikimic Acid Accumulation in Leaves

Shikimic acid accumulation patterns in glyphosate-exposed leaves of rigid ryegrass plants of RG and SG biotypes are shown in **Figure 2**. In agreement with contrasting responses of RG and SG biotypes to glyphosate dose, leaves of SG plants accumulated much larger amounts of shikimate compared to RG plants, as demonstrated by the analyses at the whole plant and leaf segment levels. The whole plant analysis indicated that these differences in shikimate accumulation were already apparent 48 h after treatment and after 96 h, a highly significant ($p < 0.001$, DF = 1, $n = 10$) 2.5-fold greater shikimate concentration was found in leaves of the SG biotype (**Figure 2A**). The leaf segment analysis showed that increased shikimate levels in glyphosate-exposed SG biotype plant leaf segments were evident from very low glyphosate concentrations and at 1000 μM, the highest concentration tested, the difference was 5.2-fold greater in SG versus RG leaf segments ($p < 0.001$, DF = 1, $n = 10$) (**Figure 2B**).

^{14}C-Glyphosate Uptake, Translocation, and Visualization

The maximum ^{14}C-glyphosate uptake for both biotypes was reached at 96 HAT, RG biotype rigid ryegrass plant leaves took up a significantly lower proportion ($p < 0.001$, DF = 1, $n = 10$) of applied ^{14}C-glyphosate compared to the SG biotype, 58 and 89%, respectively (**Figure 3**). Differential patterns of glyphosate translocation within the plant were also evident between RG and SG biotypes. From 48 HAT onward, a higher proportion of the glyphosate taken up by the treated leaf remained in its tissues in RG plants compared to SG plants, and at 96 HAT, these proportions were significantly different ($p < 0.001$, DF = 1, $n = 10$), 61.6 and 37.9% for RG and SG plants, respectively (**Figure 3**). Accordingly, compared to the RG biotype, SG biotype plants showed a significantly higher proportion ($p < 0.001$, DF = 1, $n = 10$) of absorbed glyphosate in both the roots and rest of plant at 96 HAT (**Figure 3**). Overall, uptake and translocation assays indicated that glyphosate translocation was 2.4-fold greater in SG than in RG rigid ryegrass plants. Differences between rigid ryegrass biotypes in ^{14}C-glyphosate translocation were also evidenced through phosphor imaging (**Figure 4**).

TABLE 3 | Glyphosate metabolism expressed as percentage of total glyphosate and their metabolites in R and S glyphosate biotypes of rigid ryegrass at 96 h after treatment.

Biotype	Glyphosate	AMPA	Glyoxylate
R	88.39 ± 3.15 A	8.59 ± 1.24 A	3.02 ± 0.14 B
S	86.41 ± 2.73 A	3.21 ± 0.91 B	10.38 ± 2.81 A
p	0.8714	0.0031	0.0024

Means on a column followed by the same letter were not significantly different at α = 0.05. Mean values ± standard error of the mean. AMPA, aminomethylphosphonic acid. Treated with glyphosate at 300 g ae ha^{-1} at the 3–4 leaf growth stage.

Glyphosate Metabolism

A mean proportion of 87.4% of applied glyphosate remained unmetabolized at 96 HAT in rigid ryegrass plants with no significant differences ($p = 0.8714$, DF = 1, $n = 10$) in RG versus SG biotypes (**Table 3**). However, the remaining fraction was differentially metabolized to AMPA and glyoxylate in plants of RG and SG biotypes. Whereas AMPA was the main metabolism product of the RG biotype, it was glyoxylate for the SG biotype (**Table 3**).

EPSPS Enzyme Activity

The specific activity of EPSPS in the absence of glyphosate was similar in RG and SG biotypes ($p = 0.824$, DF = 1, $n = 6$), 0.0839 ± 0.0053 and 0.0781 ± 0.0093 μmol μg^{-1} TSP min^{-1}, respectively. The concentration of glyphosate required to inhibit EPSPS activity by 50% (I_{50}) was 8.23 and 6.94 μM in RG and SG biotypes, respectively, with no significant difference ($p = 0.603$) (**Table 2**).

EPSPS Gene Sequencing

The RG biotype of rigid ryegrass did not reveal any mutation at position Pro-106 in the EPSP synthase gene (**Figure 5**).

Chemical Alternatives to Glyphosate: Effects on the Standing Population and on the Soil Seed Bank

The abundance of the RG biotype of rigid ryegrass in the original olive grove was potentially large, as denoted by the high soil cover measured in untreated plots, with mean values of 80 and 85% in years 1 and 2, respectively (**Figure 6**). *In situ* responses of the RG biotype to the different chemical treatments tested indicated that herbicide applications at the later growth stage tended to be less effective in terms of immediate effects on population size than earlier applications, with the exception of glufosinate (**Table 4**). Glyphosate-only applications at both early post-emergence and full heading led to a significant lower control effectiveness than alternative treatments within each growth stage (**Figure 7**), with the exception of full heading during the first study year, in which glyphosate effects did not differ from those

Position					100						106				110					
Consensus	TTC	TTG	GGG	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG	GCT	GCT	GTA	GTA	GCT	GCT	GGT
Translation	F	L	G	N	A	G	T	A	M	R	P	L	T	A	A	V	V	A	A	G
L. rigidum S (AF349754)	F	L	G	N	A	G	T	A	M	R	P	L	T	A	A	V	V	A	A	G
L. rigidum R (France, 2015)	F	L	G	N	A	G	T	A	M	R	S	L	T	A	A	V	V	A	A	G
L. rigidum S	F	L	G	N	A	G	T	A	M	R	P	L	T	A	A	V	V	A	A	G
L. rigidum R	F	L	G	N	A	G	T	A	M	R	P	L	T	A	A	V	V	A	A	G

FIGURE 5 | Partial gene sequence alignment of the EPSP synthase of resistant (R) and susceptible (S) rigid ryegrass biotypes.

FIGURE 6 | Untreated rigid ryegrass.

of clethodim, quizalofop, and diquat (**Table 4**). These results confirm farmer's claims regarding their inability to adequately control the local biotype of rigid ryegrass using glyphosate-only treatments. Removal of at least 85% of the RG biotype, a minimum threshold for satisfactory control, resulted from three treatments at early post-emergence, cycloxydim, flazasulfuron and flazasulfuron + glyphosate (**Figure 8**), and from the three treatments tested at tillering. At full heading, however, only glufosinate showed this effectiveness level in the second study year (**Table 4**).

As expected, high mean germination percentages, 78–83%, were recorded from floret units of mature inflorescences of untreated plants of the RG biotype of rigid ryegrass (**Table 5**), this supporting germination percentage of floret units from mature inflorescences, following after-ripening, as being an adequate proxy for the seed set. A significant interaction ($p < 0.01$) between treatment and year was found for each growth stage, except for tiller stage, so one-way ANOVAs were conducted for each study year to test for treatment effects. Compared to untreated plants, a significantly lower mean seed set was found in RG plants surviving *in situ* the different herbicide treatments, including glyphosate-only applications, although the seed set was

TABLE 4 | Effectiveness in removing the standing population and in reducing the soil seed bank of the different herbicide treatments that were tested during 2 years for *in situ* control of the R biotype of rigid ryegrass.

Treatment[a]	Effectiveness in removing the standing population (Soil cover reduction relative to untreated plots, %)[b]		Effectiveness in reducing the soil seed bank (mean seed bank reduction relative to untreated plots, %)[c]
	2013–2014	2014–2015	
Early post-emergence			
Glyphosate	33.7 E	31.7 D	60.5
Clethodim	65.3 D	75.0 C	91.4
Cycloxydim	88.7 B	90.0 B	100
Flazasulfuron	87.0 B	95.0 A	100
Flazasulfuron + glyphosate	97.0 A	100.0 A	100
Quizalofop-*p*-ethyl	82.0 C	78.3 C	92.8
Tillering			
Clethodim + glyphosate	87.0 B	88.7 B	97.1
Cycloxydim + glyphosate	92.0 A	91.7 A	100
Quizalofop-*p*-ethyl + glyphosate	87.0 B	85.0 C	95.6
Full heading			
Glyphosate	17.0 C	16.7 E	61.5
Glufosinate	83.7 A	88.3 A	99.5
Clethodim	23.7 BC	41.7 C	100
Cycloxydim	37.0 B	46.7 B	100
Quizalofop-*p*-ethyl	25.3 BC	31.7 D	100
Diquat	29.7 BC	35.0 D	50.9

[a]*Different herbicides were applied at three different growth stages.* [b]*Effectiveness at the standing population level was measured 60 days after treatment as soil cover of rigid ryegrass relative to untreated plots.* [c]*Effectiveness at the soil seed bank level was calculated as the product of mean cover reduction by mean seed set of plants surviving treatments. For each column, mean percentages of cover reduction followed by the same letter are not statistically different according to the Tukey HSD test following ANOVA.*

FIGURE 7 | Rigid ryegrass treated with 1800 g ae ha^{-1} (grams of acid equivalent per hectare) of glyphosate at early post-emergence and measured 60 days after treatment.

FIGURE 8 | Rigid ryegrass treated with 50 g ai ha^{-1} (grams of active ingredient per hectare) + 1800 g ae ha^{-1} (grams of acid equivalent per hectare) of flazasulfuron and glyphosate at early post-emergence and measured 60 days after treatment.

clearly dependent on herbicide identity (**Table 5**). The herbicides cycloxydim, which, however, is currently not authorized for use in olive groves, and flazasulfuron, either alone or in a mixture with glyphosate, prevented development of mature seeds in surviving treated plants irrespective of growth stage at treatment. Interestingly, clethodim and quizalofop-*p*-ethyl only prevented seed production in treatments at full heading, whereas at earlier growth stages, these herbicides, or their mixtures with glyphosate, did not fully prevent seed maturation (**Table 5**). Glyphosate-only applications tended to be among the treatments penalizing fewer seed sets of surviving plants, although seed set effects of quizalofop at early post-emergence, and diquat at full heading, did not differ significantly from glyphosate effects at the respective growth stages (**Table 5**). Evaluation of treatment effectiveness in terms of ability to reduce the soil seed bank, rather than on the basis of immediate effects on current population size, led to some contrasting conclusions (**Table 4**). With the exception of glyphosate-only treatments, and diquat at full heading, the different treatments tested appeared to be highly effective in reducing the soil seed bank (>90%). In addition, the results suggest that, with the referred exceptions of glyphosate and diquat, high effectiveness in seed bank reduction was generally achieved also at the most advanced growth stage (**Table 4**).

Treatment of progeny seedlings with glyphosate at the labeled field rate (720 g ae ha^{-1}) using the laboratory spray chamber was clearly useful to separate glyphosate-resistant and susceptible phenotypes (**Figure 1**). The herbicide treatment applied "*in situ*" to the RG biotype showed a significant effect on the frequency of the resistant phenotype among the progeny of surviving plants (**Figure 9**). The frequency of the resistant phenotype within the RG biotype, as evaluated by the survival response to glyphosate of the progeny of untreated plants, was 95–96% (**Figure 9**). Clethodim (66.7%) and quizalofop-*p*-ethyl (84.6%) applied at early post-emergence in the second study year, clethodim + glyphosate (50.0%) and quizalofop-*p*-ethyl+glyphosate (72.7%) at tillering in the second study year, and diquat (mean 87.9%) and glyphosate (mean 86.3%) at full heading in both study years significantly lowered the frequency of the resistant phenotype in the progeny of surviving plants (**Figure 9**). While the fraction of glyphosate-resistant seeds produced by the plants surviving glyphosate treatment, and the untreated plants did not differ at early post-emergence, as expected, this fraction was, significantly lower for plants treated at the most advanced growth stage, as previously stated.

TABLE 5 | Mean percentage seed set of rigid ryegrass plants of the R biotype surviving different herbicide treatments applied at three growth stages in field assays during two study years.

Treatment[a]	Mean seed set (%)[b]	
	2013–2014	2014–2015
Early post-emergence		
Untreated	82.0 A	78.0 A
Glyphosate	38.0 B	56.0 B
Clethodim	28.0 B	18.0 C
Quizalofop-*p*-ethyl	32.0 B	26.0 C
Tillering		
Untreated	82.0 A	78.0 A
Clethodim + glyphosate	26.0 B	12.0 B
Quizalofop-*p*-ethyl + glyphosate	28.0 B	22.0 B
Full heading		
Untreated	78.0 A	83.3 A
Glyphosate	26.0 C	48.0 B
Glufosinate	6.0 D	<0.1 C
Diquat	52.0 B	64.0 AB

[a]*Different herbicides were applied at three different growth stages in field assays.* [b]*Mean seed set was measured by percentage germination recorded in random samples of floret units from mature inflorescences of rigid ryegrass plants of the R biotype surviving the herbicide treatments. For each year, a random sample of 50 floret units was tested per experimental unit. For treatments showing non-zero response within each growth stage ANOVA was carried out accounting for the experimental design of field assays to test for treatment and year effects. The interaction was significant except for tillering and thus one-way ANOVAs for each year were carried out and post hoc Tukey tests were used to separate mean percentages. For each column and within each growth stage, treatments with the same letter did not lead to significant differences.*

DISCUSSION

Spain is the world's leading olive oil and table olive producer with a crop area of more than 2.5 million hectares in 2014, 62% of which is distributed in the southern region of Andalusia (Anonymous, 2014). Traditionally, weed control in olive groves had been based on deep-soil plowing, a management practice prone to causing severe soil erosion and fertility loss problems. Today, the soil management system mainly consists of cover crops that allow erosion reduction and a greater availability of moisture and nutrients to the crop plants. Currently, rigid ryegrass, either purposely sown or favoring its dominance in the weedy vegetation, is the preferred plant species to be used as a cover crop. Although the annual cycle of *L. rigidum* ends at early summer, farmers have the habit of applying selective herbicides after the first spring rains in order to stop weed growth and preventing the build-up of the soil seed bank. Since 1975, the most frequently used active substances were simazine and diuron (PSII herbicides), MCPA and fluroxypyr (auxinic inhibitor herbicides), among others. In 1990, however, glyphosate was commercialized for weed control in olive groves with immediate acceptance by farmers, making it an indispensable tool in perennial crop systems. Since the year 2000, farmers have sometimes used it at least twice within the same cultivation cycle without any additional alternative and/or IWM (Integrated Weed Management), which led to the emergence of glyphosate-resistant weed populations at the beginning of the first decade of the 21st century. In this work, for the first time, a detailed study has been carried out on the mechanisms behind glyphosate resistance in a *L. rigidum* biotype from Jaén province, Andalusia, with an emphasis on physiological, biochemical, and molecular bases, as well as on alternative chemical control options, both at the standing population and seed bank levels.

Dose-response assays demonstrated significantly higher LD_{50} and GR_{50} values for the RG biotype compared to a susceptible population (**Figure 1** and **Table 2**). These values were comparable to previous reports for other glyphosate-resistant rigid ryegrass biotypes (Adu-Yeboah et al., 2014; Fernandez et al., 2015, 2016).

In accordance with the differential behavior observed in dose-response assays, a contrasting pattern was found between RG and SG biotypes in shikimate accumulation in leaves following exposure to glyphosate. The lower shikimate accumulation observed in the RG biotype compared to the SG one (**Figure 2**) was consistent with the lower impact on the former, in terms of growth reduction and mortality, of increased rates of glyphosate. Low GR_{50} and LD_{50} values can result from an increased inhibition of EPSPS activity leading to a greater accumulation of shikimic acid (Gaines et al., 2010; Fernandez et al., 2015; Alcántara-de la Cruz et al., 2016a). However, the high levels of resistance to glyphosate and low shikimic acid accumulation in leaves exhibited by the RG biotype may also result from the addition of more than one NTSR and/or TSR mechanism, as has been shown in several grass weed species (Michitte et al., 2007; de Carvalho et al., 2012; Fernandez et al., 2015). In fact, relatively low levels of shikimate in leaves of glyphosate-resistant plants are not necessarily evidence of TSR mechanisms as this behavior has also been documented in cases of reduced foliar absorption, which leads to insufficient amounts of glyphosate in the target-site (Perez-Jones et al., 2007; Cruz-Hipolito et al., 2011; Alarcón-Reverte et al., 2015; Kleinman and Rubin, 2016).

Herbicide metabolism can be an effective NTSR mechanism (Saroha et al., 1998; Duke, 2012; Délye, 2013). However, a high proportion of glyphosate was found to remain un-metabolized in treated leaves of plants of both the RG and SG biotypes. Thus, metabolism does not appear to play any role in glyphosate resistance in the RG biotype. This result is consistent with previous studies in other glyphosate-resistant rigid ryegrass populations that did not demonstrate any contribution of metabolism to resistance (Fernandez et al., 2015). In fact, glyphosate metabolism does not seem to be a frequent resistance mechanism (Duke, 2011) and, to date, only sourgrass (de Carvalho et al., 2012), horseweed (Gonzalez-Torralva et al., 2012), and ragweed parthenium (Bracamonte et al., 2016) have been described as species able to transform glyphosate into non-toxic compounds.

Increased EPSPS enzyme activity is a plausible TSR mechanism in conferring glyphosate resistance. However, no differences were apparent between RG and SG biotypes either in EPSPS specific activity in the absence of glyphosate or in its inhibition response to glyphosate (I_{50}). Thus, this relationship between EPSPS basal activity and resistance to glyphosate, which has been shown to result from EPSPS gene overexpression in some *Lolium* species (Yu et al., 2007; Dayan et al., 2012; Salas

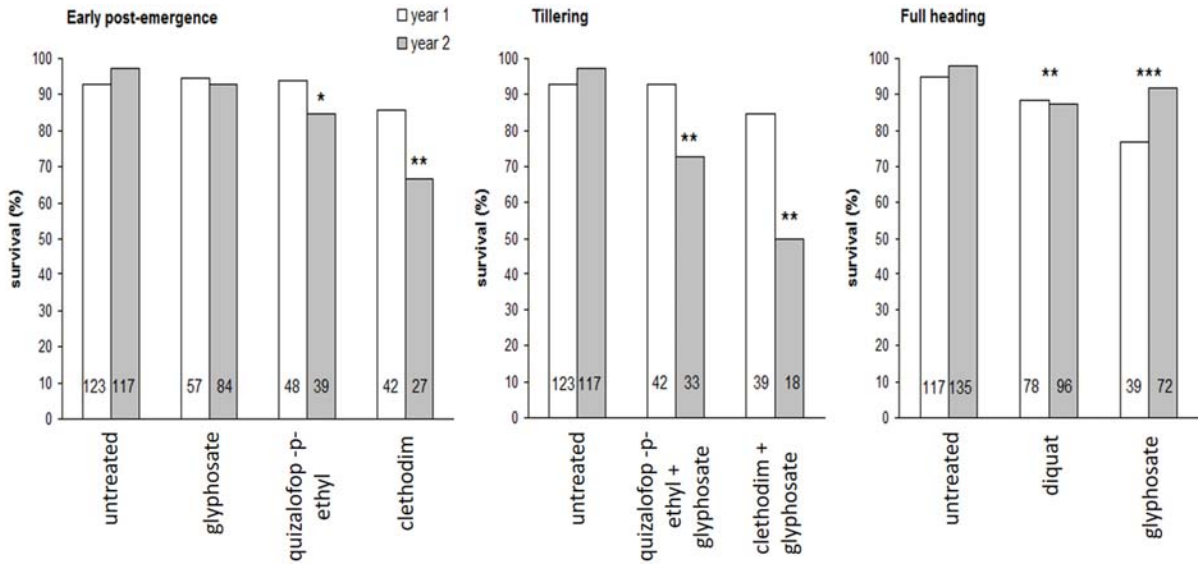

FIGURE 9 | Frequency of the R phenotype in the progeny of rigid ryegrass plants of the R biotype surviving different herbicide treatments applied at three growth stages in field assays, during the two study years. Mean values are given. Linear mixed models with binomial error distribution and logit link function were defined taking into account the experimental design to test for treatment and year effects. Asterisks indicate a significantly lower survival rate compared to untreated plants (the reference class), i.e., the corresponding parameter associated to a given treatment in the linear component of the model significantly different from 0. For early post-emergence and tillering stages a significant treatment by year interaction was found whereas for full heading only treatment effects were significant and thus they are indicated irrespective of years. For each case, the total number of plants exposed to the glyphosate treatment is indicated within bars. *$0.5 > p > 0.01$; **$0.01 > p > 0.001$; ***$p < 0.001$.

et al., 2012), is not present in the studied RG biotype. In addition, the RG biotype of rigid ryegrass did not reveal any mutation at position Pro-106 in the EPSP synthase gene, a point mutation known to endow glyphosate resistance to populations of rigid ryegrass from Australia (Wakelin and Preston, 2006) and France (Fernandez et al., 2015), and to other glyphosate-resistant weeds including the closely related species Italian ryegrass (Perez-Jones et al., 2007) and perennial ryegrass (Ghanizadeh et al., 2015), and also goosegrass (Baerson et al., 2002b). Thus, results indicate that TSR is not operating as a causal mechanism of glyphosate resistance in the studied RG biotype.

Overall, the results are in line with previous evidence of reduced uptake and translocation from other glyphosate-resistant weed populations, including rigid ryegrass (Bostamam et al., 2012; Adu-Yeboah et al., 2014; Fernandez et al., 2015), and Italian ryegrass (Michitte et al., 2007). Although impaired translocation does not occur in all glyphosate-resistant weeds, it has been considered as being the most common glyphosate resistance mechanism (Nguyen et al., 2015). Thus, it is plausible to assume that reduced uptake and translocation are the primary causal mechanisms of glyphosate resistance in the studied RG biotype of rigid ryegrass. Lower glyphosate uptake in the RG compared to SG biotype could be explained by structural differences in outer leaf surfaces (Shepherd and Griffiths, 2006; Rojano-Delgado et al., 2012; Alcántara-de la Cruz et al., 2016a), while reduced translocation could result from increased retention of glyphosate in the tips of treated leaves (de Carvalho et al., 2012; Gonzalez-Torralva et al., 2012, 2014; Adu-Yeboah et al., 2014; Bracamonte et al., 2016).

Chemical alternatives tested for *in situ* control of the RG biotype showed contrasting effects on the standing population and on the soil seed bank. Overall, treatment effectiveness was higher in terms of reduction in the contribution to the seed bank than in terms of removing the standing population (**Table 4**). This was a consequence of the markedly detrimental effect of most treatments on the seed set of surviving plants (**Table 5**). In particular, cycloxydim, flazasulfuron, and quizalofop-*p*-ethyl fully prevented seed maturation. The effect of growth stage on the ability of treatments to remove the standing population was clearly evident, with lower effectiveness at more advanced stages, a well-known, general response in chemical weed control. However, although our experimental setup did not allow for directly testing growth stage effects, treatment effects on the mean seed set of surviving plants were apparently unrelated to growth stage (**Table 5**). It should be noted that the realized contribution to the soil seed bank of plants surviving the treatments is not only determined by seed set but also by the number of florets they produced. As long as we did not measure the latter, our results represent upper estimates of the potential contribution to the soil seed bank of surviving plants, i.e., they assume that floret production was not lowered by the herbicidal treatments compared to untreated plants. Any detrimental effect of treatments on floret production would thus reinforce our findings of higher treatment effectiveness in reducing the soil seed bank than in removing the standing population.

Results of this study, focusing on the *in situ* long-term fate of the rigid ryegrass RG biotype, rather than only on immediate removal of the standing population, on which doses recommended in the product labels are based, suggest that there is a potential for implementing reduced doses of the most effective herbicide treatments. Reduced doses have been, however, associated with the rapid evolution of NTSR in outcrossing weed species like rigid ryegrass (Neve and Powles, 2005). In these cases, low detrimental doses can select for different traits conferring individually low levels of resistance but leading to increasingly resistant phenotypes through their progressive recombination allowed by outcrossing. Nevertheless, doses below labeled rates can still be recommended if target species maintain a high susceptibility (Kudsk, 2014). Reducing doses while keeping effectiveness high and alternating herbicides with contrasting modes of action can thus be a sustainable practice for management of resistance-prone rigid ryegrass populations in olive orchards in the study region.

Evaluation of the effects of "*in situ*" control treatments on the frequency of the resistant phenotype in the progeny of surviving plants indicated that most treatments significantly lowered this frequency compared to the original resistant population (i.e., a mean value of 95.5% in the progeny of untreated maternal plants), including, somewhat surprisingly, glyphosate applied at full heading. These results suggest a greater detrimental effect of these alternative herbicides, and of glyphosate applied at later growth stages, on plants of the glyphosate-resistant phenotype compared to susceptible plants within the RG population. Expression of resistance early in the plant life cycle could be at the expense of plant fitness under stressful conditions experienced at more advanced stages. Trade-offs between resistance and other functional traits have been recognized in herbicide-resistant weeds (Vila-Aiub et al., 2009), and differences in plant architecture, flowering phenology, seed dormancy depth or germination requirements, competitive ability, or resistance to diseases or pests, could explain the apparent fitness costs incurred by plants of the resistant phenotype under late stage glyphosate applications. Further research is needed to establish the existence of any such trade-offs within the RG biotype. Trade-offs can be exploited for implementing fitness-reducing management options targeting the resistant phenotype in the studied rigid ryegrass population (Vila-Aiub et al., 2005; Pedersen et al., 2007).

AUTHOR CONTRIBUTIONS

PF-M, and RDP performed the plant dose-response assays and the shikimic acid accumulation study; PF-M, and RDP carried out the EPSPS activity assays; PF-M and RDP did the ^{14}C-glyphosate absorption/translocation and metabolism study; PF-M, and RDP performed the EPSP synthase gene sequencing; FB and PF-M carried out field experiments and FB performed germination assays and data analyses. PF-M, FB, and RDP equally contributed to writing the paper.

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Hyperspectral Technologies for Assessing Seed Germination and Trifloxysulfuron-methyl Response in *Amaranthus palmeri* (Palmer Amaranth)

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Weed infestations in agricultural systems constitute a serious challenge to agricultural sustainability and food security worldwide. *Amaranthus palmeri* S. Watson (Palmer amaranth) is one of the most noxious weeds causing significant yield reductions in various crops. The ability to estimate seed viability and herbicide susceptibility is a key factor in the development of a long-term management strategy, particularly since the misuse of herbicides is driving the evolution of herbicide response in various weed species. The limitations of most herbicide response studies are that they are conducted retrospectively and that they use *in vitro* destructive methods. Development of a non-destructive method for the prediction of herbicide response could vastly improve the efficacy of herbicide applications and potentially delay the evolution of herbicide resistance. Here, we propose a toolbox based on hyperspectral technologies and data analyses aimed to predict *A. palmeri* seed germination and response to the herbicide trifloxysulfuron-methyl. Complementary measurement of leaf physiological parameters, namely, photosynthetic rate, stomatal conductence and photosystem II efficiency, was performed to support the spectral analysis. Plant response to the herbicide was compared to image analysis estimates using mean gray value and area fraction variables. Hyperspectral reflectance profiles were used to determine seed germination and to classify herbicide response through examination of plant leaves. Using hyperspectral data, we have successfully distinguished between germinating and non-germinating seeds, hyperspectral classification of seeds showed accuracy of 81.9 and 76.4%, respectively. Sensitive and resistant plants were identified with high degrees of accuracy (88.5 and 90.9%, respectively) from leaf hyperspectral reflectance profiles acquired prior to herbicide application. A correlation between leaf physiological parameters and herbicide response (sensitivity/resistance) was also demonstrated. We demonstrated that hyperspectral reflectance analyses can provide reliable information

about seed germination and levels of susceptibility in *A. palmeri*. The use of reflectance-based analyses can help to better understand the invasiveness of *A. palmeri*, and thus facilitate the development of targeted control methods. It also has enormous potential for impacting environmental management in that it can be used to prevent ineffective herbicide applications. It also has potential for use in mapping tempo-spatial population dynamics in agro-ecological landscapes.

Keywords: herbicide resistance evolution, hyperspectral imaging and sensing, precision agriculture, proximal sensing, trifloxysulfuron-methyl

INTRODUCTION

In agricultural systems, weeds are the most important biotic factor and are responsible for more than 34% of crop yield losses worldwide (Oerke, 2006), thereby constituting a serious global challenge to agricultural sustainability and food security. The noxious weed *Amaranthus palmeri* S. Watson (Palmer amaranth) is one of the economically most important weeds, affecting commodity crops, such as cotton (*Gossypium* spp.), maize (*Zea mays* L.), and soybean (*Glycine max*) (Oliver and Press, 1994; Rubin, 2000; Massinga et al., 2001). More than that, this weed is also a problem in fields of less competitive, prostrate crops, such as, watermelon (*Citrullus lanatus*) and chickpea (*Cicer arietinum*) (Rubin and Matzrafi, 2015). In view of its high seed fecundity (Keeley et al., 1987), wide range of germination temperatures (Steckel et al., 2008), and C4 photosynthetic apparatus (Wang et al., 1992), *A. palmeri* may be regarded as a "super weed" (Guttmann-Bond, 2014).

Herbicides are considered as the most efficacious and cost-effective method for weed management. In the past, *A. palmeri* has been controlled mainly with three different classes of herbicide, acetolactate synthase (ALS) inhibitors, photosystem II (PSII) inhibitors, and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (Ward et al., 2013), but optimal management strategies are yet to be developed and concerns about the evolution of herbicide resistance remain to be addressed. This paper thus focuses on two key factors in the development of a sustainable long-term weed-management strategy, namely, estimating of the population of germinating seeds and evaluating herbicide susceptibility and resistance, and offers, for the first time, a non-destructive toolbox based on hyperspectral technologies and data analyses for the prediction of seed germination and herbicide response.

Fitness characters, such as seed germination, can have a significant effect on the robustness of the infesting field population and, as a consequence, on crop yield (Awan and Chauhan, 2016; Edelfeldt et al., 2016). This effect is predicted to be more extreme in the case of an aggressive noxious weed such as *A. palmeri* (Massinga et al., 2001; Ruf-Pachta et al., 2013). A negative correlation has been found between the viability of *A. palmeri* seeds and the depths to which the seeds are buried. Sosnoskie et al. (2013) showed that the deeper the burial depth, the lower germination rate. Seed dormancy can also inhibit seed germination, as has been demonstrated in a different species of *Amaranthus*, the common waterhemp [*A. tuberculatus* (Moq) Sauer]. Common waterhemp exhibits strong primary dormancy, which may be broken within 4 months after the ripening process, depending on the dormancy level (Wu and Owen, 2015).

Over the years, the intensive use herbicides have resulted in a strong selection pressure that has led to the evolution of herbicide-resistant weeds (Rubin, 1991). Resistance to several types of herbicide, including ALS, PSII and HPPD inhibitors, have been reported for *A. palmeri* (Ward et al., 2013). In particular, recent changes in herbicide regulations in Europe have led to increased use of ALS inhibitors (Kudsk et al., 2013), which is exacerbating concerns about the evolution of ALS resistance in *A. palmeri* populations and other weeds (Sibony and Rubin, 2003; Délye et al., 2011; Nandula et al., 2012; Matzrafi et al., 2015). One of the problems in monitoring the development of herbicide resistance is that it is usually conducted retrospectively using *in vitro* destructive molecular (Délye et al., 2015), physiological (Dinelli et al., 2008; Godar et al., 2015; Kleinman et al., 2015) and/or biochemical (Edwards and Cole, 1996; Tal et al., 1996; Matzrafi et al., 2014) methods. The weed science community has therefore recognized the need for methods to detect herbicide resistance at early stages of weed emergence before the herbicide is applied (Délye et al., 2015).

A possible means to facilitate the early detection of weeds lies in hyperspectral technologies. Such technologies are already in wide use in agriculture for such diverse applications as: (1) predicting seed germination (Nansen et al., 2015); (2) distinguishing between pest-infested and non-infested seeds (Nansen et al., 2014); (3) monitoring crop responses to biotic stressors (Prabhakar et al., 2012; Nansen and Elliott, 2016); (4) assessing the leaf area index (LAI) of wheat (*Triticum aestivum*) and potato (*Solanum tuberosum*) (Herrmann et al., 2011); and (5) determining – using near infrared (NIR) – rapeseed quality, i.e., seed weight and total oil content and oil fatty acid composition (Velasco et al., 1999). In addition, weed science studies have used hyperspectral methods to distinguish between weeds and crops (Okamoto et al., 2007; López-Granados et al., 2008; Herrmann et al., 2013). The use of reflectance-based analyses can therefore be exploited to prevent ineffective or needless applications of herbicides, slow down the evolution of herbicide resistance and to map the distribution (and the possible spread) of resistant *A. palmeri* populations in agro-ecological landscapes. To the best of our knowledge this is the first study exploring a method implementing hyperspectral

means in order to estimate *A. palmeri* infestation and herbicide response.

In the current study, hyperspectral methods form the basis of a method that facilitates the use of *ex situ* and *in vivo* non-destructive methods for estimating seed germination and herbicide response, respectively. With the ultimate aim to better understand the invasiveness of *A. palmeri* and hence facilitate the development of more targeted control methods, the current study addressed four specific aims: (***i***) to examine the accuracy and utility of hyperspectral imaging to predict the germination of *A. palmeri* seeds; (***ii***) to investigate the extent to which hyperspectral reflectance data from *in vivo* leaves of young *A. palmeri* can be used to detect and assess their response to the ALS inhibitor, trifloxysulfuron-methyl; (***iii***) to spectrally assess physiological parameters prior to herbicide application; and (***iv***) to explore image processing as a tool for evaluating herbicide response. The current study is also aiming to show feasibility for spectral assessment of weed response prior to herbicides application. Ability to estimate response to herbicide will be a game changer in the field of weed management and will allow early identification of resistant weeds creating better and efficient weed management.

MATERIALS AND METHODS

Plant Material and Herbicide Treatment

Three *A. palmeri* seed populations were collected from two corn fields (designated NA1 and NA2) at Kibbutz Na'an, Israel (31°53′01″N 34°51′28″E) and from a cotton field (designated BM1) at Moshav B'ney Darom, Israel (31°49′11″N 34°41′30″E). These fields were selected for two reasons: a long history of the use of herbicides, including ALS inhibitors (trifloxysulfuron-methyl and pyrithiobac-sodium), and recent reports of herbicide resistance by the farmers. Mature seeds from 30 *A. palmeri* plants were collected in each field, and the collected seeds from each field were considered as one "population." The seeds were air dried and stored at 4°C for at least 2 months before being used in this study. A total of 120 seeds (40 from each population) were imaged individually and subsequently tested for germination, as follows. Seeds were sown into pots (7 cm × 7 cm × 6 cm) containing 100% growth mixture, constrained of tuff, coconut and kavul in varying ratios (Tuff Marom-Golan, Ram 6, Israel) and left to germinated in a net house under summer conditions (30–35°C). Germination was assessed 7–10 days after sowing (DAS) (Guo and Al-Khatib, 2003), and seed viability was recorded as "germinated" or "non-germinated" seeds.

From the original 120 seeds, we obtained 67 plants (germinated seeds), which were subsequently used in studies of susceptibility to trifloxysulfuron-methyl (Envok, 75% SL, Syngenta, Basel, Switzerland). Twenty-one days after emergence (DAE), when the plants had three to four true leaves (after leaf gas-exchange and hyperspectral leaf data measurements had been obtained; see below), individual *A. palmeri* plants were treated with trifloxysulfuron-methyl at the equivalent rate of 11.25 g ai. h^{-1} mixed with 0.15% of the surfactant alkylaryl polyether alcohol (DX spreader, 800 g ai L^{-1}, Agan Chemical Manufacturers Ltd., Ashdod, Israel). Trifloxysulfuron-methyl was applied using a chain-driven sprayer delivering 300 L ha^{-1}. The experiment was arranged in a completely randomized factorial design inside a net house under summer conditions (25/35°C, night/day). To determine the plant response to trifloxysulfuron-methyl, fresh shoot biomass was recorded 21 days after treatment (DAT), i.e., at 42 DAE. Plants were initially grouped according to their visual injury, taking under consideration of their survival odds under crop-weed competition conditions. Five plants of each seed population served as the untreated control (without herbicide application).

Leaf Gas-Exchange Measurements

At 21 DAE, leaf gas-exchange measurements were conducted with a Li-6400 portable photosynthesis and fluorescence measurement system (6400-40 leaf-chamber fluorometer; Li-Cor, Inc., Lincoln, NE, USA). All 67 plants were measured for: predicted photosynthetic rate, stomatal conductance and PSII efficiency. The measuring chamber enclosed a circular 2-cm^2 section of leaf area and calculated the gas flow on both sides of the leaf. The air flow rate was kept constant at 500 μmol m^{-2} s^{-1}, and the reference CO_2 concentration was 400 μmol CO_2 mol^{-1} air (ppm). Light intensity was monitored prior to each measurement and kept constant at 1200 μmol photons m^{-2} s^{-1} (10% blue light). Leaf gas-exchange measurements were conducted in a net house during the day (9:00–11:00 am) under summer conditions: temperatures of 30–35°C, relative humidity of 45–55%, and radiation flux of 1000–1100 μmol m^{-2} s^{-1}. To assure homogeneity, all leaf gas-exchange measurements were acquired from the third fully expanded leaf from the top of the plant. The rate of carbon assimilation (μmol CO_2 m^{-2} s^{-1}) and the rate of stomatal conductance of water vapor (mol H_2O $m^{-2}s^{-1}$) were determined. Chlorophyll a fluorescence was assessed using equation 1: The quantum yield of PSII, $ØPS_2$, was calculated as follows.

$$ØPS_2 = \frac{F'_m - F_t}{F'_m} \quad (1)$$

where F_t is the steady-state fluorescence, and F'_m is the maximal fluorescence in the light-adapted state.

Hyperspectral Imaging of Seeds

A pushbroom hyperspectral camera (PIKA II, Resonon Inc., Bozeman, MT, USA) was mounted 40 cm above the seeds, and hyperspectral images were acquired under artificial light (two 15-W, 12-V light bulbs mounted on either side of the lens), with a spatial resolution of 50 pixels per 2 mm. The main specifications of the hyperspectral camera were: Firewire interface (IEEE 1394b), 12-bit digital output, 240 spectral bands from 392 to 889 nm (spectral resolution = 2.1 nm) by 640 pixels (spatial). The objective lens had a 35-mm focal length (maximum aperture of F1.4) with a 7° field of view, optimized

for NIR and visible–NIR spectra. A piece of white Teflon (K-Mac Plastics, Grand Rapids, MI, USA) was used for white calibration. Relative reflectance with reference to the reflectance from white Teflon was determined. Colored plastic cards (green, yellow, and red) were imaged at all hyperspectral imaging events, and average reflectance profiles from these cards were used to confirm the high consistency of hyperspectral image acquisition conditions (less than 2% variance within individual spectral bands).

The hyperspectral imaging data from the seeds was processed as following. All hyperspectral imaging files were converted into ASCII code and imported into the Statistical Analysis System (SAS) software package for processing and data classification. The first 14 and last 5 spectral bands were omitted from each hyperspectral data file, as these were considered to be associated with stochastic noise. Consequently, only 221 spectral bands from 423.6 to 878.9 nm were included in the analysis (**Figure 1A**).

For the hyperspectral imaging analysis of the seeds, 120 seeds were randomly divided into three equal groups, and each group was tested as an independent validation set. This procedure was repeated three times in order to build linear discriminant analysis (LDA; Fisher, 1936) classification models. The results of the three models were averaged.

Acquisition and Analysis of Hyperspectral Leaf Reflectance Data

The hyperspectral leaf reflectance data were obtained from the adaxial side of the same leaf that had been measured earlier for gas exchange. Hyperspectral data were obtained using an Analytical Spectral Devices (ASD) spectrometer FieldSpec 4 high resolution (ASD Inc., Boulder, CO, USA) having a range of 350–2500 nm, with the optic fiber connected to a contact probe (ASD Inc.). The contact probe had a tungsten halogen light source. To obtain pure reflectance of the leaf alone, a black metal plate was placed underneath each leaf during the spectral measurement; all 67 leaves were covered in the entire field of view. The spectrometer was programmed to average 10 spectra for each sample measurement, and white reference measurements were performed several times throughout the experiment. The dark current was applied automatically by a shutter in the spectrometer in accordance with the optimization for the lighting conditions facing the white reference panel (Hatchell, 1999). The spectral output was given automatically as relative units with 1 nm intervals, where relative units were obtained by dividing each target measurement by the last acquired white reference measurement.

To facilitate analysis of the data, the edges of the spectral range that were assumed to be noisy were eliminated, and the range was set to 400–2400 nm. To assess the ability to spectrally predict photosynthetic rate, stomatal conductance and PSII efficiency, the partial least squares regression (PLS-R) method was applied. The PLS-R is a practical predictive tool for hyperspectral data (Hansen and Schjoerring, 2003; Herrmann et al., 2011) and it was chosen because it can deal efficiently with the multi-collinearity of the reflectance values of the hyperspectral data (Wold and Eriksson, 2001; Atzberger et al., 2010). A PLS-R model was constructed for each of the measured plant properties, namely, photosynthetic rate, stomatal conductance and PSII efficiency, and each model was cross-validated using the Venetian blinds method. Each model was assessed in terms of its coefficient of determination (R^2), the root mean square error of calibration (RMSEC) and the RMSE of cross-validation (RMSECV).

To assess the ability to assign hyperspectral leaf data to different classes of herbicide response, PLS discriminant analysis (PLS-DA) was applied to allow maximal separation among the predefined classes (in this case, sensitive, moderate response, and resistant to the herbicide). This method has been used previously to differentiate between broadleaf weeds, grass weeds and wheat (Herrmann et al., 2013). To combine the PLS (numerical method) with the DA (categorical method), each class was assigned a binary artificial sequence of arbitrary numbers. This sequence was assigned to all the class samples; the size of the sequence was set by the number of classes (Musumarra et al., 2004; Xie et al., 2007). Spectral samples were used to build a PLS-DA model that was cross-validated using the Venetian blinds method. The cross validation results for the model are presented in the Results section. The classification quality was assessed by the accuracy figures presented in a confusion matrix and the matrix's Cohen's Kappa as presented and defined by Cohen (1960). The PLS-R and PLS-DA models and their post processing were run in a Matlab 7.6 (MathWorks, Natick, MA, USA) environment using the PLS toolbox (Eigenvector Research Inc., Wenatchee, WA, USA).

Herbicide Response Bulk Analysis of Different *A. palmeri* Populations

To determine whether the plants could be bulked instead of being analyzed as three different populations, two different statistical analyses (Tukey–Kramer and a leave-one-out cross-validation) were performed. The Tukey–Kramer test was performed using JMP Pro 12 (SAS Institute Inc., Cary, NC, USA). In the leave-one-out analysis, the average was calculated three times with different population set aside each time (Equation 2).

$$\overline{X} = \frac{X_1 \times n_1 + X_2 \times n_2}{(n_{\text{total}} - n_3)} \quad (2)$$

where $\overline{X}$ is the one-population-out average, X_i is the population average, n_i is the number of plants in the population, and i describes the tested population. According to both the Tukey–Kramer test and the cross-validation test, there were no significant differences between the three populations (Supplementary Table S1).

Assessing Plant Response to Trifloxysulfuron-methyl Using Digital Imaging Technology

To assess plant response to trifloxysulfuron-methyl, all plants (treated and untreated) were photographed 21 DAT. Photographs were taken with an off-the-shelf digital camera (Canon, PowerShot SX20 IS®) placed on a tripod, positioned at a 45°

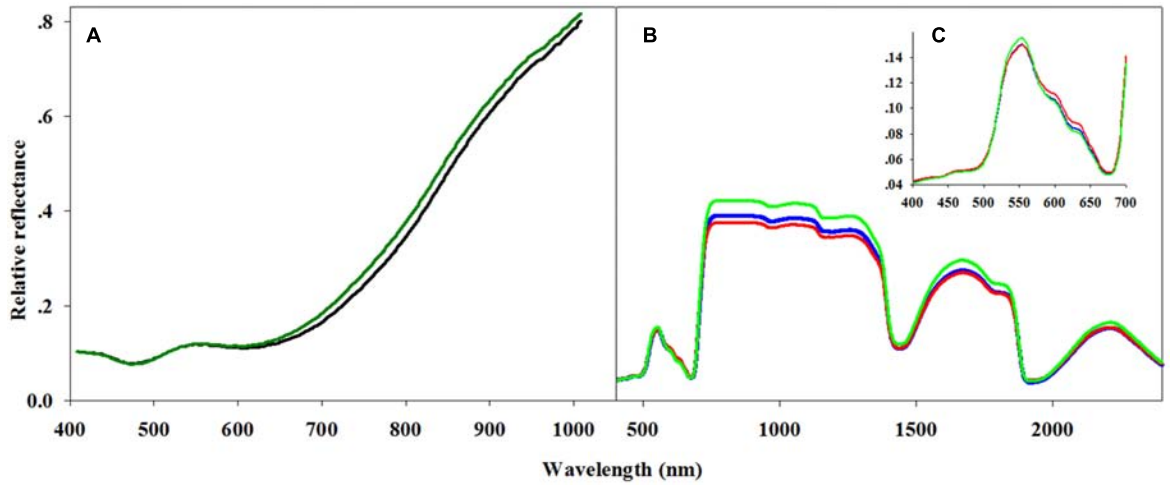

FIGURE 1 | Averaged spectra for hyperspectral analysis of seeds and leaves. (A) Two seed reflectance classes: germinating (dark green line) and non-germinating seeds (black line), **(B)** three classes, resistant (green line), moderate (blue line) and sensitive (red line), of general leaf reflectance, and **(C)** with a focus on the 400–700 nm spectral region covering the visible spectrum.

angle from the pot, at a distance of 1.2 m and against a black background. The 8-bit JPEG images analysis and processing were performed using Matlab and the public-domain software ImageJ (NIH)[1]. To assess plant weight based on the images, the first parameter to be analyzed was the mean gray value (MGV), and the thresholds were determined to include all green organs based on the hue, saturation and brightness (HSB) of an 8-bit JPEG image. The MGV was calculated from the average gray scale value of the pixels in the selected area for the HSB threshold using Equation 3.

$$gray\ value = 0.299\mathrm{R} + 0.587\mathrm{G} + 0.114\mathrm{B} \quad (3)$$

where R, G, and B stand for the three spectral regions: red, green, and blue, respectively. In all the 8-bit JPEG images, the setting of the different color components in each pixel was determined on the basis of the R, G, and B 8-bit (2^8) intensity graduations values, ranging from 0 to 255.

The second parameter to be determined was the area fraction (AF), which was calculated in Matlab based on images from all plants. The thresholds were determined to include all shoot tissue pixels based only on the brightness channel. AF was calculated as the sum of all of the pixels in the selected area (*SA*) divided by the total number of pixels in the image (*totA*; Equation 4).

$$AF = \frac{SA}{totA} \quad (4)$$

Data obtained from the photographs and data of shoot biomass (fresh weight, FW) were analyzed to determine the correlation between plant weight and the AF or MGV values. The data were analyzed using SigmaPlot software (ver. 10, Systat Software Inc., San Jose, CA, USA). A non-linear regression model [polynomial, linear (Equation 5)] was developed to analyze the correlation of the recorded weights from the different plants with the different AF and MGV values.

$$f = y_0 + a{*}x \quad (5)$$

where y_0 – the value of AF or MGV measured with ImageJ, a – the slope of the curve and x – the shoot FW (% of control).

DNA Extraction and Molecular Studies to Detect Target Site Resistance to ALS Inhibitors

Mutations in the *ALS* gene can endow herbicide resistance due to structural modifications in the herbicidal target site (Tranel et al., 2016). To detect structural substitutions, the *ALS* gene was sequenced and analyzed. A section of leaf tissue (3 cm^2) was excised from each treated plant. Each leaf section was placed in its own microtube. DNA was extracted using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and diluted 10-fold before further use. Primers were used to identify the gene and locate the common point mutations that can endow altered target sites. Known primers were used to sequence the *ALS* gene from *A. palmeri* (Sibony and Rubin, 2003; Manor, 2011).

All polymerase chain reaction (PCR) amplifications were performed in 25 μL with a final concentration of 0.20 μM of each dNTP and 0.25 μM of each primer. The cycling program began with 4 min at 94°C, followed by 37 cycles, each consisting of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C. The program ended with a final step of 4 min at 72°C. PCR products were separated on agarose gels (1.5%) to confirm the amplicon size, and each strand was sequenced using the same specific primers (Supplementary Table S2). Sequence analyses and alignment were performed using the BioEdit software (Hall, 1999). The obtained sequences were compared to known sequences of the *ALS* genes from *Arabidopsis thaliana* (X51514).

[1]http://rsb.info.nih.gov/ij

TABLE 1 | Seed distribution according to populations and germination model validation results.

Population	Germinating	Non-germinating	Total
NA1 (# of samples)	14	26	40
NA2 (# of samples)	30	10	40
BM1 (# of samples)	23	17	40
Total (# of samples)	67	53	120
Accuracy	81.21%	77.3%	
Standard deviation of accuracy	2.56	2.64	

n = 120; Kappa = 0.58.

RESULTS

Hyperspectral Seed Imaging for Germination Test

Seed germination was recorded 7–10 DAS (Supplementary Table S3) and data were correlated with data from reflectance measurements. Based on the LDA classification method, 67 seeds were identified as germinating and 53 as non-germinating, with accuracy (the ability to correctly identify each class) rates of 81.2% for the identification of germinated seeds and 77.3% for the identification of non-germinated seeds (**Table 1**). **Table 1** presents the distribution of the seed samples in terms of germination success among the three populations. The accuracies are of the ability to correctly identify each of the two classes.

Grouping the *A. palmeri* Plants according to Their Response to Trifloxysulfuron-methyl

Individual plants were grouped according to their response to trifloxysulfuron-methyl, as sensitive, moderate response, or resistant, according to whether they accumulated 0–20%, >20 to ≤40%, or >40%, respectively, of the biomass of the untreated control (Supplementary Table S3). The method of dividing the plants into different groups according to their response to the herbicide was examined and validated through the use of a chi-square test ($P > 0.75$). Out of the 67 plants used in the study, 13 plants were classified as resistant, 30 as moderate response, and 24 as sensitive (**Table 2**). This grouping method also reduces the effect contributed by the initial genetic differences and highlights the effect of environmental factors on herbicide response.

To eliminate the possibility of a target site resistance mechanism, we sequenced the *ALS* genes of 5–10 individuals from each response group. No alteration of the *ALS* gene that could be associated with target site resistance was found (Supplementary Figure S1).

Determination of the Response of *A. palmeri* to Trifloxysulfuron-methyl Using Hyperspectral Leaf Data

Using PLS-DA, we created a classification model that distinguishes between the three classes of herbicide response (sensitive, moderate response, resistant) based on the full spectral range (400–2400 nm; **Figure 1B**). The attempt to distinguish between the three classes based on cross-validation had a total accuracy of 50.7% (**Table 3**). For distinguishing solely between sensitive and resistant individuals (i.e., two classes), the total accuracy increased to 86.5% (**Table 4**).

Variable importance in projection (VIP) was used to explore the importance of the connection between spectral regions and the plants' herbicide response (Supplementary Figure S2 and Table S4). VIP values show the importance of each wavelength to the model (Cohen et al., 2010). This method was applied for the two-class PLS-DA classification model as presented by Wold et al. (1993). The two-class VIP model (Supplementary Figure S2 and Table S5) shows the VIP values and their peaks at 400–700 and 1850–2000 nm (**Figures 1B,C**). Therefore, a PLS-DA classification model of the same two classes was applied for each individual spectral region. Examination of the cross-validation

TABLE 2 | Distribution of *A. palmeri* population response groups under trifloxysulfuron-methyl treatment.

		Resistant	Moderate	Sensitive	Total count (% of total)
NA1	Count	4	5	5	14
	Total %	5.97	7.46	7.46	20.90
	Col %	30.77	16.67	20.83	
	Row %	28.57	35.71	35.71	
NA2	Count	4	14	12	30
	Total %	5.97	20.90	17.91	44.78
	Col %	30.77	46.67	50.00	
	Row %	13.33	46.67	40.00	
BM1	Count	5	11	7	23
	Total %	7.46	16.42	10.45	34.33
	Col %	38.46	36.67	29.17	
	Row %	21.74	47.83	30.43	
Total Count		13	30	24	67
Total %		19.40	44.78	35.82	

n = 67; (Prob > 0.7514).

TABLE 3 | Confusion matrix for distingushing between three response groups (resistant response, moderate response and sensitive response).

	Resistant	Moderate	Sensitive	Total predicted as	User accuracy (% correct)
Resistant	8	11	2	21	38.1
Moderate	4	7	3	14	50
Sensitive	1	12	19	32	59.4
Total actual class	13	30	24	67	
Producer accuracy (% correct)	61.5	23.3	79.2		50.7

Confusion matrix for the 400–2400 nm spectral range in the PLS-DA cross-validation model. n = 67, Kappa = 0.27.

results from the visible spectral range showed a higher level of total accuracy: 89.2% (**Table 4**).

Relationship between Physiological Characteristics and the Response of *A. palmeri* to Trifloxysulfuron-methyl

Evolutionary changes contributing to herbicide resistance can be correlated with different adaptive traits. We tested the differences in three physiological variables – photosynthetic rate, stomatal conductance, and PSII efficiency – in corelation with plants' response to the herbicide. Calibration and cross-validation data sets were fitted against measured data to determine the correlation between different herbicide response groups and data sets (**Figures 2B,D,F**). The obtained R^2 values for the calibration and cross-validation analyses were 0.71 vs. 0.61 for photosynthetic rate, 0.68 vs. 0.59 for stomatal conductance, and 0.71 vs. 0.60 for PSII efficiency (**Figures 2A,C,E**). The strong significant correlation between the measured and the predicted values indicates an actual relationship between these productivity traits and herbicide response (**Figures 2A,C,E** and **Table 5**). Herbicide response was found to correlated with higher physiological capacities. The resistant plant group exhibited significantly higher ($p \leq 0.05$) mean values for all three productivity traits, as compared to the sensitive group: 27.6 vs. 17.66 for photosynthetic rate, 0.14 vs. 0.1 for stomatal conductance, and 0.34 vs. 0.28 for PSII efficiency (Supplementary Table S6).

TABLE 4 | Confusion matrix for distingushing between two classes (resistant and sensitive).

	Resistant	Sensitive	Total predicted as	User accuracy (% correct)
Full spectral range 400–2400 nm				
Resistant	11	3	14	78.6
Sensitive	2	21	23	91.3
Total actual class	13	24	37	
Accuracy (% correct)	84.6	87.5		86.5
Visible spectral range 400–700 nm				
Resistant	10	1	11	90.9
Sensitive	3	23	26	88.5
Total actual class	13	24	37	
Accuracy (% correct)	76.9	95.8		89.2

Confusion matrix for the 400–2400 nm and 400–700 nm spectral ranges in the PLS-DA cross-validation model. 400–2400 nm (n = 37, Kappa = 0.71); 400–700 nm (n = 37, Kappa = 0.75).

Response of *A. palmeri* Plants to Trifloxysulfuron-methyl Assessed Using Imaging Technology

All plants were photographed digitally (**Figure 3A**) to allow area measurement of MGV (**Figure 3B**) and AF (**Figure 3C**). So as to refer only to the productive traits of the plant (defined by the green tissue), the thresholds based on HSB values were adjusted in the pictures of surviving plants. Initial variables for the ImageJ software were: hue: 45–115; saturation: 22–255; and brightness: 68–255. MGV and AF were determined using the macro record for the threshold area and batch-processing for the rest of the images. MVG was found to be highly correlated with the measured biomass ($R^2 = 0.84$; **Figure 3D**). So as to refer to the entire plant shoot, the brightness channel was used in an adjusted range of 0.50–1 (equivalent to 128–255 nm). The data analysis revealed a strong correlation between AF measured under these conditions and measured biomass ($R^2 = 0.95$; **Figure 3E**). Data shown here indicates that the AF parameter is more suitable for the prediction of absolute plant biomass, whereas plant survival and health are better predicted with the MGV.

Description of Germination Prediction and Herbicide Control of *A. palmeri*

We propose a bi-model (**Figure 4**) that uses reflectance data from seed imaging and hyperspectral data for leaves together with leaf physiological characteristics to predict both germination and herbicide response in a weed population. The first step is to obtain samples for spectral measurements: if there are weeds growing in the field, they are spectrally measured, and seed samples are collected for laboratory experiments. Seeds are cleaned of soil, imaged indoors, and transferred to soil-filled pots for germination in order to obtain germination validation data. Plants are then grown under controlled conditions for leaf hyperspectral measurements, followed by herbicide application for validation purposes. The analyses are based on validation of the germination obtained by germination tests as well as response to herbicide obtained by examination of plant biomass and survival rate at 21 DAT. In the current study, plants were grown in pots and measured in a net house, allowing validation of both germination and herbicide application. The outputs of

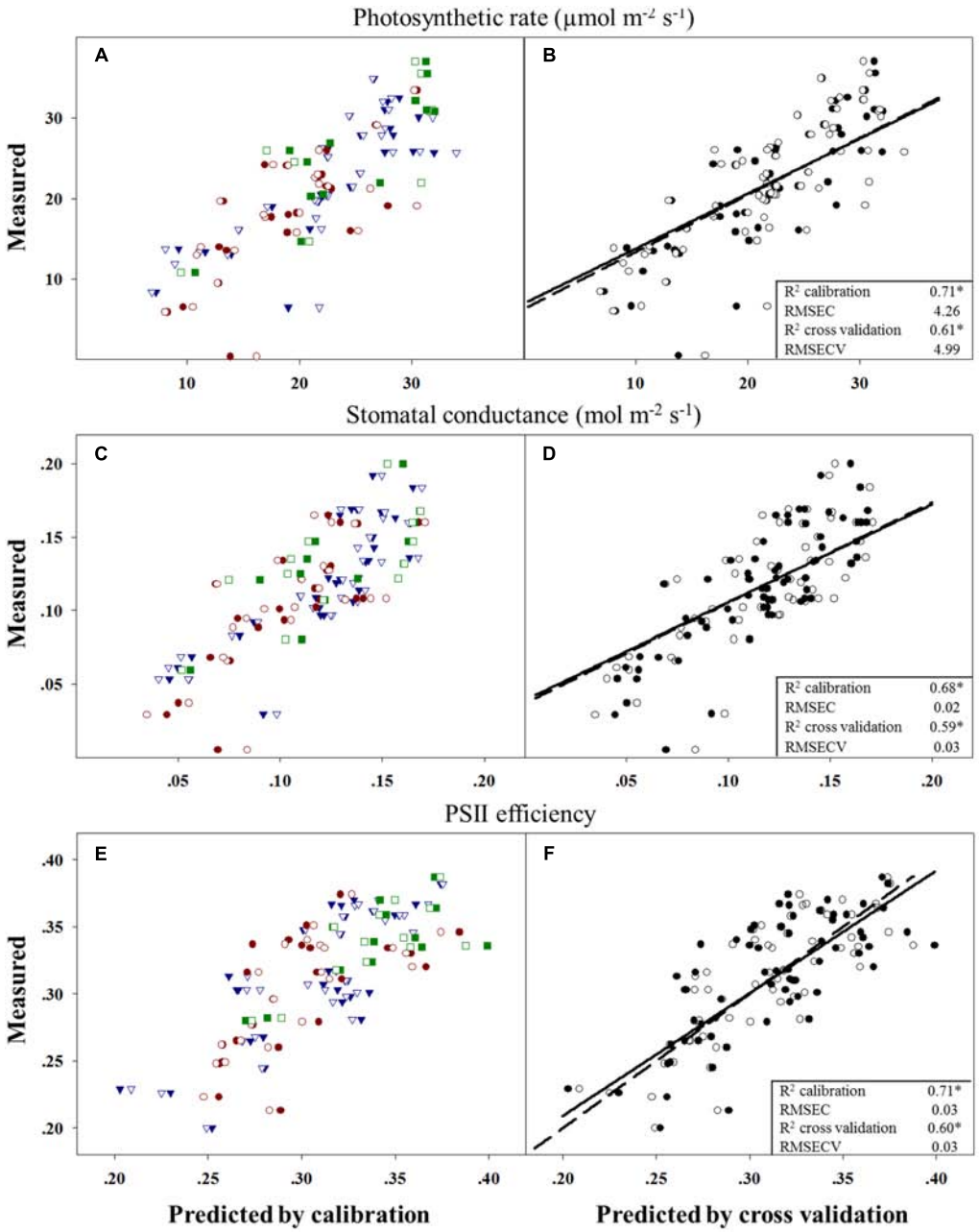

FIGURE 2 | Correlation between measured and predicted values in the calibration to (filled shapes/full line) and cross-validation (open shapes/dashed line) of all three physiological variables. Measured vs. predicted values for all 67 plants divided into the three response groups and by prediction method: moderate response (blue), sensitive (red) and resistant (green) **(A,C,E)**. Data for parameter comparisons conducted using the two methods: **(B)** photosynthetic rate, **(D)** stomatal conductance and **(F)** PSII efficiency (PSIIE). $n = 67$; $^*p < 0.001$.

the model enable both prediction of germination and response to herbicide.

DISCUSSION

In this study, we present novel, non-destructive methods for the estimation of seed germination and herbicide response in *A. palmeri* prior to herbicide application. At present, resistance is detected retrospectively (Steckel et al., 2008; Goggin et al., 2016; Rey-Caballero et al., 2016), and methods for the detection of herbicide resistance are based on time-consuming processes such as pre- or post-emergence herbicide application and heredity tests (Burgos et al., 2012). These methods result in at least one season of yield loss, often unnecessary multiple applications of herbicide, and long-term damage reflected in the enrichment of

TABLE 5 | Output of PLS-R cross-validation models for photosynthetic rate, stomatal conductance and photosystem II efficiency.

Model name	Photosynthetic rate	Stomatal conductance	Photosystem II efficiency
R^2 calibration	0.709*	0.684*	0.707*
RMSEC	4.26	0.023	0.027
Latent variable	6	6	6
R^2 cross-validation	0.610*	0.590*	0.595*
RMSECV	4.99	0.027	0.032

*n = 67; *p < 0.001.*

the seed bank with resistant seeds. Early detection of herbicide resistance may slow its evolution and can serve as a jumping-off point for developing alternative management practices to slow the spread of the phenomenon.

Seed hyperspectral imaging was found to be ∼80% accurate for germination prediction. An examination of the hyperspectral data obtained from leaves had 86.5% total accuracy for classification, based on two response groups (sensitive and resistant) instead of three (sensitive, intermediate, and resistant). When the spectral range was reduced to visible, the accuracy still remained relatively high, ∼89.2%. The resistant response group had higher mean values for all three physiological variables (photosynthetic rate, stomatal conductance, and PSII efficiency) than the sensitive group (Supplementary Table S6), which provided further support for the novel methodology presented in the current study. In most cases, due to its dominance, target site resistance divides the population into two phenotypic groups (sensitive and resistant). Sequencing individuals from all response groups, haven't reveal any known substitutions associated with resistance to ALS inhibitors. Similar cases of non-target site resistance to ALS inhibitors have previously been reported, and there is evidence that the involvement of a single gene encoding for cytochrome P450 enzymes can endow this resistance (Yamada et al., 2000; Gion et al., 2014). This type of resistance mechanism can be correlated with the effect of one gene with two alleles, creating three levels of response to the herbicide (sensitive, moderate, and resistant). Plants' response to trifloxysulfuron-methyl can also be endowed by other non-target site resistance mechanisms but our results eliminate the possibility of a target site resistance mechanism in our plants, reinforcing the validity of our three group analysis method. Further study is needed to better understand the correlation

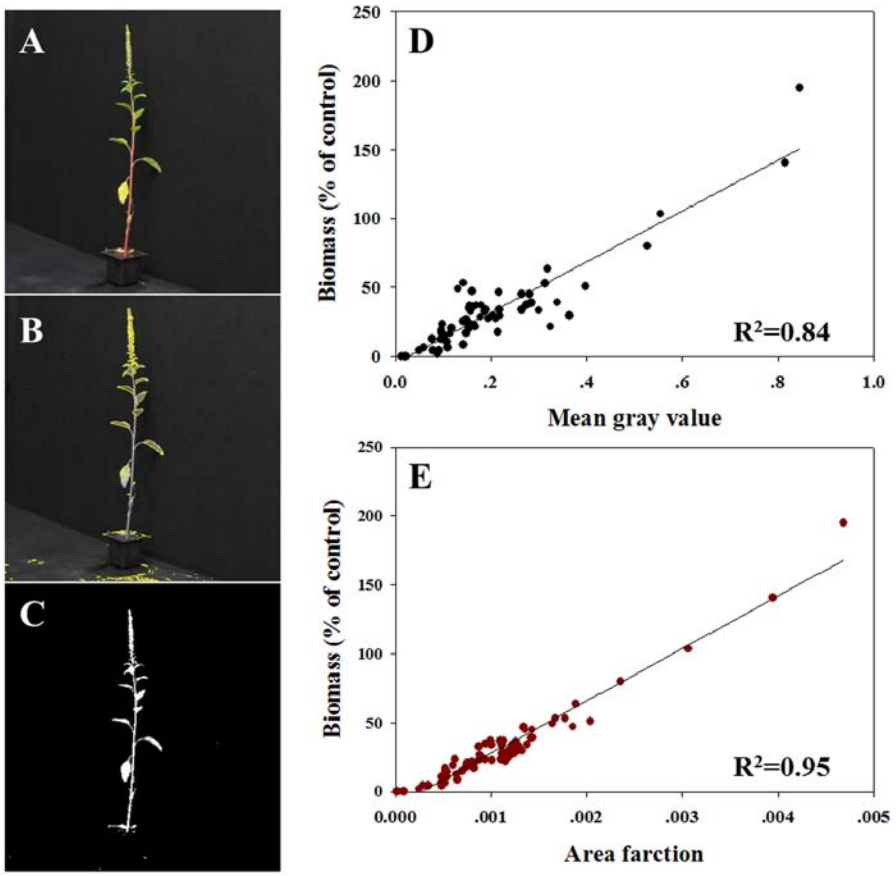

FIGURE 3 | Correlation between biomass (% of control) to two different parameters measured using ImageJ software. (A) Example of an actual digital photograph of a plant; **(B)** example of a picture of the selected area used for MGV assessment; **(C)** example of a picture of the selected area used for AF analysis; **(D)** MGV per plant vs. biomass, and **(E)** AF where all pixels per plant vs. biomass.

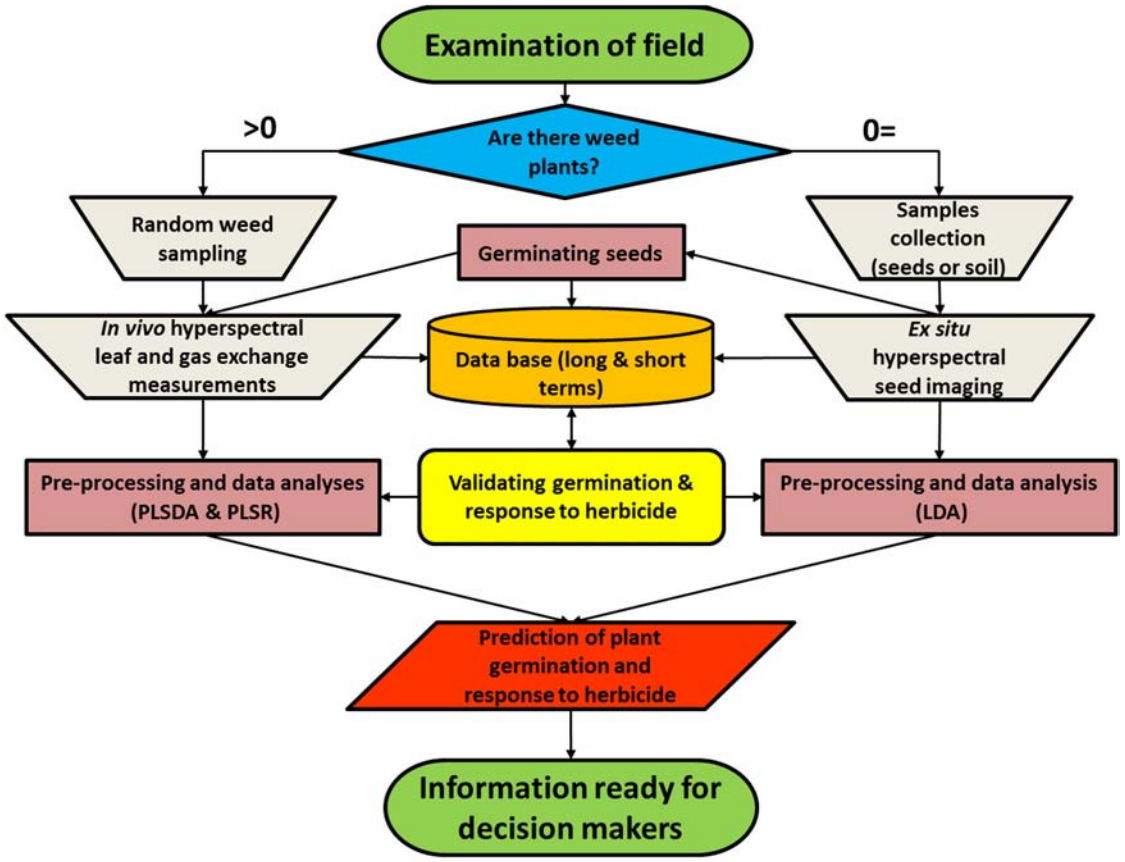

FIGURE 4 | A bi-model showing the process of data collection from a specific weed population to produce predictions of plant germination and herbicide response.

between herbicide response and different physiological traits. Hyperspectral analyses might be an efficient tool for achieving these goals.

In agriculture, hyperspectral techniques are already being applied to detect different traits in food products (ElMasry et al., 2007; Kamruzzaman et al., 2012; Ignat et al., 2014). Two particular studies describe work that impinges on our own: one reports the potential of NIR spectroscopy for the simultaneous analysis of seed weight, total oil content and oil fatty acid composition in intact single seeds of rapeseed (Velasco et al., 1999), and the other describes the use of NIR to discriminate between viable and empty seeds of *Pinus patula* Schiede and Deppe (Tigabu and Odén, 2003). In light of the above work, we hypothesized that the sequence of events leading up to germination and the accompanying changes in the contents of metabolites in the seeds would allow us to distinguish between *A. palmeri* seeds that are ready to germinate and a seeds that are not ready to germinate (dormant) or are non-viable. In the current study, we have shown a robust model (described below) for the detection of germination ability of *A. palmeri*.

In weed science, hyperspectral imaging has previously been used for site-specific weed management, particularly for weed crop classification (López-Granados, 2011; Herrmann et al., 2013; Shapira et al., 2013) or as a part of decision support system for herbicide application (Tellaeche et al., 2008; Lati et al., 2011). We could not find any reference to the use of this technology for early detection of herbicide response in young weeds (phenological stage of 3–4 true leaves). The model presented in the current study (**Figure 4**) merges seed and leaf assessment by hyperspectral technologies. Each of the model branches (i.e., seeds and plants) can be operated alone, but for optimal comprehensive weed management it is recommended that both branches of the model be applied. The output can be used by variety of decision makers. All the information acquired is entered into a database, which in the current era of big data will have a variety of immediate and potential uses: The database will find utility for applying data mining techniques for each run of the model as well as for long-term data collection and analyses that can also produce a spatial distribution of herbicide resistance. The imaging techniques described here can be used to predict seed germination, giving the farmer an indication of the following year's field population and an evaluation of weed infestation in the field. Chemical weed control can be applied both pre- and post-weed emergence (Yadav et al., 2016); certain compounds, such as pendimethalin (tubulin interaction inhibition), can serve for both purposes (Riches et al., 1997;

Yaduraju et al., 2000). The low benefit that is derived from pre-emergence herbicidal treatment is related largely to the uncertainty about the subsequent year's weed infestation rates. The seed data presented here can be used in a pre-season decision support system determining whether herbicides should be applied pre- or post-emergence. The non-destructive hyperspectral leaf methodology can provide immediate results and recommendations for the current season to prevent unnecessary herbicide applications and also to prevent the adding of the current year's herbicide resistant seeds to the seed bank. Here we propose a confirmed model to estimate *A. palmeri* population responses to trifloxysulfuron-methyl. This model is flexible as it can be adapted (after fitting modified parameters) to other troublesome weed species or crop tolerance to herbicides. The model can include safety as well as maintenance applications, that is, highway weed control and for removing weeds from fences as well as from parks and gardens. As the availability of hyperspectral sensors, computing power and machine learning techniques increases, we envisage that hyperspectral technologies determining resistance to a specific herbicide or herbicides will find ever-increasing application in weed control; for example, a sprayer with a hyperspectral 'eye' and a digital 'brain' would be able to deliver the most effective herbicide in real time. Such a system could also include non-destructive measurements for a variety of agricultural uses and the ability to collect seeds for future genetic studies.

CONCLUSION

In a world in which crop resources are decreasing, input investments in agriculture are increasing, and technology (e.g., optical sensors) is becoming more readily available and cost effective, the proposed hyperspectral detection methods for herbicide response could have a significant impact on the optimal exploitation for agriculture of semi-arid areas and in other resource-poor environments. The current study may be regarded as a 'feasibility check' of an integrated model that can predict both ecological fitness of a field population (e.g., seed germination) and the response to a specific herbicide. The proposed system can be applied to prevent ineffective and unnecessary use of chemicals, thereby reducing costs and, more importantly, minimizing the overload of unnecessary chemicals in the environment. The proposed toolbox could also serve as a powerful tool for herbicide development by improving accuracy of dosages and timing and increasing the probability of early detection of responses to herbicides in weeds as well as in crops. This methodology can also be applied in weed-infested non-arable lands and for other weed species and other herbicides.

AUTHOR CONTRIBUTIONS

MM, IH, CN, TK, YZ, TI, DS, BR, AK, and HE all contributed to the current study and to writing the paper. MM, HE, and BR conceived and designed the study. CN constructed the seeds hyperspectral imaging system and analyzed the data. IH, AK, and TI designed methodology of leaf data collection. IH and MM obtained the leaf spectral measurements. YZ and MM obtained the leaf gas exchange measurements. IH analyzed the leaf spectral data. TK conducted and analyzed RGB images.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00474/full#supplementary-material

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Physiological and Molecular Characterization of Hydroxyphenylpyruvate Dioxygenase (HPPD)-inhibitor Resistance in Palmer Amaranth (*Amaranthus palmeri* S.Wats.)

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Herbicides that inhibit hydroxyphenylpyruvate dioxygenase (HPPD) such as mesotrione are widely used to control a broad spectrum of weeds in agriculture. *Amaranthus palmeri* is an economically troublesome weed throughout the United States. The first case of evolution of resistance to HPPD-inhibiting herbicides in *A. palmeri* was documented in Kansas (KS) and later in Nebraska (NE). The objective of this study was to investigate the mechansim of HPPD-inhibitor (mesotrione) resistance in *A. palmeri.* Dose response analysis revealed that this population (KSR) was 10–18 times more resistant than their sensitive counterparts (MSS or KSS). Absorbtion and translocation analysis of [^{14}C] mesotrione suggested that these mechanisms were not involved in the resistance in *A. palmeri*. Importantly, mesotrione (>90%) was detoxified markedly faster in the resistant populations (KSR and NER), within 24 hours after treatment (HAT) compared to sensitive plants (MSS, KSS, or NER). However, at 48 HAT all populations metabolized the mesotrione, suggesting additional factors may contribute to this resistance. Further evaluation of mesotrione-resistant *A. palmeri* did not reveal any specific resistance-conferring mutations nor amplification of *HPPD* gene, the molecular target of mesotrione. However, the resistant populations showed 4- to 12-fold increase in *HPPD* gene expression. This increase in *HPPD* transcript levels was accompanied by increased HPPD protein expression. The significant aspects of this research include: the mesotrione resistance in *A. palmeri* is conferred primarily by rapid detoxification (non-target-site based) of mesotrione; additionally, increased *HPPD* gene expression (target-site based) also contributes to the resistance mechanism in the evolution of herbicide resistance in this naturally occurring weed species.

Keywords: mesotrione, resistant mechanism, target-site, non-target-site, metabolism, absorption and translocation, HPPD expression

INTRODUCTION

Mesotrione is a synthetic triketone herbicide chemically known as 2-[4-(methylsulfonyl)-2-nitrobenzoyl]-1,3-cyclohexanedione and biochemically inhibits 4-hydroxyphenylpyruvate dioxygenase (HPPD). This enzyme is important in the catabolism of tyrosine and anabolism of plastoquinones, tocopherols, and subsequently carotenoid biosynthesis (Beaudegnies et al., 2009). Plastoquinone plays a vital role in two significant pathways: (a) as an essential component of photosynthetic electron transfer from photosystem II (PS II) to photosystem I in the process of generating ATP, and (b) acts as an important cofactor for phytoene desaturase, a key enzyme in the carotenoid biosynthesis pathway. Carotenoids are light harvesting molecules, and protect plants from photo oxidation by quenching the triplet chlorophyll and prevent the formation of destructive singlet oxygen (Siefermann, 1987).

4-hydroxyphenylpyruvate dioxygenase-inhibitors are a relatively new class of chemistry discovered about three decades ago and are widely used in agriculture for weed management. HPPD-inhibitors are broadly classified into three chemical families: isoxazoles (e.g., isoxaflutole and pyrasulfotole), pyrazolones (e.g., topramezone), and triketones (e.g., mesotrione and tembotrione) depending on the chemical structure and properties (Lee et al., 1998). Upon treatment with these herbicides, susceptible plants exhibit characteristic bleaching symptoms as a result of loss of carotenoid synthesis and eventually leading to lipid peroxidation of cell membranes. Mesotrione is one of the most widely used HPPD-inhibiting herbicides that selectively control many broad-leaved weeds, including *Amaranthus palmeri*, and some grasses in corn (*Zea mays* L.) when applied post as well as pre-emergence herbicide (Mitchell et al., 2001). Rapid metabolism, via ring hydroxylation mediated by cytochrome P450 monooxygenase(s) combined with reduced absorption of mesotrione has been attributed to selectivity of this herbicide in corn (Mitchell et al., 2001). The differential selectivity of mesotrione and many herbicides such as sulfonylureas (ALS-inhibitors) and triazines (PS II-inhibitors) between crops and weeds is attributed to the ability of the crops to rapidly detoxify these compounds by cytochrome P450 monooxygenases or glutathione *S*-transferases (GSTs) (Hawkes et al., 2001). On the other hand, the differential selectivity of mesotrione between monocot and dicot species is attributed to HPPD enzyme in monocots being less sensitive to the inhibitors. Tobacco, a dicot species, is highly sensitive to mesotrione, however, when transformed with a *HPPD* gene from wheat, showed tolerance to this herbicide (Hawkes et al., 2001). Transgenic soybeans tolerant to mesotrione, tembotrione, and isoxaflutole have been developed with an herbicide-insensitive maize HPPD to increase the selectivity and spectrum of weed control (Siehl et al., 2014). Mesotrione and other HPPD-inhibitors are important in controlling several ALS- and PS II-inhibitor resistant weed biotypes (Sutton et al., 2002). It is also important to preserve the effectiveness and extend the use of these herbicides as no herbicides with new modes of action have been introduced in the last 20 years (Duke, 2012), and new herbicide-resistant traits are being stacked in crops to control weeds.

Palmer amaranth (*A. palmeri* S. Wats.) is one of the most economically important weeds in corn, soybean (*Glycine max* L.), cotton (*Gossypium* spp.), sorghum (*Sorghum bicolor* L.), and many other cropping systems throughout the United States (Ward et al., 2013; Chahal et al., 2015). Infestation of Palmer amaranth can significantly decrease the quality, and cause huge yield losses ranging from 63 to 91% depending on the density and duration of interference in different crops (Ward et al., 2013). Management of Palmer amaranth is possible using several herbicide chemistries, however, repeated and extensive use of herbicides resulted in the evolution of resistance to multiple herbicides with various modes of action such as 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, acetolactate synthase (ALS)-, PS II-, microtubule-, more recently to protoporphyrinogen oxidase (PPO)- and HPPD-inhibitor herbicides (Heap, 2017). Currently, two weed species in the Amaranthaceae family, common waterhemp (*A. tuberculatus*) and Palmer amaranth, have evolved resistance to several HPPD-inhibiting herbicides which offer a feasible option to manage other herbicide-resistant weeds including glyphosate-resistant Palmer amaranth (Norsworthy et al., 2008). HPPD-inhibitor resistant waterhemp was first reported in Illinois (IL) in 2009 (Hausman et al., 2011). Detoxification mediated by cytochrome P450 monooxygenases has been reported to confer mesotrione resistance in this waterhemp population (Ma et al., 2013).

In central Kansas (KS), a Palmer amaranth population with resistance to HPPD-inhibitors was first documented in Stafford County and subsequently confirmed in 2012 (Thompson et al., 2012). Later, HPPD-inhibitor resistant Palmer amaranth populations were also found in the nearby state of Nebraska (NE) in a corn field, which had a history of continuous use of HPPD-inhibitors (Sandell et al., 2012). Interestingly, the field in KS where HPPD-inhibitor-resistant Palmer amaranth was found, had no previous history of applications of HPPD-inhibitors, but did have a long history of PS II- and ALS-inhibiting herbicides. This population was initially found resistant to Huskie® (Bayer Crop Science), a mixture of pyrasulfotole (HPPD-inhibitor) and bromoxynil (PS II-inhibitor) and is also resistant to several other HPPD-inhibitors such as mesotrione, tembotrione, and topramezone and was also found to be resistant to atrazine, a widely used PS-II inhibitor (Lally et al., 2010; Thompson et al., 2012). The mechanism of HPPD-inhibitor resistance in the Palmer amaranth populations from KS or NE is unknown. The objectives of this research were to determine the mechanism(s) of resistance to mesotrione in the HPPD-inhibitor resistant Palmer amaranth populations from KS and NE.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Three mesotrione 'resistant' Palmer amaranth populations from Kansas (KS) and Nebraska (NE), designated as KSR, KSR2, NER and five mesotrione 'susceptible' populations from Mississippi

(MS), KS, and NE, designated as MSS, KSS, KSS II, KSS III, and NES, respectively, were used in this study. KSR seed was derived by crossing male and female plants of Palmer amaranth from KSR2 that survived 105 g ai ha^{-1}, field use rate of mesotrione (Callisto^{TM}, Syngenta Crop Protection) under greenhouse conditions to generate a more homogeneous resistant population. However, KSR2 seed was collected from Palmer amaranth plants which survived a HPPD-inhibitor application in a field in Stafford County, KS (Thompson et al., 2012) that had wheat-sorghum crop rotation. Seed of NER was collected from Palmer amaranth that survived mesotrione application in a corn field in NE (Sandell et al., 2012). NES population is also provided by Sandell et al. (2012) and MSS by Syngenta. The mesotrione-susceptible populations were selected based on their sensitivity to mesotrione at field recommended rate (i.e., completely killed at field rate) relative to resistant populations. The three susceptibles from KS comes from three distinctly separated locations. KSS (Thompson et al., 2012), KSS II (37°31′05.74″ N and 097°29′42.43″ W), KSS III (37°59′24.0″ N and 100°49′12.0″ W) are from fields in Riley, Reno, and Finney Counties in KS, respectively. Seeds of mesotrione-susceptible and -resistant Palmer amaranth were germinated in small trays (25 cm × 15 cm × 2.5 cm) with commercial potting mixture (Miracle Gro). Seedlings 2–3 cm tall, were transplanted into small pots (6 cm × 6 cm × 6.5 cm) in the greenhouse, maintained at 25/20°C and 15/9 h photoperiod, supplemented with 250 μmol m^{-2} s^{-1} illumination provided with sodium vapor lamps. When the plants reached 5–6 cm tall, they were transferred to a growth chambers maintained at 32.5/22.5°C, 15/9 h photoperiod, 60–70% relative humidity. Light in the growth chamber was provided by fluorescent bulbs delivering 550 μmol m^{-2} s^{-1} photon flux at plant canopy level. Plants were watered as needed regularly both under greenhouse as well as growth chamber conditions.

Mesotrione Dose Response Assay

Mesotrione-resistant (KSR) and -susceptible (MSS and KSS) Palmer amaranth were grown under greenhouse and growth chamber conditions as described above. Initially, the KSR and KSR2 Palmer amaranth populations were screened with the commercial field application rate of 105 g ai ha^{-1} mesotrione to determine the frequency of resistant individuals in the population before determining the level of resistance by dose response assay. The frequency of resistance was 90–95% and 60–70% in KSR and KSR2, respectively (data not shown). For the dose response analysis, when the Palmer amaranth plants (MSS, KSS, and KSR) were 10–12 cm tall with 8–10 leaves, mesotrione was applied at 0, 6.5, 13.125, 26.25, 52.5, 105 (1X), 210, 315, 420, and 840 g ai h^{-1}, where 1X represents the field recommended rate of mesotrione. This stage (8–10 leaves) is the phenological stage at which most farmers in KS and NE apply mesotrione to control Palmer amaranth. Required adjuvants, crop oil concentrate (COC, Agridex) and ammonium sulfate (AMS, Liquid N-Pak; Winfield) at 1% v/v and 1% w/v (8.5 lb/100 gal = 1% w/v), respectively, were included, respectively, in all the treatments to enhance droplet-to-leaf surface contact. Treatments were applied with a bench-type track sprayer (Generation III, DeVries Manufacturing, RR 1 Box 184, Hollandale, MN, USA) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., P.O. Box 7900, Wheaton, IL, USA) delivering 187 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. Following treatment, plants were returned to the same growth chambers (within 30 min after treatment). Treatments were arranged in a completely randomized design with five replications and the experiment was repeated three times. Treated plants were clipped off at the soil surface and immediately weighed (aboveground fresh biomass) 3 weeks after treatment (WAT). Harvested plants were packed in paper bags and oven (Precision Scientific Thelco Laboratory Oven) dried at 60°C for a week before measuring dry biomass.

Absorption of [^{14}C] Mesotrione and Translocation of [^{14}C] Compounds

Greenhouse grown seedlings (as described above) of KSR and MSS and KSS Palmer amaranth were moved to growth chamber 2–3 days before applying [^{14}C] mesotrione to allow the plants to acclimate. Ten to twelve centimeters tall (8–10 leaf stage) plants were treated with a total of 3.3 kBq of [phenyl-U-^{14}C]-labeled mesotrione with specific activity of 781 M Bq g^{-1}. Unlabeled mesotrione was added to the radioactive solution to obtain 105 g ai ha^{-1} mesotrione in a carrier volume of 187 L. Additionally, COC (Agridex) and AMS (Liquid N-Pak; Winfield) were added at 1% v/v and 1% w/v, respectively, to this mixture to enhance droplet-to-leaf surface contact. A total volume of 10 μL was applied as 10 1 μL droplets on the upper surface of the fourth youngest leaf. The treated plants were returned to the same growth chamber. Plants were harvested at 48 and 72 hours after treatment (HAT) and separated into treated leaf (TL), leaves above the treated leaf (ATL), and leaves below the treated leaf (BTL) and wrapped in a single layer of tissue paper. Treated leaves were washed with 5 mL wash solution (10% methanol and 0.05% Tween) for 60 s in a 20 mL scintillation vial to remove any unabsorbed herbicide. Radioactivity in the leaf rinsate was measured using liquid scintillation spectrometry (LSS: Tricarb 2100 TR Liquid Scintillation Analyzer; Packard Instrument Co., Meriden, CT, USA). Plant parts were oven (Precision Scientific Thelco Laboratory Oven) dried at 60°C for 48 h and total radioactivity absorbed was quantified by combusting using a biological oxidizer (OX-501, RJ Harvey Instrument) and LSS. Total [^{14}C] mesotrione absorption was determined as; % absorption = (total radioactivity applied – radioactivity recovered in wash solution) × 100/total radioactivity applied. Herbicide translocation was determined as; % translocation = 100 – % radioactivity recovered in treated leaf, where % radioactivity recovered in treated leaf = radioactivity recovered in treated leaf × 100/radioactivity absorbed. Six replications were included in each treatment and the experiment was repeated.

Metabolism of Mesotrione in Whole Plant and Treated Leaves

KSR, NER and MSS, KSS and NES Palmer amaranth populations were grown as described previously for [^{14}C] mesotrione absorption and translocation experiments. Twenty microliter of

[^{14}C] mesotrione containing 7.2 kBq was applied on 10–12 cm tall (8–10 leaf stage) plants as 10 1μL droplets on the adaxial surface of fully expanded fourth and fifth youngest leaves. [^{14}C] mesotrione and its metabolites were extracted as described in Godar et al. (2015). Treated leaves were harvested 4, 8, 16, 24, 48, and 72 HAT and washed with wash solution to remove unabsorbed herbicide. Whole plant tissue including the washed treated leaves or only the treated leaves were then frozen in liquid nitrogen and homogenized using a mortar and pestle. [^{14}C] mesotrione and its metabolites were extracted with 15 ml of 90% acetone at 4°C for 16 h. The samples were centrifuged at 5,000 × *g* for 10 min and supernatant from each sample was concentrated at 45°C for 2–3 h with a rotary evaporator (Centrivap, Labconco) until a final volume of 500–1000 μL of extract was reached. The extract was then transferred to a 1.5 mL microcentrifuge tube and centrifuged at high speed (10,000 g) for 10 min at room temperature. The total radioactivity in each sample was measured by LSS and samples were normalized to 0.05 KBq/50 μL (3000 dpm/50 μL) amount of [^{14}C]-labeled compounds by diluting the samples with acetonitrile:water (50:50, v/v) prior to HPLC analysis.

Total extractable radioactivity in 50 μL was resolved into parent [^{14}C] mesotrione and its polar metabolites by reverse-phase HPLC (Beckman Coulter, System Gold) following the protocol optimized previously in our laboratory (Godar et al., 2015). Reverse-phase HPLC was performed with a Zorbax SB-C18 column (4.6 mm × 250 mm, 5-μm particle size; Agilent Technologies) at a flow rate of 1 mL min^{-1}. The radioactivity in the sample was detected using radio flow detector LB 5009 (Berthold Technologies). The whole plant metabolism experiment had three replicates for each treatment and the experiment was repeated. Similarly, the experiment where metabolism of mesotrione in only TL was performed also included three replicates and was repeated.

RNA Extraction, cDNA Synthesis, and *HPPD* Gene Expression

In this study, the KSR, NER and MSS, KSS, KSS II, KSS III, NES Palmer amaranth plants were not treated with mesotrione, however, adjuvants COC (1% v/v) and AMS (0.85% w/v) were applied to 10–12 cm tall plants. Above ground plant tissue was harvested 24 h after treatment and frozen in liquid nitrogen and stored at −80°C for RNA isolation. The frozen tissue was homogenized in liquid nitrogen using a pre-chilled mortar and pestle to prevent thawing, and transferred 100 mg tissue into a 1.5 mL microcentrifuge tube. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). The quality and quantity of total RNA was determined using agarose gel (1%) electrophoresis and spectrophotometer (NanoDrop 1000, Thermo Scientific), respectively, and RNA was stored at −80°C.

For cDNA synthesis, 1 μg of total RNA was treated with DNase 1 enzyme (Thermo Scientific, Waltham, MA, USA) to remove any genomic DNA (gDNA). cDNA was synthesized from 1 μg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and was diluted in 1:5 ratio for gene expression study. Quantitative PCR/real-time PCR (qPCR/rtPCR) was used to determine *HPPD* gene expression in all samples. The qPCR reaction mix consisted of 8 μL of SYBR Green mastermix (Bio-Rad Inc., Hercules, CA, USA), 2 μL each of forward and reverse primers (5 μM), and 20 ng cDNA to make the total reaction volume of 14 μL. *HPPD* gene expression was normalized using either *β-tubulin* or carbamoyl phosphate synthetase (*CPS*) as a reference gene. qPCR (CFX96 Touch^{TM} Real-Time PCR Detection System, Bio-Rad Inc.) was performed at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 30 s and 60°C for 1 min (Ma et al., 2013). A meltcurve profile was included following the thermal cycling protocol to determine the specificity (no primer dimers, no gDNA contamination, and no non-specific product) of the qPCR reaction. Primer sequences used were: HPPD forward and reverse (F 5′-CTGTCGAAGTAGAAGACGCAG-3′ and R 5′-TACATACCGAAGCACAACATCC-3′); *β-tubulin* forward and reverse (F 5′-ATGTGGGATGCCAAGAACATGATGTG-3′ and R 5′-TCCACTCCACAAAGTAGGAAGAGTTCT-3′); and CPS forward and reverse (F 5′-ATTGATGCTGCCGAGGATAG-3′ and R 5′-GATGCCTCCCTTAGGTTGTTC-3′). The *HPPD*:*β-tubulin* and *HPPD:CPS* expression was determined using the $2\Delta C_T$ method, where C_T is threshold cycle and ΔC_T is $C_{T_{\text{Reference gene (}\beta\text{-tubulin, or CPS)}}} - C_{T_{\text{Target gene (HPPD)}}}$. HPPD gene expression was studied using three biological replicates and three technical replicates for each biological replicate. The experiment was repeated three times and the average value ± standard error of total biological replicates was used to show the expression fold.

Protein Extraction, SDS–pAGE, and Western Blotting

Above ground plant tissue (0.5 g) from 10 to 12 cm tall Palmer amaranth from KSR, NER and MSS, KSS, KSS II, KSS III and NES was homogenized in liquid nitrogen and added to 20 mL extraction buffer [50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, and 0.038 g PMSF, one tablet of Pierce Protease Inhibitor (Thermoscientific), 1 g insoluble PVPP]. The extraction and purification procedure was developed by modifying the methods of Wang et al. (2006) and Wu et al. (2014). In short, homogenates were centrifuged at 4°C, 10 min, 12000 × *g* (Beckman J2-HC centrifuge, USA) and supernatant was collected. One milliliter of TCA (100%) was added to 10 ml of supernatant and incubated for 1 h at 4°C. Samples were centrifuged as before, and the supernatant was discarded. Two milliliter of methanol (100%) was added to the pellet, tubes were vortexed vigorously for 60 s and centrifuged (4°C, 10 min, 12000 × *g*). Supernatant was discarded and acetone (2 ml; 80%) was added to the pellet, vortexed and then centrifuged (4°C, 10 min, 12000 × *g*). Pellet was air dried to remove the remaining acetone and 2 ml phenol (equilibrated with Tris-HCL; pH 8.0, Sigma) was added, vortexed at high speed for 30–60 s and centrifuged (4°C, 10 min, 12000 × *g*) and the supernatant was collected. Proteins were precipitated by adding 2 mL ammonium acetate (0.1 M in methanol) to the supernatant and incubated overnight at −20°C. Next, the sample was centrifuged (4°C, 10 min, 12000 × *g*) and the supernatant was discarded. Pellet

was washed with methanol (100%) followed by acetone (80%) and finally air dried. Dried samples were resuspended in 200 μL SDS-Sample buffer and the protein concentration in the extract was determined using the RED 660^{TM} Protein Assay (G-Biosciences).

To resolve proteins in the samples by SDS gel electrophoresis, samples were incubated at 95°C for 5 min. Next, 50 μg of total protein was resolved by electrophoresis on 11% polyacrylamide gel (90 min at 120 V) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) at 150 V for 1 h or 30 V overnight. The PVDF membrane was blocked with 5% non-fat dry milk at room temperature for 30 min and then washed three times in TBST. The membranes were incubated with a rabbit polyclonal HPD antibody (Novus biologicals; dilution 1:500) in TBST at 4°C overnight. The membrane was washed three times with TBST and incubated in with donkey anti-rabbit HRP conjugated polyclonal antibody (Jackson Immuno Research Laboratories Inc; dilution 1:50,000) at room temperature for 1 h. After three more washes, membranes were exposed to an HRP substrate solution (Luminata^{TM}, Millipore) and image detection was carried out using a G-BOX (Syngene).

DNA Extraction and *HPPD* Gene Amplification

DNA extraction for *HPPD* gene amplification was performed on the same plant samples used for RNA extraction, cDNA, and *HPPD* gene expression. gDNA was extracted from the frozen leaf tissue (100 mg) using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The quality and quantity of gDNA was determined using agarose gel (0.8%) electrophoresis and spectrophotometer (NanoDrop 1000, Thermo Scientific) and DNA was stored at −20 or −80°C. The following forward and reverse primers (F 5′-CTGTCGAAGTAGAAGACGCAG-3′ and R 5′-TACATACCGAAGCACAACATCC-3′) were used to amplify the *HPPD* gene from Palmer amaranth populations.

Statistical Analysis

All the experiments were conducted in a completely randomized design, and the data from all experiments were combined for each study before performing statistical analysis as there was no interaction between the experiments and treatments.

Dose-response data (expressed as percentage of the untreated control) were analyzed using 'drc' package in R 3.1.2 (Ritz et al., 2015). The three-parameter log-logistic model as shown below was used to show the relationship between herbicide rate and biomass, $Y = d/[1+\exp\{b[\log(x) - \log(GR_{50})]\}]$ where Y is the response (dry biomass or plant health) expressed as percentage of the untreated control, d is asymptotic value of Y at upper limit, b is the slope of the curve around GR_{50} (the herbicide rate giving response halfway between d and the lower asymptotic limit which was set to 0), and x is the herbicide rate. Resistance index (R/S) was calculated as GR_{50} ratio between the MSS or KSS and the KSR populations.

Absorption and translocation data, expressed as percentage of applied and absorbed, respectively, metabolism data, and qPCR (*HPPD* gene expression) data were analyzed using one-way ANOVA in R 3.1.2 and the means were compared using Tukey's HSD test. The time course of mesotrione metabolism by MSS and KSR Palmer amaranth populations was fitted with a three-parameter Weibull regression.

RESULTS

Mesotrione Dose Response Assay to Determine the Level of Resistance

The HPPD-inhibitor-resistant and -susceptible Palmer amaranth populations were derived from different locations. To determine their level of resistance to mesotrione, we conducted dose response assays with these populations. We found a variation in the level of resistance to mesotrione at individual plant level in all populations, especially the KSR2 (**Figure 1A**). This variation is a reflective of genetic variability within and among the populations because the experiments were conducted under controlled environmental conditions (growth chambers) eliminating changes in environmental conditions. Since KSR2 showed extreme variation at 105 g ai ha^{-1} mesotrione, the population was not used further in the dose response analysis. The amount of mesotrione required to reduce plant growth to 50% (GR_{50}) 3 WAT was ∼151 g ai ha^{-1} for KSR compared to 15 and 8 g ai ha^{-1} for MSS and KSS, respectively (**Figure 1B**). However, all the surviving resistant individuals showed injury (bleached) symptoms on shoot meristem at all doses of mesotrione and 3 WAT the injured plants did not recover to phenotype of untreated plants, even at low doses of 52.5 g ai ha^{-1} mesotrione. The KSR was 10 and 18 times more resistant compared to MSS and KSS, respectively (**Figure 1B** and **Table 1**). In a different study, the NER Palmer amaranth showed 4- to 14-fold resistance relative to NES in response to mesotrione, tembotrione, and topramezone applications (Sandell et al., 2012).

Absorption of [^{14}C] Mesotrione and Translocation of [^{14}C] Compounds

The resistance/higher tolerance to mesotrione and other HPPD-inhibiting herbicides can arise through a variety of mechanisms. First, we tested if there is a difference in absorption by measuring how much [^{14}C] mesotrione was absorbed by the resistant and susceptible plants. Absorption of [^{14}C] mesotrione in KSR at 48 and 72 HAT was 71 and 69% (as % of total applied), which was not significantly different from the susceptible populations (76 and 74% in MSS and 69 and 77% in KSS, at 48 and 72 HAT, respectively; **Figures 2A,B**, $P > 0.05$).

Resistance can be derived if the plants have reduced translocation of the herbicide. Since, [^{14}C] mesotrione after application can be translocated to ATL, BTL or roots or stay in TL as [^{14}C] mesotrione or its metabolites and it is difficult to separate specific [^{14}C]'s, it is more appropriate to say translocation of [^{14}C] compounds. Data analysis showed no significant differences in the translocation of [^{14}C] compounds to ATL or BTL from TL at 48 HAT between resistant or susceptible populations. KSR (37% expressed as % of total [^{14}C] mesotrione absorbed) showed translocation that was in between both susceptible populations MSS (29%) and KSS

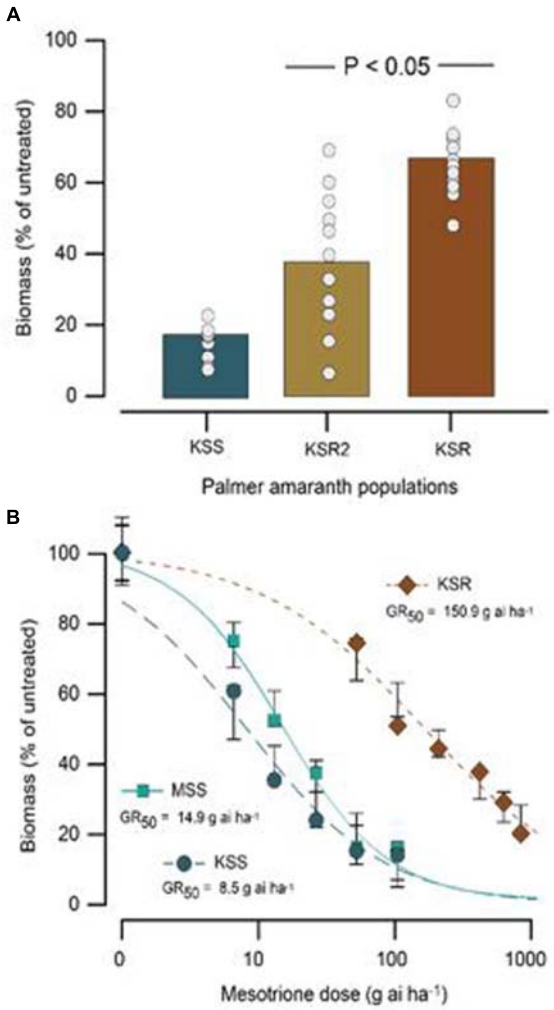

FIGURE 1 | Response of susceptible (MSS and KSS) and resistant (KSR) Palmer amaranth populations 3 weeks after treatment (WAT) with the herbicide mesotrione. (A) Individual plant aboveground dry biomass variability in response of KSR compared to the original KSR2 population to 105 g ai ha^{-1} rate of mesotrione, bar indicates the average from 12 individual samples (circles). **(B)** Non-linear regression analysis of aboveground dry biomass of MSS, KSS, and KSR populations at different doses of mesotrione. Symbols are averages of 12 replicates fitted with a three-parameter log-logistic model; model parameters are shown in **Table 1**.

TABLE 1 | Summary parameters describing the response of MSS and KSS (susceptible) and KSR (resistant) Palmer amaranth aboveground dry biomass to rates of mesotrione 3 weeks after treatment (WAT).

Population	Regression parameters[b]		GR_{50}[b]	R/S[c]	R/S[d]
	b	*d*	g ai ha^{-1}		
MSS	1.13	100.8 (4.0)	14.9 (1.7)	1	1.76*
KSS	0.95	100.6 (4.8)	8.5 (1.4)	0.6*	1
KSR	0.69	100.9 (4.1)	150.9 (25.9)	10.1**	17.8**

[a]*Abbreviations: WAT, weak after treatment; b, relative slope around GR_{50}; d, upper limit of the response; GR_{50}, mesotrione rate causing 50% reduction in aboveground dry biomass; R/S, resistance index [ratio of GR_{50} of MSS or KSS (susceptible) and KSR (resistant) populations].*
[b]*Values in parenthesis are ±1 standard error.*
[c]*RS values based on MSS population.*
[d]*RS values based on KSS population.*
*,**R/S is significantly greater than 1 at $P < 0.001$, $P = 0$, repectively.*
*The response was fitted with a three-parameter log-logistic model; fitted curves are shown in **Figure 1B**.*

(55%) populations (**Figure 2C**, $P > 0.05$). This suggests that there is an underlying genetic variation in the ability of Palmer amaranth to translocate mesotrione that does not correlate with resistance. This variation is likely responsible for the significant difference we observed in the translocation of [^{14}C] mesotrione between the MSS and KSS. Furthermore, the significant difference disappeared at 72 HAT where the KSR, MSS and KSS had 39, 33, and 39%, respectively, of [^{14}C] mesotrione translocated from the TL, to the above and below treated plant parts (**Figure 2D**, $P > 0.05$). In addition, because of rapid metabolism of mesotrione in resistant plants (**Figure 3**) it was not possible to say whether there were any differences in the translocation of mesotrione between resistant and susceptible Palmer amaranth. However, assuming that the major metabolites of mesotrione move in a similar way as the parent molecule, translocation appears to be similar. Thus, neither difference in mesotrione absorption nor translocation contributed substantially to mesotrione resistance in KSR Palmer amaranth.

Metabolism of [^{14}C] Mesotrione

Some weeds also have been shown to acquire resistance by increasing their ability to metabolize specific herbicides. To test for a role of metabolism based resistance in the KSR population, we measured how much [^{14}C] mesotrione was metabolized into other polar compounds over time. The input [^{14}C] mesotrione resolved at peak retention time of about 18.1 by reversed-phase HPLC with no other peaks observed (data not shown). This indicates that peaks at 13.1 and 14.3 retention times observed in plant lysates are products derived from mesotrione metabolism (**Figure 3**). These peaks gradually increased with decrease in input [^{14}C] mesotrione in all the populations indicating that the metabolites might be hydroxylated products of mesotrione (Ma et al., 2013). To determine the % of mesotrione remaining, we quantified the amount of radioactivity of the 18.1 peak as fraction of total radioactivity. As early as 4 HAT we observed significant differences with more than 70% of input parent [^{14}C] mesotrione still being detected in susceptible samples, while in KSR plants ∼50% of parent [^{14}C] mesotrione was metabolized (data not shown). At 24 HAT, KSR, and NER metabolized much more parent compound (>90%) compared to MSS, KSS and NES (**Figures 3A–E**) ($P < 0.01$), which still showed about 28, 30, and 50%, respectively, of parent [^{14}C] mesotrione. This amount of mesotrione was sufficient to injure the plant and subsequently kill the susceptible plants 3 WAT. The half-life T_{50} is the amount of time taken for 50% of the parent input [^{14}C] mesotrione to degrade or metabolize inside the plant through enzymatic transformation. It was found that T_{50} for MSS and KSR was 14.6 and 5.9 h, respectively, indicating that KSR metabolizes the mesotrione 2.5 times faster compared to

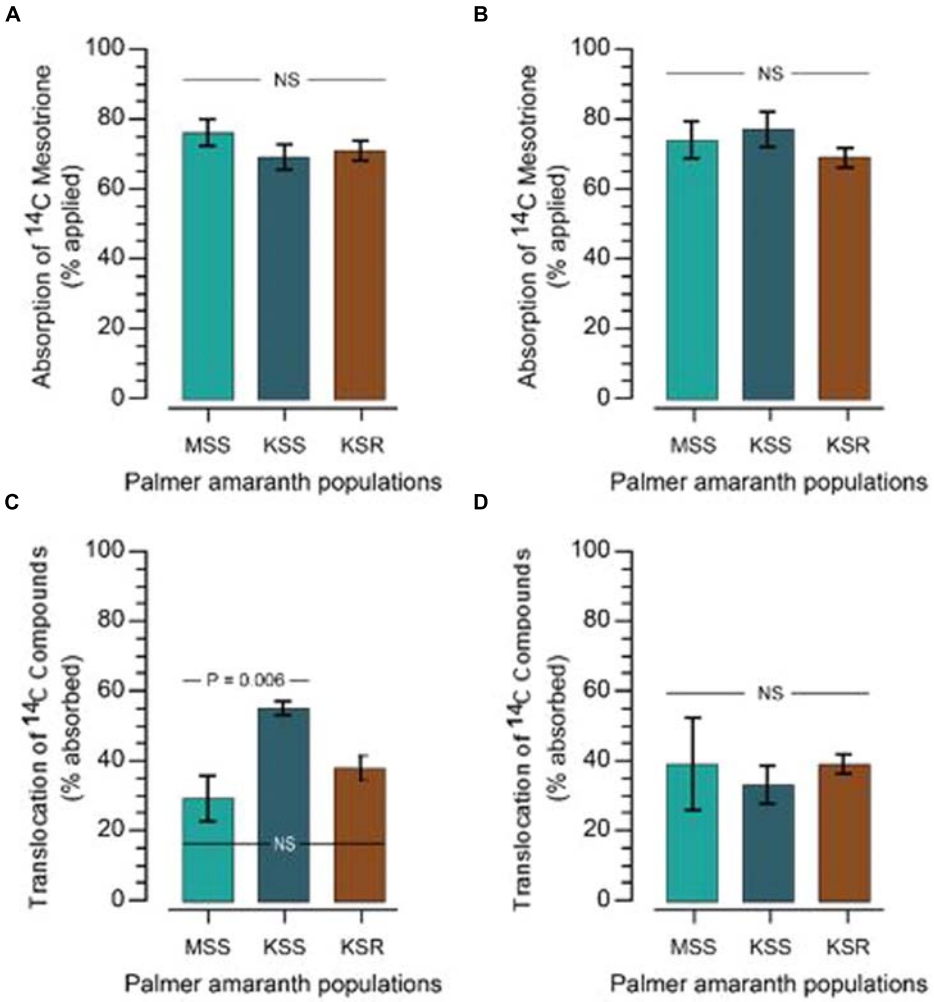

FIGURE 2 | [^{14}C] mesotrione absorption and translocation in resistant and susceptible Palmer amaranth populations. (A,B) Absorption of [^{14}C] mesotrione; **(C,D)** translocation of [^{14}C] compounds in resistant (KSR) and susceptible (MSS and KSS) Palmer amaranth populations. Absorption and translocation in the plant was measured at 48 **(A,C)** and 72 **(B,D)** hours after treatment (HAT) with liquid scintillation spectrometry (LSS). Data were analyzed using one-way ANOVA and the means were compared using Tukey's HSD test. Error bars represent standard error of means (n = 6) at each time point. NS, non-significant at α = 0.05.

the MSS (**Figure 4** and **Table 2**). These metabolism data indicate that mesotrione metabolism is contributing significantly to the resistance in Palmer amaranth. However, interestingly, both resistant and susceptible Palmer amaranth populations were able to completely metabolize parent [^{14}C] mesotrione by 48–72 HAT (data not shown) further suggesting that rapid metabolism alone may not solely conferring resistance to mesotrione in KSR or NER.

Analysis of *HPPD* Gene Expression

We tested for possible mutation or amplification of the *HPPD* gene conferring resistance to mesotrione in Palmer amaranth. However, our data did not show any mutations or amplification of the *HPPD* gene in this population (**Figure 5A**). Therefore, we hypothesized that, in addition to rapid metabolism, increased expression of the *HPPD* gene may possibly contribute to mesotrione resistance in KSR or NER. To test this idea, mRNA levels of the *HPPD* gene in all mesotrione-resistant and -susceptible Palmer amaranth individuals were determined. Since genetic variation as well as variability in the degree of sensitivity to mesotrione exists, there was 1- to 2.5-fold variation in *HPPD* gene expression among the five susceptible populations (MSS, KSS, KSS II, KSS III, and NES). *HPPD* mRNA levels in KSR and NER (normalized against *β-tubulin* and *CPS)* was at least 12-fold and 8- to 12-fold higher, respectively, compared to MSS (**Figure 5B**, $P < 0.001$). When compared to the other four susceptible populations, KSS, KSS II, KSS III, and NES, *HPPD* gene expression relative to *β-tubulin* or *CPS* was least 4- to 9-fold more in KSR and NER (**Figure 5B**, $P = 0.001$). These data indicate that the basal mRNA levels for HPPD are strongly upregulated in resistant populations. This increase in *HPPD* gene expression is likely to an important role in the initial response of resistant Palmer amaranth when mesotrione is applied.

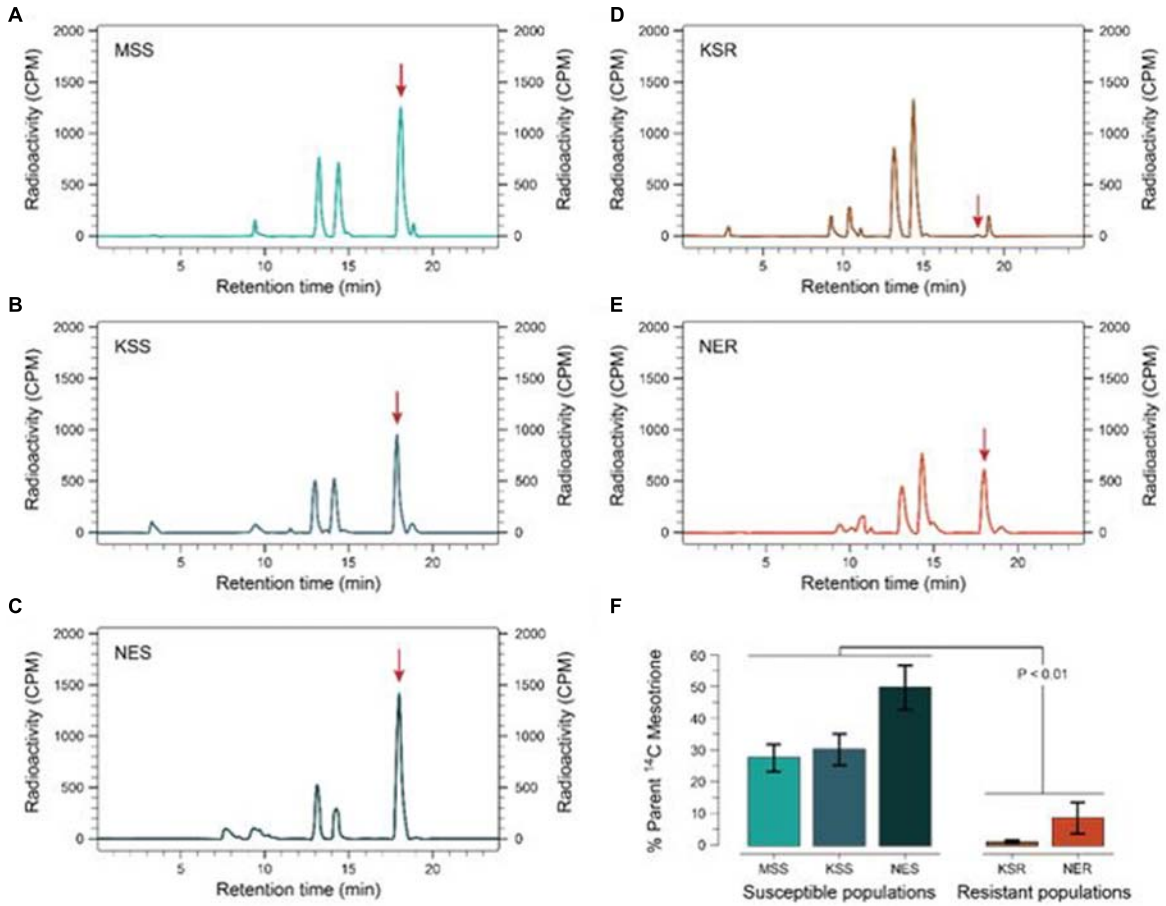

FIGURE 3 | Metabolism of [^{14}C] mesotrione in resistant and susceptible Palmer amaranth populations harvested at 24 HAT. Reverse-phase HPLC chromatograms of plants treated with [^{14}C] mesotrione and harvested **(A)** MSS **(B)** KSS **(C)** NES **(D)** KSR, and **(E)** NER. Peak retention time around 18.1 min is the mesotrione (input) and other peaks 13.1 and 14.3 min are the major metabolites of [^{14}C] mesotrione. **(F)** Represents the amount of [^{14}C] mesotrione input remaining as percentage of total in the resistant (KSR and NER) and susceptible populations (MSS, KSS and NES) 24 HAT. Data were analyzed using one-way ANOVA and the means were compared using Tukey's HSD test. Error bars represent the standard error of means of 6–9 biological replicates.

HPPD Protein Expression in Mesotrione-Resistant Palmer Amaranth

To investigate whether the *HPPD* mRNA transcript abundance correlates with increased HPPD protein levels, we next conducted immunoblot analysis. No antibody is available against Palmer amaranth HPPD; however, *Amaranthus* HPPD is 35% identical with human HPPD. Therefore, we used a human HPPD antibody to test if there is cross-reactivity with the Palmer amaranth *HPPD* protein. As shown in **Figure 5**, the antibody recognized HPPD in human cell lysates (HEK lysate). In the Palmer amaranth lysate, a protein with molecular weight of about 48 kDa was detected, which is consistent with the anticipated size of *Amaranthus* HPPD. The protein could be detected in both susceptible and resistant Palmer amaranth populations, however, KSR or NER lysates showed more HPPD protein as compared to MSS, KSS, KSS II, KSS III, or NES lysates at 50 μg protein concentrations (**Figure 6**). The differences in the HPPD protein between the KSR and NER can be explained because plants in the KSR population are more uniform with their response to mesotrione, while NER is a field collected population segregating and exhibiting variation in plant to plant response to mesotrione application. Since a polyclonal HPPD antibody was used, non-specific and cross hybridization occurred due to the cross-reactivity of the antibody with other proteins in the sample. In all, our data indicate that the increased mRNA levels observed in the resistant populations are translated into increased protein levels.

DISCUSSION

4-hydroxyphenylpyruvate dioxygenase-inhibiting herbicides are relatively new group of herbicides which effectively control a broad spectrum of broadleaf and some grass weeds. Mesotrione is a triketone developed for pre- and post-emergence control of many broadleaf weeds along with some grass weeds in corn. To date, only two weeds species, belonging to the same botanical family, Amaranthaceae, have evolved resistance to HPPD-inhibitors, namely waterhemp and Palmer amaranth (Hausman et al., 2011; Thompson et al., 2012; Heap, 2017).

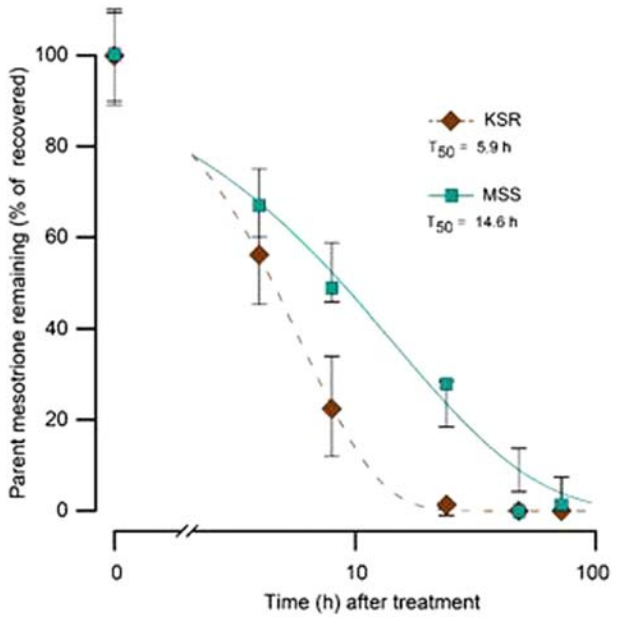

FIGURE 4 | The time course of [^{14}C] mesotrione metabolism (T_{50}) in the treated leaves MSS (susceptible) and KSR (resistant) Palmer amaranth populations across 4, 8, 16, 24, 48, and 72 HAT. Error bars represent the standard error of means of 6–9 biological replicates.

TABLE 2 | Summary parameters describing the time course of mesotrione metabolism by MSS (susceptible) and KSR (resistant) Palmer amaranth populations.

Population	Regression parameters[a]		T_{50}[a]	R/S
	b	*d*	*h*	
MSS	0.74	99 (5.0)	14.6 (1.9)	1
KSR	1.33	100 (5.0)	5.9 (0.5)	2.45*

b, relative slope around T_{50}; d, upper limit of the response; T_{50}, time taken to metabolize 50% of recovered mesotrione; R/S, resistance index [ratio of T_{50} of MSS (susceptible) and KSR (resistant) populations].

[a]*Values in parenthesis are ±1 standard error.*

**R/S is significantly greater than 1 at $P < 0.01$.*

The response was fitted with a three-parameter Weibull regression; fitted curves are shown in ***Figure 4****.*

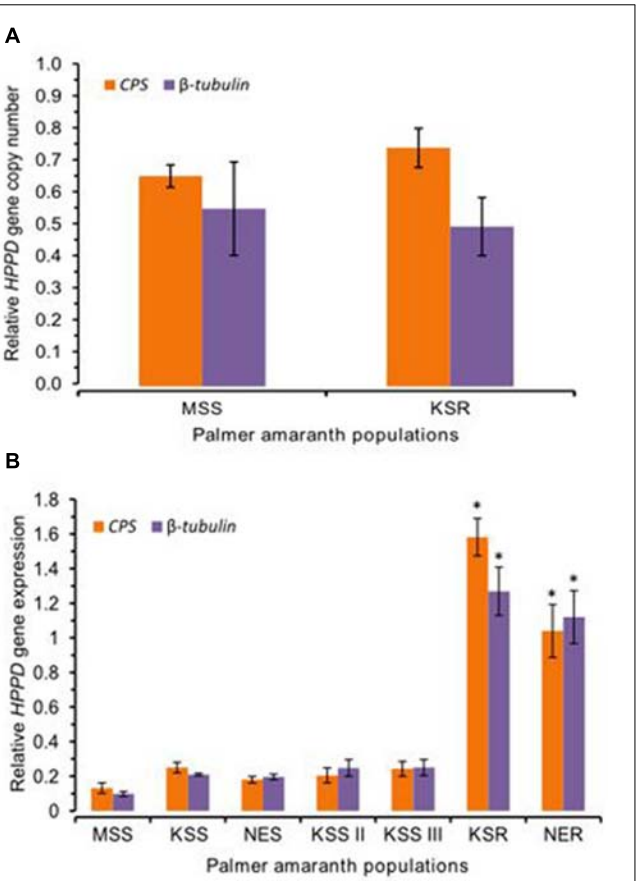

FIGURE 5 | 4-hydroxyphenylpyruvate dioxygenase (*HPPD*) gene amplification and expression. (A) *HPPD* genomic copy number in MSS (susceptible) and KSR (resistant) Palmer amaranth relative to two reference genes *CPS* and *β-tubulin*. Error bars represent the standard error of means of 12 biological replicates**. (B)** The amount of *HPPD* gene expression in the susceptible Palmer amaranth populations (MSS, KSS, KSS II, KSS III, and NES) and resistant Palmer amaranth populations (KSR and NER). The amount of *HPPD* gene expression was normalized to the corresponding level of two reference genes, *β-tubulin* and *CPS*. Data were analyzed using one-way ANOVA and the means were compared using Tukey's HSD test. Bars represent the means ± SE of 6–9 biological replicates. Asterisks above error bars represent significant difference in *HPPD* gene expression compared to corresponding to each susceptible population MSS, KSS, KSS II, KSS III, or NES at $\alpha = 0.05$.

Plant species can evolve resistance to herbicides essentially via two main mechanisms, (a) non-target-site based involving decreased absorption, reduced translocation and/or enhanced metabolism of herbicides and (b) target-site based as a result of mutations in the target gene or increased levels of the target protein, enabled through gene amplification or transcriptional upregulation. Absorption and translocation of mesotrione was similar for mesotrione-resistant and -susceptible Palmer amaranth populations in this research (**Figure 2**) and, thus, did not appear to contribute to resistance. However, greater sensitivity observed in KSS (GR_{50} 8 g ha^{-1}) in the dose response assay compared to MSS (GR_{50} 15 g ha^{-1}) might have resulted from increased translocation of mesotrione (**Figure 2C**). The absorption of [^{14}C] mesotrione in Palmer amaranth is consistent and corresponds to the mean absorption of radio labeled mesotrione across different time points as reported in waterhemp population from IL (Ma et al., 2013). Once absorbed, these herbicides generally translocate via both xylem and phloem (Mitchell et al., 2001; Beaudegnies et al., 2009) to other parts of the plant. However, the translocation of [^{14}C] mesotrione data showed no significant differences contributing to mesotrione resistance.

Plants can detoxify both exogenous and endogenous compounds through a large family of enzymes known as cytochrome P450 monooxygenases. However, the degree to which each plant can metabolize and degrade xenobiotic chemicals is a major contributor to their survival and in the

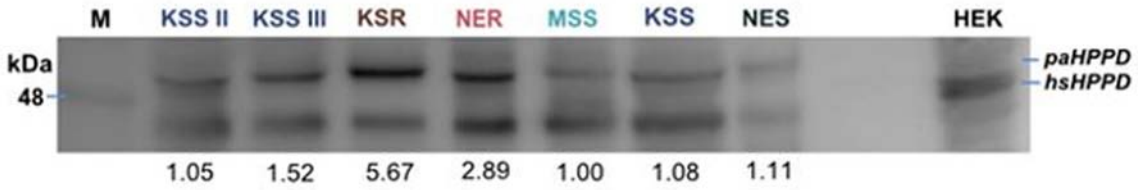

FIGURE 6 | Protein lysates of indicated populations were resolved by 11% SDS–PAGE and immunoblotted for the presence of HPPD in mesotrione-susceptible (MSS, KSS, NES, KSS II, and KSS III) and -resistant (KSR and NER) Palmer amaranth populations using a rabbit polyclonal antibody against human HPD antibody at 50 μg protein concentration. Last lane contains lysate derived from HEK 293 cells (human embryonic kidney cells) and was used as a positive control. Blot shows one individual plant from each population at 50 μg. M represents the marker. The blots were also quantified and MSS is normalized to 1 and other populations were calculated relative to MSS (numbers shown below the blot for each lane).

evolution of resistance. For example, crops like corn, wheat, rice, and sugarcane have a natural tolerance to several groups of herbicides (e.g., HPPD-, ALS-inhibitors) conferred by cytochrome P450 detoxification mechanism (Kreuz et al., 1996; Mitchell et al., 2001). Enhanced detoxification, likely by cytochrome P450 monooxygenases as the mechanism of mesotrione resistance, has been reported in waterhemp population from IL (Ma et al., 2013). The data presented here suggest that Palmer amaranth resistance to mesotrione results, primarily, from the ability to rapidly metabolize this herbicide (**Figure 3**). Our data shows a strong correlation between the rate of mesotrione degradation and the degree of susceptibility or resistance. Resistant Palmer amaranth (KSR) was able to detoxify 50% of mesotrione (T_{50} 5.9 h; **Figure 4**) in a short time compared to corn (T_{50} 11.9 h) and waterhemp (T_{50} 12 h) (Ma et al., 2013). Similarly, waterhemp susceptible to mesotrione required about 30 h (T_{50}) which is about two times slower than susceptible Palmer amaranth. However, our data also suggest that the susceptible individuals also completely metabolize mesotrione by 48–72 HAT indicating that detoxification of mesotrione alone may not be the only mechanism of resistance in Palmer amaranth. In weeds, oxidation, hydroxylation, or dealkylation of different herbicides, by cytochrome P450s has been reported to be one of the major non-target-site mechanisms confirming resistance to herbicides in both broadleaf and grass weed species (Powles and Yu, 2010).

Recently a rice cytochrome P450 gene, CYP72A31 has been identified to confer resistance to ALS-inhibiting herbicides in both rice and *Arabidopsis* (Saika et al., 2014). Previously Pan et al. (2006) reported involvement of rice CYP81A6 in imparting resistance to PS II- and ALS-inhibiting herbicides. Furthermore, when wheat CYP71C6v1 cDNA was cloned and expressed in yeast, ALS inhibiting herbicides were metabolized via phenyl ring hydroxylase (Xiang et al., 2006). Transcriptomic analysis of diclofop-resistant rigid ryegrass (*Lolium rigidum*) revealed involvement of three Cytochrome P450 genes, a nitronate monooxygenase (NMO), three GST, and a glucosyl transferase (GT) in detoxification of diclofop (Gaines et al., 2014). However, the specific role of cytochrome P450s in detoxification of mesotrione is unknown and might not suffice to induce agriculturally significant resistance. Especially, since it seems to only be temporal difference, as all populations are able to fully metabolize mesotrione in 48 h. Though primary, faster degradation of mesotrione alone may not be significant for resistance of Palmer amaranth at recommended field rates or higher.

In addition to the non-target mechanism of rapid detoxification of mesotrione, the target-site based resistance mechanism(s) such as mutation or amplification of HPPD were also tested in our KSR populations. Sequencing of the *HPPD* gene did not show any mutations (unpublished) or amplification in this population. On the other hand, we found a significant increase in *HPPD* gene and protein expression (**Figures 5B**, **6**) in mesotrione-resistant populations, suggesting that the resistant plants have a sufficiently high amount of HPPD enzyme available for maintaining the function of carotenoid biosynthetic pathway even when exposed to field rate of mesotrione. Biochemically, mesotrione and other HPPD-inhibiting herbicides act as competitive inhibitors of the HPPD enzyme involved in the conversion of 4-hydroxyphenylpyruvate (HPP) to 2,5-dihydroxyphenylacetate (homogentisate) (Beaudegnies et al., 2009). In the model plant, *Arabidopsis thaliana*, constitutive over expression of *HPPD* that was 10-fold higher than the wild type plants showed increased tolerance to sulcotrione, a triketone herbicide (Tsegaye et al., 2002). Similarly, heterologous expression of barley *HPPD* in tobacco also resulted in 10-fold higher resistance to sulcotrione (Falk et al., 2003).

Interestingly, a combined resistance through detoxification and target site upregulation has been observed to insecticides in mosquitoes. Here, it has been reported that the insects upregulate metabolic enzymes, esterases, GSTs, or cytochrome P450 monooxygenases through changes/mutations in the *cis*/*trans*-acting elements, gene regulation or via amplification of the genes encoding these enzymes (Xianchun et al., 2007). For example, in southern house mosquito (*Culex quinquefasciatus*), *CYP9M10* is overexpressed to 260-fold higher in a pyrethroid-resistant compared to a susceptible strain via two mechanisms. Two copies of a large fragment of ∼100 kb containing the *CYP9M10*, flanked by MITE (a transposable element) of about 0.2 kb upstream of duplicated copies were found. Since only two copies of this cytochrome cannot explain the 260-fold upregulation, the *cis*-acting and promoter regions were sequenced and it was discovered that there was a *cis*-acting mutation which

mediated increased expression (Itokawa et al., 2010). To our knowledge, this is the first case of Palmer amaranth that naturally evolved mesotrione resistance because of increased target-site gene expression without gene amplification. Increased gene expression can occur without increase in gene copies via changes in the *cis* or *trans*-acting elements, alterations in the promoter region of the gene or post-transcriptional mechanisms that regulate gene expression (Gallie, 1993; Carino et al., 1994; Chung et al., 2007). Glyphosate-resistant junglerice (*Echinochloa colona*) showed enhanced basal *EPSPS* activity of 1.4-fold compared to the susceptible plants, possibly through such changes (Alarcón-Reverte et al., 2015). Similar molecular process could be involved in *A. palmeri* that confer resistance to mesotrione. Experiments are in progress in our laboratory to investigate the genetics of non-target-site based (metabolism) and target-site based (increased *HPPD* gene expression) resistance to mesotrione using forward genetics approach in our Palmer amaranth population.

In addition to herbicide selection pressure, availability of extensive genetic variability, high growth rate and fecundity, adaptation to wide ecological conditions in Palmer amaranth (Knezevic et al., 1997), metabolic resistance and increased *HPPD* gene expression provides an adaptive advantage to survive and spread under diverse environmental stresses. However, the fitness of such herbicide-resistant Palmer amaranth is not known and investigation of fitness costs associated with the resistance trait can help predict the dynamics of evolution and spread of mesotrione resistance in other populations. Furthermore, transcriptome analysis of mesotrione-resistant Palmer amaranth with multiple mechanisms will be a valuable genetic resource: (a) to identify and characterize the precise role of specific cytochrome P450s and other target and non-target genes in mesotrione resistance and (b) in the research and development of novel herbicides and herbicide tolerant crops.

The mesotrione-resistant Palmer amaranth populations used in this study are also resistant to atrazine and chlorsulfuron (ALS-inhibitor), two widely used herbicides in corn production. In general, HPPD-inhibitors are a viable option to manage weeds that are resistant to PS-II and ALS-inhibitors in corn. As Palmer amaranth is a troublesome weed in corn, evolution of resistance to HPPD-inhibitors in this weed will leave fewer herbicide options for management. As no new herbicide modes of action have been discovered in more than two decades, it is increasingly important to effectively and efficiently use currently available herbicides for sustainable agricultural production. More importantly, the non-target-site based mesotrione resistance in Palmer amaranth may exhibit cross resistance to other known and unknown herbicides that are yet to be discovered. Hence, the weed management strategies in regions with Palmer amaranth and other weeds should include diversified tactics to effectively prevent evolution and spread of multiple herbicide resistance.

AUTHOR CONTRIBUTIONS

MJ conceived and supervised the work. SN designed, planned and performed the experiments and analyzed the data. AG performed the statistical analysis and interpretation of the results. PW and JR contributed in western blotting experiment and protein expression analysis. CT provided the seed, and DP and CT revised the manuscript critically.

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A KASP Genotyping Method to Identify Northern Watermilfoil, Eurasian Watermilfoil, and Their Interspecific Hybrids

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The invasive aquatic plant Eurasian watermilfoil (*Myriophyllum spicatum* L.) can hybridize with the related North American native species northern watermilfoil (*M. sibiricum* Kom.). Hybrid watermilfoil (*M. spicatum* × *M. sibiricum*) populations have higher fitness and reduced sensitivity to some commonly used aquatic herbicides, making management more difficult. There is growing concern that management practices using herbicides in lakes with mixed populations of watermilfoil species may further select for hybrid individuals due to the difference in herbicide sensitivity. Accurate and cost-effective identification of rare hybrid individuals within populations is therefore critical for herbicide management decisions. Here we describe KASP assays for three SNPs in the ITS region to genotype individuals from both parental watermilfoil species and their hybrid, using synthesized plasmids containing the respective sequences as positive controls. Using KASP we genotyped 16 individuals from one lake and 23 individuals from a second lake, giving a highly accurate picture of *Myriophyllum* species distribution dynamics. We identified one hybrid individual among 16 samples from one lake, a discovery rate of <10%. Discriminant analysis showed that while a single SNP was generally sufficient for genotyping an individual, using multiple SNPs increased the reliability of genotyping. In the future, the ability to genotype many samples will provide the ability to identify the presence of rare individuals, such as a less common parental species or the inter-specific hybrid. Lakes with complex species distribution dynamics, such as a low proportion of hybrids, are where herbicide application must be carefully chosen so as not to select for the more vigorous and less herbicide-sensitive hybrid individuals.

Keywords: genotyping, KASP, invasive, aquatic weed, hybridization

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INTRODUCTION

The invasive aquatic plant Eurasian watermilfoil (*Myriophyllum spicatum* L.) was introduced to the United States from Asia during the 1940s (Couch and Nelson, 1988; Moody et al., 2016). After introduction, this submersed species spread rapidly throughout the USA, forming dense monotypic mats that have caused economic and ecological damage to infested lakes, streams, and reservoirs (Eiswerth et al., 2000; Olden and Tamayo, 2014). The decrease in native plant diversity that occurs after *M. spicatum* invasion is an alarming ecological impact (Madsen et al., 1991).

Like many aquatic plants, *M. spicatum* is a perennial that often reproduces asexually, a strategy that has the advantage of cloning better-adapted genotypes in stable environments (Philbrick and Les, 1996). *M. spicatum* uses a very simple form of asexual reproduction called autofragmentation. Autofragmentation usually occurs soon after flowering when stems that are relatively fragile break off from the parent plant and float away as structures that can start new colonies (Grace, 1993). One characteristic of these shoot pieces that adds to the success of autofragmentation is the development of adventitious roots. *M. spicatum* has retained the ability to reproduce sexually using a common strategy of many aquatic angiosperms. Modified stems project from the vegetative mat and terminate in monecious flowers above the water surface. This allows for flowers to be wind-pollinated. Sexual reproduction and subsequent seed production are often overlooked as an important survival strategy for *M. spicatum*. Seed production can be an insurance against local extinction because there is some seed dormancy (Grace and Wetzel, 1978). Sexual reproduction can also produce new genotypes that could be better adapted to changing environments.

Environmental change for aquatic plants is usually thought of in terms of salinity, pH, or turbidity, but not necessarily annual applications of aquatic herbicides. It is now apparent that *M. spicatum* has hybridized with the related North American species northern watermilfoil (*M. sibiricum* Kom.) (Moody and Les, 2007; Zuellig and Thum, 2012; Grafe et al., 2015). In the absence of herbicide selection pressure, hybrid watermilfoil (*M. spicatum* × *M. sibiricum*) populations appear to have higher fitness, manifested as a faster and more aggressive growth rate than either parental species in both laboratory and field conditions (LaRue et al., 2013; Hovick and Whitney, 2014). Some hybrid populations are also less sensitive to several commonly used aquatic herbicides, including 2,4-D, fluridone, norflurazon, and topramazone (LaRue et al., 2013; Berger et al., 2015). There is growing concern that current management practices in lakes with mixed populations of watermilfoil species, which rely heavily on herbicide application, may further select for hybrid populations due to the difference in herbicide sensitivity.

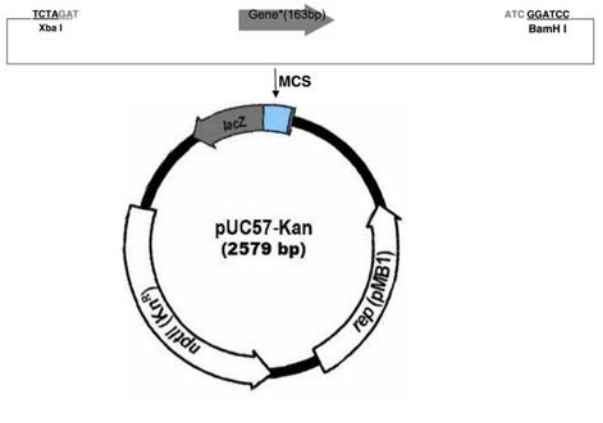

FIGURE 1 | Plasmid map illustrating the cloning strategy for the two plasmid inserts in the pUC57-Kan plasmid (GenScript). The cloning strategy and insert size are identical for the two plasmids, so a generic map is given that represents the strategy for both plasmids.

Several methods to accurately identify *M. spicatum*, *M. sibiricum*, and *M. spicatum* × *M. sibiricum* hybrid individuals using morphological characteristics have been proposed. Morphological characteristics, while sufficient to distinguish between *M. spicatum* and *M. sibiricum*, are no longer reliable once hybrid individuals are present, as the hybrid characteristics are often intermediate between the two species (e.g., the number of pinnae or leaflet pairs) (Coffey and McNabb, 1974; Moody and Les, 2007).

Sufficient genetic variation exists between the two species that genotyping is an accurate method for species identification (Moody and Les, 2002; Sturtevant et al., 2009). Current methods rely on 23 intra-genic polymorphic single nucleotide polymorphisms (SNPs) within the first and second nuclear ribosomal internal transcribed spacer regions (ITS1 and ITS2) of *M. spicatum* and *M. sibiricum* (Moody and Les, 2002). Of these SNPs, 11 clearly distinguish between *M. spicatum* and *M. sibiricum*. When a single individual is heterozygous for both alleles of a single SNP, it indicates the individual is an inter-specific hybrid. A hybrid individual will also be heterozygous for the remaining 10 SNPs due to linkage of the SNPs within the ITS regions.

Single nucleotide polymorphism genotyping in these species has been performed using several methods. Originally, the ITS region was amplified via polymerase chain reaction (PCR), the PCR products were cloned, and multiple clones were sequenced to determine their genotype (Grafe et al., 2015). This process requires the longest time and highest cost per sample of available methods. Subsequently, genotyping was streamlined with the development of a PCR restriction fragment length polymorphism (PCR-RFLP) assay using either a *Bmt*I or *Fsp*I restriction digest that cut at base pair (bp) 274 or 551 of the ITS amplicon, respectively. By eliminating the cloning and sequencing for species identification with the PCR-RFLP assay, Grafe et al. (2015) were able to substantially decrease the amount of time

TABLE 1 | The full sequence of the plasmid inserts for *M. sibiricum* and *M. spicatum* positive plasmid controls.

Plasmid	Gene name	Length (bp)	Vector name	Sequence (5′-3′)
1	M_Sib_Positive_Control	163	pUC57-Kan	CATGACGAACTTAGCACACCGCTAGCCGACTTGTGCGGCAGCGGCGTTGCAAACTTCGATACCTA CAAAGCCCACCCTTCAAGGATATGGTGCTGCGGAAGCAGATATTGGATAACTCAGCCTTTGTTGCG TCGTGCCCGCCGTGCCCCTTGGAGCTCAGCAT
2	M_Spi_Positive_Control	163	pUC57-Kan	CATGACGAACTTAGCACACCACTAGCCGACTTGTGCGGCAGCGGCGTTGCAAACTTCGATACCTA CAAAGCCCACCCTTCAAGGATAAGGCGCTGCGGAAGCAGATATTGGATAACTCAGCCTTTGTTGCG CCGTGCCCGCCGTGCCCCTTGGAGCTCAGCAT

and money per sample required for positive species identification of individual watermilfoil specimens. The higher throughput enabled larger sample sizes, providing a more accurate estimate of *Myriophyllum* species distribution dynamics.

Recent advances in SNP genotyping provide more cost-effective and accurate results than PCR-RFLP. Currently, the Kompetitive Allele Specific PCR (KASP) assay is a common technique for genotyping SNPs. This assay is used in several fields of study, including plant breeding, disease identification, and species identification (Semagn et al., 2014). KASP is able to discriminate between two alleles of a SNP using a common reverse primer paired with two forward primers, one specific to each allele. Each forward primer also has a nucleotide sequence that hybridizes to either the HEX or FAM fluorophore quencher. Amplification proceeds using stringent conditions to permit forward primers to bind only if they are perfectly complementary to the template sequence. Fluorophores are released from the quencher molecule when a forward primer is incorporated in a PCR product, causing the released fluorophore to fluoresce. This fluorescence is detected at the end of the assay using a real-time PCR machine, and the proportion of fluorescence from HEX, FAM, or both indicates the genotype of the sample.

Kompetitive Allele Specific PCR genotyping has several advantages compared to other genotyping assays. KASP assays are more convenient, as they are both faster and less expensive. Eighty or more individuals can be genotyped simultaneously on a standard 96-well plate, giving a much more accurate view of the *Myriophyllum* species distribution dynamics, and increasing the likelihood of detecting a rare hybrid individual. KASP assay design is very flexible, as useful SNPs are not limited to available restriction enzyme recognition sites, and primers can even cover stretches of sequence containing multiple SNPs by incorporating degenerate or mixed bases into the primer sequence. KASP assays are quantitative and therefore amenable to statistical analysis, such as assigning probabilities to genotyping calls. Data from

TABLE 2 | Kompetitive Allele Specific PCR primers for SNPs 118, 363, and 478 in the *Myriophyllum* ITS region.

Primer name	Primer sequence (5′-3′)	OligoAnalyzer 3.1 predicted melting temperature
SNP 118 (G/A)		
M. sibiricum FP-118	CATGACGWACTTAGCACACCG	55.9°C
M. spicatum FP-118	CATGACGAACTTAGCACACCA	55.2°C
Universal RP-118	TAGGTATCGAAGTTTGCAACGC	55.5°C
SNP 363 (A/G)		
M. sibiricum FP-363	CAATATCTGCTTCCGCAGCA	55.6°C
M. spicatum FP-363	CAATATCTGCTTCCGCAGCG	56.6°C
Universal RP-363	CAAAGCCCACCCTTCAAGGA	57.7°C
SNP 478 (T/C)		
M. sibiricum FP-478	GATAACTCAGCCTYTGTTGCGT	56.4°C
M. spicatum FP-478	GATAACTCAGCCTTTGTTGCGC	56.9°C
Universal RP478	ATGCTGAGCTCCAAGGGGCA	61.8°C
5′ FAM TAG (*M. sibiricum*)	GAAGGTGACCAAGTTCATGCT	
5′ HEX TAG (*M. spicatum*)	GAAGGTCGGAGTCAACGGATT	

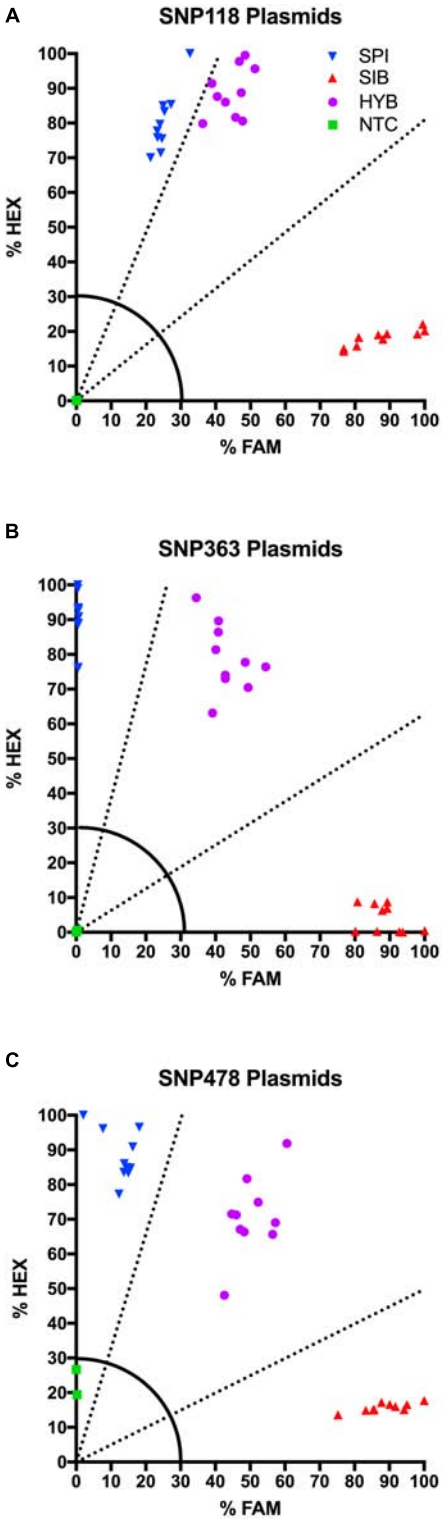

FIGURE 2 | Results of KASP assays for plasmids containing the *M. sibiricum* positive control (▼), *M. spicatum* positive control (▲), 1:1 mixture of the two to represent hybrids (●), and no-template controls (■). SNPs 118, 363, and 478 are shown in panels **(A–C)**, respectively. Dashed lines represent cutoffs for making genotyping calls. The solid quarter circle line is the cutoff for no amplification.

TABLE 3 | Kompetitive Allele Specific PCR SNP genotyping calls and probability of accuracy (Prob) for eight known *M. spicatum* (M. spi, dark gray) biotypes and eight known hybrid (Hyb, light gray) watermilfoil (*M. spicatum* × *M. sibiricum*) biotypes.

Sample	All three SNPs		SNP 118		SNP 363		SNP 478	
	Call	Prob	Call	Prob	Call	Prob	Call	Prob
Norway	M. spi	1.00	M. spi	0.76	M. spi	1.00	M. spi	1.00
Hayden	Hyb	1.00	Hyb	1.00	Hyb	1.00	Hyb	1.00
Mattoon	Hyb	1.00	Hyb	1.00	Hyb	1.00	Hyb	1.00
Houghton	Hyb	1.00	Hyb	1.00	Hyb	1.00	Hyb	1.00
CSU KCK	M. spi	1.00	M. spi	0.99	M. spi	1.00	M. spi	1.00
Alpine 2	Hyb	1.00	Hyb	1.00	Hyb	1.00	Hyb	1.00
Alpine 3	Hyb	1.00	Hyb	1.00	Hyb	1.00	Hyb	1.00
Richard Farm	Hyb	1.00	Hyb	1.00	Hyb	1.00	Hyb	1.00
4BC	M. spi	1.00	M. spi	0.95	M. spi	1.00	M. spi	1.00
St Helens	M. spi	1.00	M. spi	0.89	M. spi	1.00	M. spi	1.00
Jeff	Hyb	1.00	Hyb	1.00	Hyb	1.00	Hyb	1.00
Hall	M. spi	1.00	M. spi	0.95	M. spi	1.00	M. spi	0.99
Stoney 2	M. spi	1.00	M. spi	0.78	M. spi	1.00	M. spi	0.98
Fawn	M. spi	1.00	M. spi	0.95	M. spi	1.00	M. spi	1.00
Alpine 1	Hyb	1.00	Hyb	1.00	Hyb	1.00	Hyb	1.00
Hanbury	M. spi	1.00	M. spi	0.99	M. spi	1.00	M. spi	1.00

multiple SNP genotyping assays can be integrated into a single model, increasing the robustness of species diagnostics.

Here we describe KASP assays for three SNPs in the ITS region to genotype individuals from both parental watermilfoil species and their hybrid, using synthesized plasmids containing the appropriate sequences as positive controls. Using these three KASP assays, we genotyped 23 and 16 individuals, respectively, from two lakes, giving a highly accurate picture of *Myriophyllum* species distribution dynamics in each case. Discriminant analysis showed that while a single SNP was generally sufficient for genotyping an individual, using multiple SNPs increased the reliability of genotyping.

MATERIALS AND METHODS

Plant Collection

Several previously identified *M. spicatum* biotypes and known inter-specific watermilfoil hybrid (*M. spicatum* × *M. sibiricum*) biotypes (eight biotypes each) were harvested from aquaponics cultures maintained in the CSU Weed Research lab. Unknown *Myriophyllum* individuals were collected from the following two lakes in northern Colorado: Rainbow Lake located at 40.506758, −104.989224, and Walleye Lake at 40.505680, −104.982883. Individual stems (Rainbow, $n = 23$; Walleye, $n = 16$) were collected from each lake by rake throws. A single leaf from a stem was used for extraction and therefore a tissue sample is expected to represent a unique individual. Tissue samples were stored in sealed bags with damp paper towels at 4°C until DNA extraction.

Plant DNA Extraction

DNA was extracted from 50 mg of watermilfoil leaf tissue using a modified CTAB method (Doyle, 1991). All steps were performed at room temperature (22°C) unless otherwise indicated and all chemicals were of molecular biology grade. In brief, tissue was initially ground to a fine powder with a metal bead in 500 μL of 2x CTAB buffer (2% CTAB, 1% PVP, TRIS-EDTA pH 5) using a TissueLyser II (Qiagen, Germantown, MD, USA) at 30 oscillations/second for 1 min. Ground samples were incubated at 65°C for 1 h, after which 500 μL of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The samples were slowly rocked on an orbital shaker for 15 min. Samples were centrifuged at 10,000 × *g* for 5 min. The upper phase was transferred to a new tube, to which 500 μL of chloroform:isoamyl alcohol (24:1) was added. The samples were again centrifuged at 10,000 × *g* for 5 min. The upper phase was transferred to a new tube and nucleic acids were precipitated using 0.1 volumes of 3 M sodium acetate and 2.5 volumes of 100% ethanol. Samples were precipitated at 4°C for 15 min and then centrifuged at 15,000 × *g* for 15 min. The resulting pellets were re-suspended in 50 μL of sterilized water. DNA concentrations and quality were assessed using a spectrophotometer (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA). Samples were subsequently diluted to 5 ng/μL for use in all KASP assays.

Plasmid Design

Two plasmids were designed as positive controls for the KASP assay. Plasmid inserts consisted of the sequence within the ITS region complementary to the genotyping primers, with all inter-primer sequence removed (**Figure 1** and **Table 1**). The complete oligonucleotides were synthesized by GenScript (Piscataway, NJ, USA) in the pUC57-Kan plasmid.

Control plasmids were transformed into DH5α *E. coli* cells using a standard heat transformation protocol (provided by GenScript) and selection on LB plates using 50 μg/mL kanamycin. Individual colonies were transferred to a numbered patch plate and allowed to grow at 37°C for 16 h.

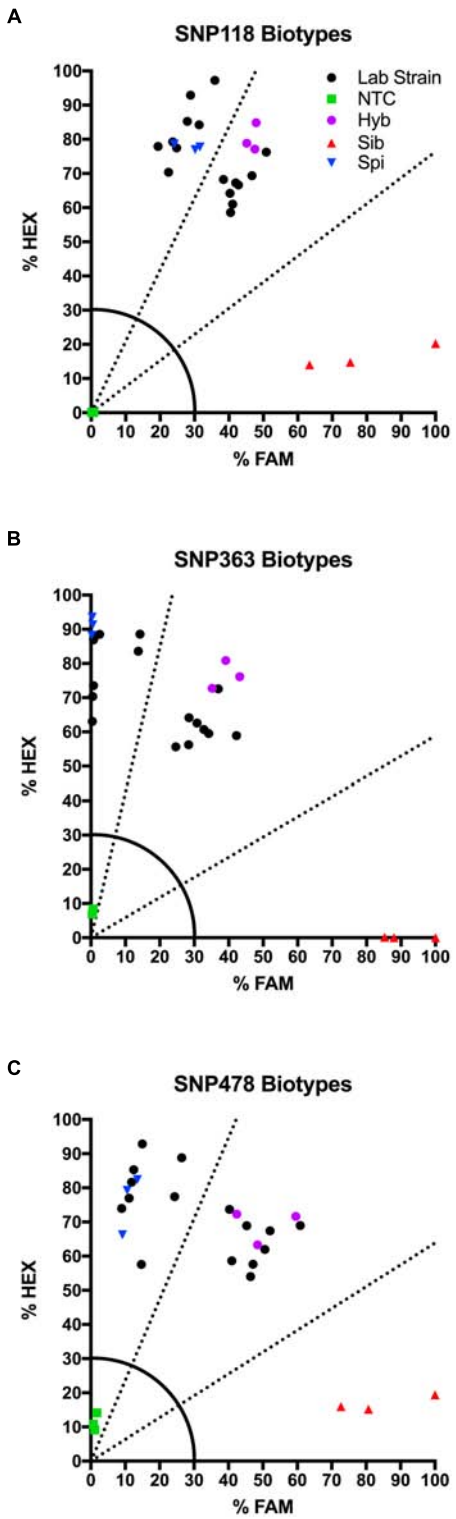

FIGURE 3 | Results of KASP assays for SNPs 118 (A), 363 **(B)**, and 478 **(C)** from 16 lab biotypes (eight known inter-specific hybrids and eight known *M. spicatum* biotypes; ●), as well as the *M. sibiricum* positive control (▼), *M. spicatum* positive control (▲), a 1:1 mixture of the two to represent hybrids (●), and no-template controls (■). Dashed lines represent cutoffs for making genotyping calls. The solid quarter circle line is the cutoff for no amplification.

E. coli DNA Extraction

DNA was extracted from cultures grown from ten colonies on each patch plate. A toothpick was dipped into the *E. coli* colony and used to inoculate 1 mL of LB containing 50 μg/mL kanamycin. After incubating for 16 h at 37°C with shaking, the *E. coli* cultures were pelleted by centrifugation at 8,000 × *g*. DNA was extracted from the pellets using Qiaprep Spin Miniprep kit (Qiagen, Germantown, MD, USA) according to the manufacturer's recommendations. DNA concentrations and quality were assessed using a NanoDrop 2000 spectrophotometer. Extracted plasmids were subsequently diluted to 5 pg/μL for use in all KASP assays. A 1:1 mixture of the diluted plasmids was used in KASP assays to simulate an inter-specific hybrid.

Primer Design

Three primer sets were designed for the KASP assay to distinguish three diagnostic SNPs at bp 118, 363, and 478 in the Internally Transcribed Spacer (ITS) region (Moody et al., 2016; **Table 2**). For each primer set, the forward primer for *M. spicatum* was appended at the 5′ end with the sequence complementary to the HEX fluorophore quencher, while the forward primer for *M. sibiricum* was appended at the 5′ end with the sequence complementary to the FAM fluorophore quencher. The forward primers for *M. sibiricum* at SNPs 118 and 478 spanned sequences containing SNPs that discriminate between sub-populations, which required the use of degenerate bases in the primers. Degenerate bases are indicated according to the universal IUPAC code (Cornish-Bowden, 1985). The forward primers for SNPs 118 and 478 in *M. sibiricum* designed to amplify these degenerate bases were an equal blend of the two possible alleles at the degenerate SNP.

KASP Assay

A primer master mix including forward and reverse primers for a single SNP was made according to the KASP assay manufacturer's recommendations (LGC Genomics, Beverly, MA, USA). After all primers were re-suspended in Tris-HCl, pH 8.3, at 100 μM, a primer master mix was assembled with 18 μL of the *M. spicatum* forward primer, 18 μL of the *M. sibiricum* forward primer, 45 μL of the common reverse primer, and 69 μL of 10 mM Tris-HCl, pH 8.3. KASP master mixes were made for each SNP assay with 432 μL LGC Genomics Master Mix (which includes polymerase, dNTPs, buffer, and HEX- and FAM-tagged oligonucleotides) and 11.88 μL of the appropriate primer master mix.

Kompetitive Allele Specific PCR reactions were assembled in a 96-well plate with 4 μL of master mix and either 4 μL water (no-template control), 4 μL genomic DNA at 5 ng/μL, or 4 μL of plasmid DNA at 5 pg/μL. Reactions were performed in a Bio-Rad CFX Connect (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the following standard KASP PCR program: activation at 94°C for 15 min, then 10 touchdown cycles of 94°C for 20 s (denaturing), 61–55°C for 60 s (dropping 0.6°C per cycle, for annealing and elongation), 23°C for 30 s (to permit accurate plate reading), followed by 26 cycles of 94°C for 20 s, 55°C for 60 s, 23°C for 30 s. Fluorescence was tracked in real-time with plate reads at the end of every amplification cycle.

Fluorescence data from the cycle showing the greatest distinction between clusters without any background amplification was used for genotyping, which was determined to be cycles 22–24 of the amplification phase.

Data Analysis

Due to slight variations in maximum fluorescence and fluorescence in the no-template controls between plates, HEX and FAM fluorescence for each data point were transformed as a percentage of the maximum fluorescence for each fluorophore within a plate. Maximum fluorescence is defined as the highest FAM or HEX signal from any reaction in a 96-well plate. Cutoffs for genotyping calls on unknown samples were drawn by calculating the point halfway between the mean (x,y) coordinate of the control (plasmid) hybrid and either the control *M. sibiricum* or *M. spicatum* clusters, then drawing a line from that point to the origin (0,0). Additionally, a zone of "no amplification" was defined as 30% based on the maximum fluorescence observed in no-template controls. The 30% cut-off was selected because technical variation at low fluorescence values is more likely to influence genotyping calls than at high fluorescence values, and based on the observation that none of our no-template controls exceeded 30%. A quarter circle at 30% around the axis intercept was used to define this zone. Genotypes were assigned to unknown samples based on the sector of the plot where their fluorescence values occurred. If a sample fell within the bounds of a zone it was assigned that genotyping call.

Once each sample (experimental samples as well as plasmid controls) was assigned a genotype based on its zone, linear discriminant analysis was performed in JMP 12.2 (SAS Institute Inc., Cary, NC, USA) to evaluate the probability of that individual having its assigned genotype. Genotyping results from each SNP were first assessed independently, then using all three SNPs combined to provide more robust probabilities.

RESULTS

We developed three KASP primer sets that distinguish between the native *M. sibiricum* and the invasive *M. spicatum* species as well as inter-specific hybrids. Our KASP primers utilize the previously identified SNPs at base pairs 118, 363, and 478 of the ITS region (**Table 2**). We tested the primer sets on plasmids containing known sequences, on known lab biotypes of *M. spicatum* and hybrids, and on unknown *Myriophyllum* individuals harvested from two lakes in northern Colorado. We assigned genotypes manually, and then measured the reliability of the genotyping calls using discriminant analysis to assign probabilities to calls from each SNP individually as well as using all three SNPs together.

TABLE 4 | Kompetitive Allele Specific PCR SNP genotyping calls and probability of accuracy (Prob) for 23 unknown watermilfoil individuals from Rainbow Lake. M. spi (dark gray) = *M. spicatum*.

Sample	All three SNPs		SNP 118		SNP 363		SNP 478	
	Call	Prob	Call	Prob	Call	Prob	Call	Prob
Plant 1	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 2	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 3	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 4	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 5	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 6	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 7	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 8	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 9	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 10	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 11	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 12	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 13	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 14	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 15	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 16	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 17	M. spi	1.00	M. spi	0.98	M. spi	1.00	M. spi	1.00
Plant 18	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 19	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 20	M. spi	1.00	M. spi	0.88	M. spi	1.00	M. spi	1.00
Plant 21	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 22	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 23	M. spi	1.00	**M. spi**	**0.085**	M. spi	1.00	M. spi	1.00

Bolded font indicates that the genotyping call made by the point's location does not agree with the linear discriminant analysis.

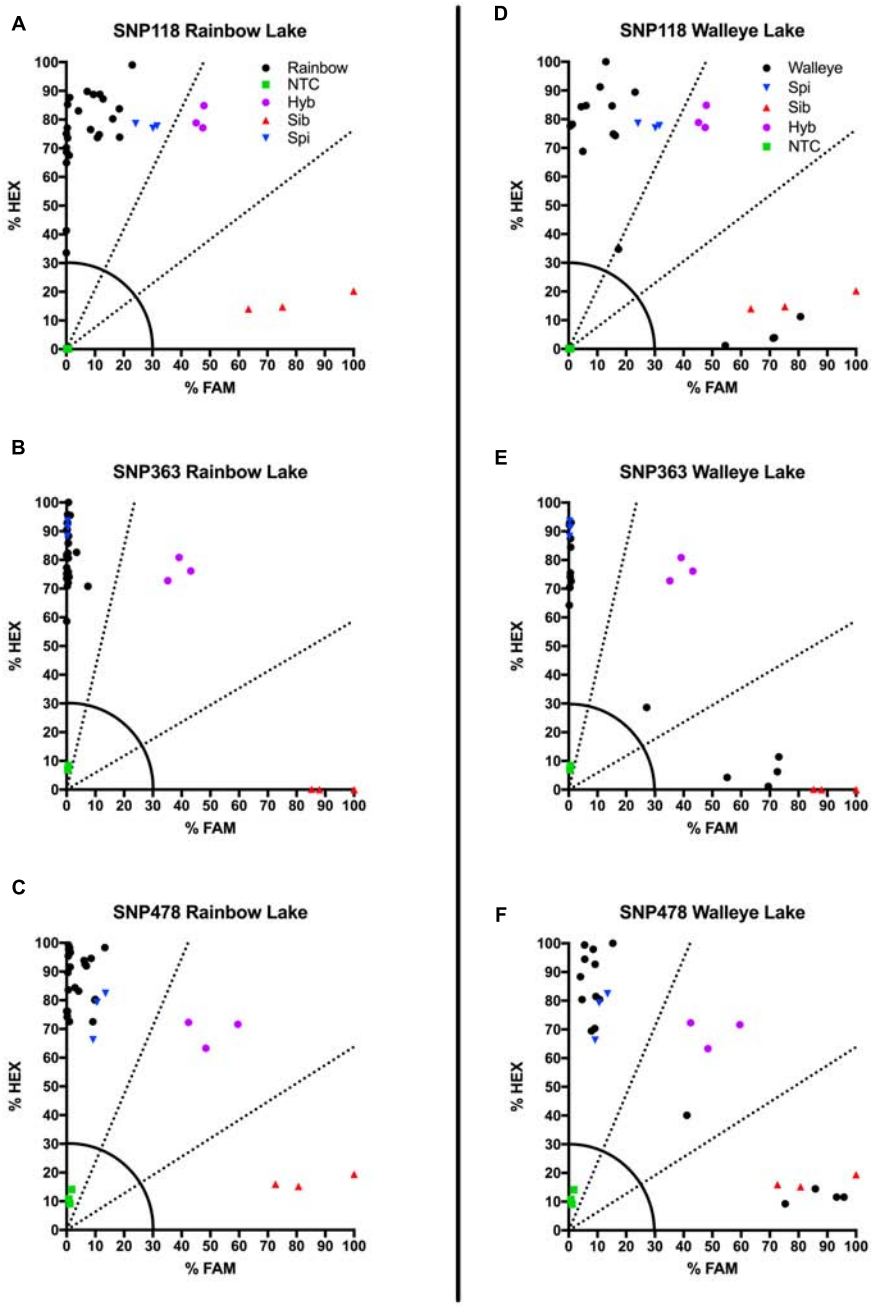

FIGURE 4 | Results of KASP assays from wild collections of unknown watermilfoil individuals from Rainbow Lake for SNP 118 (A), SNP 363 **(B)**, and SNP 478 **(C)**, and from Walleye Lake for SNP 118 **(D)**, SNP 363 **(E)**, and SNP 478 **(F)**. Samples are represented as follows: *M. sibiricum* positive control (▼), *M. spicatum* positive control (▲), a 1:1 mixture of the two to represent hybrids (●), no template controls (■), and unknown watermilfoil samples (●). Dashed lines represent cutoffs for making genotyping calls. The solid quarter circle line is the cutoff for no amplification.

KASP Assays on Plasmids

We developed two plasmids (**Figure 1**) to serve as positive controls for the KASP-PCR. Plasmid controls were ideal because they allow for rapid generation of DNA of a known genotype and eliminate the need to maintain both species of *Myriophyllum* as well as the inter-specific hybrid in hydroponic culture as positive genotyping controls.

The plasmid DNA performed consistently from assay to assay and allowed us to more accurately characterize unknown individuals in the KASP assay. For SNP 118, SNP 363, and SNP 478, we tested ten distinct *E. coli* colonies. All ten samples containing a given genotype formed a tight, distinct cluster on the HEX-FAM *x-y* plot with fluorescence values well above the 30% cut-off (**Figure 2**). SNP 118 had a very clear *M. sibiricum*

cluster, but the *M. spicatum* and the 1:1 synthetic hybrids were relatively close to each other, due to increased FAM fluorescence for the *M. spicatum* samples (**Figure 2A**). However, there was no overlap between the *M. spicatum* and the synthetic hybrid sample clusters. SNP 363 and SNP 478 show obvious separation of the fluorescence signal from each of the three possible genotypes, with the *M. spicatum* plasmids having almost exclusively HEX signal, *M. sibiricum* plasmids having almost exclusively FAM signal, and the 1:1 mixture of each genotype having both HEX and FAM signals (**Figures 2B,C**). No plasmid had an ambiguous call or fell below the 30% fluorescence threshold for any of the three SNPs. This test confirmed the utility of plasmids as internal positive controls for plant genotyping assays.

KASP Assays on Lab Biotypes

We tested several biotypes of *Myriophyllum* that are maintained in aquaponics culture at CSU. These biotypes were originally collected from various locations in North America (**Table 3**). The KASP results from all three SNP primer sets showed that eight of these biotypes clustered with the *M. spicatum* plasmid control, with high HEX signal and minimal FAM signal (Norway, CSU KCK, 4BC, St Helens, Hall, Stoney 2, Fawn, Hanbury), while eight clustered with the 1:1 synthetic hybrid mixture of *M. spicatum* and *M. sibiricum* plasmid controls, with approximately equal HEX and FAM fluorescent signals (Hayden, Mattoon, Houghton, Alpine 2, Alpine 3, Richard Farm, Jeff, Alpine 1) (**Table 3** and **Figure 3**). The genotyping calls from the KASP assay matched the known genotypes of the samples exactly.

The probability that a genotype call was correct was calculated by performing discriminant analysis on the corrected fluorescence data for each SNP separately and for all three SNPs together (**Table 3**). Particularly for SNP118, several individuals had a reduced probability that the genotype was correct (e.g., Norway or Stoney 2). However, when all three SNPs were considered together, the probability was 100% for each genotype call (**Table 3**). These results confirm that all three SNPs are strongly linked and co-inherited and therefore the three SNPs can be used together to provide accurate genotyping, as would be expected for SNPs in the ITS2 region.

KASP Assays on Rainbow and Walleye Lake

We also tested our assay on wild, unknown individuals from two lakes in northern Colorado, Rainbow Lake ($n = 23$) and Walleye Lake ($n = 16$). For Rainbow Lake, all sampled individuals were identified as the invasive *M. spicatum*, as the fluorescence signal from all three SNPs for each individual was predominantly the HEX wavelength (**Table 4** and **Figures 4A–C**). Only the genotyping call of *M. spicatu*m for plant 23 for SNP 118 was unsupported by the linear discrimination analysis ($P = 0.085$). The analysis assigned this call as "No amplification" with $P = 0.856$. However, when all three SNPs were considered together, a call of *M. spicatum* for Plant 23 was predicted with $P = 1.0$. Samples from Walleye Lake were identified as *M. spicatum*, *M. sibiricum* and inter-species hybrid genotypes, with 11 individuals showing predominantly HEX fluorescence and clustering with the *M. spicatum* plasmid controls, while four individuals (plants 2, 3, 8, and 12) showed predominantly FAM fluorescence and clustered with the *M. sibiricum* plasmid controls (**Table 5** and **Figures 4D–F**). Additionally, one individual (plant 1) was identified as the hybrid genotype, as all three SNPs showed unambiguous dual HEX and FAM fluorescence and clustered with the artificial hybrid (**Table 5** and **Figures 4D–F**).

TABLE 5 | Kompetitive Allele Specific PCR SNP genotyping calls and probability of accuracy (Prob) for 16 unknown watermilfoil individuals from Walleye Lake.

Sample	All three SNPs		SNP 118		SNP 363		SNP 478	
	Call	Prob	Call	Prob	Call	Prob	Call	Prob
Plant 1	Hyb	1.00	Hyb	0.49	Hyb	1.00	Hyb	1.00
Plant 2	M. sib	1.00	M. sib	1.00	M. sib	1.00	M. sib	1.00
Plant 3	M. sib	1.00	M. sib	1.00	M. sib	1.00	M. sib	1.00
Plant 4	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 5	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 6	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 7	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 8	M. sib	1.00	M. sib	1.00	M. sib	1.00	M. sib	1.00
Plant 9	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 10	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 11	M. spi	1.00	M. spi	0.99	M. spi	1.00	M. spi	1.00
Plant 12	M. sib	1.00	M. sib	1.00	M. sib	1.00	M. sib	1.00
Plant 13	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 14	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 15	M. spi	1.00	M. spi	1.00	M. spi	1.00	M. spi	1.00
Plant 16	M. spi	1.00	M. spi	0.99	M. spi	1.00	M. spi	1.00

M. spi (dark gray) = M. spicatum; Hyb (light gray) = inter-specific hybrid (M. spicatum × M. sibiricum); M. sib (white) = M. sibiricum.

DISCUSSION

Discriminant analysis verified the accuracy of the genotyping calls, with SNP 118 producing a few lower-confidence genotype calls (for plants 20 and 23 from Rainbow Lake and plant 1 from Walleye Lake) but 100% probability of a correct call when data from all three SNPs were considered simultaneously. The forward primers for both SNP 118 and SNP 478 used one degenerate base each; however, the calls for SNP 478 appear to be much more accurate than for SNP 118. The degenerate bases in each case were required to allow efficient amplification of ITS sequences, because SNPs exist in the forward primers that distinguish between different sub-populations of *M. sibiricum,* but not between *M. spicatum* and *M. sibiricum*. Performance differences in the three KASP markers may be attributed to the type and location of the degenerate base. For the SNP118 forward primer, a W indicates an A or T which is either a purine or pyrimidine, respectively, while the forward primer for SNP478 contains a Y indicating a C or T, which are both pyrimidines. Degenerate bases may lower optimal annealing temperature of the primer, which in turn lowers the ability of the primer set to distinguish between our two species. The use of multiple SNPs together for genotype identification overcomes inefficiencies of any one marker, as they are always co-inherited.

The ability to genotype dozens of individuals provides the ability to identify the presence of rare individuals, such as a less common parental species or the inter-specific hybrid. Lakes with complex species distribution dynamics, such as low proportion of hybrids, are where herbicide application must be carefully chosen so as not to select for the more vigorous and less herbicide-sensitive hybrid individuals. Only recently were hybrids suspected (Moody and Les, 2002), and then determined to be more invasive and less herbicide sensitive (LaRue et al., 2013). The exact distribution, population sizes, and proportions of hybrid, *M. sibiricum*, and *M. spicatum* are largely unknown, in part due to the cost and limited throughput of current genotyping methods. The genetic diversity of the two *Myriophyllum* species in the ITS region has been explored by Sturtevant et al. (2009), including characterization of the various within-species ITS genotypes that exist. Our KASP markers are useful to distinguish between *M. spicatum*, *M. sibiricum* and their interspecific hybrid, but not to distinguish within-species genetic variation.

Monitoring has been conducted in some areas of North America (e.g., Zuellig and Thum, 2012; Grafe et al., 2015; Moody et al., 2016), but the distribution of the invasive species on the continental scale remains undetermined. With the ability to genotype hundreds of individuals rapidly and inexpensively using KASP, aquatic weed managers will be able to make more informed decisions about herbicide type and application rates, such as choosing specific herbicides and rates to control rare hybrid individuals only when they are confirmed to be present. Appropriate sampling structures would be critical, including spatially dispersed locations within a lake, to avoid redundant sampling of any clonal plants and ensuring detection of potentially rare hybrid individuals. Larger data sets comprised of accurate genotyping data will allow modeling of *Myriophyllum* species distribution dynamics, testing the hypothesis that increased selection pressure from herbicide application favors hybrid individuals due to their decreased herbicide sensitivity. Lakes can be genotyped using KASP both before and after herbicide applications to quantify shifts in species distribution dynamics toward invasive *M. spicatum* or hybrid watermilfoil individuals.

SOURCES OF MATERIALS

Lake material was collected from two of the lakes at the Swift Ponds at Colorado Youth Outdoors: Rainbow Lake (40.506758, −104.989224) and Walleye Lake (40.505680, −104.982883).

AUTHOR CONTRIBUTIONS

EP, MF, KK, SN, and TG designed the experiments; EP, MF, and KK performed the experiments; EP, MF, and TG analyzed the results; and EP, MF, KK, SN, and TG wrote and approved the manuscript.

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Nucleotide Diversity at Site 106 of *EPSPS* in *Lolium perenne* L. ssp. *multiflorum* from California Indicates Multiple Evolutionary Origins of Herbicide Resistance

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The repeated evolution of herbicide resistance in weeds is an ongoing problem in agricultural regions across the world, and presents a unique system in which to study the origins and spread of adaptive traits across heterogeneous landscapes. *Lolium perenne* ssp. *multiflorum* (Lam.) (Italian ryegrass) is a widespread grass weed of agricultural crops that has repeatedly evolved resistance to herbicides across the world. In California, resistance to glyphosate has become increasingly common. To identify the mechanisms conferring glyphosate resistance in California populations of *L. perenne* and to gain insights into the evolutionary origins and spread of resistance in the region, we investigated the frequency of target-site mutations conferring resistance to glyphosate combined with the frequency of resistant individuals in 14 populations. A region of *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) was sequenced in 401 individuals to assay for target site mutations. Seven unique alleles were detected at codon site 106, four of which have been previously shown to confer target-site-based resistance to glyphosate. Four different resistance alleles were detected, indicating that resistance to glyphosate has evolved multiple times in the region. In two populations, no *EPSPS* mutations were detected despite the presence of resistant plants, strongly suggesting that non-target-site-based mechanisms confer resistance to glyphosate in these populations. It is likely that resistance to glyphosate in these 14 California populations of *L. perenne* derives from at least five evolutionary origins, indicating that adaptive traits can evolve repeatedly over agricultural landscapes.

Keywords: herbicide resistance, weed populations, *EPSPS* mutations, mechanism, evolutionary origins

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INTRODUCTION

The phenomenon of agricultural weeds evolving in response to agricultural practices presents an ideal opportunity to study plant adaptation across landscapes. Agricultural landscapes are comprised of fields and the lands between them, and management by different growers or agencies across time and space results in a heterogeneous landscape of environments for weeds. Weed management practices such as tillage, hand weeding, and herbicide applications are strong selection pressures for the evolution of adaptive traits that allow plants to survive those management

strategies (Barrett, 1983; Powles and Yu, 2010; Owen et al., 2011; Delye et al., 2013). In particular, repeated applications of herbicides with the same site of action selects for rare mutant resistant individuals which, if present, can result in a rapid increase in the frequency of resistance alleles and resistant plants in the population until the infestation becomes uncontrollable with those herbicides (Jasieniuk et al., 1996; Neve et al., 2009).

Repeated evolution of resistance to glyphosate, the most widely used herbicide worldwide (Baylis, 2000; Benbrook, 2016), has been particularly problematic, occurring in 36 weed species on six continents to date (Heap, 2016). Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the shikimate pathway, which results in the accumulation of shikimate and plant death (Holländer and Amrhein, 1980; Steinrücken and Amrhein, 1980; Herrmann and Weaver, 1999). Following the widespread adoption of glyphosate-resistant transgenic crops and the associated increases in glyphosate use, many weed populations evolved multiple mechanisms of resistance to glyphosate (Baylis, 2000; Powles, 2008; Heap, 2016).

Non-synonymous mutations resulting in four different amino acid substitutions at codon site 106 of *EPSPS* have been shown to confer resistance to glyphosate in several weed species (Kaundun et al., 2011; Shaner et al., 2011). The mutations at site 106 result in an altered EPSPS enzyme that is not bound by glyphosate while retaining affinity for the PEP substrate, allowing plants to survive field-applied doses of the herbicide (Funke et al., 2006). All known mutations conferring target-site-based resistance occur at site 106 (Shaner et al., 2011). Although individuals with mutations at both sites 102 and 106 have been shown to confer a heightened level of resistance in goosegrass in Malaysia (Yu et al., 2015), non-synonymous mutations at site 102 alone do not confer resistance to glyphosate (Funke et al., 2006).

Resistance to glyphosate is also commonly conferred by altered translocation of glyphosate through the plant or by amplification of the *EPSPS* gene (Powles and Yu, 2010; Shaner et al., 2011). The first identified and most frequently cited mechanism of glyphosate resistance is altered translocation, where glyphosate is prevented from reaching its target site, the EPSPS enzyme, by translocation away from meristems and actively growing points and sequestration in the vacuole (Wakelin et al., 2004; Ge et al., 2010, 2012). The genetic basis of this mechanism of resistance to glyphosate is not currently known, although ATP-binding cassette (ABC) transporters and a tonoplast-intrinsic protein (TIP) have been implicated in glyphosate resistance in *Conyza canadensis* (Peng et al., 2010; Yuan et al., 2010). Recently, *EPSPS* gene amplification has been identified as a mechanism of resistance to glyphosate in multiple weed species (Sammons and Gaines, 2014). Resistant individuals with this mechanism may contain two to over 100 copies of *EPSPS* as a result of tandem gene duplication or a mobile genetic element, with correlative high *EPSPS* expression and resistance levels (Gaines et al., 2010; Jugulam et al., 2014; Wiersma et al., 2015).

Lolium perenne ssp. *multiflorum* is a diploid, self-incompatible, obligately outcrossing grass weed that infests a wide range of crops worldwide (Fearon et al., 1983; Charmet et al., 1996). Populations of *L. perenne* ssp. *multiflorum* and the closely related *Lolium perenne* ssp. *rigidum* have evolved resistance to herbicides with 12 different modes of action on six continents (Heap, 2016). In California, resistance to glyphosate in *L. perenne* ssp. *rigidum* was first identified in 1998 in an almond orchard (Simarmata et al., 2003), and later confirmed in multiple populations of *L. perenne* ssp. *multiflorum* across the Central Valley (Jasieniuk et al., 2008). Proline-to-alanine (P106A) or proline-to-serine (P106S) substitutions at the site corresponding to codon 106 of the *EPSPS* gene were identified in resistant plants (Jasieniuk et al., 2008; Simarmata and Penner, 2008). To date, non-target-site-based resistance to glyphosate has not been identified in California populations of *L. perenne* ssp. *multiflorum*. In 2013, populations of *L. perenne* ssp. *multiflorum* containing glyphosate-resistant plants were identified in northwestern California after 2 years of failed control with glyphosate. These populations are separated geographically from the Central Valley and may have evolved resistance independently through the same or a different mechanism.

While resistance to glyphosate has evolved repeatedly in multiple species and within species across different regions around the world (Heap, 2016), it is not always clear how the adaptive trait evolves and spreads among populations of a species within an agricultural region. Early population genetic models predicted that gene flow likely contributes to the spread of target-site-based resistance across a landscape to a greater degree than do novel mutations, as mutation rates are generally assumed to be low (Jasieniuk et al., 1996). Recently, studies of neutral genetic variation in weed populations, combined with patterns of phenotypic variation in resistance, have provided support for the spread of herbicide resistance through both gene flow (Delye et al., 2010a; Okada et al., 2013, 2014) and independent origins (Kuester et al., 2015). However, in weeds with highly outcrossing mating systems and high genetic diversity within populations, such as *L. perenne*, genetic differentiation between populations is often low (Balfourier et al., 1998; Kubik et al., 2001; McGrath et al., 2007; Wang et al., 2009), making it difficult to determine whether a trait shared by two populations is derived from a common origin.

Analysis of genetic diversity and population structure of California *L. perenne* ssp. *multiflorum* with microsatellite markers did not reveal whether the glyphosate resistance trait had originated once and then spread within and among populations, or spread as a result of multiple independent evolutionary origins (Karn and Jasieniuk, 2017). However, extensive population admixture did indicate the potential for resistance spread through gene flow. In this study, we examined genetic variation in *EPSPS* at codon site 106 where target-site mutations conferring resistance to glyphosate have previously been identified (Sammons and Gaines, 2014; Heap, 2016). If multiple alleles known to confer resistance are detected at this locus, then logically they must be derived from separate mutation events and, consequently, we can conclude that resistance has evolved multiple times. However, if all resistance alleles are identical, a single mutation event with subsequent gene flow through pollen or seed dispersal may have spread resistance among populations in the region. If resistance is observed in populations but resistant individuals do not contain any *EPSPS*

mutations at codon site 106, resistance may be conferred by non-target-site-based mechanisms.

In addition to contributing basic knowledge on how adaptive traits originate and spread across landscapes, increased understanding of the evolution of herbicide resistance is needed to mitigate its impacts on agriculture (Neve et al., 2014). One possible strategy for the management of resistance with a single evolutionary origin relies on limiting the spread of resistance while using resistance management practices to control already resistant populations (Neve et al., 2009; Beckie, 2011). In contrast, when populations of a weed species have multiple independent origins of herbicide resistance, successful management requires the implementation of practices that reduce factors contributing to selection for resistance, in addition to limiting spread and controlling already resistant populations (Neve et al., 2009; Norsworthy et al., 2012).

The goal of this study was to examine the evolutionary origins of glyphosate resistance across a landscape by investigating *EPSPS* target site mutations in *L. perenne* ssp. *multiflorum* populations in northwest California. Specifically, we asked the following questions (i) which *EPSPS* alleles at site 106 are associated with glyphosate resistance in *L. perenne* populations in northwest California?, (ii) has resistance evolved more than once across the landscape?, and (iii) is there evidence that non-target-site-based resistance is present in California populations of *L. perenne*? To address these questions, we phenotyped individuals from multiple populations for resistance or susceptibility to glyphosate, sequenced the codon at *EPSPS* site 106 where target-site resistance mutations occur in both resistant and susceptible individuals, and assessed the frequency of different *EPSPS* alleles present in populations.

MATERIALS AND METHODS

Plant Material and Glyphosate Resistance

Italian ryegrass populations in orchards and vineyards from Sonoma and Lake Counties where growers reported difficulty controlling plants with glyphosate, and from surrounding areas where predominantly susceptible populations may be experiencing gene flow with resistant plants, were sampled (**Table 1**). One additional population from Butte County in the Central Valley, from an area identified as containing evolved resistance to glyphosate more than 10 years ago (Simarmata et al., 2003; Jasieniuk et al., 2008), served as a comparison with the populations from Sonoma and Lake Counties where resistance has evolved more recently. In each of the populations, leaf tissue was collected for DNA extraction, and mature seeds were collected for resistance testing, from 30 to 40 randomly sampled individuals.

To test for resistance to glyphosate, eight seeds from each sampled plant were germinated on moistened filter paper in Petri dishes at 20°C and a 12-h photoperiod. Germinated seedlings were transplanted into 8 cm × 8 cm square pots filled with UC soil mix (sand, compost, and peat in 1:1:1 ratio with 1.8 kg m^{-3} dolomite) with two seedlings per pot and grown in the greenhouse at 27/15°C with ambient light conditions. At the tillering stage, individual plants were divided into genetically identical clones following the method described by Boutsalis (2001) and grown in the greenhouse to the two to three leaf stage. One clone of a genotype was treated with water, which served as a control. The second clone was treated with glyphosate (Roundup PowerMax, Monsanto, St. Louis, MO, USA) at the rate of acid equivalent 1681 g ha^{-1}, which is twice the recommended (label) field rate for the control of annual *L. perenne* plants under 6″ tall. All treatments were applied in an enclosed cabinet track sprayer equipped with an 8002E nozzle (TeeJet, Spraying Systems Co., Wheaton, IL, USA) delivering 200 L ha^{-1}. Three weeks after glyphosate treatment, we scored each plant as alive or dead, and characterized the percentage of resistant plants in each population by the percentage of plants surviving glyphosate treatment of the total number of plants treated. Plants from a previously characterized susceptible reference seed collection (Jasieniuk et al., 2008) were included during each herbicide application to confirm herbicide activity.

Detection of Target-Site Mutations

DNA was extracted from leaf tissue of all individuals sampled in the field following the CTAB method (Doyle and Doyle, 1987). Extracted DNA was quantified and diluted to 25 ng μL^{-1}. The following primers used for PCR amplification of the region surrounding site 106 of *EPSPS* were designed from the *L. perenne* ssp. *multiflorum* GenBank sequence available at the time of genotyping (accessed on January 18, 2011): F: 5′-AACCGGATCCTCCTCCTCT-3′ and R: 5′-TGCCAAGGAAACAATCAACA-3′. *EPSPS* alleles were amplified in PCR reactions consisting of 25 ng DNA template, 1× Qiagen PCR buffer (Valencia, CA, USA), 0.25 mM additional $MgCl_2$, 0.4 μM forward and reverse primers, 0.125 mM DNTPs, and 0.5 units Taq polymerase. The PCR program consisted of an initial denaturing period of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C, and a final extension of 10 min at 72°C. PCR products were cleaned to remove excess nucleotides with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) solution according to the manufacturer's instructions prior to amplification with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer instructions. Sequencing PCR products were precipitated in an ethanol and sodium acetate wash and resuspended in 10 μL highly deionized formamide.

Sequencing was performed with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were edited with Sequencing Analysis Software v5.1 (Applied Biosystems), and aligned with Geneious Software (Auckland, New Zealand). Usable DNA sequences at site 106 were obtained from 401 individuals. The region corresponding to amino acid site 106, where point mutations conferring resistance to glyphosate were previously identified, was scanned for mutations. Individuals with single peaks at each base position were recorded as homozygous for that base, while individuals with multiple overlapping peaks at a base position were recorded as heterozygous for those two bases.

RESULTS

Resistance to Glyphosate

Of the 1949 individuals tested for resistance to glyphosate at 1681 g ae ha^{-1}, 38% survived herbicide treatment. All sampled populations contained some individuals that survived (**Table 1**). Within populations, resistance to glyphosate, estimated as the percentage of individuals surviving glyphosate treatment per population, varied from 9.7 to 89.0% (**Table 1**). Population 1, sampled from the area where glyphosate resistance was first reported in California (Simarmata et al., 2003), contained 73.8% resistant individuals. Three populations (populations 12, 13, and 14) from an area where growers reported possible resistance contained 85–89% resistant individuals, while a population (population 15) bordering the area contained 21% resistant individuals, confirming glyphosate resistance in the region. In the southern portion of the studied area, populations show a gradient of survivorship ranging from 9.7% survivorship in the southern end to 40.6% in the northern end of Sonoma County (**Table 1**).

EPSPS Alleles

The region of the *EPSPS* gene encoding codon site 106 was sequenced from DNA of 401 field-sampled plants. Seven different alleles were identified at site 106 (**Table 2**). Three of these (CCA, CCC, and CCT) encode the wild-type susceptible proline allele (P106). The other four alleles contain non-synonymous mutations at site 106, which result in amino acid substitutions from proline to threonine (P106T), serine (P106S), leucine (P106L), and alanine (P106A). These four mutant resistance alleles were detected in 20.2% of all individuals genotyped and account for 11% of the 802 alleles detected across all populations.

The most common allele in all populations was P106 encoded by CCA, accounting for 655 of 802 alleles detected (**Table 2**), even in populations where the majority of individuals also contained a resistant allele. Most individuals containing a resistant allele were heterozygous, with one copy of a resistant allele and one copy of a susceptible allele (**Table 3**). Eight out of 81 individuals (10%) with resistance alleles were homozygous for the P106T allele, all of them in populations 13 or 14. No individuals were found to be heterozygous for two different resistance alleles, despite the presence of multiple types of resistance alleles in some populations.

Within populations, the frequency of different alleles varies widely across the studied area. The existence of four separate alleles that confer resistance to glyphosate indicates multiple independent evolutionary origins of resistance. Of the four resistance alleles, P106T encoded by ACA is the most common and was found in 8 of the 14 populations (**Table 2**). P106T was detected at high frequencies (>50% of individuals) in Lake County populations 12, 13, and 14, which are located very near each other in the northern part of the sampled area (**Figure 1**). This suggests a common origin of the allele in this area, with subsequent spread to other nearby populations. The other three resistance alleles are found at lower frequencies in only a small number of populations each (**Table 2**), and are distributed mostly in the southern end of the studied area, and in population 1 (**Figure 1**). Population 1 is from an area that has had evolved glyphosate resistance for the longest period of time and contains three of the four different resistance alleles (**Table 2** and **Figure 1**). The three susceptible wild-type alleles, CCA, CCC, and CCT, all code for the same amino acid and would be expected to be selectively neutral. However, only the CCA version of P106 was found in all populations (**Table 2**).

For many populations, the frequency of individuals with glyphosate-resistant phenotypes correlates roughly with the frequency of individuals with resistance alleles (**Table 3**).

In all but one population, the frequency of resistant individuals is higher than the frequency of resistance alleles. In populations 10 and 15, no resistance alleles were detected despite

TABLE 1 | *Lolium perenne* ssp. *multiflorum* populations sampled in northwest California and the numbers of genotyped and phenotyped individuals and frequencies of glyphosate-resistant plants in each.

Population ID	Cropping system	County	Latitude (N)	Longitude (W)	N_S	N_G	N_P	%R
1	Orchard	Butte	39.8	−121.98	32	23	128	73.8
2	Vineyard	Sonoma	38.23	−122.52	30	27	128	9.7
3	Vineyard	Sonoma	38.24	−122.42	37	28	171	22.5
4	Vineyard	Sonoma	38.24	−122.36	33	18	212	29.1
6	Vineyard	Sonoma	38.359	−122.502	34	31	123	22.7
7	Vineyard	Sonoma	38.214	−122.457	33	32	65	32.1
8	Vineyard	Sonoma	38.587	−122.829	31	31	176	26.6
9	Vineyard	Sonoma	38.662	−122.825	33	29	150	31.6
10	Vineyard	Sonoma	38.673	−122.811	32	30	55	35.2
11	Vineyard	Sonoma	38.761	−122.976	41	39	166	40.6
12	Vineyard	Lake	38.989	−122.821	20	18	91	85.1
13	Orchard	Lake	38.997	−122.834	36	34	186	89
14	Orchard	Lake	38.996	−122.84	31	31	153	87.9
15	Orchard	Lake	39.086	−122.943	30	30	145	20.6

N_S, number of individuals sampled for leaf tissue and seeds from each population; N_G, number of individuals genotyped; N_P, number of progeny phenotyped for response to glyphosate; % R, percentage of individuals surviving treatment with glyphosate at 1678 g ae ha^{-1}.

TABLE 2 | Numbers of different alleles and amino acid substitutions detected in 401 *Lolium perenne* ssp. *multiflorum* individuals genotyped at codon site 106 of *EPSPS*.

Population	CCA P106	CCC P106	CCT P106	ACA P106T	GCA P106A	TCA P106S	TTA P106L	Total detected
1	29	4	1	2	3	7	0	46
2	46	5	0	2	0	1	0	54
3	52	3	0	0	0	1	0	56
4	30	3	0	1	0	1	1	36
6	56	3	0	0	0	0	3	62
7	55	8	0	0	0	0	1	64
8	54	6	1	1	0	0	0	62
9	48	7	2	0	1	0	0	58
10	55	5	0	0	0	0	0	60
11	68	5	2	3	0	0	0	78
12	24	1	0	11	0	0	0	36
13	40	0	0	28	0	0	0	68
14	39	1	0	22	0	0	0	62
15	59	1	0	0	0	0	0	60
Total	655	52	6	70	4	10	5	802

Two EPSPS alleles were detected per individual.

TABLE 3 | Frequencies of glyphosate-susceptible or -resistant allelic genotypes at site 106 and the frequencies of resistant phenotypes within sampled *Lolium perenne* ssp. *multiflorum* populations in northwest California.

	% S	% R				% RR	% R	Total % R	% R	
Population	P106	P106T	P106A	P106S	P106L	Alleles	Alleles	Alleles	Individuals	N_G
1	47.8	8.7	13.0	30.4	0.0	0.0	52.2	52.2	73.8	23
2	88.9	7.4	0.0	3.7	0.0	0.0	11.1	11.1	9.7	27
3	96.4	0.0	0.0	3.6	0.0	0.0	3.6	3.6	22.5	28
4	83.3	5.6	0.0	5.6	5.6	0.0	16.7	16.7	29.1	18
6	90.3	0.0	0.0	0.0	9.7	0.0	9.7	9.7	22.7	31
7	96.9	0.0	0.0	0.0	3.1	0.0	3.1	3.1	32.1	32
8	96.8	3.2	0.0	0.0	0.0	0.0	3.2	3.2	26.6	31
9	96.6	0.0	3.4	0.0	0.0	0.0	3.4	3.4	31.6	29
10	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	35.2	30
11	92.3	7.7	0.0	0.0	0.0	0.0	7.7	7.7	40.6	39
12	38.9	61.1	0.0	0.0	0.0	0.0	61.1	61.1	85.1	18
13	26.5	73.5	0.0	0.0	0.0	8.8	64.7	73.5	89	34
14	45.2	54.8	0.0	0.0	0.0	16.1	38.7	54.8	87.9	31
15	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	20.6	30

% S, the percentage of genotyped individuals with two susceptible alleles; % R, the percentage of genotyped individuals with one or two resistance alleles of the specified type; % RR alleles, the percentage of genotyped individuals homozygous for a resistance allele; % R alleles, the percentage of genotyped individuals heterozygous for a resistance allele; Total % R alleles, the total percentage of genotyped individuals with one or two resistance alleles; % R individuals, the percentage of phenotyped individuals surviving glyphosate treatment at 1681 g ae ha^{-1}; N_G, the number of individuals genotyped in each population.

the presence of 35.2 and 20.6% resistant individuals, respectively (**Table 3**), suggesting non-target-site-based mechanisms of resistance.

DISCUSSION

Target-Site-Based Resistance

Resistance to glyphosate in northwest California populations of *L. perenne* ssp. *multiflorum* is commonly conferred by *EPSPS* target-site mutations at the site corresponding to codon 106, based on the results of this study. The four non-synonymous mutations identified in the sampled populations have been previously shown to confer glyphosate resistance in *L. perenne* ssp. *multiflorum* and ssp. *rigidum* (Wakelin and Preston, 2006; Jasieniuk et al., 2008; Kaundun et al., 2011; Sammons and Gaines, 2014). The presence of four separate resistance alleles indicates at least four separate evolutionary origins of target-site-based glyphosate resistance across the agricultural landscape. Moreover, the distribution of resistance alleles suggests that resistance either evolved independently in the southern and northern extremes of the studied area, and/or there has been long-distance gene flow

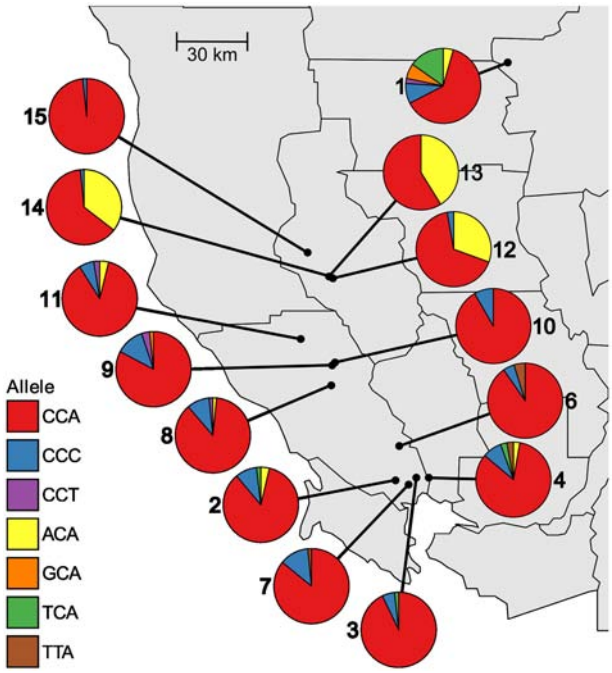

FIGURE 1 | Distribution and frequencies of *EPSPS* alleles at site 106 in 14 sampled populations of *Lolium perenne* ssp. *multiflorum* in northwest California.

between these areas through movement of resistant weed seed. Local spread of resistance through gene flow is also likely, as demonstrated by the shared presence of some resistance alleles in closely located populations.

Most resistance alleles detected can be explained by a single nucleotide substitution from a susceptible allele, except for the P106L allele encoded by TTA, which requires two nucleotide substitutions from CCA or three from CCC or CCT. It is also possible that the P106L allele is a result of a single nucleotide substitution from the P106S allele encoded by TCA. P106L and P106S do co-occur in one population. However, both alleles are relatively uncommon, making exact determinations of the origins of allele P106L difficult.

The number of individuals genotyped in this study does not allow detection of rare alleles ($p < 0.05$) within populations. Very large sample sizes are required to detect rare genetic variants and diminishing returns of increasing sample sizes makes detection of all rare alleles in a population impractical (Marshall and Brown, 1975). The threshold for the desired level of allele detection varies with the goal of the study, e.g., collection of rare adaptive genotypes for breeding or maintenance of representative germplasm for conservation (Crossa and Vencozsky, 2011). To be 95% confident in detecting at least one copy of all alleles with frequency $p > 0.05$, it is necessary to sample 59 unrelated gametes, or approximately 30 diploid outcrossing individuals, while only 15 individuals are required to detect all alleles with frequency $p > 0.1$ (Marshall and Brown, 1975). The goal of this study was to detect mutant alleles that confer glyphosate resistance in *L. perenne* populations. In orchard and vineyard populations with frequencies of resistant plants high enough to be of concern to weed management, the alleles conferring the resistance trait likely are also common. However, it is possible that populations, which currently are predominantly susceptible, contain rare resistance alleles that may increase in frequency in the future due to selection, and this study may not have detected those alleles.

Non-Target-Site-Based Resistance

Most sampled populations contained a higher frequency of resistant individuals than resistant *EPSPS* alleles, indicating that non-target-site-based mechanisms may underlie resistance in some individuals. The differences in frequencies of resistant phenotypes and resistant genotypes may also be partially due to stochastic effects associated with the plants used for phenotyping versus those used for genotyping, or due to error in the estimation of allele frequencies from the relatively low number of plants genotyped. Both methodological issues could result in estimates of genotype frequencies deviating from their actual values in populations, and would have an equal probability of skewing estimates to be either higher or lower than the actual genotype frequencies. However, 13 of the 14 sampled populations have higher frequencies of resistant plants than resistance alleles (**Table 3**), strongly suggesting a biological cause rather than stochastic effects. Non-target-site mechanisms of glyphosate resistance have been identified in *L. perenne* populations from other agricultural areas in the United States as well as worldwide (Perez-Jones et al., 2007; Preston et al., 2009; Salas et al., 2012, 2015). In addition to the differences in frequencies of resistant phenotypes and genotypes, further support for the presence of non-target-site-based resistance in this study comes from populations 10 and 15, in which no resistance alleles were detected despite the populations containing 35.2% and 20.6% resistant individuals, respectively (**Table 3**). It is highly unlikely that resistant *EPSPS* alleles could be present at those frequencies in a population without being detected in 30 genotyped individuals. It is far more likely that these populations contain individuals that are resistant to glyphosate through a non-target-site-based mechanism. Individuals with a non-target-site-based mechanism of resistance would be phenotyped as resistant but genotyped as containing only P106 alleles.

Altered translocation of glyphosate away from growing tissue and overexpression of the EPSPS enzyme through gene duplication have both been identified as the mechanisms underlying resistance to glyphosate in populations of *L. perenne* in other agricultural regions (Wakelin et al., 2004; Perez-Jones et al., 2007; Salas et al., 2012, 2015). The genetic basis of altered translocation is not currently known, thus adaptive genetic variation associated with the mechanism could not be analyzed here. *EPSPS* gene duplication was not detected in California populations (Putta and Jugulam, 2015, personal communication). It is also possible that individual plants have multiple mechanisms of resistance, containing both target-site-based and non-target-site-based mechanisms of resistance. Little is known about whether separate mechanisms of resistance to the same herbicide may confer an increased level of

resistance, or if a fitness cost to one or both herbicides may affect the frequencies of one or both mechanisms in future generations. Future physiological and genetic studies will assess the relative importance of target-site and non-target-site-based mechanisms of resistance to glyphosate in *L. perenne* populations of California.

The presence of both target-site and non-target-site resistance in a region indicates that the evolution of herbicide resistance can be quite complex across an agricultural landscape. A similar pattern of multiple target-site alleles with additional non-target-site-based mechanisms conferring herbicide resistance was observed for *Alopecurus myosuroides* and *L. perenne* ssp. *rigidum* evolving resistance to ACCase-inhibitors (Delye et al., 2010b; Malone et al., 2014), and indicates this may be common in weeds across agricultural landscapes. Resistance traits in populations located near each other may have separate or shared evolutionary histories through novel mutation of resistance alleles or spread through gene flow. Single populations may contain heterogeneous mixtures of individuals with distinct resistance traits. This is especially important to recognize considering that due to experimental limitations, studies of herbicide resistance in weeds often investigate only a small number of populations or only a few individual plant lines from a larger number of populations, and may result in studies concluding resistance is more uniform than it is in reality.

CONCLUSION

Resistance to glyphosate has evolved repeatedly in populations of *L. perenne* ssp. *multiflorum* across the agricultural landscape of northwest California. Four distinct alleles at codon site 106 of the *EPSPS* gene confer target-site resistance to glyphosate. The distribution of *EPSPS* alleles among and within populations reveals a complex evolutionary history of the resistance trait, with multiple independent mutation events together with local spread of resistance through gene flow. Recently, non-target-site-based resistance is becoming evident in some populations within the region further complicating identification of the evolutionary origins and processes underlying resistance. It is clear, however, that long-term successful management of glyphosate-resistant *L. perenne* will require the adoption of strategies to manage currently resistant populations while also reducing the selection pressure for future evolution of glyphosate resistance.

AUTHOR CONTRIBUTIONS

EK and MJ have made substantial, direct and intellectual contributions to the work, and approved it for publication.

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Unraveling the Transcriptional Basis of Temperature-Dependent Pinoxaden Resistance in *Brachypodium hybridum*

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Climate change endangers food security and our ability to feed the ever-increasing human population. Weeds are the most important biotic stress, reducing crop-plant productivity worldwide. Chemical control, the main approach for weed management, can be strongly affected by temperature. Previously, we have shown that temperature-dependent non-target site (NTS) resistance of *Brachypodium hybridum* is due to enhanced detoxification of acetyl-CoA carboxylase inhibitors. Here, we explored the transcriptional basis of this phenomenon. Plants were characterized for the transcriptional response to herbicide application, high-temperature and their combination, in an attempt to uncover the genetic basis of temperature-dependent pinoxaden resistance. Even though most of the variance among treatments was due to pinoxaden application (61%), plants were able to survive pinoxaden application only when grown under high-temperatures. Biological pathways and expression patterns of members of specific gene families, previously shown to be involved in NTS metabolic resistance to different herbicides, were examined. Cytochrome P450, glucosyl transferase and glutathione-*S*-transferase genes were found to be up-regulated in response to pinoxaden application under both control and high-temperature conditions. However, biological pathways related to oxidation and glucose conjugation were found to be significantly enriched only under the combination of pinoxaden application and high-temperature. Analysis of reactive oxygen species (ROS) was conducted at several time points after treatment using a probe detecting H_2O_2/peroxides. Comparison of ROS accumulation among treatments revealed a significant reduction in ROS quantities 24 h after pinoxaden application only under high-temperature conditions. These results may indicate significant activity of enzymatic ROS scavengers that can be correlated with the activation of herbicide-resistance mechanisms. This study shows that up-regulation of genes related to metabolic resistance is not sufficient to explain temperature-dependent pinoxaden resistance. We suggest that elevated activity of enzymatic processes at high-temperature may induce rapid and efficient pinoxaden metabolism leading to temperature-dependent herbicide resistance.

Keywords: ACCase inhibitors, climate change, CYP450, glutathione-*S*-transferase, metabolic resistance, reactive oxygen species, RNA-seq, temperature-dependent response

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INTRODUCTION

Anthropogenic greenhouse gas emissions and climate change pose risks to long-term food security due to their detrimental effects on agriculture productivity (Myers et al., 2017). To feed the 9.6 billion people expected by 2050 (FAOSTAT, 2017) a significant increase in cereal-grain yield will be needed (reviewed by Tester and Langridge, 2010). In the long-term (2030–2050), climatic changes in the Middle East are expected to affect mean temperatures by 1–2°C (Parry et al., 2007; Nelson et al., 2009). However, greater risks to food security may be posed by changes in between-year and within-year variability and the increasing frequency and severity of extreme weather events (Gornall et al., 2010; Lelieveld et al., 2016; Stott, 2016). These environmental changes will affect the development and productivity of both crops and weeds. Weed infestation has already been acknowledged as a major factor causing yield reduction in various crops such as maize (*Zea mays*, Soltani et al., 2016), rice (*Oryza sativa*, Chauhan and Johnson, 2011; Chauhan and Opena, 2012), and hazelnut (*Corylus avellana,* Kaya-Altop et al., 2016).

Since their introduction in 1940, herbicides are the most cost-effective and efficient practice for weed control. In recent years, this method of weed control has become less efficient due to the evolution of herbicide-resistant weeds (Heap, 2017). Herbicide resistance is a consequence of strong selection pressure imposed by repeated application of the same herbicide to a weed population. Herbicide resistance can result from modification of the target site (TS) or via other mechanisms involved in non-target site (NTS) resistance (Rubin, 1991). Mechanisms of TS resistance have been well studied; they involve structural changes at herbicide-binding sites or increased expression of target proteins. NTS resistance can be endowed through reduced absorption (Koger and Reddy, 2005), reduced translocation (Feng et al., 2004) or sequestration (Kleinman and Rubin, 2016). The underlying mechanisms involved in NTS resistance are still not thoroughly understood (Délye, 2013).

Acetyl-CoA carboxylase (ACCase) inhibitors are commonly used to control grass weeds in various crops. In most plants, two isoforms of the ACCase enzyme, heteromeric and homomeric, exist in different cell compartments (i.e., cytosol and plastids; Roesler et al., 1997). In grass species, only the homomeric form of the enzyme is present and ACCase inhibitors function by blocking this form (Sasaki and Nagano, 2004). NTS resistance to ACCase inhibitors is endowed mostly by herbicide detoxification (Kaundun, 2014). The first two crucial phases of detoxification are mediated by members of the cytochrome P450 (CYP450) enzyme family (Manabe et al., 2007; Gaines et al., 2014; Iwakami et al., 2014) and both glutathione-*S*-transferase (GST; Cummins et al., 1999, 2013; Skipsey et al., 2011) and glucosyl transferase (GT; Baerson et al., 2005; Gardin et al., 2015; Xu et al., 2015) play key roles in the conjugation of the herbicide. Different genes from all three families (CYP450, GST, and GT) have been found to be up-regulated in herbicide-resistant grass weed populations such as *Lolium rigidum* (Gaines et al., 2014; Duhoux et al., 2015), *Alopecurus myosuroides* (Gardin et al., 2015), and *Eleusine indica* (An et al., 2014; Chen et al., 2015).

Environmental conditions such as temperature can affect the retention, penetration and movement of herbicides through the plant and can also modify plants growth and development, indirectly affecting herbicide activity within the plant (e.g., Hammerton, 1967; Caseley, 1989; Rubin, 1991; Sundby et al., 1993; Robinson et al., 2015). Temperature may modify the response of plants to herbicides with different modes of action (HRAC, 2017). This phenomenon has been demonstrated in the effect of paraquat (group D) on *Hordeum glaucum* (Lasat et al., 1996), the effect of glyphosate (group G) on *Conyza* sp. (Kleinman et al., 2015), the effect of mesotrione (group F) on *Amaranthus palmeri* (Godar et al., 2015), and the effect of pinoxaden (group A) on *Brachypodium hybridum* (Matzrafi et al., 2016).

Brachypodium, a small annual grass species, native to the Mediterranean region, is a valuable model system for a variety of biological processes and genome organization in cereals (reviewed by Kellogg, 2015). In recent years, it has emerged as a powerful model plant for the study of herbicide resistance in grass weeds (e.g., Gressel et al., 1983; Matzrafi et al., 2014; Frenkel et al., 2017). Recently, we demonstrated that elevated temperatures result in increased tolerance to ACCase inhibitors in various grass weed species (Matzrafi et al., 2016). Here, we employed a system biology approach to uncover the transcriptional basis of temperature-dependent NTS resistance mechanism. We hypothesized that temperature-dependent herbicide detoxification is facilitated by enhanced enzymatic efficiency at elevated temperatures. Previously, we have identified a *B. hybridum* accession, presenting temperature-dependent resistant to pinoxaden (Matzrafi et al., 2016). The aims of the current study were to: (i) characterize the transcriptional differences between pinoxaden-treated and untreated plants under different temperatures, (ii) elucidate the biological processes that are associated with temperature-dependent herbicide detoxification, and (iii) examine the role of metabolism-related genes known to be involved in herbicide resistance in temperature-dependent pinoxaden resistance in *B. hybridum*.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *B. hybridum* accession BrI-782 (temperature-dependent NTS-resistant to the ACCase inhibitor pinoxaden; Matzrafi et al., 2014) were germinated in trays filled with growth mixture (Pele-Shacham, Israel). The trays were placed in a dark, cold room (16°C) until germination. After emergence, uniform seedlings were transplanted into pots (7 cm × 7 cm × 6 cm) containing similar growth mixture and transferred to a phytotron where they were kept under natural Mediterranean growth conditions [10/16°C (night/day), 10 h of light]. Two temperature regimes were used in this study: control [10/16°C (night/day)] and high temperature [28/34°C (night/day)].

At the three-leaf stage (BBCH scale 13; Hong et al., 2011), plants were treated with either water (control) or the recommended dose of the ACCase inhibitor pinoxaden (Axial®,

50 g L^{-1} pinoxaden + 11.25 g L^{-1} cloquintocet-mexyl, EC, Syngenta, Switzerland; X = recommended dose of 30 g ai ha^{-1}). The treatment was applied using a chain-driven sprayer delivering 300 L ha^{-1}. One hour after treatment (HAT), plants were moved back to the phytotron and each plant was assigned to one of two temperature regimes: control or high. In each room, 10 plants (five treated with pinoxaden and five treated with water) were kept for 21 days after treatment (DAT). Survival rates were visually assessed and shoot fresh weight was measured.

Sample Preparation and RNA Sequencing

Samples of fresh shoot tissue were collected from treated and untreated plants at 24 HAT (**Figure 1A**), immediately frozen in liquid nitrogen and stored at −80°C. RNA-seq analysis was conducted using three plants from each of the following treatments: control (C), pinoxaden application (X), high-temperature (H) and the combination of pinoxaden and high-temperature (HX; **Figure 1A**). Total RNA was extracted using a Plant/Fungi Total RNA Purification Kit (Norgen Biotek Corp., Canada). Total RNA was treated with TURBODNase® (RNase-Free; Ambion, Warrington, United Kingdom) to eliminate DNA contamination. RNA was quantified using a NanoDrop (ND-1000) spectrophotometer (Thermo Scientific, Wilmington, DE, United States) and RNA integrity and quality were assessed with a 2100 Bioanalyzer (Agilent Technologies Inc., Germany). Additional data concerning sample quality and other parameters are presented in Supplementary Table S1.

cDNA libraries were generated using a NEBNext Ultra Directional RNA Prep Kit (New England Biolabs, Ipswich, MA, United States), following the manufacturer's instructions. After verifying their quality, libraries were indexed with six-nucleotide barcodes and sequencing was performed on the Illumina HiSeq2000 machine using multiplexing for generating 50 base-paired (bp) end reads. Sequencing was carried out at the Technion Genome Center (Haifa, Israel).

RNA-Sequencing Analysis

B. hybridum transcript levels were obtained from HiSeq2000 machine using a custom computational pipeline. Briefly, 50 bp end reads were trimmed and quality-filtered using Trimmomatic (Bolger et al., 2014) and then mapped to the *B. distachyon* genome (International Brachypodium Initiative, 2010), version 3.0 using Tophat (Kim D. et al., 2013). Tophat was run with a maximum intron length of 10,000 bases to reduce the likelihood of false positives (Walters et al., 2013). The mean percentages of overall mapped reads and multiple mapped reads across samples were 80 and 5%, respectively. Mapped reads were then checked for overlap with JGI v3.1[1] gene exons using htseq-count (Anders et al., 2015). The mean percentage of reads overlapping (a known feature across samples) was 90%. We then used DESeq2 (Love et al., 2014) to detect differentially expressed genes (DEGs) among samples from the different experimental treatments (high-temperature, pinoxaden and their combination) and the control. Data is presented as log2 of fold-change values (log2FC). For DEG analysis we considered genes that were significant at false discovery rate (FDR) ≤ 5%.

Functional Annotation of Transcriptome Analysis

Differentially expressed genes were analyzed using hierarchical clustering and divided into parallel plots using the JMP (ver. 12) statistical package (SAS Institute Inc., Cary, NC, United States). Genes that were highly up-regulated under HX treatment were annotated with MapMan software (Thimm et al., 2004) using a functional data base containing 32,031 different assigned identifiers, with more than 35 general biological processes matching the *B. distachyon* genome (International Brachypodium Initiative, 2010). Pathway-enrichment analysis was performed with FunRich, a functional enrichment analysis software tool[2] (FDR ≤ 0.05; Pathan et al., 2015), using MapMan annotation as reference data (Thimm et al., 2004).

Quantitative PCR

RNA samples used for RNA-seq analysis ($n = 3$) as well as additional three plants that were grown and treated with the plants selected for RNA-seq analysis were used for qPCR validation ($n = 6$). First-strand cDNA was synthesized using qScript™ cDNA Synthesis Kit (Quanta Biosciences Inc., United States), following the manufacturer's instructions. qPCR was carried out using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences Inc., United States) in a PikoReal RT-PCR system (Thermo Fisher Scientific Inc., United States). Gene-specific primers were designed using Primer3 software (Supplementary Table S2). PCR mixtures included 2 μL of cDNA (diluted by four), 8 μL of SYBR Mix, and 300 nM of each primer in a final volume of 10 μL. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used for the normalization and calibration processes. Transcript values were relatively tested compared to the housekeeping gene *S*-adenosylmethionine decarboxylase (SamDC, BRADI2G02580; Hong et al., 2008), whose expression was not affected by the herbicide and\or high-temperature treatment.

ROS Staining and Imaging

Plants of *B. hybridum* accession BrI-782 were grown under controlled conditions, as described above. At the three-leaf stage, plants were treated with the recommended dose of pinoxaden as described above. Treated and untreated plants were transferred back to controlled conditions or subjected to a high-temperature regime. The experiment was conducted three times using three replicates for each treatment. ROS measurements were taken at 2, 8, and 24 HAT. Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was used as a probe for H_2O_2/peroxides as described previously (Pena et al., 2012). A 50 μM stock of H_2DCF-DA was prepared

[1] http://genome.jgi.doe.gov

[2] http://www.funrich.org

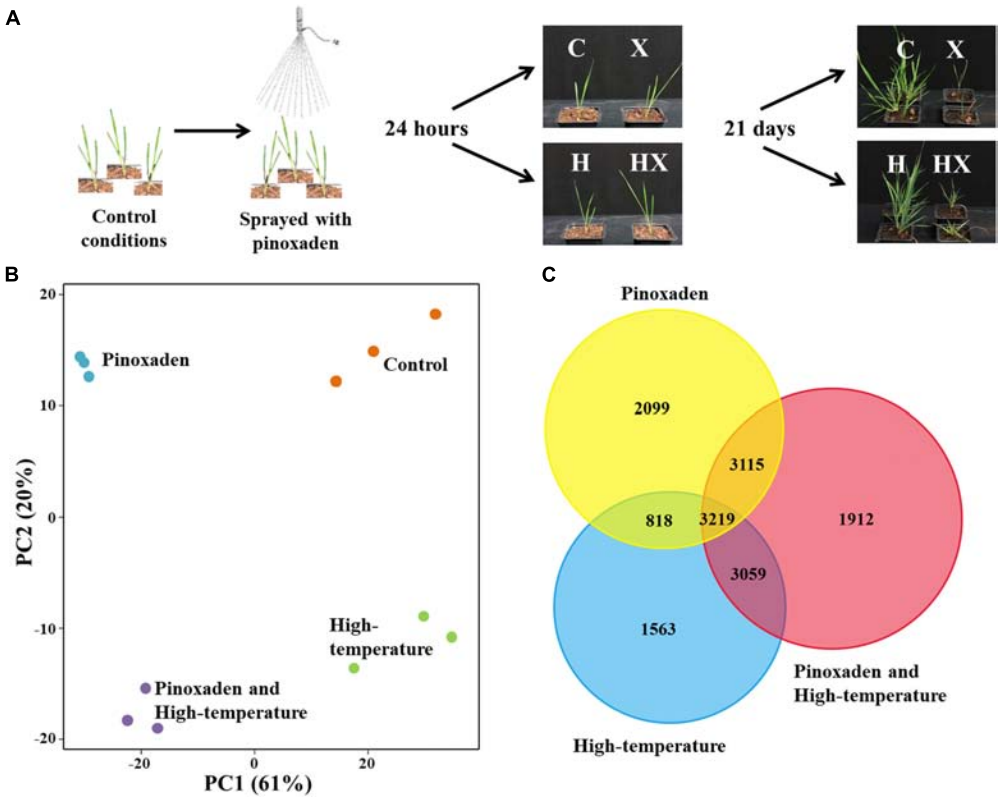

FIGURE 1 | (A) Experimental design and final phenotypes of *Brachypodium hybridum* plants (accession BrI-782) grown under control (C, 10/16°C day/night) and high-temperature (H, 28/34°C) conditions. Plants were sprayed with pinoxaden (X) or water at the three-leaf stage; RNA-seq samples were taken 24 h after application. Plant survival rates were determined 21 days after pinoxaden application. **(B)** Principal-component (PC) analysis of rlog transformed gene expression data, generated by RNA-sequencing. Each treatment contains three biological repeats and is indicated by a different color. **(C)** Venn diagram of differentially expressed genes (DEGs) from all three treatments.

in DMSO, diluted (1:9) in a 10 mM Tris-HCl (pH 7.4) and non-ionic surfactant (Spreader L77, ADAMA-Agan, Israel) at 0.05% v/v was added. The second leaf of each plant was cut and covered with aluminum foil, to prevent light exposure for 30 min, during which time the leaf was kept at room temperature. Leaves were than washed three times with 10 mM Tris-Hcl (pH 7.4). Fluorescent stereoscope images were captured using a Nikon SMZ1500 (Nikon, Japan) zoom stereoscope, with an excitation filter of 450–490 nm and a barrier filter of 510 nm, which was used to detect H_2DCFDA green fluorescence. Images were captured with a color camera (DS RI1, Nikon, Japan) operated with NIS Elements V3 software (Nikon, Japan). Exposure times were equal for all samples. Autofluorescence was not observed in unstained controls at the exposure time used. Leaf fluorescence was quantified using ImageJ software (ver. 1.63; U.S. National Institutes of Health).

Accession Number

Raw sequencing files of mRNA sequencing are available at the short read archive of the National Center for Biotechnology Information (https://trace.ncbi.nlm.nih.gov/Traces/sra) under accession number PRJNA360668.

RESULTS

As a native Mediterranean temperate grass species, *B. hybridum* grows during the winter season (November to April). Under control conditions (10/16°C, night/day), which mimic the Mediterranean winter, BrI-782 plants showed a higher sensitivity to pinoxaden that was manifested by lower shoot fresh weights (21%) and a lower survival rate, as compared with treated plants grown under high-temperature (**Figure 1A** and Supplementary Table S3). We hypothesized that the significant differences in plant response to pinoxaden under contrasting temperature regimes may be the result of temperature-dependent transcriptional modifications. In order to test our hypothesis, we used RNA-seq to detect the temperature-dependent differences in transcripts, pathways and mechanisms between pinoxaden-sensitive and pinoxaden-resistant plants.

RNA-Seq Data

A total of 257 million raw reads were generated from the 12 libraries (three biological replicates for each of the four treatments). After removing reads containing adaptor or ploy-N and low-quality reads, 208 million clean reads were obtained with 13.4–18.6 million reads per sample. In the absence of a

fully sequenced *B. hybridum* genome, the annotation procedure was carried using the *B. distachyon* database. Our *B. hybridum* samples scored 80.6% mapped reads and 87.2% featured contigs (Supplementary Table S1). Principal-component analysis was used to assess the variability among biological replicates and treatments. Within each treatment, the three biological replicates showed a high degree of similarity to one another and formed an independent cluster. Among the treatments, application of herbicide either alone or in combination with high-temperature resulted in a separation of the control and high-temperature treatments. This separation explained 61% of the total experimental variance (**Figure 1B**).

The number of DEGs between each treatment and control conditions ranged between 11,305 for the combination of herbicide and high-temperature, 9,253 for the herbicide application, and 8,659 for the high-temperature treatment (Supplementary Table S4). A search for common DEGs among all treatments yielded 3,219 genes. The combined herbicide and high-temperature treatment was very similar to both the herbicide application and the high-temperature treatment with 3,115 and 3,059 common DEGs, respectively (**Figure 1C**). The similarity of the combination treatment to the other two single treatments correlates with the dual effect of both stress factors together. The combination of herbicide and high-temperature had the highest number of up-regulated DEGs (5,377), as compared to the herbicide application (4,415) and the high-temperature treatment (3,822; Supplementary Table S4).

Biological Process Analysis

The pattern of gene expression was analyzed using hierarchical clustering of 3,129 common DEGs across all treatments. DEGs were classified into 25 groups by parallel plots (Supplementary Table S5), which enabled us to identify specific trends in gene expression. Assuming that over-expression of DEGs can lead to enhanced herbicide detoxification (Délye, 2013), we focused on groups that included DEGs that were up-regulated mainly in the combined treatment (groups 4 and 14 in **Figures 2A,B**, respectively; Supplementary Table S5). Up-regulation of genes from both plots was slightly different, as plot 4 showed greater differences in the combined treatment, as compared with plot 14 (**Figure 2**). Enrichment analysis was conducted to identify key biological processes related to temperature-dependent pinoxaden detoxification. Both groups included genes associated with protein, RNA, stress, and transport (**Figures 2A,B**). Examination of specific pathways within each category showed that both groups were enriched with genes associated with oxygenase activity ($P = 0.03$, $P < 0.01$, respectively) as well as conjugation-related pathways, including genes associated with UDP-glucosyl and glucuronyl transferase ($P < 0.01$) and GST ($P < 0.01$; **Figures 2A,B**). Genes associated with ABC transporters and multidrug resistance, representative of the final stage in herbicide detoxification, were also significantly enriched ($P = 0.05$; **Figure 2B**).

Candidate Metabolic-Resistance Genes

Herbicide metabolism is generally composed of four different phases, systematically rendering the herbicide molecule into a non-toxic product (Carvalho et al., 2009). We identified DEGs that are involved in these processes (FDR ≤ 0.05) and used qPCR to validate their expression levels under the different treatments (Supplementary Figure S1). Two DEGs, Bradi2g44160 and Bradi2g44200, were annotated as members of the CYP72A subfamily, which are associated with the first phase of herbicide metabolism. Genes of this subfamily were recently reported to be up-regulated in *L. rigidum* plants resistant to an ACCase inhibitor (Gaines et al., 2014). In our study, these genes were highly up-regulated ($\log_2$FC > 7.9) under both the herbicide application and the combination of herbicide and high-temperature treatments (**Figure 3A** and Supplementary Table S6). Other genes involved in CYP450 catalytic activity encode for NADP enzymes (Davydov, 2001), which were also found to play a role in the process of ROS quenching (Buchanan et al., 2006). NAD(P)-linked oxidoreductase (Bradi3g48197) was up-regulated with $\log_2$FC values of 3.3 and 4.6 under high-temperature and combination of herbicide and high-temperature treatments, respectively (Supplementary Table S5). Additional CYP450 genes, CYP96A10 (Bradi3g19220) and CYP79B2 (Bradi1g15695), were up-regulated only in the combination of herbicide and high-temperature, but at lower levels than the CYP72A genes ($\log_2$FC < 2.6; **Figure 3A** and Supplementary Table S6).

With regard to the second phase of herbicide metabolism, DEGs involved in GT processes, UDP-glucosyl and glycosyl transferase (UDPGT); UDPGT88A1 (Bradi2g49067), UDPGT73B5 (Bradi2g44820), and UDPGT85A2 (Bradi3g46855) were up-regulated under herbicide application and the combination of herbicide and high-temperature (**Figure 3B** and Supplementary Table S7). Another major group of second-phase-related genes, GSTs, were also examined. GST DEGs (Bradi3g31777, Bradi3g61100 and others) were up-regulated in response to both herbicide treatments (herbicide application and combination of herbicide and high-temperature; **Figure 3C** and Supplementary Table S8). The trends observed in the RNA-seq experiment for these genes were also observed in the qPCR analysis (Supplementary Figure S1).

ROS Accumulation in Response to Different Treatments

CYP450 activity, which produces ROS, is also regulated by the accumulation of ROS (Davydov, 2001; Narusaka et al., 2004; McIntosh et al., 2014). Peroxidases are known to act as part of ROS neutralization mechanisms (Meunier and Bernadou, 2000). A high expression of two DEGs (Bradi5g24200 and Bradi4g25660), annotated as peroxidases, was detected only under the combination of herbicide and high-temperature ($\log_2$FC > 3.5; **Figure 3D** and Supplementary Table S9). The multiprotein bridging factor 1C (MBF) was previously described as a key regulatory element linking ROS signaling to stress responses (Miller et al., 2008). MBF (Bradi1g37080) showed a higher expression level under the combination of herbicide and high-temperature, as compared with the other treatments (herbicide application and high-temperature treatments) with $\log_2$FC values of 8.1, 6.6 and 7.4, respectively (Supplementary Table S5). This trend can be correlated with greater activity of

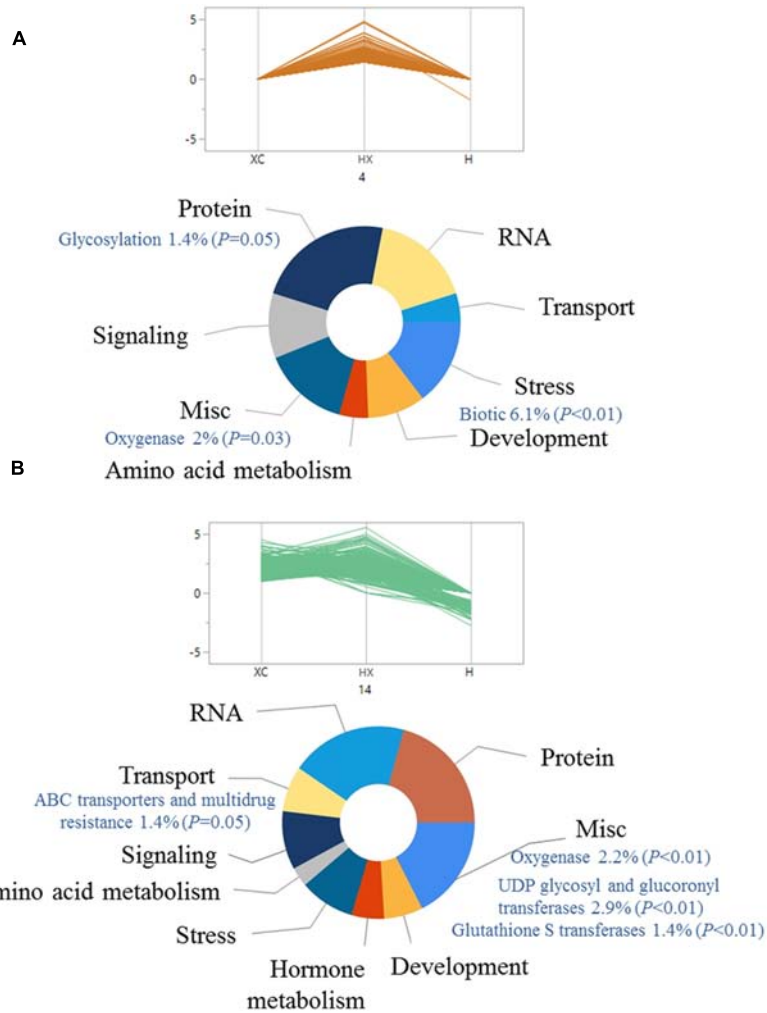

FIGURE 2 | Functional classification of significantly (FDR ≤ 0.05) up-regulated genes in pinoxaden-treated plants under high-temperature regime, compared with the two other treatments (pinoxaden and high-temperature). All genes were divided into 25 groups using hierarchal clustering analysis according to the expression pattern of all three treatments. Two clusters, **(A)** 4 and **(B)** 14, were chosen as representative trends for up-regulated genes. Doughnut charts were produced based on MapMan annotations and the FunRich platform.

CYP450 enzymes, leading to more ROS neutralization, as part of the herbicide-resistance mechanism.

To learn more about the role of ROS in the herbicide-resistance mechanism, ROS staining was conducted and the patterns of ROS accumulation among the different treatments were examined. ROS quantities did not differ significantly among the different treatments at 2 and 8 HAT. However, at 24 HAT, plants subjected to the combination of herbicide and high-temperature had ROS levels that were significantly lower than those detected under control conditions (**Figure 4** and Supplementary Table S10).

DISCUSSION

Elevated temperatures have been shown to have a direct effect on the level of NTS herbicide resistance in various weed species (Godar et al., 2015; Kleinman et al., 2015; Matzrafi et al., 2016). Here, we describe for the first time the transcriptional basis of a temperature-dependent NTS herbicide-resistance mechanism in *B. hybridum* and reveal the importance of temperature in herbicide-related metabolic processes. Biochemical analysis of temperature-dependent pinoxaden detoxification suggests two main stages of oxidation and glucose conjugation as key steps in the resistance mechanism (Matzrafi et al., 2016). Exploring transcriptional-enriched pathways that were differentially regulated under the combination of herbicide and high-temperature revealed up-regulation of several known herbicide defense-related genes (e.g., oxygenases, GST, GT, and ABC transporters; **Figures 2A,B**). The enrichment of these pathways reinforces previous findings and indicates a higher rate of pinoxaden metabolism at high-temperature. Members of the CYP72A subfamily that were previously identified as components of the mechanism of resistance to ACCase and acetolactate

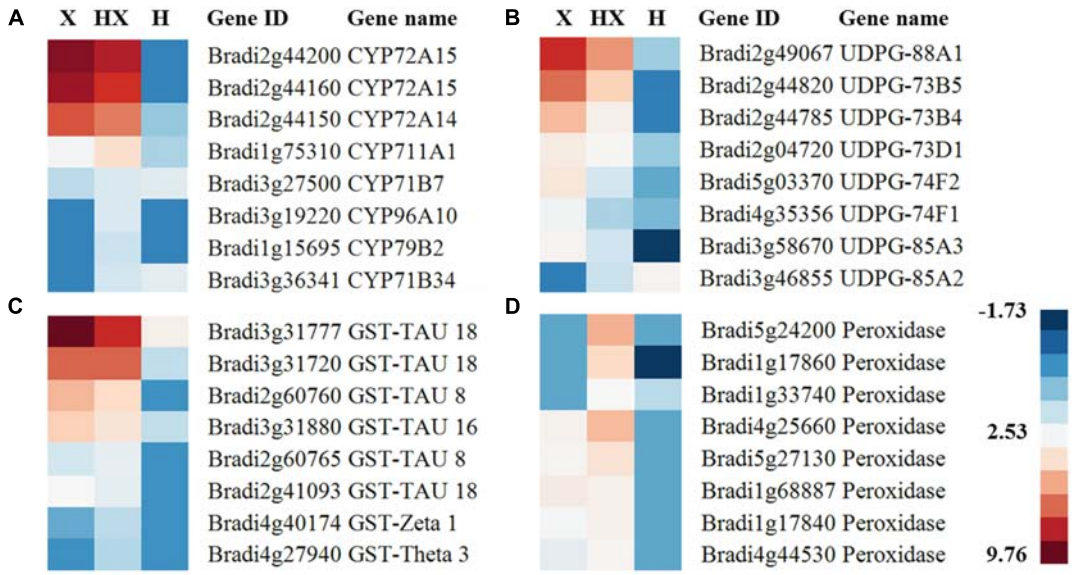

FIGURE 3 | Heatmap of herbicide-associated genes among the different treatments relative to control conditions. X, pinoxaden; H, high-temperature, and HX, combination of high-temperature and pinoxaden. **(A)** Cytochrome P450, **(B)** UDP-glucosyl and glucuronyl transferase, **(C)** glutathione-*S*-transferase, and **(D)** peroxidase. Blue and red colors represent low and high relative expression compared with the mean value of expression across all samples, respectively. Scale is $\log_2$ of mean expression value.

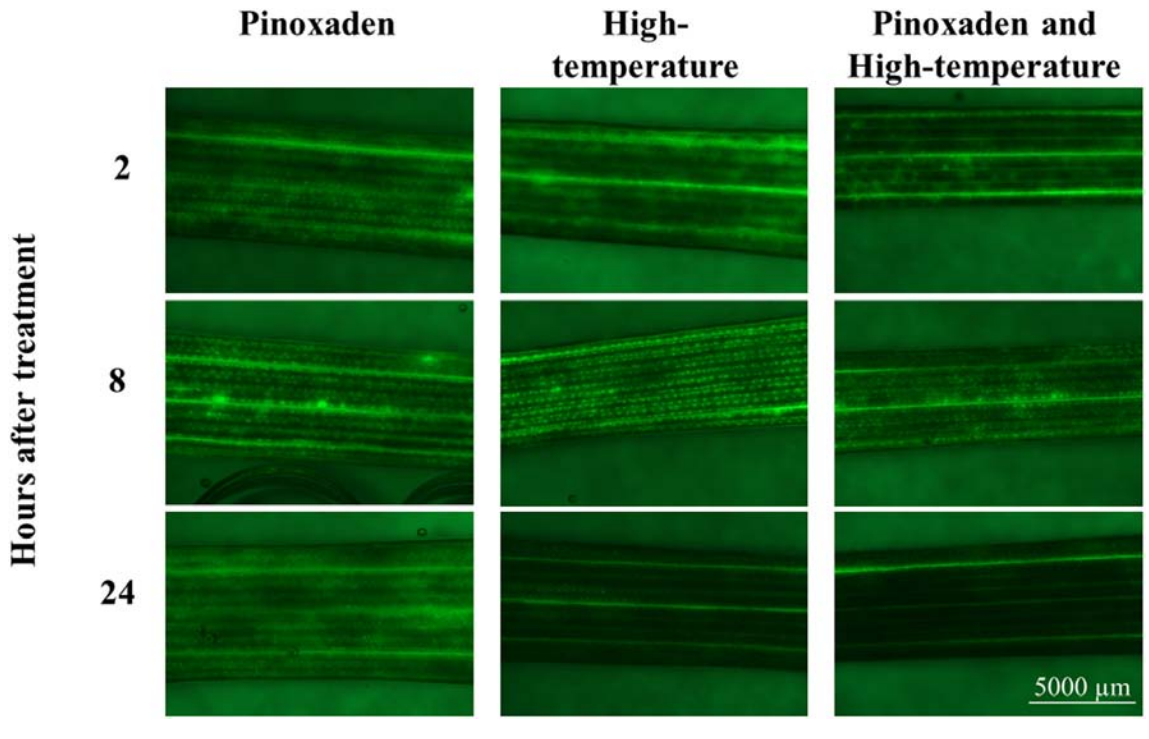

FIGURE 4 | Visual presentation of the accumulation of reactive oxygen species using H_2DCF-DA dye. Plants were treated with: pinoxaden, high-temperature (28/34°C) and high-temperature and pinoxaden application. Photos were taken 2, 8, and 24 h after herbicide application.

synthase inhibitors (Gaines et al., 2014; Saika et al., 2014) were also found to be highly expressed in pinoxaden-treated plants under both temperature regimes (**Figure 3A**). The abundance of genes from the CYP72A subfamily identified in different studies involving different inhibitors and weed species (Imaishi and Matumoto, 2007; Gaines et al., 2014; Iwakami et al., 2014;

Saika et al., 2014) supports the assumption of their role in this mechanism of herbicide resistance. Previous studies suggested the involvement of GT activity in resistance to ACCase inhibitors (Menendez and De Prado, 1996; Brazier et al., 2002), which function mainly at the second phase of herbicide detoxification (McFadden et al., 1989; Baerson et al., 2005). Expression levels of different transcripts encoding CYP72A and UDPGT genes that were detected in our work can be associated with the formation of specific pinoxaden metabolites previously found in *B. hybridum* plants (Matzrafi et al., 2016). The detection of these DEGs following pinoxaden application in both temperature treatments (herbicide application and the combination of herbicide and high-temperature) suggests that the observed differences in plant response to pinoxaden are not solely due to the up-regulation of these genes. Further investigation of the role of the 2,099 DEGs that were found to be expressed only under pinoxaden treatment is needed to help to shed light on their role in herbicide response.

The catalytic activity of CYP450 enzymes includes the splitting of O_2 molecules to form a hydroxylated product (Munro et al., 2013). In this process, ROS are formed and transformed into H_2O (Davydov, 2001; McIntosh et al., 2014). Both NADP and peroxidases were previously suggested to play a role in CYP450 catalytic activity and the quenching of ROS (Davydov, 2001; Buchanan et al., 2006). The up-regulation of NADP and peroxidases that was unique to the combination of herbicide and high-temperature treatment can be correlated with the rapid metabolism of pinoxaden leading to herbicide resistance. The direct damage caused by ROS following the application of herbicides that function as photosystem I and II inhibitors (Fujii et al., 1990; Fuerst and Norman, 1991) is well known. In studies of other types of herbicides, this phenomenon is less familiar, but over time, as in almost any other stress response, ROS will form. The significant reduction in ROS content 24 HAT under the combination of herbicide and high-temperature, as compared with all other treatments, may indicate more efficient metabolic activity of ROS scavengers at high-temperature.

Recent studies have suggested a new approach for understanding ROS activity, as ROS have been found to act as beneficial elements in the context of many physiological functions, such as programmed cell death and cellular proliferation (Mittler, 2016). In previous studies, ROS were shown to have an effect on the expression pattern of CYP450 enzymes, inducing the expression of CYP81D8 in *Arabidopsis* (Desikan et al., 2001). Over-expression of additional plant defense-related genes such as GSTs [involved in antioxidant stress, lipid peroxidation (Cummins et al., 2013), detoxification of ACCase inhibitors (Edwards and Cole, 1996; Cummins et al., 1999)], and MBF, which is involved in transcriptional co-activation of ROS signaling (Miller et al., 2008), were also detected the combination of herbicide and high-temperature treatment. Herbicide metabolism processes can also be associated with several chaperoning components related to regulatory elements and activating genes. Several HSP related DEGs, mainly HSP20 (Bradi3g58590 and Bradi1g44230), were highly expressed in response to high-temperature and combination of herbicide and high-temperature (Supplementary Table S5). It can be hypothesized that this high expression is part of a general stress response (Waters, 2013), yet variation in the level of expression can suggest different activation rate of downstream resistance related components. High expression of regulatory element such as NAC related genes (Bradi1g63600 and Bradi4g44000), involved in response to biotic and abiotic stress responses (Puranik et al., 2012), was also found. Activators such as protein kinase (Bradi1g65930) and Calcium-binding (Bradi1g53830 and Bradi4g30100) related DEGs (Supplementary Table S5) were highly expressed under herbicide application. These genes can be correlated to the function of peroxidases and other enzymes related to herbicide detoxification processes (Kim Y.S. et al., 2013; Alberto et al., 2016). Our results suggest an orchestrated regulation, at the transcriptional and biochemical levels that facilitates temperature-dependent pinoxaden resistance, which is activated upon application of pinoxaden at high-temperature. These factors encourage a rapid and efficient pinoxaden detoxification that eventually results in plant survival.

Monooxygenation activity of CYP72A genes has been widely discussed in the context of herbicide resistance (Prall et al., 2016). However, only plants subjected to high-temperature survived pinoxaden treatment. Thus, the expression of CYP450 genes may not be related to their monooxygenation activity. On the other hand, temperature has been shown to play a key role in the enzymatic efficiency of CYP450 (Puntarulo and Cederbaum, 1989; Puchkaev et al., 2002). These findings help to explain the differences in plant survival between herbicide application and the combination of herbicide and high-temperature, as pinoxaden was rapidly metabolized only under high-temperature.

CONCLUSION

Our previous (Matzrafi et al., 2016) and current results on temperature-dependent herbicide resistance have emphasized the evolutionary effect of climate change on herbicide resistance and, by extension, global agriculture. We propose that temperature-dependent pinoxaden resistance may be affected more by enzymatic efficiency than by gene regulation. It is suggested that the over-expression of metabolic-related genes (CYP72A, GST and GT) is not sufficient for effective herbicide metabolism, as was previously suggested. While previous studies, quantified levels of gene expression based on comparisons between sensitive and resistant populations (i.e., different genetic background), here we used sensitive and resistant plants with the same genetic background (Accession BrI-782). It could be argued that the differences found in the current study were not detected in previous studies, due to the differences in genetic backgrounds. Thus, our strategy enabled to improve our understanding on NTS resistance mechanism under climate change. Exploring the mechanism of temperature-dependent resistance in various weed species may reveal further hub genes and up-stream regulators that affect NTS resistance.

AUTHOR CONTRIBUTIONS

MM designed and conducted experiments. MM and LS-M analyzed the data and drafted the paper. BR and ZP designed experiments and wrote the paper. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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Different Mutations Endowing Resistance to Acetyl-CoA Carboxylase Inhibitors Results in Changes in Ecological Fitness of *Lolium rigidum* Populations

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Various mutations altering the herbicide target site (TS), can lead to structural modifications that decrease binding efficiency and results in herbicide resistant weed. In most cases, such a mutation will be associated with ecological fitness penalty under herbicide free environmental conditions. Here we describe the effect of various mutations, endowing resistance to acetyl-CoA carboxylase (ACCase) inhibitors, on the ecological fitness penalty of *Lolium rigidum* populations. The TS resistant populations, MH (substitution of isoleucine 1781 to leucine) and NO (cysteine 2088 to arginine), were examined and compared to a sensitive population (AL). Grain weight (GW) characterization of individual plants from both MH and NO populations, showed that resistant individuals had significantly lower GW compared with sensitive ones. Under high temperatures, both TS resistant populations exhibited lower germination rate as compared with the sensitive (AL) population. Likewise, early vigor of plants from both TS resistant populations was significantly lower than the one measured in plants of the sensitive population. Under crop-weed intra-species competition, we found an opposite trend in the response of plants from different populations. Relatively to inter-population competition conditions, plants of MH population were less affected and presented higher reproduction abilities compared to plants from both AL and NO populations. On the basis of our results, a non-chemical approach can be taken to favor the sensitive individuals, eventually leading to a decline in resistant individuals in the population.

Keywords: ACCase inhibitors, competition, germination, *Lolium rigidum*, ecological fitness penalty, target-site resistance

INTRODUCTION

Major parts of cultivated land in the world (~70%) are occupied by cereal crop-plants such as bread wheat (*Triticum aestivum*), corn (*Zea maize*), and rice (*Oryza sativa*) (FAOSTAT, 2016). Rigid ryegrass (*Lolium rigidum* Gaud.) is among the worldwide most noxious grass weed species infesting winter cereal-crops worldwide (Heap, 2017). For example, field studies in Australia have shown that *L. rigidum* can cause yield reductions of more than 40% (Pannell et al., 2004). Chemical

control is the most cost efficient method to reduce the yield losses associated with weeds infestation. However, in recent years, increasing abundance of herbicide-resistant weed populations endanger food security for the ever-increasing world population. Herbicide resistance of *Lolium* species has been reported in various habitats; agricultural fields, orchards, vineyards, road sides, and more (Heap, 2017). Notably, *L. rigidum* was found to be the species that developed resistant to the highest number of different modes of action (MOA; Heap, 2017), which can be consequence of the obligate outcrossing nature of this specie (Mccraw et al., 1983).

Fatty acid biosynthesis is a crucial stage in the formation of different organelles, waxes and other secondary metabolites (Harwood, 1988). Acetyl CoA carboxylase (ACCase) is a key enzyme in the first step of fatty acids synthesis, it catalysis the formation of malonyl-CoA from acetyl-CoA (Roesler et al., 1997). Two isoforms of ACCase, heteromeric and homomeric, are present in plants. While most plants have both forms in different cell compartments (cytosol and plastids), in the Gramineae species, only the homomeric form of the enzyme is present in both compartments (Sasaki and Nagano, 2004). This fact facilitated the creation of this unique group of selective and highly efficient ACCase inhibitors targeted to damage only grass weeds. Three different chemical groups are classified as ACCase inhibitors: aryloxyphenoxypropionates (AOPP), cyclohexanediones (CHD), and phenylpyrazoline (PPZ) (HRAC, 2017). Their mechanism of action, blocking only the *in vivo* activity of the plastidic homomeric ACCase form, exist in grass weeds only (Sasaki and Nagano, 2004).

Herbicide resistance has two sub categories of target site (TS) and non-target site (NTS) resistance. TS resistance is the result of structural modification, or over-expression of a specific gene encoding for the target protein (Délye et al., 2013a). NTS resistance can occur due to reduced translocation (González-Torralva et al., 2012), modification in subcellular distribution (Kleinman and Rubin, 2016), herbicide detoxification (Matzrafi et al., 2016) or other mechanisms (reviewed by Délye et al., 2013a). To date, seven different substitution were reported in the ACCase gene sequence, which result in various structural modifications reducing the binding efficiency of the herbicide molecule reviewed by Kaundun, 2014).

Fitness penalty associated with herbicide resistance was previously demonstrated in various weed species for both TS (Menchari et al., 2008; Shergill et al., 2016; Frenkel et al., 2017) and NTS (Vila-Aiub et al., 2005a,b) resistance mechanisms. In most cases, TS resistance to ACCase inhibitors via alteration of the ACCase enzyme will inevitably lead to a penalty in plant performance. Changes in morpho-physiological traits such as seed germination rate (Vila-Aiub et al., 2005b), biomass production (Menchari et al., 2008) and reproductive abilities (Papapanagiotou et al., 2015), were reported in association with TS resistance to ACCase inhibitors. The level of fitness penalty can be affected by different mutations and plant species. A substitution of isoleucine 2041 to asparagine in the *Hordeum glaucum* ACCase gene, resulted in reduced vegetative biomass and seed production, while substitution of isoleucine 1781 to leucine/valine did not carry any fitness penalty (Shergill et al., 2016). On the other hand, in *Setaria viridis* alteration in position 1781 led to higher fitness in the resistant (i.e., mutant) plant compared to the wild type (Wang et al., 2010).

While the level of herbicide resistant associated with TS mutations is not affected by environmental conditions (Vila-Aiub et al., 2009), we hypothesize that environmental conditions will have a major effect on the developmental and reproductive performances of TS resistance plants, under herbicide-free conditions. Here we characterized the differences in ecological fitness penalty caused by TS mutations (isoleucine 1781 to leucine and cysteine 2088 to arginine) in *L. rigidum* populations. Our specific aims were to: (**i**) define the effect of different mutations in the ACCase gene sequence on grain features, (**ii**) test the effect of environmental conditions on ecological fitness penalty, and (**iii**) characterize the competition ability of each population.

MATERIALS AND METHODS

Plant Material

Seeds of the sensitive (Alumim, AL) *L. rigidum* population were collected from an organic wheat field where no herbicides were applied. Seeds of two TS resistant *L. rigidum* populations, Ma'oz Haim (MH, substitution of isoleucine 1781 to leucine) and Nahal Oz (NO, substitution of cysteine 2088 to arginine), were collected follow failures of ACCase inhibitors to control *L. rigidum* plants: clodinafop-propargyl in wheat field and clethodim in a carrot field, respectively. In each field, seeds from 20 to 30 random mature plants were collected into a paper bag and termed as "population". The seeds were separated, air-dried and stored at 4°C until used.

Seeds from each population were germinated in trays filled with a commercial growth mixture (Tuff Merom Golan, Israel). Trays were placed in a controlled growth room (16°C) to break the seeds' dormancy, and at two-leaf stage, seedling were transplanted into plastic pots (7 cm × 7 cm × 6 cm, one plant per pot) containing the same growth mixture. Plants were placed in a controlled greenhouse (18/25°C night / day) and watered as needed. TS-resistant plants from MH and NO populations were selected three times with the same herbicide that was used in the field (clodinafop-propargyl and clethodim, respectively). Individual plants that survived herbicide application (5–6) from each population were grown in cages covered with air breathing nylon screens to prevent foreign pollination. Plants of the sensitive population (AL) were grown under the same conditions without herbicide application. Seeds of all three populations were harvested, air-dried and stored at 4°C as described above. The same germination procedure was used for all experiments.

Response of *L. rigidum* Populations to ACCase Inhibitors

Three herbicides, representing the three chemical groups of the ACCase inhibitors were used for this study, when X = the recommended rate. Aryloxyphenoxypropionate (Fop) – diclofop-methyl (Iloxan®, 360 g L^{-1} EC, Bayer, Germany; X = 720 g a.i. ha^{-1}), phenylpyrazole (Den) – pinoxaden (Axial®, 45 g L^{-1} + cloquinotocet-mexyl 11.25 g L^{-1} EC, Syngenta,

Switzerland; X = 30 g a.i. ha^{-1}) and cyclohexandione (Dim) – cycloxydim (Focus®, 100 g L^{-1} EC, BASF; Germany, X = 100 g a.i. ha^{-1}), were used. Plants (3–4 leaves stage) sprayed with increasing rates (0, 0.25X, 0.5X, X, 2X, 4X, and 8X) of the three herbicides, in order to quantify the level of resistance under controlled conditions. Herbicides applied using a chain-driven sprayer delivering 300 L ha^{-1}. Plant shoot fresh weight (FW) was recorded 21 days after treatment (DAT).

Target Site Resistance Validation

Fresh leaf tissue (∼200 mg) of plants from both MH and NO populations that survived herbicide application, and from sensitive control plants of AL population, were sampled and kept under –80°C until used. DNA extracted using the Puregene DNA isolation kit (Gentra, MN, United States) according to the manufacturer's instructions. For sequence analysis, primers were designed using known sequence of *L. rigidum* (DQ184640.1), as described previously (Matzrafi et al., 2014 and Supplementary Table S1). Specific regions in the ACCase gene sequence were amplified and PCR products were sequenced to locate the common point mutations that might endow TS resistance. Sequence analyses and alignment were performed using Bioedit software (Hall, 1999). The obtained sequences were compared to the known ACCase gene sequence of *Alopecurus myosuroides* (AJ3107671).

Grain Characterization

Sixty grains from TS resistant (MH and NO) and sensitive (AL) *L. rigidum* populations were weighed on a microbalance (M2P, Sartorius, Göttingen, Germany) to obtain grain weight (GW). The same 60 grains from each population were photographed using a binocular (SZX16, Olympus, Tokyo, Japan) and analyzed to obtain grain area parameters. Image analysis and processing were performed using the Matlab software (MathWorks, Natick, Massachusetts, United States) and the public-domain software ImageJ[1] (NIH). Pictures were converted into gray scale, and mean gray value (MGV) was calculated from the average gray scale value of pixels in the selected area using equation 1:

$$\mathrm{MGV} = 0.2989 \times \mathrm{R} + 0.5870 \times \mathrm{G} + 0.114 \times \mathrm{B} \quad (1)$$

where R, G, and B stands for the three spectral regions: red, green, and blue, respectively. Based on the MGV, a threshold was selected to include all grain pixels (MGV > 0.13), and minimum size of 5000 pixels was defined to create non-disturbed analysis. Suitable pixels gained the value of 1 and transformed into a binary picture as white pixels. The ratio between the lengths of each pixel/micron was calculated using ImageJ software so that the data on white pixels in the picture were converted into grain area according to equation 2:

$$16\mu\mathrm{m}^2 = 0.25\,\mathrm{pixel}_{(\mathrm{length})}/1\mu\mathrm{m} \rightarrow \mathrm{pixel}_{\mathrm{area}} \quad (2)$$

Follow the grains weight analysis, all 180 seeds (60 from each population) were sown, one per pot (7 cm × 7 cm × 6 cm), and placed in a greenhouse (19/25°C, night/day). At 3–4 leaves stage, plants were sprayed with diclofop-methyl (2X). Survival rate (plants that produced new leaves) was recorded 21 DAT.

[1]http://rsb.info.nih.gov/ij

Characterization of Seed Emergence Rate

Five seeds from each population were placed into one pot (9 cm × 9 cm × 10 cm, filled with growth mixture) and covered with a thin layer of the same mixture (0.25 cm). Pots were placed in a controlled dark growth chamber under two temperature regimes: control conditions (10/16°C night/day) and high temperature (19/25°C). Three replicates were used for each population × temperature combination. Seedling emergence (appearance of the coleoptile) recorded daily over 14 days.

Characterization of Inter-Population and Intra-species Competition

Seedlings of the sensitive (AL) and resistant (MH and NO) *L. rigidum* populations, and bread wheat *cv*. Zahir (Hazera, Israel) were transplanted into 4.3 L pot (25cm × 15cm × 11.5cm) filled with a mixture of 80% brown–red degrading sandy soil (Rhodoxeralf; composed of 76% sand, 8% silt and 16% clay) and 20% of growth mixture. Each population was arranged either alone (i.e., inter-population competition) or together with wheat (i.e., intra-species competition), with total 28 plants per pot (to mimic field density of 746 plants/m^2), three replications for each combination. Plants were grown in a controlled greenhouse (16/25°C night/day) under short day (10 h) conditions.

Pots were photographed with an RGB digital camera (Canon PowerShot SX20 IS, Canon, Melville, NY, United States), in an overhead view (53 cm from the camera lens to the surface of the box). Plants were photographed over 35 days in intervals of 7 days. Images were analyzed to estimate vigor differences between different populations using the Matlab software. In any 8-bit JPEG image setting of different color components of individual pixel was done using the values obtained from each of the three color channels; red (R), green (G), and blue (B). To quantify the vegetation area cover, we cropped the image to the pot size and created a binary matrix in the same size. Pixels that had the highest value in the green channel (G > R and G > B) were assigned to the array 1 in the binary matrix, others (G < B or G < R) were assigned as 0. The proportion of the vegetation area in the pot was calculated as sum of 1 array in the matrix divided by the size of the matrix as described in equation 3:

$$\%\,\mathrm{Foliage\ cover} = \frac{\text{black pixels in selected area}}{\text{total selected area}} \times 100 \quad (3)$$

Plants from both competition categories were harvested 64 days after transplanting, the numbers of tillers and spikes were recorded and all aboveground biomass was oven-dried (80°C for 48 h) and then weighed. Since each population had different plant architecture, in order to be able to compare between populations under the intra-species competition, values were calculated relatively to inter-population competition standards.

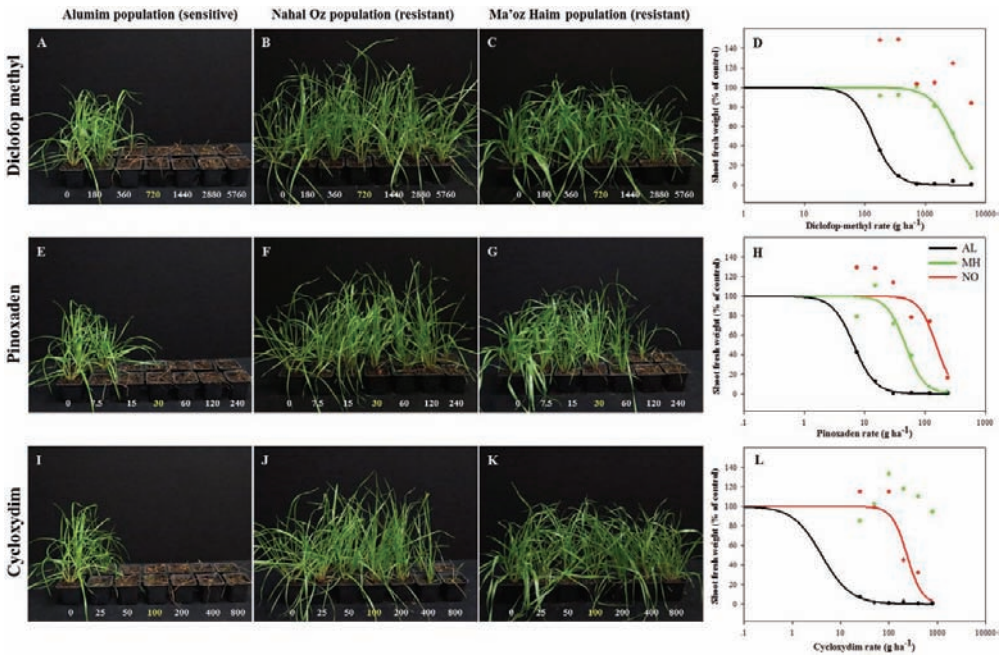

FIGURE 1 | Effect of application of increasing rates of different ACCase inhibitors: diclofop-methyl **(A–D)**, pinoxaden **(E–H)**, and cycloxydim **(I–L)**, on the survival rate and shoot fresh weights of plants from sensitive (AL, black) and target-site resistant (MH, green and NO, red) *Lolium rigidum* populations.

Statistical Analyses

The JMP Pro 13 (SAS Institute Inc., Cary, NC, United States) was used for all statistical analyses. Differences between treatments were examined using different tests as specified in each experiment. Analysis of variance (ANOVA) performed to examine the effect of single variants and the interactions between different treatments. Dose-response curves were constructed by plotting the shoot DW data 21 DAT, from the different populations as a percentage of untreated control (UTC). All the data including, seed emergence, foliage cover, GW, and area, were analyzed using SigmaPlot (ver. 10) software (Systat Software Inc., San Jose, CA, United States). A nonlinear curve model [sigmoidal logistic, three parameters; Seefeldt et al., 1995] was adjusted to analyze the effects of the tested herbicides in the different experiments, as described in Equation 4:

$$Y = \frac{a}{1 + (\frac{x}{x_0})^b} \quad (4)$$

In the model, if $b > 0$, then a describes the upper limit of Y. $X_0 = ED_{50}$ and b describes the slope of the curve in ED_{50}. The resistance index (RI) was calculated as the ratio of the ED_{50} value of the resistant accession to the ED_{50} of the sensitive one.

RESULTS

Herbicide Resistance in *Lolium rigidum* Populations

Individuals from all three *L. rigidum* populations were analyzed for their response to herbicides from three chemical classes of ACCase inhibitors: diclofop-methyl, pinoxaden, and cycloxydim. Plants of the sensitive population (AL) did not survive treatments with more than one quarter of the recommended rate of all three herbicides (diclofop methyl = 180, Pinoxaden = 7.5, and cycloxydim = 25 a.i. ha^{-1}) (**Figure 1**). Plants from both MH and NO populations showed high survival rates in response to increasing rates of diclofop-methyl up to 8X (**Figures 1B–D**). Additionally, plants from NO population did not show 50% decrease in shoot FW and ED_{50} value could not be extracted under diclofop-methyl treatment (**Figure 1D** and Supplementary Table S2). The same trend (no ED_{50}) was shown in the response of plants from MH population to cycloxydim (**Figure 1L** and Supplementary Table S2). Under pinoxaden treatment, plants from MH and NO populations showed high RI values (7.14 and 23.1, respectively; **Figures 1E–H** and Supplementary Table S2).

DNA sequencing of specific sections in the ACCase gene showed substitutions in both heterozygote and homozygote forms in plants from MH and NO populations. MH plants showed substitution of isoleucine 1781 to leucine, and NO plants had substitution of cysteine 2088 to arginine (Supplementary Figure S1).

Grain Characterization

A correlation between fitness penalties and grain characteristics have been reported for various plant species (e.g., Wang et al., 2010; Shergill et al., 2016). Comparison of grains from each population revealed high differences among grains of TS populations compared to grains of the sensitive one. GW and surface area of individuals from MH population was significantly lower compared to grains of AL population (1.79 mg and 4.55

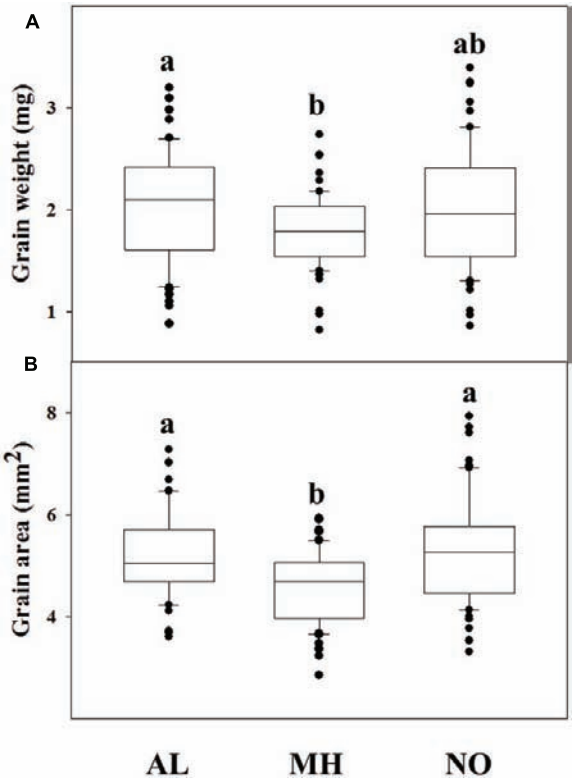

FIGURE 2 | Grain parameters of 60 individuals from different *Lolium rigidum* populations. **(A)** grain weight and **(B)** grain area in of the sensitive (AL) and target-site resistant (MH and NO) *L. rigidum* populations. Different letters indicate significant differences between populations by Tukey-HSD ($P \leq 0.05$).

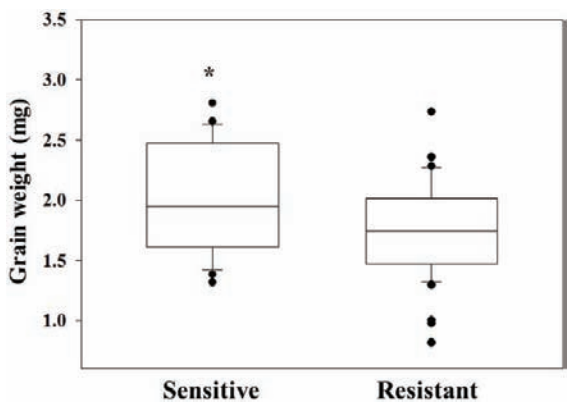

FIGURE 3 | Grain weight of herbicide sensitive (AL) and target-site resistant (MH and NO) *Lolium rigidum* populations. Data is mean [sensitive (n = 22) and resistant (n = 45)]. *indicate significant differences between the two groups using Student t-test at $P \leq 0.05$.

mm^2 *vs.* 2.05 mg and 5.19 mm^2, respectively; **Figures 2A,B**). The NO population grains showed lower values, but not significant, in measured GW (1.99 mg) and area (5.28 mm^2), compared to grains of AL population (**Figures 2A,B** and Supplementary Table S3).

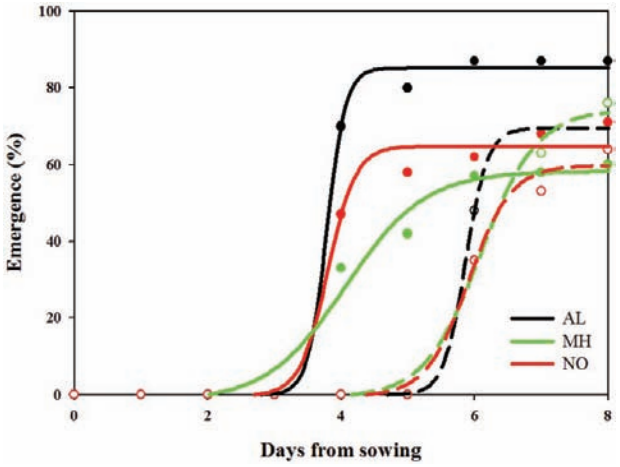

FIGURE 4 | Effect of temperature on seedling emergence rate of sensitive (AL, black) and target-site resistant (MH, green and NO, red) *Lolium rigidum* populations. Temperature regimes: control 10/16°C (open circles, dash line) and high temperature treatment 19/25°C (close circles, full line).

The same grains were germinated in pots and sprayed at 3–4 leaf stage with 2X of diclofop-methyl. Twenty-one DAT plant survival rate and FW were recorded. Grain of plants that were found to be resistant to diclofop-methyl showed significantly lower GW than what was measured in sensitive plants (1.75 mg *vs.* 2 mg, respectively, **Figure 3** and Supplementary Table S2). Notably, only 38.09% of the individuals in the NO population were diclofop-methyl resistant as compared to 94.74% in the MH population (Supplementary Table S4).

Seedling Emergence Rate

In general, seedling emergence in all *L. rigidum* populations started earlier under high (19/25°C) compared to control (10/16°C) temperature regimes (**Figure 4** and Supplementary Table S5). Under high temperatures, seedlings from both AL, and NO populations emerged faster and at higher percentage as compared to seedlings from MH population. Emergence of seeds from all three populations under the favorable temperature regime (control) was similar (**Figure 4** and Supplementary Table S5).

Effect of Inter- and Intra- Species Competition of Ecological Fitness Penalty

TS resistance to various herbicide MOAs had been reported to be associated with reduction in competition abilities, which can affect plants ecological fitness (e.g., Chun et al., 2013; Shergill et al., 2016). Here, plants from TS-resistant and -sensitive populations of *L. rigidum* were tested for their inter-population and intra-species (with wheat) competition abilities. Plants of AL population grown under inter-population competition showed higher foliage cover from the second week of the experiment, eventually leading to significant differences in coverage comparing to plants of both MH and NO populations

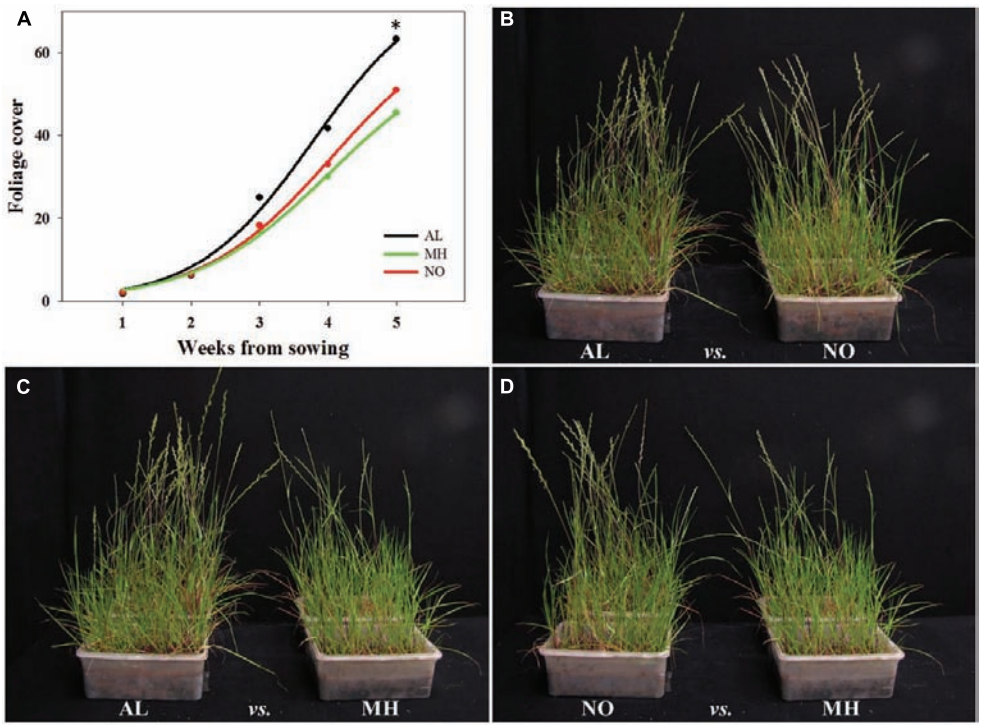

FIGURE 5 | Visual assessment of inter-population competition between sensitive (AL) and target-site resistant (MH and NO) *Lolium rigidum* populations, in an herbicide-free environment. **(A)** Biomass production of sensitive (AL, black) and target-site resistant (MH, green and NO, red) *L. rigidum* populations, estimated every week (total five weeks) by image analysis and presented as foliage cover. Photographs showing the inter-population competition between *L. rigidum* populations: **(B)** AL *vs.* NO, **(C)** AL *vs.* MH and **(D)** NO *vs.* MH. *indicate significant difference between populations at $P \leq 0.05$.

(63.26 cm^2 $plant^{-1}$ *vs.* 45.46 cm^2 $plant^{-1}$and 50.99 cm^2 $plant^{-1}$, respectively; **Figure 5A** and Supplementary Table S6).

Under inter-population competition, plants from MH population showed reduced tillers numbers (2.26 *vs.* 3.30 and 3.05 tillers $plant^{-1}$ for AL and NO plants, respectively) and vegetative aboveground growth (10.33 g $plant^{-1}$ *vs.* 16.42 g $plant^{-1}$ and 11.92 g $plant^{-1}$ for AL and NO plants, respectively; **Table 1** and **Figures 5B–D**). Plants from MH population also presented significantly lower productivity with less spikes (0.6 $plant^{-1}$) compared to plants from AL (1.6 $plant^{-1}$) and NO (1.5 $plant^{-1}$) populations (**Table 1**). Furthermore, plants of MH population were shorter (34.9 cm) compared to individuals from both AL and NO (44.0 and 41.5 cm, respectively). Plants from the sensitive population of AL ranked the highest score in each of the morpho-physiological parameters under inter-population competition (**Table 1**).

Under intra-species competition of *L. rigidum* and wheat plants, the values of different parameters were calculated as a ratio relative to the inter-population competition. Plants of MH population showed less sensitivity to intra-species competition

TABLE 1 | The effect of inter-population and intra-species competition, on dry weight biomass production, plant height, number of tillers, and number of spikes, on untreated plants of sensitive (AL) and target-site (TS) resistant (MH and NO) *Lolium rigidum* populations.

Population	Dry weight, g $plant^{-1}$	Plant height, cm	No. of tillers, $plant^{-1}$	No. of spikes, $plant^{-1}$
Inter-population				
AL	16.4 ± 1.9a	44.0 ± 3.7a	3.3 ± 0.1a	1.6 ± 0.3a
NO	11.9 ± 2.2a	41.4 ± 3.8a	3.0 ± 0.2a	1.5 ± 0.2a
MH	10.3 ± 1.1a	34.9 ± 3.4a	2.3 ± 0.1b	0.6 ± 0.1b
Intra-species (relative value[1])				
AL	0.06 ± 0.1a	1.0 ± 0.1a	0.9 ± 0.1a	0.8 ± 0.1b
NO	0.11 ± 0.2a	1.1 ± 0.1a	0.8 ± 0.1a	1.0 ± 0.2b
MH	0.05 ± 0.1a	1.2 ± 0.1a	1.0 ± 0.1a	1.6 ± 0.09a

Different letters indicate significant differences ($P \leq 0.05$) between plants from three Lolium rigidum populations by Tukey LSD test.[1]*Intra-species competition calculated as a relative value in comparison to the average value that was recorded for different parameters in the inter-population competition.*

than plants of AL and NO populations in most parameters (**Table 1**). This advantage was strongly pronounced in differences found in the number of spikes, were plants of MH population showed a significant increase (1.57) in productivity compared to plants from both AL and No population (0.79 and 0.98, respectively; **Table 1**).

DISCUSSION

ACCase inhibitors have been used in modern agriculture since the 1970s of the previous century and played important role in selective control of grass weeds (Kaundun, 2014). Selection pressure caused by repeated- and/or mis-application unravel different TS and NTS resistance ecotypes (Délye et al., 2013a), and can eventually lead to the evolution of resistant individuals in a field population (Rubin, 1991). Moreover, obligate outcrossing grass weed species, such as *A. myosuroides* and *Lolium* species, contributes to the distribution of these resistance traits (Mccraw et al., 1983; Yang et al., 2008). TS resistance to ACCase inhibitors has been reported to be associated with fitness penalty (Vila-Aiub et al., 2005b; Menchari et al., 2008; Kukorelli et al., 2013). Here we used a comprehensive approach to characterize the effect of different mutations in the ACCase gene on the ecological fitness of resistant *Lolium* species populations throughout the plants' life cycle.

Examining of grains from *Lolium* populations with different herbicide response, individuals of the sensitive AL population showed the highest GW compared to ones from both MH and NO populations (**Figure 2**). Moreover, the same trend of lower GW was found among sensitive and resistant plants from the same TS resistant population. Notably, Sabet Zangeneh et al. (2016) reported that TS resistance *L. rigidum* populations (Ile 1781 to Leu and Ile 2041 to Asn) had no significant differences in thousand seeds' weight compared with a sensitive population. Seed weight can affect the level of dormancy, germination rate and early seedling establishment. Under agro-ecosystems, species with larger seed weight will have advantage coping with adverse establishment conditions such as burial, competition, low soil moisture, and nutrients imbalance (e.g., Leishman, 2000; Van Etten et al., 2016). In agreement, the sensitive population (AL), with greater seed weight showed faster seed emergence under both temperature regimes compared to both TS resistant populations (Supplementary Table S5). Smaller grains size of resistant plants can also explain the decrease in their inter-population competitions abilities (**Figure 3**), as was also reported previously (Van Etten et al., 2016).

Analysis of the association between seed weight and herbicide response in the two TS resistant populations showed that while most (94.7%) of plants from MH population were resistant, only 38.1% of plant from the NO population were recorded as such (Supplementary Table S4). Interestingly, all individuals of NO population that were treated with diclofop-methyl in the dose response experiments had survived the treatment. Furthermore, compared with the MH population, higher resistance was found in NO population at the maximum rate of 8X (**Figures 1B–D** and Supplementary Table S2). These results suggest that the NO population is in transition phase compared to MH population that is more uniform in its herbicide response at the population level.

Weed competitiveness is associated with resource capture, for example light interception that is considered major limiting factor for weed growth under conventional cropping systems (Holt, 1995). Environmental factors such as: radiation, temperature and competition were previously shown to affect seed dormancy (Sbatella and Wilson, 2010), grain yield production (Frenkel et al., 2017) and fertility (Gramshaw, 1972; Pedersen et al., 2007). Plants' canopy height has been suggested as good indicator of light interception, especially for grasses (Cudney et al., 1991; Seavers and Wright, 1999). Plants from MH and NO populations showed reduce plant height, lower biomass production and reproductively compared to plants of AL population (**Table 1**). This trend is further emphasized by the observed accelerated vegetative development and number of tillers/spikes of AL plants compared to plant of TS-resistant populations (**Figure 5C** and **Table 1**).

The sensitive population (AL) exhibited higher abilities compared to the TS resistant MH population, in term of seedling vigor, plant height, biomass and reproductive capacities (**Table 1**). Interestingly, these advantages were reduced under changing competition and temperature conditions, suggesting high phenotypic plasticity as consequence of genotype × environment interactions. The TS resistant population of MH showed less phenotypic plasticity under changing environmental conditions. It can be proposed that narrow genetic variation, caused by 94% resistant individuals, enables less plasticity in the response to environmental changes. Under competition with wheat plants, reproductive abilities of MH plants were less affected compared to both AL and NO plants (**Table 1**). This specific population has been subjected to a strong selection for ACCase inhibitors alongside competition with wheat plants. It may have facilitated the evolution of a dual advantage, herbicide resistance/crop-weed competition abilities. Plants competition play a key role in weed community, mainly due to limited resources (Navas, 2012). The ability of a plant to grow and survive in response to resource depletion due to competition with neighboring plants is pronounced by its competitive abilities.

Normally, ACCase inhibitors are applied in the beginning of the growing season at a critical period for weed control (Knezevic et al., 2002). Our results suggests, that the sensitive plants that emerge earlier under high temperatures are exposed and controlled by the herbicide, whereas resistant individuals will germinate later in season, and thus, will eventually escape herbicide application. This can be further exacerbated under the projected climate change scenarios, which predict an aggravation in the intensity and frequency of extreme events, such as temperature fluctuations (Easterling et al., 2000). Environmental factors such as temperature and water availability are highly important factors in seed germination, both rate and percentage (Alvarado and Bradford, 2002). Membrane composition was found to play a key role in the response of seeds to environmental cues which influence the germination processes (Nishida and Murata, 1996). The activity of the ACCase enzyme play a crucial role in fatty acid biosynthesis thus on lipid content, membrane

structure, and other essential cell components (Harwood, 1988; Price et al., 2003). It can be suggested that the differences found between sensitive and TS-resistant population in seedling ability to emerge is associated with changes in fatty acids composition derived by the altered ACCase enzyme activity. In previous studies, high lipid content was shown to be correlated with higher and faster germination rate in several weed species (Gardarin et al., 2011). It was also suggested that seeds with higher soluble content, such as higher lipid content, can have altered water adsorbent ability than other seeds and will eventually germinate faster (Nonogai, 2006). More studies are needed to test the interaction between the ACCase alteration, fatty acid composition and plant ecological fitness.

CONCLUSION

Fitness penalty is inevitable phenomenon associated with TS modifications (Vila-Aiub et al., 2009). Moreover, our results, as well as previous studies (Menchari et al., 2008; Délye et al., 2013b; Jang et al., 2013), demonstrate that different substitutions in the same target gene would not necessarily have the same effect on the level of fitness penalty. In the current study, we show that while under specific environmental conditions the effect of mutation in the target gene on plant fitness can be minimal, under altered environmental conditions the level of fitness penalty can increased. Thus, we suggest that in order to understand the effect of TS resistance on fitness of weed population, plants need to be tested under different environmental conditions and growth stages.

Understanding the interaction between different TS-resistance ecotypes and environmental conditions could serve as a powerful tool for developing improved weed management techniques. Using integrated weed management combining non-chemical and chemical approached that can favor the sensitive ecotypes, a gradual dilution of the resistance individual proportion in the weed population can be achieved.

AUTHOR CONTRIBUTIONS

MM, OG, and ZP designed the experiments. MM and OG conducted the experiment. MM, OG, BR, and ZP analyzed data and wrote the paper. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01078/full#supplementary-material

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Fitness Outcomes Related to Glyphosate Resistance in Kochia (*Kochia scoparia*): What Life History Stage to Examine?

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A fast-spreading weed, kochia (*Kochia scoparia*), has developed resistance to the widely-used herbicide, glyphosate. Understanding the relationship between the occurrence of glyphosate resistance caused by multiple EPSPS gene copies and kochia fitness may suggest a more effective way of controlling kochia. A study was conducted to assess fitness cost of glyphosate resistance compared to susceptibility in kochia populations at different life history stages, that is rate of seed germination, increase in plant height, days to flowering, biomass accumulation at maturity, and fecundity. Six kochia populations from Scott, Finney, Thomas, Phillips, Wallace, and Wichita counties in western Kansas were characterized for resistance to field-use rate of glyphosate and with an *in vivo* shikimate accumulation assay. Seed germination was determined in growth chambers at three constant temperatures (5, 10, and 15 C) while vegetative growth and fecundity responses were evaluated in a field study using a target-neighborhood competition design in 2014 and 2015. One target plant from each of the six kochia populations was surrounded by neighboring kochia densities equivalent to 10 (low), 35 (moderate), or 70 (high) kochia plants m^{-2}. In 2015, neighboring corn densities equivalent to 10 and 35 plants m^{-2} were also evaluated. Treatments were arranged in a randomized complete block design with at least 7 replications. Three kochia populations were classified as glyphosate-resistant (GR) [Scott (SC-R), Finney (FN-R), and Thomas (TH-R)] and three populations were classified as glyphosate-susceptible (GS) [Phillips (PH-S), Wallace (WA-S) and Wichita (WI-S)]. Of the life history stages measured, fitness differences between the GR and GS kochia populations were consistently found in their germination characteristics. The GR kochia showed reduced seed longevity, slower germination rate, and less total germination than the GS kochia. In the field, increases in plant height, biomass accumulation, and fecundity were not clearly different between GR and GS kochia populations (irrespective of neighbor density). Hence, weed management plans should integrate practices that take advantage of the relatively poor germination characteristics of GR kochia. This study suggests that evaluating plant fitness at different life history stages can increase the potential of detecting fitness costs.

Keywords: kochia, ecological fitness, glyphosate resistant, seed germination, vegetative growth and fecundity

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INTRODUCTION

Differences in the relative fitness of herbicide-resistant (HR) and -susceptible (HS) weed genotypes influences the dynamics of mixed HR and HS populations by shifting their proportions over time (Gressel and Segel, 1982). Under field conditions, HR and HS plants interact with each other and with other plant species. The biotype with greater fitness is expected to out-compete the relatively less fit biotype. Since many mutations in plants have deleterious, pleiotropic effects on their likelihood of survival and/or reproduction (Roles and Conner, 2008), it is generally assumed that herbicide resistance mutations will be associated with an initial cost to a plant's fitness (Vila-Aiub et al., 2015). The resource-based allocation theory suggests that there is a trade-off between allocation of resources for plant growth and for plant defense (herbicide resistance) against environmental stress (Herms and Mattson, 1992; Gassmann, 2005).

The assumption that HR plants are less fit than HS plants in the absence of herbicide application is mostly based on early studies of triazine resistance (Vila-Aiub et al., 2015). These studies indicated a marked reduction in the vegetative and reproductive success of triazine-resistant biotypes relative to susceptible biotypes when triazine herbicides were not applied (as reviewed by Jasieniuk et al., 1996). The most common mechanism for triazine resistance, mutation in the quinone B binding domain of D1 protein in PS II, decreases the binding affinity for triazine molecules to the protein (Devine and Shukla, 2000). This mutation reduces efficiency of PS II, which results in reduced plant fitness. Although, fitness cost has been widely detected in triazine-resistant plants, this cannot be generalized for resistance to other herbicide modes of action, such as glyphosate, a 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) inhibitor. A genetic mechanism responsible for glyphosate resistance in kochia has been reported to be amplification of EPSPS gene which translates to overproduction of EPSPS enzyme in plant (Jugulam et al., 2014; Godar et al., 2015), and it is expected that more metabolic energy will be required for enzyme production at the detriment of other plant biological functions or developmental processes compared to normal EPSPS production in a susceptible individual.

Efforts are still being made to identify how evolution of glyphosate resistance in weeds can result in differential fitness, and reports so far have shown that fitness differences between GR and GS cannot be generalized across plant species (Vila-Aiub et al., 2014; Wang et al., 2014; Kumar and Jha, 2015). Reports also suggest that GR plants' fitness varied by mechanism of glyphosate resistance (Preston and Wakelin, 2008; Preston et al., 2009), genetic background of weed species (Giacomini et al., 2014), and environmental stress, such as competition for resources (Davis et al., 2009; Shrestha et al., 2010).

Accurate quantitative estimates of the relative fitness of resistant and susceptible plants in the absence of herbicide have been difficult to obtain (Jasieniuk et al., 1996; van Etten et al., 2016). A more appropriate comparison to estimate differential fitness of resistant and susceptible plants is to compare plants within the same weed population (Pedersen et al., 2007) or use of isogenic lines (Vila-Aiub et al., 2015) to reduce the effect of genetic background on fitness analysis. However, studies have generally compared the fitness of resistant and susceptible plants from very different and geographically separate populations (Jacobs et al., 1988; Stowe and Holt, 1988; Holt, 1990; Thompson et al., 1994; Shrestha et al., 2010; van Etten et al., 2016), and of these, some compared any resistant and only one susceptible population (Mortimer et al., 1992; Marshall et al., 1994; Shrestha et al., 2010). To control confounding effects of plant response to resistance or susceptible traits and genetic background resulting from environmental experience, care must be taken to select populations from the same geographic area or growing conditions (Jasieniuk et al., 1996), and several resistant and susceptible populations should be compared (Cousens et al., 1997; Strauss et al., 2002; van Etten et al., 2016).

Kochia produces protogynous flowers where the stigmas emerge before anther development and this necessitates outcrossing. Thus, cross pollination within and among plants is common in kochia. Repeated use of glyphosate and outcrossing among populations would potentially promote development of glyphosate resistance in kochia populations. Maintaining homogeneity in kochia through inbreeding over generations has been difficult perhaps due to inbreeding depression as observations showed that such seeds either have low viability or improperly formed (Esser, 2014). In this study, multiple kochia populations were collected from fields in an irrigated crop rotation of grain sorghum and glyphosate-resistant (GR) soybean in western Kansas. These populations were compared in a common garden to check for differences in the original environment of these populations. A qPCR study showed that individuals in our GR populations had higher EPSPS gene copy number (the mechanism responsible for glyphosate resistance) while the GS populations had normal EPSPS gene copy numbers (Osipitan, 2016).

Measuring fitness throughout a plant's life cycle has also been recommended (Vila-Aiub et al., 2015; van Etten et al., 2016). Examining a single life history stage may not be sufficient to identify differential fitness for resistance traits (Pedersen et al., 2007; Lamego et al., 2011; Lehnhoff et al., 2013). Subjecting each of these life history stages to either abiotic or biotic environmental stress has helped identify fitness differences between HR and HS weed biotypes. For example, the influence of temperature on rate of weed seed germination has been widely explored to explain the differences between HR and HS weed biotypes (Thompson et al., 1994; Park et al., 2004; Vila-Aiub et al., 2005; Elahifard and Mijani, 2014; Tang et al., 2015). Many research studies have also used densities or crowding to subject plant biotypes to competition in order to measure their fitness through vegetative and reproductive responses (Légère et al., 2000; Vila-Aiub et al., 2005; Shrestha et al., 2010; Travlos and Chachalis, 2013). Two main competition designs used to evaluate differential growth and/or fecundity of HR and HS weed populations include target-neighborhood design (Vila-Aiub et al., 2005, 2009a) and replacement series (Légère et al., 2000; Shrestha et al., 2010; Travlos and Chachalis, 2013). The advantage of target-neighborhood over replacement series design is the ability of the former to allow the study of competition effect

or response under varying densities with two or more biotypes being compared.

The objectives of this study were to (1) characterize six kochia populations from western Kansas into glyphosate-resistant (GR) and glyphosate-susceptible (GS) populations and (2) evaluate fitness costs as a result of evolution of glyphosate resistance in these kochia populations at three life history stages: seed germination, plant vegetative growth, and fecundity.

MATERIALS AND METHODS

Six kochia populations were collected from different fields in western Kansas. Kochia seeds were collected from one suspected GR population in Thomas County in 2007 and seeds from five other populations were collected in 2012, including suspected GR populations in Scott and Finney Counties and suspected GS populations from Phillips, Wallace and Wichita Counties. Seeds were harvested from more than 10 mature plants per site and bulked in separate bags for each population. Seeds were stripped off plants and cleaned using an air column separator. Efforts were made to ensure that seeds from the 10 plants for each population were equally bulked. To minimize loss of seed quality, the collected seeds were stored in cold (≤ -5 C).

Characterization of Populations to GR and GS: Glyphosate Discriminatory Dose Assay

Seeds of each kochia population were sown in 28 by 6 by 8 cm flats filled with moisture control potting mix (Miracle Gro, Marysville, OH) and grown in the Department of Agronomy—Weed Science greenhouse at Kansas State University, Manhattan, KS. The greenhouse was maintained at 25/20 C day/night, photoperiod of 15/9 h day/night enhanced with 120 μmol m^{-2} s^{-1} illumination provided by sodium vapor lamps, and about 60% relative humidity. Once 2–3 cm tall, individual seedlings were transplanted into 8.5 by 8.5 by 7 cm plastic pots filled with the same potting mix. Glyphosate (Roundup WeatherMax, Monsanto Company, St. Louis, MO) at a field use rate (1X, 0.84 kg ae ha^{-1}) and double the field use rate (2X, 1.68 kg ae ha^{-1}) along with 2% v/v liquid ammonium sulfate were applied to individual plants when they reached 8–10 cm in height. Glyphosate was applied in a cabinet spray chamber (Research Track Sprayer, De Vries Manufacturing, P.O. Box 184, Hollandale, MN). The spray chamber had a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., P.O. Box 7900, Wheaton, IL) that delivered a spray volume of 168 L ha^{-1} using a pressure of 220 kpa with a speed of 4.8 km h^{-1}. Glyphosate treatments were replicated 10 and 7 times for first and second runs, respectively. There were also five untreated plants for each population as controls in each run. The study was laid out in a completely randomized design on experimental benches in the greenhouse.

Plant survival and injury score were recorded at 4 week after glyphosate treatment. Survival was measured as percentage of living plants compared to total plants sprayed. Plant injury score was on the scale of 1–9, where 1 represented no injury and 9 represented completely injured or dead plants. Injury score response of populations to glyphosate treatment were tested with a one-way ANOVA in R version 3.2.3 (R Core Team, 2015) and Tukey's Honestly Significant Difference ($\alpha = 5\%$) was used to compare injury score means among populations.

Characterization of Populations to GR and GS: Shikimate Accumulation Assay

The six kochia populations were assessed for shikimate accumulation to further validate the characterization of GR and GS populations. In plants, glyphosate inhibits production of the aromatic amino acids (tryptophan, phenylalanine and tyrosine) in the shikimic acid pathway causing a build-up of shikimate-3-phosphate, a substrate of EPSPS and its dephosphorylated state, shikimate (Shaner et al., 2005). Both GR and GS plants are expected to accumulate shikimate after exposure to glyphosate but the levels are much higher in GS plants (Wiersma et al., 2015). A measure of shikimate accumulation was determined following the procedure developed by Shaner et al. (2005) and modified by Godar (2014). Plants of each population were grown in the greenhouse in a similar method and condition described above. Ten representative plants that were 10 cm tall were selected from each population. Four 5-mm leaf disks were collected from two fully-expanded young leaves from each plant. Shikimate accumulation in plants from the six kochia populations were analyzed using one-way ANOVA to test if there was difference among populations and the means were compared using Tukey's Honestly Significant Difference ($\alpha = 5\%$).

Germination Study

Seeds were stored in cold (≤ -5 C) until germination study was conducted in 2015. The TH-R kochia population was not included in the germination study because of seed age difference (collected in 2007) compared with other kochia populations which were of the same age (all collected in 2012). Germination rates of the five populations were evaluated at three constant temperatures of 5, 10, and 15 C in darkened growth chambers. A conducive temperature for kochia germination was 10 C (Everitt et al., 1983). Three petri-dishes (100 by 15 mm) of 30 seeds for each of the five populations were placed into one of the three growth chambers set at 5, 10, or 15 C. Dishes within a chamber were arranged in a completely randomized design with three replications. The dishes contained one filter paper (Whatman No 2) soaked with 3 ml of distilled water and moistened with additional water as needed throughout the study. The temperature in each growth chamber was constantly monitored with an internal thermometer. A seed was considered germinated when the radicle was about 2 mm long. Germination counts were done every 12 h for the first 144 h (6 d) and subsequent germination counts were done every 24 h for a total of 21 d. Germinated seeds were removed after each count. The initial 12 h observation interval was to capture the lag and rapid germination stages, while the 24 h observation interval would capture remaining germination. After the final germination count, un-germinated seeds in the petri dishes were re-wetted 7 days later. The viability of the re-wetted seeds was tested through finger press for hardness after 78 h at 24 C (a modified procedure of Davis et al., 2005). Relatively hard seeds were considered

viable while easily ruptured seeds were considered unviable. Both germinated seeds and hard seeds after wetting accounted for the total viable seeds.

A non-linear three parameter logistic regression model was used to describe germination dynamics fit in R version 3.2.3 (R Core Team, 2015):

$$y = d/[1 + (t/t_o)^b] \quad (1)$$

where y is cumulative germination (%) at time t (h), d is maximum cumulative germination, t_o is time (h) required to reach 50% of maximum cumulative germination, and b is slope of function around t_o. Analysis of variance was conducted to determine the effect of temperature on parameter estimates among populations and parameter estimates were ranked based on Tukey's Honestly Significant Difference ($\alpha = 5\%$) in R V 3.2.3 (R Core Team, 2015).

Vegetative Growth and Fecundity Study

The vegetative and seed production fitness of the six kochia populations were evaluated in a field experiment which was very different from greenhouse or partial field studies (where plants were placed in pots in an open field) used in most ecological fitness experiments (Vila-Aiub et al., 2009a; Shrestha et al., 2010; Wang et al., 2014; Kumar and Jha, 2015). The field experiment was conducted at the Kansas State University Department of Agronomy Ashland Bottoms Experiment Field (39.12577 N 96.6365 W) near Manhattan, KS in 2014 and 2015. The two locations were within 1 km of each other. The soil series of the field was a Wymore, which is a moderately well drained silty clay loam soil formed in loess (Natural Resources Conservation Service - US Department of Agriculture, 2016). In 2014, results of a soil analysis were pH (7.1), nitrogen (17 ppm), phosphorus (26.5 ppm), potassium (339 ppm), organic matter (2.5%), and cation exchange capacity (13.1 meq 100 g^{-1}). In 2015, the soil analysis results were pH (6.2), nitrogen (78.9 ppm), phosphorus (47.1 ppm), potassium (529 ppm), organic matter (2.5%), and cation exchange capacity (13.1 meq 100 g^{-1}).

The density treatment design was a modified form of target-neighborhood method used by Vila-Aiub et al. (2009a). The distribution of target-neighbor plants was illustrated in **Figure 1**. In 2014, one target plant from each of the six kochia populations was surrounded by its own population of neighboring kochia at densities equivalent to 10 (low), 35 (moderate), or 70 (high) plants m^{-2}. The two treatment factors were six kochia populations and three neighbor densities. Treatments were in a randomized complete block design with 10 replications in 2014. Individual plot size was 0.6 m by 0.6 m and spacing between plots was 1 m. Seeds were coated with Laponite RD® gel of a rate of 0.012 g ml^{-1} of water (Colquhoun et al., 2001) to prevent displacement of the shallowly-sown kochia seeds by wind and to create a moist environment for seed germination. A 12 ml syringe was used to place 10 ml of solution containing approximately 10 kochia seeds on the soil surface. After kochia germination, seedlings were thinned to the respective proportions of target plant to neighbor densities. For the 10 and 35 neighbor plants m^{-2} treatment, the distance from target to neighbor was 15 cm while for the 70 neighbors m^{-2} treatment, the distance from target to the immediate neighbor and between neighbors was about 10 cm.

In 2015, five kochia populations were used as targets excluding the WA-S population to allow efficient management of plots because of additional treatments. Four kochia neighbor density treatments were evaluated by adding a no neighbor treatment. A complementary study in 2015 included corn as a neighbor at densities of 0, 10, and 35 corn plants m^{-2} to evaluate effect of crop density on the target plants. Two treatment factors in the kochia-only study were five kochia populations and four kochia neighbor densities while in the corn-neighbor study, two treatments factors were five kochia populations and three corn densities. Both studies were conducted within the same randomized complete block design with seven replications. Individual plot size was 0.6 m by 0.6 m and spacing between plots was 3 m. In 2015, seedlings were grown in the greenhouse and transplanted into the experiment rather than direct seeding. In the greenhouse, kochia seeds were shallowly sown in 25 by 15 by 2.5-cm plastic trays filled with commercial potting mixture (Miracle Gro, Marysville, OH). The trays were placed in a no-hole runoff catchment tray and water was added to the tray for sub-irrigation. The greenhouse was maintained at 25/20 C day/night, 60% relative humidity, and 15/9 h day/night photoperiod, supplemented with 120 μmol m^{-2} s^{-1} illumination provided by sodium vapor lamps. Kochia seedlings of about 5 cm tall were transplanted into the field in their respective target plot. In the case of corn as neighbors, corn seeds were directly sown in the field and the respective kochia seedlings (about 5 cm tall) as target plants were transplanted at corn emergence. For the 10 and 35 plants m^{-2}, the target to neighbor distant was about 15 cm while for the 70 plants m^{-2}, the distance from target to the immediate neighbor and between neighbors was about 10 cm.

The neighbor densities at the commencement and end of each trial were recorded, and the neighbor biomass was collected at the end of the season. Kochia target plant height, stem diameter at base of the plant, and plant canopy width (as a measure of the widest canopy) were collected biweekly starting from 3 weeks after planting in 2014 or at transplanting in 2015. Days to first flowering were recorded, from sowing in field or greenhouse, as a phenological variable. Target plants were harvested at 130 and 120 days after establishment in the field in 2014 and 2015, respectively. Fresh weight of the harvested target plants were recorded, plants placed in oven at 40 C for about 72 h and dry weights recorded. Seeds were collected by stripping them off the plants and cleaned using an air column separator. Total and 1000-seed weight per plant were measured. Total seed number (TS) was calculated for each plant:

$$TS = (SW_T/SW_{1000}) \times 1000 \quad (2)$$

where SW_T is total seed weight (g $plant^{-1}$) and SW_{1000} is weight of 1000 kochia seed (g). Growing degree days (GDD) for each day after sowing of seeds were calculated as recommended by Schwinghamer and Van Acker (2008):

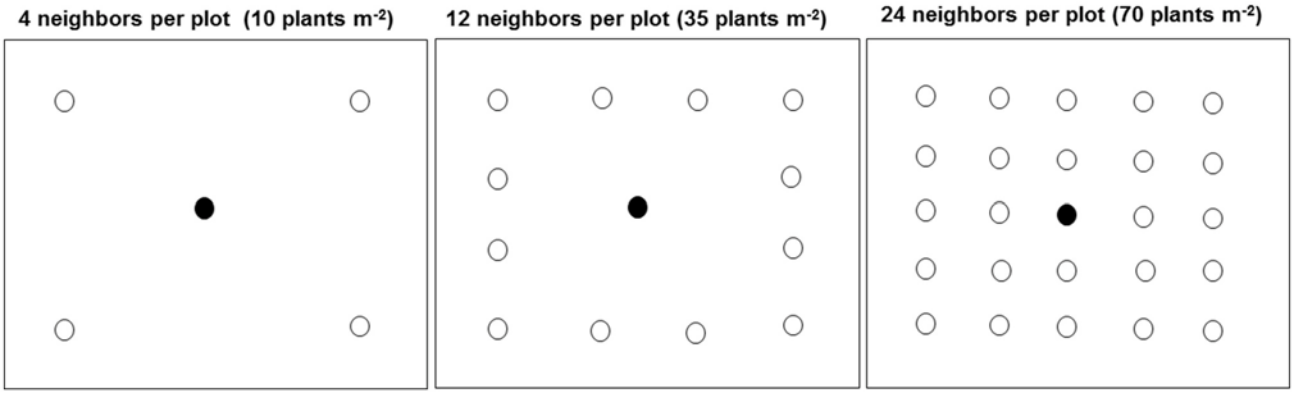

FIGURE 1 | Distribution of target and neighbor kochia plants at different densities in each plot (0.6 by 0.6 m) in the field. The closed and open circles were the target and neighboring plants respectively. For the 10 and 35 plants m^{-2}, the target to neighbor distant was about 15 cm while for the 70 plants m^{-2}, the distance from target to the immediate neighbor and between neighbors was about 10 cm.

$$GDD_{daily} = [(T_{max} + T_{min})/2] - T_{base} \quad (3)$$

$$\text{Cumulative GDD} = \sum_{i=1}^{n} \text{GDDdaily} \quad (4)$$

where T_{max} is the maximum daily air temperature, T_{min} is the minimum daily air temperature, and T_{base} is the base temperature at which plant growth and development was deemed not to occur (0 C); n is the number of days elapsed from sowing date, and GDD_{daily} was a nonnegative value (daily GDD values that were negative were replaced by 0). A base temperature of 0 C seemed reasonable given that kochia has been known to emerge early in the spring (Dille et al., 2017) and 0 C has been used previously as a biologically justifiable base temperature for modeling the germination and emergence of kochia (Schwinghamer and Van Acker, 2008).

Growth of kochia populations over time was modeled using plant height as a function of cumulative GDD with the following three-parameter sigmoid function:

$$H = a/[1 + \exp(-(x - c)/b)] \quad (5)$$

where H is the target plant height (cm) at cumulative GDD (x), while parameter *a* is the final height (cm), parameter *c* is the cumulative GDD required to attain 50% of the maximum height, and parameter *b* is the slope of the curve at the inflection point (and near 50% cumulative GDD). The growth regression curve and analysis were done using SigmaPlot V.12.3 (Systat Software, Inc).

Due to changes in the neighbor density between the commencement and end of the field trial, analysis of covariance (ANCOVA) was used to analyze the effects of the factors (neighbor densities and populations) on the fitness variables where density at the end of the season was considered as the random effect using the generalized linear mixed model GLIMMIX procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC), which did not necessarily assume normal distribution of data, and was found suitable for the collected fitness data.

RESULTS

Characterization of Kochia Populations to GR and GS: Discriminatory Glyphosate Dose Assay

Based on visual assessment of survival at 4 week after glyphosate application, all kochia plants from Scott (SC-R) and Thomas (TH-R) populations survived and 77% of kochia plants from Finney (FN-R) population survived the field use rate of glyphosate (**Table 1**). One out of 17 plants from the Wichita (WI-S) population survived while no plants survived from the Phillips (PH-S) and Wallace (WA-S) populations at the field use rate of glyphosate. The average injury score corresponded to the survival rating for each of these populations (**Table 1**). Thus, it was confirmed that SC-R, FN-R and TH-R populations were GR, while PH-S, WA-S, and WI-S populations were identified as GS in response to the field use rate of glyphosate. When treated with double the field rate of glyphosate, all kochia plants from the three GS populations did not survive, while 12, 30, and 13% of kochia plants from the SC-R, FN-R, and TH-R populations survived, respectively (**Table 1**). This suggests that the resistant populations were still segregating for glyphosate resistance.

Characterization of Kochia Populations to GR and GS: Shikimate Accumulation Assay

The average shikimate accumulation in each GS population (43 ng μL^{-1}) was significantly greater than for each of the GR populations (18 ng μL^{-1}) as shown in **Figure 2**. Generally, at a discriminating dose of 100 μM, it is expected that there will be differential shikimate accumulation between GR and GS individuals (Shaner et al., 2005; Gaines et al., 2010). The use of shikimate accumulation had been previously used as a rapid nondestructive method for characterization of GR and GS kochia biotypes (Jugulam et al., 2014; Godar et al., 2015; Kumar et al., 2015). Overall, these results confirm that three of the kochia populations were truly GR including those from SC, FN, and TH counties, while the other three populations from PH, WI, and

TABLE 1 | Survival (% of total individuals) and injury score at 4 wk after herbicide treatment of six kochia populations from western Kansas.

Herbicide[b]	Mode of action	Rate (kg ha^{-1})	Populations					
			SC-R[a]	FN-R	TH-R	PH-S	WA-S	WI-S
			% Survival					
Glyphosate[c]	EPSPS inhibitor	0.84 ae	100	77	100	0	0	7
		1.68 ae	12	30	13	0	0	0
			Injury score					
Glyphosate	EPSPS inhibitor	0.84 ae	1.1 B[d]	2.0 B	1.0 B	8.9 A	8.9 A	8.8 A
		1.68 ae	6.0 AB	6.6 AB	3.6 B	9.0 A	9.0 A	9.0 A

Injury score was on the scale of 1–9, where 1 represents no injury and 9 represents completely injured (or dead). Total number of individuals in each population from two trials were 17.
[a]SC-R, FN-R and TH-R were suspected glyphosate resistant populations from Scott, Finney, and Thomas Counties, respectively, while PH-S, WA-S and WI-S were expected glyphosate susceptible populations from Phillips, Wallace and Wichita Counties, respectively in western Kansas.
[b]Herbicide treatments were applied to 8- to 10-cm tall kochia plants.
[c]with ammonium sulfate at 2% v/v.
[d]Injury score followed by the same letter within a row (among populations) are not significantly different based on Tukey's honestly significant difference at $P < 0.05$.

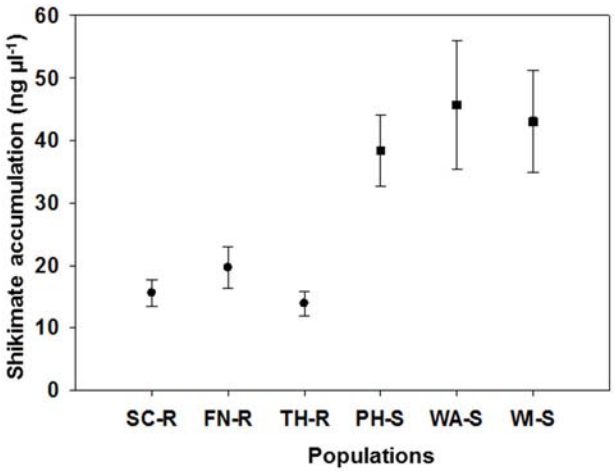

FIGURE 2 | Mean (and standard error) for shikimate accumulation (ng ul^{-1}) of 10 representative plants for each of six kochia populations. Suspected glyphosate-resistant populations were from Scott (SC-R), Finney (FN-R), and Thomas Counties (TH-R) while suspected glyphosate-susceptible populations were from Phillips (PH-S), Wallace (WA-S) and Wichita Counties (WI-S) in western Kansas.

WA counties were GS. The mechanism of glyphosate resistance in the three GR populations was confirmed to be an increase in EPSPS gene copy (Osipitan, 2016).

Germination Characteristics

The regression model (Equation 1) for each population fit the germination data as all parameter estimates were significantly different from zero ($P < 0.001$) on the basis of *t*-tests (data not shown) and coefficient of determination (R^2) was sufficient (0.56–0.94) except when there was poor germination (**Table 2**). Maximum cumulative seed germination increased with increase in temperature from 5 to 15 C while time required to reach 50% of the maximum cumulative germination was shorter with increase in temperature for all populations (**Figure 3**, **Table 2**). None of the GR populations germinated at 5 C, while the GS populations had maximum cumulative germination ranging from 4 to 16% of total seed (**Figure 3**, **Table 2**). At 10 and 15 C, the two GR populations had greater maximum cumulative germination than the GS populations (**Figure 3**, **Table 2**). The maximum germination in respect to percent of total seeds for PH-S was the least (44% for 10 C and 56.3% for 15 C) compared to other GS populations. At low temperature (5 C), the time required to reach 50% of the maximum cumulative germination could not be estimated for the GR populations due to very low germination however, among the GS populations there was no difference in required time to attain 50% germination. The differences among the GR and GS populations were more obvious as temperature increased from 10 to 15 C (**Table 2**). At 15 C, the GR populations consistently required more time to reach maximum germination than the GS populations. Generally, viability test after final germination count, showed that GR populations had more nonviable seeds than the GS populations (data not shown).

Vegetative Growth and Fecundity

Effect of Neighbor Density on Target Plant Height over Time

The three-parameter sigmoid model (Equation 5) adequately fit the data for plant height over time. The rate of change in plant height as measured by the slope (b estimate) was not different among populations under the influence of low or high kochia neighbor densities in both 2014 and 2015 (**Figure 4**, **Tables 3**, **4**). Similarly, there was no difference in the rate of change in stem diameter or canopy spread among populations over time (data not shown).

At low neighbor density, the estimated final plant height (parameter "a") was different among populations in 2014 (**Figure 4A**, **Table 3**) but not different in 2015 (**Figure 4C**, **Table 3**). At high neighbor density in both years, there were differences in estimated final plant height among populations (**Figures 4B,D**, **Table 4**), however, ANCOVA showed that there was an interaction between populations and densities for the observed final plant height (**Table 5**).

TABLE 2 | Parameter estimates obtained from logistic regression model (Equation 1) to describe the germination dynamics in % of total seed for each kochia population.

Populations	Parameter estimates[b]								
	b			d			t_0		
	5 C	10 C	15 C	5 C	10 C	15 C	5 C	10 C	15 C
				%			hours		
SC-R[a]	*n*[c]	−14.1	−5.5	*n*	6.6 C[d]	18.4 C	*n*	243 A	122 A
FN-R	*n*	−10.6	−2.7	*n*	7.4 C	13.7 C	*n*	195 A	148 A
PH-S	−2.6	−1.9	−3.6	4.4	43.8 B	56.3 B	1092	200 A	68 C
WA-S	−5.3	−3.5	−2.9	16.1	71.4 A	84.8 A	214	99 B	54 D
WI-S	−2.4	−2.2	−2.9	4.2	57.5 A	71.2 A	976	313 A	95 B

[a] *SC-R and FN-R were glyphosate-resistant kochia populations from Scott and Finney Counties, respectively while PH-S, WA-S and WI-S were glyphosate-susceptible populations from Phillips, Wallace and Wichita Counties, respectively.*

[b] *Parameter estimates: b is the slope, d is the maximum % cumulative germination (of total seeds), and t_0 is time (hours) required to reach 50% of maximum cumulative germination.*

[c] *No parameters could be estimated due to very poor germination.*

[d] *Within a column (across populations), parameter estimates with different letters were significantly different ($P < 0.05$), while parameter estimates with no letters had significant different differences among populations. Comparison of estimates among population was conducted using Tukey's honestly significant difference test ($\alpha = 5\%$).*

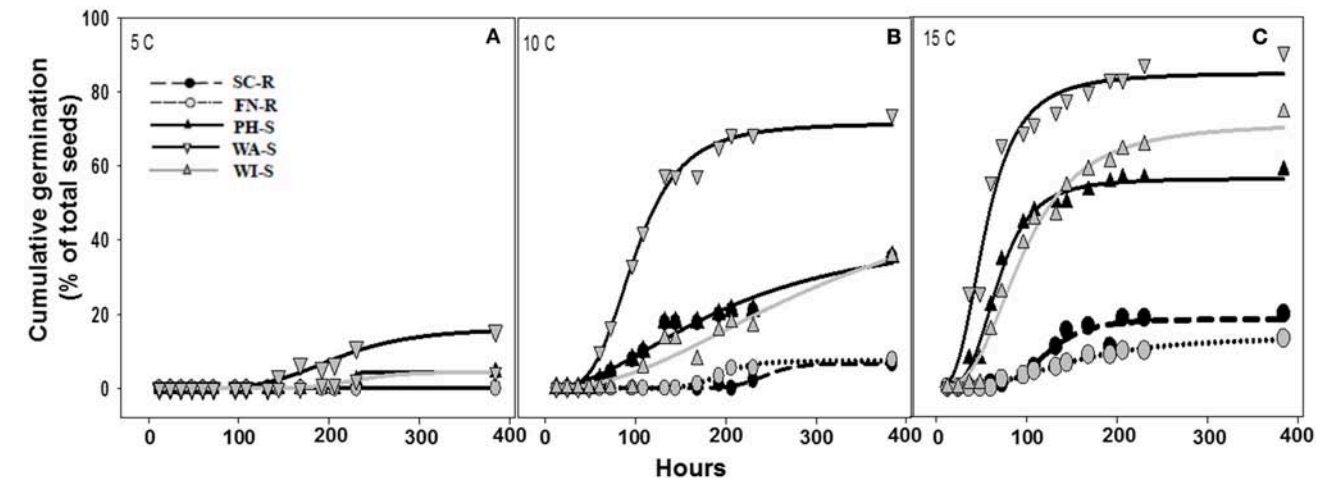

FIGURE 3 | Cumulative germination as a percentage of 30 seed **(A–C)** at one of three constant temperatures of 5, 10, and 15 C. Germination dynamics of glyphosate-resistant and -susceptible kochia populations are modeled with Equation 1. Parameter estimates for each model are included in **Table 2**. Plots with solid lines and triangle symbols represent the GR populations while those with broken lines and circle symbols represents the GS populations.

The recorded differences in final plant height were not consistent among GR and GS populations. For instance, in competition with low neighbor density, TH-R was the shortest among populations at the end of the season and was about 68% of the tallest recorded population (SC-R) in 2014 (**Figure 4A**, **Table 3**). While in competition with high neighbor density averaged over years, TH-R and WI-S plants were about 55 and 30% of the average height attained by SC-R (130 cm), and 58 and 29% of the average height attained by PH-S (124 cm), while three populations (SC-R, PH-S, and FN-R) had similar heights at harvest (**Figures 4B,D**, **Table 4**).

Phenology estimate in the model showed that thermal time, cumulative GDD required to attain 50% of maximum height, was not different among populations and the cumulative GDD of each population fall between 1,265 to 1,894 GDD in 2014 and 1,422 to 2006 GDD in 2015 across neighbor densities (**Tables 3**, **4**). Going by these estimates, there was no evidence that temperature required for increase in height differed among the populations.

Effect of Neighbor Density on Target Plant Biomass, Days to Flowering, and Fecundity

Analysis of covariance of growth and reproductive measurements at the end of season showed that there were interactions between the two main factors of kochia population and kochia neighbor density for target plant biomass in both years, as well as for seed weight and number of seeds produced per plant in 2015 (**Table 5**). Across populations, as neighbor density increased, plant biomass decreased by 80% in 2014 and 85% in 2015 (**Table 6**) while total seed weight and number per plant were reduced by 71 and 80%, respectively (**Table 7**) in 2015. The differences among populations were significant at high neighbor density for these

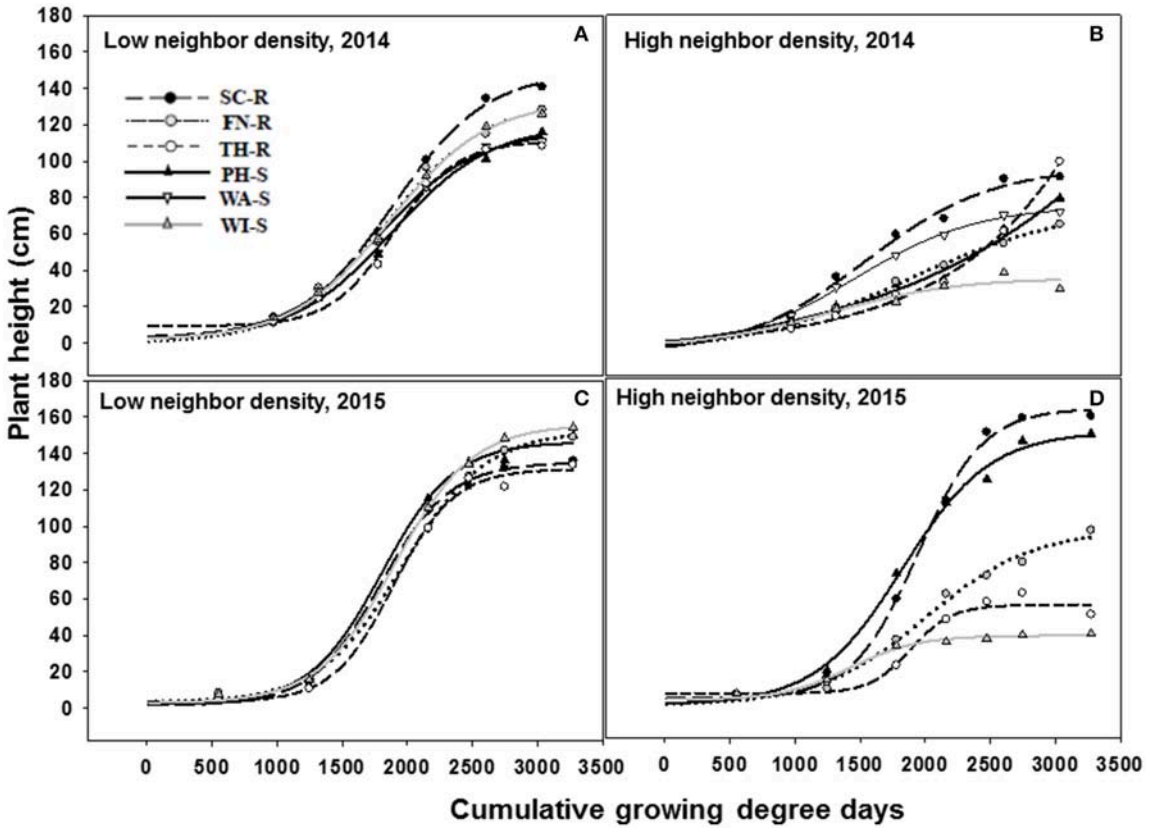

FIGURE 4 | Increase in kochia plant height (cm) as a function of cumulative growing degree days (Tbase = 0 C, from the date of planting) at low (10 plants m^{-2}) and high (70 plants m^{-2}) kochia neighbor density using a three parameter sigmoid model (Equation 5) in 2014 and 2015. Resistant populations are represented by broken lines and mean values in circles while the susceptible populations are represented by solid lines and mean values in triangles. **(A–D)** represents low neighbor density for 2014, high neighbor density for 2014, low neighbor density for 2015, and high neighbor density for 2015 respectively. **Tables 3, 4** shows the parameter estimates of the model.

measurements (**Tables 7**, **8**), however, the differences among populations were not in respect to glyphosate resistance.

Target plant biomass at harvest showed no differences among populations at low neighbor density but there were differences at high neighbor density; differences were not consistent among GR and GS populations (**Table 6**). Similarly, the effects of kochia neighbor density on total seed weight and seed number per plant were significant at high density where SC-R and PH-S had similar total seed weights (13.1–14.8 g $plant^{-1}$) and these were greater than the seed weight of FN-R, TH-R, and WI-S (**Table 7**). The impact of high neighbor density compared to low neighbor density on plant biomass and seed production among populations might have been exaggerated. At low density, the proximity of target to neighbor was 15 cm while at high density it was only 10 cm, hence there was confounding effect of target to neighbor distance and number of neighbors at each density level. However, the evaluation of proximity effect may not be necessary for this study, as the crowding treatment for all populations was similar and this study was more concerned about the response of each population to the crowding level.

The type of neighbor (kochia or corn) had an effect on target plant height, biomass, seed weight, and seed number (**Table 8**), such that corn suppressed target kochia plant variables more than kochia as a neighbor. The impact of type of neighbor on the populations was not influenced by the density of neighbor (**Table 8**). The ANCOVA of pooled populations for GR or GS showed that there was no difference for plant height, stem diameter, plant width, days to flowering, seed weight, 1000-seed weight, number of seed per plant, or reproductive effort based on resistance (**Table 9**).

DISCUSSION

The influence of temperature on speed and level of germination of GR and GS kochia populations is important in agronomy, as it relates to cohort establishment (periodic emergence of plants of the same species per unit land area in a growing season) and optimum time of weed control. Because GR populations had delayed germination at constant low temperatures, an early season weed management strategy with no effective supplemental weed control for subsequent cohorts will likely increase the proportion of GR individuals within a kochia population over time. Also, it is proposed that in this case, mixed populations GS individuals would have competitive advantage for limited

TABLE 3 | Parameter estimates (±SE) and coefficient of determination (R^2) for plant height of each kochia population and year in response to low neighbor density when Equation 5 was fit to data.

Year	Population	Parameter estimates (±SE)			R^2
		a	b	c	
		cm		CGDD	
2014	SC-R	146 (21)	360 (112)	1,894 (108)	0.80
	FN-R	133 (36)	381 (220)	1,841 (223)	0.61
	TH-R	102 (15)	242 (106)	1,891 (107)	0.67
	PH-S	118 (21)	381 (138)	1,881 (133)	0.73
	WA-S	115 (26)	371 (176)	1,779 (180)	0.58
	WI-S	131 (30)	373 (186)	1,864 (183)	0.63
	P-value	0.038	NS	NS	
2015	SC-R	133 (11)	268 (79)	1,793 (84)	0.89
	FN-R	149 (26)	323 (146)	1,961 (155)	0.68
	TH-R	128 (10)	233 (67)	1,893 (72)	0.84
	PH-S	144 (12)	278 (72)	1,802 (77)	0.85
	WI-S	153 (8)	298 (46)	1,904 (49)	0.94
	P-value	NS	NS	NS	

Parameter a represents plant height at harvest, b represents the slope of the curve, and c represents the cumulative growing degree days at 50% of the maximum height.
CGDD, Cumulative growing degree days.
NS, Not significant ($P > 0.05$).
Estimates with different letters are significantly different ($\alpha = 0.05$) within a column.

TABLE 4 | Parameter estimates (SE) and coefficient of determination (R^2) for plant height of each population and year in response to high neighbor density when Equation 5 was fit to data.

Year	Population	Parameter estimates (±SE)			R^2
		a	b	c	
		cm		CGDD	
2014	SC-R	102 (28)	4817 (244)	1545 (244)	0.59
	FN-R	80 (77)	634 (902)	1866 (826)	0.29
	TH-R	97 (24)	927 (213)	1276 (787)	0.48
	PH-S	99 (28)	1576 (662)	1629 (600)	0.45
	WA-S	79 (38)	469 (417)	1466 (425)	0.34
	WI-S	38 (13)	483 (311)	1265 (294)	0.53
	P-value	0.016	NS	NS	
2015	SC-R	159 (17)	251 (81)	1944 (89)	0.82
	FN-R	96 (26)	411 (235)	2006 (247)	0.63
	TH-R	48 (14)	158 (198)	1889 (227)	0.24
	PH-S	149 (21)	335 (121)	1827 (128)	0.74
	WI-S	35 (13)	260 (313)	1422 (366)	0.31
	P-value	<0.001	NS	NS	

Parameter a represents plant height at harvest, b represents the slope of the curve and c represents the cumulative growing degree days at 50% of the maximum height.
CGDD, Cumulative growing degree days.
NS, Not significant ($P > 0.05$).
Estimates within a column with different letters are significantly different ($\alpha = 0.05$).

TABLE 5 | *P*-value of analysis of covariance for fitness variables of populations at varying kochia neighbor densities.

Fitness variables	Population		Density		Population by density	
	2014	2015	2014	2015	2014	2015
Biomass at harvest (g)	NS	0.025	0.001	0.001	0.012	0.003
Stem diameter at harvest (cm)	NS	NS	NS	NS	NS	NS
Plant width at harvest (cm)	–	NS	–	0.001	–	NS
Days to flowering	0.004	0.017	0.032	NS	NS	NS
Total seed weight (g plant^{-1})	NS	0.048	0.001	0.001	NS	0.013
1,000 seed weight (g)	NS	NS	NS	NS	NS	NS
Total seed number (plant^{-1})	NS	0.001	0.001	0.001	NS	0.016
Reproductive Effort	NS	NS	NS	NS	NS	NS

NS, Not significant at $p > 0.05$.

TABLE 6 | Means of target kochia biomass (g plant^{-1}) at different levels of kochia neighbor densities.

Year	Population	Kochia neighbor densities			*P*-value
		Low	Moderate	High	
		g plant^{-1}			
2014	SC-R	1556	86	87 a	0.002
	FN-R	1430	389	127 a	0.01
	TH-R	561	161	211 a	NS
	PH-S	1077	561	71 ab	NS
	WA-S	1118	254	318 a	NS
	WI-S	2302	57	17 b	0.001
	P-value	NS	NS	0.04	
2015	SC-R	315	181	66 a	NS
	FN-R	577	121	39 b	0.001
	TH-R	241	218	33 b	0.001
	PH-S	354	162	46 ab	0.04
	WI-S	477	141	19 c	0.001
	P-value	NS	NS	0.001	

NS, Not significant.
Estimates with different letters are significantly different ($\alpha = 0.05$) within a column.

resources through earlier seedling emergence with subsequent early canopy cover and root growth, reducing the growth and potential seed production of GR individuals in the absence of glyphosate applications.

The GR populations not only have delayed germination but also possess reduced ability to germinate at constant temperatures. In this study, the difference in total germination among GR and GS populations during the period of observation may be attributed to either differential seed viability or dormancy. Differences in seed viability are more likely as GR and GS populations had differential total germination even under ideal temperatures (10 C). Noting that this study was conducted 3 years after the seeds were collected suggests that seeds from GR populations may lose viability sooner than GS populations.

TABLE 7 | Means of seed weight (g plant^{-1}) and seed number (# plant^{-1}) at different levels of kochia neighbor densities in 2015.

	Seed weight				Seed number			
	Kochia neighbor densities				Kochia neighbor densities			
Population	Low	Moderate	High	*P*-value	Low	Moderate	High	*P*-value
SC-R	63.50	37.40	13.1 a	0.16	146,112	47,663	16,074 a	0.02
FN-R	120.57	50.72	1.7 b	0.001	199,970	74,123	3,037 b	0.001
TH-R	65.31	65.32	1.32 b	0.001	41,086	62,626	1,105 b	0.001
PH-S	82.50	34.51	14.8 a	0.05	113,074	70,713	16,323 a	0.01
WI-S	74.60	39.01	0.82 b	0.001	80,591	22,358	1,294 b	0.001
P-value	NS	NS	0.001		NS	NS	0.001	

NS, Not significant.
Estimates with different letters are significantly different ($\alpha = 0.05$) within a column.

TABLE 8 | Analysis of covariance for target response under intra-specific (kochia) and interspecific (corn) neighbors for 10 and 35 plants m^{-2} neighbor densities.

Fitness variables	Type of neighbor			Type of neighbor by density
	Corn	Kochia	*P*-value	*P*-value
Plant height at harvest (cm)	128	143	0.02*	NS
Biomass at harvest (g plant^{-1})	239	292	0.04*	NS
Seed weight (g plant^{-1})	29.9	63.9	0.02*	NS
Seed number (# plant^{-1})	45,300	85,703	0.006*	NS

**Significant difference at $\alpha \leq 0.05$.*
NS, Not significant.

Consequently, in a segregating population or a seed mixture of GR and GS kochia individuals, the frequency of the GR to GS may decrease over time in the absence of glyphosate application. Wakelin and Preston (2006) reported that at the end of 3 years, there was an observable decrease in proportion of surviving GR individuals in seeds of a segregating population of ryegrass (*Lolium rigidum*) from a cross between GR and GS parents. Most recently, van Etten et al. (2016) reported a negative correlation between GR and seed quality or seed germination in a study that examined 43 naturally-occurring populations of tall morningglory (*Ipomoea purpurea*) that varied in their level of GR. This was similar to an earlier report by Debban et al. (2015) that GR lines of tall morningglory had fewer viable seeds than GS lines.

The underlying factor contributing to differences in germination characteristics of GR and GS populations is yet to be understood. The GR kochia populations were known to have high EPSPS gene copies (Osipitan, 2016) and EPSPS gene copies in kochia were generally known to be highly functional for production of EPSPS enzymes (Godar, 2014; Kumar et al., 2015; Gaines et al., 2016). However, the reduced longevity or viability of GR seeds may not be unconnected with the postulation that metabolic energy cost associated with overproduction of EPSPS protein can be detrimental to other protein production since protein production or repair is known to be important to long term seed viability, particularly when seed are stored in dry state (Shen-Miller, 2002).

Any weed management strategy that is timely to remove both early and later emerging kochia seedlings, as well as a strategy that delays the germination of seeds to another growing season, can help reduce the frequency of GR individuals within a population on the field. One such strategy may include a residual soil-applied herbicide that would remove earlier germinating GS seedlings, and continue to be present for later germinating GR seedlings. Another strategy is the use of tillage to bury kochia seed to a soil depth that impedes seedling establishment (Schwinghamer and Van Acker, 2008) such that even when seed are repositioned (through tillage) to soil depth conducive for germination in a subsequent cropping season, many of the GR seeds could have lost their viability.

It was expected that overproduction of EPSPS enzyme that endowed GR in kochia and the consequent metabolic cost of production of this enzyme at the expense of other protein production needed for plant growth and fecundity will be a trade-off for plant fitness and this trade-off was expected to be more obvious under competition with neighbors for resources. However, in this field study, reduced growth rate and less fecundity were not observed, even under competition. This is similar to a previously reported greenhouse study by Kumar and Jha (2015). Different study environments (field and greenhouse) have now demonstrated that GR in kochia endowed by overproduction of EPSPS appears to have no associated cost with growth and seed production.

Differences among kochia populations in response to varying levels of neighbor competition were not clearly related to glyphosate resistance, but rather showed some consistency in direction of inherent ability of the populations to capture resources in the presence of neighbors. One GR population (TH-R) and one GS population (WI-S) consistently showed less vegetative growth and seed production at high neighbor density while there was no difference in growth and seed production of other populations (both GR and GS). If just two populations, such as SC-R vs. WI-S or TH-R vs. PH-S were compared for plant height, biomass accumulation or seed production, the differences could have been erroneously attributed to evolution of GR in the populations. The results suggest the importance of evaluating more than just two populations for fitness comparison, to check for consistency and allow validity. It is obvious that

TABLE 9 | *P*-values of the analysis of covariance for pooled populations [Resistant (R) vs. Susceptible (S)], neighbor density and interaction.

Fitness variables	R vs. S		Density		R vs. S by density	
	2014	2015	2014	2015	2014	2015
Plant height at harvest (cm)	NS	NS	0.001**	0.001**	0.02*	NS
Biomass at harvest (g plant^{-1})	NS	NS	0.001**	0.001**	NS	NS
Stem diameter at harvest (cm)	NS	NS	0.001**	NS	NS	NS
Days to flowering	NS	NS	0.03*	NS	NS	NS
Total seed weight (g plant^{-1})	NS	NS	0.001**	0.001**	NS	NS
1,000 seed weight (g)	NS	NS	NS	NS	NS	NS
Total seed number	NS	NS	0.001**	0.001**	NS	NS
Reproductive effort	NS	NS	NS	NS	NS	NS

*Significant (≤ 0.05).
**Highly significant (≤ 0.01).

factor(s) other than GR trait resulted in differences of final plant height, biomass accumulation, and seed production among these populations. These differences could be as a result of their diverse genetic background (Mengistu and Messersmith, 2002; Giacomini et al., 2014) or adaptive features that evolved due to their original environmental conditions (Jasieniuk et al., 1996).

Use of pure-lines or isogenic lines collected from inbreeding of several filial generations has been recommended and has been previously used as reliable plant material for fitness studies (Vila-Aiub et al., 2009b; Giacomini et al., 2014; Kumar and Jha, 2015). Many studies used a single pair comparison between resistant and susceptible individuals of such lines to arrive at conclusion on fitness studies. This study did not evaluate such inbred lines. Kochia, as an obligate outcrossing species, demonstrates significant inbreeding depression, which may also mask fitness differences. Another approach may be to conduct comparisons among several pairs of resistant and susceptible inbred lines developed from different populations.

CONCLUSIONS

Fitness costs as a result of evolution of herbicide resistance in plants cannot be generalized. Differences in methodology and interpretation of studies that attempt to quantify fitness costs or evolutionary trade-offs associated with evolved resistance to herbicide are very diverse. It is also very likely that identification of fitness costs will vary based on weed species, the mechanism of resistance involved, and the genetic background through which resistance is expressed. Nevertheless, conducting these studies to identify differences in life history traits had shown a great potential in detecting fitness cost.

Of the life history stages measured, fitness difference between the GR and GS kochia populations was consistently found in their germination characteristics. The GR populations showed less seed longevity, slower germination rate, and less total germination than the GS populations. Plant growth, days to flowering, and seed production were not necessarily different among GR and GS populations. In general, this study suggests that any weed management strategy that delays the germination and emergence of seeds can help reduce the frequency of GR individuals in a kochia population in the field. Otherwise, once the GR individuals germinate and become seedlings, they grow, and reproduce even under intense competition with either kochia 'or' crop neighbors. Thus, agronomic practices that would take advantage of differences in the seed biology of GR and GS should be explored to help minimize or reverse the evolution of GR in kochia populations.

AUTHOR CONTRIBUTIONS

JD secured the funding and was major advisor for the graduate PhD degree of OO, the first author. JD initiated the study, supervised the study, provided laboratory and field materials needed for the study, review and edited manuscript drafts. OO modified the study design, conducted the study, analyzed and interpreted the data, and drafted the manuscript.

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Interaction of 2,4-D or Dicamba with Glufosinate for Control of Glyphosate-Resistant Giant Ragweed (*Ambrosia trifida* L.) in Glufosinate-Resistant Maize (*Zea mays* L.)

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Glyphosate-resistant (GR) giant ragweed is a problematic broadleaf weed in crops including maize and soybean in the Midwestern United States. Commercialization of crops with 2,4-D or dicamba and glufosinate resistance will allow post-emergence (POST) applications of these herbicides. Therefore, information is needed on how 2,4-D/dicamba will interact with glufosinate in various rate combinations. The objectives of this study were to evaluate the interaction of glufosinate plus 2,4-D and/or dicamba for control of GR giant ragweed, and to determine their effect on GR giant ragweed density, biomass, maize injury, and yield. Field experiments were conducted in 2013 and 2014 in a field infested with GR giant ragweed in Nebraska, United States. The treatments included POST applications of glufosinate (450 or 590 g ai ha^{-1}), 2,4-D, or dicamba at 280 or 560 g ae ha^{-1} applied alone and in tank-mixtures in glufosinate-resistant maize. The results showed that dicamba applied alone resulted in 56 to 62% and 73 to 83% control at 14 and 28 days after treatment (DAT), respectively, and ≥95% control at 60 DAT or at harvest compared to 17 to 30% and 57 to 73% control with 2,4-D applied alone at 280 and 560 g ai ha^{-1}, respectively. Glufosinate tank-mixed with 2,4-D and/or dicamba consistently provided ≥89% control of GR giant ragweed, except that control with glufosinate plus 2,4-D varied from 80 to 92% at 60 DAT and at harvest. The comparison between the observed and expected control (determined by Colby's equation) suggested an additive interaction between glufosinate and 2,4-D or dicamba for control of GR giant ragweed. Contrast analysis also indicated that GR giant ragweed control with glufosinate plus 2,4-D or dicamba was either consistently higher or comparable with individual herbicides excluding 2,4-D applied alone. Herbicide programs, excluding 2,4-D at 280 g ae ha^{-1}, resulted in ≥80% reduction in GR giant ragweed density. Tank-mixing glufosinate with 2,4-D or dicamba showed an additive effect and will be an additional tool with two effective modes of action for the management of GR giant ragweed in maize.

Keywords: glyphosate resistant, herbicide interaction, tank-mixture, weed control

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INTRODUCTION

Multiple herbicide-resistant crops, such as 2,4-D or dicamba plus glyphosate and/or glufosinate-resistant soybean, will be planted in the United States in the near future (Green, 2016). This technology will provide an additional tool for the management of glyphosate-resistant (GR) weeds (Diggle et al., 2003; Green et al., 2008; Vink et al., 2012; Craigmyle et al., 2013a,b). Moreover, new herbicides recently registered or labeled in maize and soybean are pre-mixtures of existing herbicides with multiple effective modes of action (Chahal et al., 2015; Ganie et al., 2015; Sarangi and Jhala, 2017). Herbicide pre-mixtures or tank-mixtures are typically based on the assumption that any rare individual in a weed population naturally insensitive or less sensitive to one herbicide active ingredient in an herbicide mixture should not be able to express a fitness advantage and survive in the presence of additional effective herbicide active ingredient(s) (Lagator et al., 2013). Therefore, herbicide active ingredients with different modes of action present in an herbicide mixture should have a common weed control spectrum, similar efficacy, and persistence, along with different metabolic pathways to effectively reduce the selection pressure and delay the evolution of herbicide-resistant weeds (Wrubel and Gressel, 1994). The commercialization of multiple herbicide-resistant crops will increase the use of herbicide mixtures with auxinic herbicides (2,4-D or dicamba) plus glufosinate to effectively control weeds, including GR weeds, in maize, soybean [*Glycine max* (L.) Merr.], and cotton in the United States (*Gossypium hirsutum* L.) (Vink et al., 2012; Craigmyle et al., 2013a,b; Merchant et al., 2013).

Synthetic auxin herbicides such as 2,4-D and dicamba are important systemic herbicides for the control of broadleaf weeds (Vink et al., 2012; Peterson et al., 2016). Synthetic auxin herbicides cause an up-regulation of auxin responses in plants leading to a disturbance in the balance of natural plant growth hormones that interrupts normal growth and differentiation; triggers abnormal unregulated cell division; causes uncontrolled growth; and causes damage to chloroplasts, membranes, and vascular tissues (Grossmann, 2010). Dicamba and 2,4-D have been successfully used for over 40 and 70 years, respectively, to control broadleaf weeds primarily in cereal crops and non-crop areas (Behrens et al., 2007; Peterson et al., 2016). The recent development of 2,4-D and dicamba-resistant crops will provide an opportunity to apply these herbicides post-emergence (POST) (Vink et al., 2012; Craigmyle et al., 2013a,b; Merchant et al., 2013). As of January 2017, 18 and 6 weed species worldwide have evolved resistance to 2,4-D and dicamba, respectively, including three species resistant to 2,4-D and two species resistant to dicamba in the United States (Heap, 2017).

Glufosinate is an important broad-spectrum contact herbicide that can be used in tank-mixture with 2,4-D, dicamba and/or glyphosate in the newly developed multiple herbicide-resistant maize, soybean, and cotton (Vink et al., 2012; Barnett and Steckel, 2013; Craigmyle et al., 2013a,b; Merchant et al., 2013). Glufosinate inhibits the activity of glutamine synthetase, an enzyme responsible for the synthesis of glutamine from glutamate plus ammonia, resulting in the buildup of ammonia in cells and the disruption of the plant's nitrogen metabolism (Wendler et al., 1990; Wild and Wendler, 1991). Toxic concentration of ammonia in the cells usually disrupts the cell's chloroplast structure, prevents normal photosynthesis and photophosphorylation, and eventually destroys the cells (Devine et al., 1993; Hinchee et al., 1993). Glufosinate is effective for the control of a wide spectrum of weeds, including broadleaf and grass weeds (Steckel et al., 1997). Additionally, glufosinate is also effective for control of certain weed species such as morningglory (*Ipomoea* spp.) and hemp sesbania [*Sesbania exaltata* (Raf.) Rydb. ex A.W. Hill], which are naturally less sensitive to glyphosate (Corbett et al., 2004). Previous studies have reported that synthetic auxin herbicides (2,4-D or dicamba) plus glufosinate provided effective control of broadleaf weeds: for example, Palmer amaranth [*Amaranthus palmeri* (L.) Wats.] control improved from 89 to 97% with glufosinate plus 2,4-D or dicamba compared to less than 83% control with the respective herbicides applied alone (Merchant et al., 2013). Craigmyle et al. (2013b) reported that control of Asiatic dayflower (*Commelina communis* L.) and common waterhemp (*Amaranthus rudis* Sauer) was 68 to 92% and more than 95%, respectively, with a tank-mixture of glufosinate and 2,4-D compared to glufosinate or 2,4-D applied alone (0 to 22% and 75 to 95% control, respectively).

Giant ragweed (*Ambrosia trifida* L.) is an important summer annual broadleaf weed found in wastelands, roadsides, fence-lines, and agronomic crops including maize, soybean, and cotton (Abul-Fatih and Bazzaz, 1979; Bassett and Crompton, 1982; Johnson et al., 2006). Giant ragweed has a competitive advantage over crops and other annual weed species due to its early emergence, large leaf area, rapid growth rate, high plasticity, and ability to regulate its resource utilization in response to changing environmental factors (Abul-Fatih and Bazzaz, 1979; Bazzaz and Carlson, 1979). Management of giant ragweed has become complicated due to its extended emergence pattern in the eastern maize belt of the United States (Schutte et al., 2008, 2012) and due to the evolution of giant ragweed biotypes resistant to ALS-inhibitors and/or glyphosate in the Midwestern United States (Johnson et al., 2006; Norsworthy et al., 2010, 2011; Jhala et al., 2014; Kaur et al., 2014). Nevertheless, effective integrated management options for GR giant ragweed based on preplant tillage followed by PRE and/or POST applications of herbicide-mixtures have been reported in maize and soybean (Ganie et al., 2016, 2017). Recent research in Nebraska has reported that giant ragweed is sensitive to synthetic auxin herbicides and can be effectively controlled by a preplant application of 2,4-D (Jhala et al., 2014; Kaur et al., 2014; Ganie et al., 2016). Similarly, glufosinate plus 2,4-D or dicamba provided greater than 90% control of GR giant ragweed (Barnett and Steckel, 2013). Vink et al. (2012) reported 100% control of GR giant ragweed with preplant followed by POST applications of glyphosate plus dicamba in dicamba-tolerant soybean.

Use of herbicide tank-mixtures or pre-mixtures has become a common reactive approach for the management of herbicide-resistant weeds (Green, 1991; Buttel, 2002; Hart and Pimentel, 2002; Beckie and Reboud, 2009). Typically, herbicide mixtures that provide improved weed control while allowing a reduced dose of the component herbicides are considered

economically viable (Gressel, 1993). However, reduced doses of the component active ingredients in an herbicide mixture may affect synergistic interactions and result in disproportionate performance of the component herbicides (Green, 1991). In addition, the exposure of weed populations to the lower herbicide doses usually used in herbicide mixtures may result in selection for generalist type mutation(s) or non-target site mechanisms, providing resistance to all the herbicide active ingredients present in the mixture (Neve and Powles, 2005). Relatively large differences in the efficacy of the component herbicide in a mixture exposes weed population to a higher selection pressure of the better performing partner, and likely reduce the potential of the herbicide tank-mixture to delay the evolution of herbicide resistance (Beckie and Reboud, 2009).

Previous research has shown that a specific rate of the constituent active ingredients is needed for herbicide mixtures with synergistic interaction. For example, Hugie et al. (2008) reported that a threshold mesotrione rate was needed to attain synergism between mesotrione and atrazine. Therefore, research is needed to determine the effect of individual herbicide rates on the type of interaction (additive, synergistic, or antagonistic) between glufosinate with 2,4-D and/or dicamba. The objectives of this study were to evaluate the efficacy of 2,4-D and/or dicamba plus glufosinate for control of GR giant ragweed, and to determine the effect of herbicide rate combinations on the type of interaction between 2,4-D and/or dicamba plus glufosinate on giant ragweed control, density, biomass, maize injury, and yield. We hypothesized that an additive interaction between synthetic auxins (2,4-D or dicamba) and glufosinate for GR giant ragweed control will be achieved when constituent herbicides are used at the labeled rate in tank-mixture.

MATERIALS AND METHODS

Field Experiments

Field experiments were conducted at Clay Center (40.52°N, 98.05°W) and David City (41.25°N, 97.13°W), Nebraska in 2013 and 2014, respectively, in growers' fields infested with GR giant ragweed. Giant ragweed biotypes from these sites were confirmed resistant to glyphosate in 2011, with 14-fold resistance compared to glyphosate-susceptible biotypes included for comparison (Rana et al., 2013). The density of GR giant ragweed at these sites varied from 18 to 30 plants m^{-2}. The soil type at Clay Center was fine, smectitic, mesic Vertic Argiaquolls (Butler series) with a silt loam texture (17% sand, 58% silt, 25% clay), 2.5% organic matter, and a pH of 6.5. The soil type at David City was fine, smectitic, mesic Udic Argiustolls (Hastings series) with a silty clay loam texture (18% sand, 50% silt, 32% clay), 2.1% organic matter, and a pH of 5.4. The experiment was arranged in a randomized complete block design with 18 treatments and four replications. The treatments included POST applications of glufosinate (450 or 590 g ai ha^{-1}) (Liberty 280, Bayer Crop Science, Research Triangle Park, NC, United States), 2,4-D amine (280 or 560 g ae ha^{-1}) (2,4-D Amine, Winfield Solutions, LLC, St Paul, MN, United States), and dicamba (280 or 560 g ae ha^{-1}) (Clarity, BASF Corporation, Research Triangle Park, NC, United States) alone and in tank-mixtures (**Table 1**). Treatment with no herbicide application served as a non-treated control for comparison. Maize seeds (Cv. "Pioneer 1151AM" in 2013 and "Mycogen 2V709" in 2014) with resistance to both glyphosate and glufosinate were planted on May 16, 2013 and May 17, 2014. The seeds were planted 3 cm deep at a density of 79,000 seeds ha^{-1}. Individual plots were 3 m wide and 9 m long with four maize rows spaced 76 cm apart.

Herbicide treatments were applied as POST (June 5, 2013 and June 6, 2014) on 8 to 12 cm tall (4 to 6 leaf stage) giant ragweed plants. Herbicides were applied with a CO_2-pressurized backpack sprayer equipped with a four-nozzle boom fitted with XR11015 flat-fan nozzles (TeeJet, Spraying Systems Co., Wheaton, IL, United States) and calibrated to deliver 140 L ha^{-1} at 276 kPa. The experimental location was under rainfed conditions in 2013 and irrigated conditions in 2014.

Data Collection

Data were collected for visual assessments of giant ragweed control with treatments compared to non-treated control on a scale of 0 to 100% (0 being no control and 100 being complete control) at 14, 28, and 60 days after POST herbicide treatments (DAT), and before maize harvest. Herbicide-injury symptoms including slight bending of the maize plants with 2,4-D or dicamba, and chlorotic spots characteristic of glufosinate on maize canopy were recorded on a scale of 0 to 100% (0 being no injury and 100 being plant death) at 14 and 21 DAT. Giant ragweed density was recorded from three randomly selected 0.25 m^2 quadrats per plot at 60 DAT. Glyphosate-resistant giant ragweed biomass was assessed from three randomly selected 0.25 m^2 quadrats per plot at 60 DAT. Giant ragweed plants that survived herbicide treatments were cut at the stem base close to the soil surface, placed in paper bags, dried in an oven for 72 h at 50°C, and weighed (g). Maize was harvested using a plot combine and yields were adjusted to 15% moisture content (Harrison et al., 2001). Giant ragweed biomass data were converted into percent biomass reduction compared to the non-treated control (Sarangi et al., 2017) as:

$$\text{Biomass reduction (\%)} = \left[\frac{(C-B)}{C}\right] \times 100 \qquad (1)$$

where C is the biomass of the non-treated control replicates and B is the biomass of an individual treated experimental unit.

Statistical Analysis

Data were subjected to ANOVA using the PROC GLIMMIX procedure in SAS version 9.3 (SAS Institute Inc, Cary, NC, United States). The treatments with zero response variables were not included in the analyses. Before analyses, data were tested for normality of residuals using the PROC UNIVARIATE procedure in SAS, which suggested that data does not follow a Gaussian distribution. Therefore, visual estimates of giant ragweed control, and biomass reduction data were arcsine square-root transformed before analysis; however, back-transformed data are presented with mean separation based on the transformed data. When the ANOVA indicated that treatment effects

were significant, means were separated at $P \leq 0.05$ using Fisher's protected least significant difference (LSD) test. Single degree-of-freedom contrast statements were used to compare herbicide programs with 2,4-D, dicamba, or glufosinate applied alone vs. their tank-mixtures. Specific contrast statements were used to compare 2,4-D vs. dicamba, glufosinate plus 2,4-D or dicamba vs. alone application of these herbicides, and glufosinate plus 2,4-D plus dicamba vs. glufosinate plus 2,4-D or dicamba. To determine the type of interaction (additive, synergistic, or antagonistic) between herbicide programs, the Colby equation was used to calculate the expected values (Colby, 1967):

$$E = (X + Y) - \left(\frac{XY}{100}\right) \quad (2)$$

where E is the expected control of giant ragweed with application of herbicides A + B in tank-mixture, and X and Y are the observed control with the application of herbicides A and B, respectively, at specific rates. The statistical differences between the expected and observed values of control were determined by the t-test in R (R statistical software, R Foundation for Statistical Computing, Vienna, Austria[1]). The herbicide combination was considered synergistic if the expected mean was significantly lower than the observed mean. If the expected mean was greater than the observed mean, the herbicide combination was considered antagonistic (Colby, 1967).

[1]http://www.R-project.org

RESULTS

Year-by-treatment interactions for visual estimates of giant ragweed control, density, and aboveground biomass reduction were not significant ($P \geq 0.05$); therefore, data were combined over 2 years. However, year-by-treatment interaction for maize yield was significant; therefore, yield is presented separately for both years.

Giant Ragweed Control

The application of 2,4-D at 280 and 560 g ae ha^{-1} resulted in 30 and 57% control of GR giant ragweed at 14 DAT, respectively (**Table 1**). However, dicamba resulted in comparable giant ragweed control (56 to 62%) with both rates (280 and 560 g ae ha^{-1}) (**Table 1**). Averaged across application rates, GR giant ragweed control with 2,4-D was 44% compared to 59% control with dicamba (**Table 2**). In contrast, GR giant ragweed control with glufosinate at 450 or 560 g ai ha^{-1} was 87 to 92% (**Table 1**). Herbicide programs including glufosinate plus 2,4-D or dicamba, and glufosinate plus dicamba plus 2,4-D tank-mixed at various rates provided ≥90% giant ragweed control

TABLE 1 | Observed and expected control of glyphosate-resistant giant ragweed with 2,4-D, dicamba, and glufosinate applied alone or in tank-mixtures in glyphosate plus glufosinate-resistant maize in field experiment conducted in 2013 and 2014 in Nebraska, United States.[a,b]

Herbicide treatment	Rate	Giant ragweed control[c]				Expected control based on Colby's equation[d,e]	
		14 DAT	28 DAT	60 DAT	At harvest	14 DAT	60 DAT
	g ae or ai ha^{-1}	%					
Non-treated control	–	0	0	0	0	–	–
2,4-D	280	30 d	20 f	17 e	18 f	–	–
2,4-D	560	57 c	73 e	71 d	66 e	–	–
Dicamba	280	56 c	73 e	97 ab	95 abc	–	–
Dicamba	560	62 c	83 de	99 a	99 a	–	–
Glufosinate	450	92 ab	89 bcd	70 d	79 cde	–	–
Glufosinate	590	87 b	85 dc	83 bcd	87 abcde	–	–
Glufosinate + 2,4-D	450 + 280	96 a	91 abcd	80 dc	81 cde	94	75
Glufosinate + 2,4-D	450 + 560	95 a	93 abc	92 abcd	91 abcd	97	91
Glufosinate + 2,4-D	590 + 280	94 ab	91 abcd	80 dc	84 bcde	91	86
Glufosinate + 2,4-D	590 + 560	93 ab	93 abc	82 bcd	86 bcde	94	95
Glufosinate + dicamba	450 + 280	95 a	96 a	89 abcd	92 abcd	96	99
Glufosinate + dicamba	450 + 560	95 a	96 a	97 ab	98 ab	97	100
Glufosinate + dicamba	590 + 280	95 a	95 ab	97 ab	92 abcd	94	100
Glufosinate + dicamba	590 + 560	96 a	97 a	94 abc	95 abc	95	100
Dicamba + 2,4-D	280 + 140	66 c	84 de	94 abc	98 ab	–	–
Glufosinate + dicamba + 2,4-D	450 + 280 + 140	90 ab	93 abc	90 abcd	94 abc	99	98
Glufosinate + dicamba + 2,4-D	590 + 280 + 140	92 ab	94 ab	92 abcd	94 abc	96	99
p-value		<0.0001	<0.0001	<0.0001	<0.0001		

[a]*Abbreviation: DAT, days after treatment.* [b]*Data from non-treated control plots were excluded from the analysis.* [c]*Means followed by the same letter within a column are not statistically different according to Fisher's protected LSD at $P \leq 0.05$.* [d]*Expected values of giant ragweed control were determined by the Colby equation: $E = (X+Y) - (\frac{XY}{100})$, where E is the expected control of giant ragweed with application of herbicides A + B in tank-mixture, and X and Y are the observed control with the application of herbicides A and B, respectively, at specific rates.* [e]*The observed and expected control at 14 and 60 DAT were compared using a t-test that suggested no statistical differences, indicating that 2,4-D and/or dicamba showed an additive interaction with glufosinate.*

TABLE 2 | Contrast statements to compare herbicide programs for control of glyphosate-resistant giant ragweed in glyphosate plus glufosinate-resistant maize in a field experiment conducted in 2013 and 2014 in Nebraska, United States.[a,b]

Treatment	Giant ragweed control[c] (%)			
	14 DAT	28 DAT	60 DAT	At harvest
2,4-D vs. dicamba	44 vs. 59**	47 vs. 78**	44 vs. 98**	42 vs. 97**
Glufosinate + 2,4-D vs. 2,4-D alone	95 vs. 44**	92 vs. 47**	84 vs. 44**	86 vs. 42**
Glufosinate + 2,4-D vs. glufosinate alone	95 vs. 90*	92 vs. 87 NS	84 vs. 77 NS	86 vs. 83 NS
Glufosinate + dicamba vs. dicamba alone	95 vs. 59**	96 vs. 78**	94 vs. 98 NS	94 vs. 97 NS
Glufosinate + dicamba vs. glufosinate alone	95 vs. 90*	96 vs. 87**	94 vs. 77**	94 vs. 83*
Glufosinate + 2,4-D + dicamba vs. glufosinate + 2,4-D	91 vs. 95 NS	94 vs. 92 NS	91 vs. 84*	94 vs. 86*
Glufosinate + 2,4-D + dicamba vs. glufosinate + dicamba	91 vs. 95 NS	94 vs. 96 NS	91 vs. 94 NS	94 vs. 94 NS

[a]*Preplanned single degree of freedom contrast statements were performed to compare treatments with herbicides used alone (2,4-D, dicamba or glufosinate) versus treatment combinations of 2,4-D and/or dicamba plus glufosinate, and three-way versus two-way combinations of 2,4-D, dicamba, and glufosinate.* [b]**Significant with* $P \leq 0.05$; ***significant with* $P \leq 0.01$, *NS, non-significant or* $P \geq 0.05$. [c]*Abbreviation: DAT, days after treatment.*

in contrast to 66% control with dicamba plus 2,4-D at 14 DAT (**Table 1**).

Herbicide programs excluding 2,4-D at 280 g ae ha^{-1} resulted in ≥73% GR giant ragweed control at 28 DAT; for example, dicamba at 560 g ae ha^{-1} and glufosinate at 450 or 590 g ai ha^{-1} resulted in 83 to 89% control of GR giant ragweed. At 28 DAT, control with 2,4-D plus dicamba improved to 84% compared to the previous rating (66%), though ≥91% control was achieved with glufosinate plus 2,4-D or dicamba (**Table 1**). Irrespective of the individual herbicide rate in the tank-mixtures evaluated in this study, glufosinate plus dicamba provided 95 to 97% control compared to 91 to 93% control with glufosinate plus 2,4-D (**Table 1**). Similarly, the contrast analysis suggested that control of GR giant ragweed with glufosinate plus 2,4-D or dicamba was greater compared to glufosinate, 2,4-D or dicamba applied alone at 14 and 28 DAT except that the contrast between glufosinate plus 2,4-D vs. glufosinate was not significant ($P > 0.05$) at 28 DAT (**Table 2**).

Control of GR giant ragweed at 60 DAT and at harvest was less than or equal to 18% with 2,4-D applied alone at 280 g ae ha^{-1} compared to 66 to 71% control when applied at 560 g ae ha^{-1} (**Table 1**). Irrespective of the application rate, dicamba provided an effective control of GR giant ragweed ranging from 95 to 99% at 60 DAT or at harvest (**Table 1**). Giant ragweed control improved from 70 to 79% with glufosinate applied at 450 g ai ha^{-1} to 83 to 87% control at 590 g ai ha^{-1} (**Table 1**). Moreover, tank-mixing glufosinate with dicamba resulted in 89 to 98% giant ragweed control compared to glufosinate plus 2,4-D (80 to 92%), with limited difference among treatments (**Table 1**). For example, glufosinate (450 or 590 g ai ha^{-1}) plus 2,4-D (280 g ae ha^{-1}) resulted in 80% giant ragweed control compared to 97% control with glufosinate at 450 or 590 g ai ha^{-1} tank-mixed with dicamba at 560 or 280 g ae ha^{-1}, respectively, at 60 DAT (**Table 1**). In contrast, three way tank-mixtures of glufosinate plus dicamba plus 2,4-D provided comparable control of giant ragweed at 60 DAT or at harvest ranging from 90 to 94% regardless of glufosinate application rate (**Table 1**). However, the contrast analysis showed that giant ragweed control with glufosinate plus 2,4-D, glufosinate plus dicamba, and glufosinate plus 2,4-D plus dicamba was better compared to 2,4-D, glufosinate, and glufosinate plus 2,4-D, respectively, at 60 DAT or at harvest (**Table 2**). Additionally, the contrasts between 2,4-D vs. dicamba were significant ($P \leq 0.01$) indicating that dicamba provided greater GR giant ragweed control compared to 2,4-D (**Table 2**).

The expected values of giant ragweed control for herbicide mixtures including glufosinate plus 2,4-D and/or dicamba at 14 and 60 DAT determined by Colby's equation were not different compared to observed values (**Table 1**), indicating that tank-mixtures of glufosinate plus 2,4-D and/or dicamba at the rates used in this study showed an additive interaction for control of GR giant ragweed.

Giant Ragweed Density and Aboveground Biomass Reduction

Glyphosate-resistant giant ragweed density and aboveground biomass reduction were in consensus with the visual assessment of control at 60 DAT (**Tables 1**, **3**). The highest giant ragweed density with an average of 20 plants m^{-2} was recorded in the untreated control. Among herbicide programs, the highest giant ragweed density with an average of 11 plants m^{-2} was observed with 2,4-D applied at 280 g ae ha^{-1}, while the remaining treatments resulted in ≥80% reduction in giant ragweed density (2 to 4 plants m^{-2}), including a 100% reduction with dicamba at 560 g ae ha^{-1} (**Table 3**). Among herbicide treatments, the contrast analysis of GR giant ragweed density indicated differences ($P \leq 0.05$) only between 2,4-D (8 plants m^{-2}) vs. dicamba (2 plants m^{-2}), and glufosinate plus 2,4-D (3 plants m^{-2}) vs. 2,4-D (8 plants m^{-2}) (**Table 4**). Among all herbicide programs, the lowest aboveground biomass reduction was 38% with 2,4-D at 280 g ae ha^{-1} (**Table 3**). Most of the herbicide treatments resulted in ≥80% reduction in aboveground biomass of GR giant ragweed, with the exception of 68, 74, and 78% biomass reduction with glufosinate (590 g ai ha^{-1}) plus 2,4-D (560 g ae ha^{-1}), glufosinate (590 g ai ha^{-1}), and glufosinate (450 g ai ha^{-1}) plus 2,4-D (280 g ae ha^{-1}), respectively (**Table 3**). Similarly, contrast statements for the aboveground biomass reduction were significant ($P \leq 0.05$) only between 2,4-D vs. dicamba and glufosinate plus 2,4-D vs. 2,4-D, and glufosinate plus dicamba vs. glufosinate (**Table 4**).

Maize Injury and Yield

Herbicide treatments including 2,4-D and dicamba alone at the higher rate (560 g ae ha^{-1}) or tank-mixed with glufosinate resulted in 2 to 12% maize injury at 14 DAT (data not shown); however, the injuries were transient and did not result in yield reduction. Maize yields were lower under rainfed conditions in 2013 compared to irrigated conditions in 2014; therefore, yield data were presented separately for both years. Among herbicide treatments, 2,4-D at 280 g ae ha^{-1} resulted in the lowest maize yield in both years (**Table 3**). Glufosinate (450 g ai ha^{-1}) plus dicamba (560 g ae ha^{-1}) resulted in the highest maize yield (10,783 and 12,416 kg ha^{-1} in 2013 and 2014), respectively; however, the yield was comparable among most of herbicide treatments with the exception of glufosinate at 450 g ai ha^{-1} plus 2,4-D at 280 g ae ha^{-1} (5,035 kg ha^{-1}) in 2013 and glufosinate at 450 g ai ha^{-1} (7,246 kg ha^{-1}) in 2014 (**Table 3**). Contrast analysis of yield in 2013 suggested that average maize yield with glufosinate plus dicamba was 9,749 kg ha^{-1} compared to the average yield (6,344 kg ha^{-1}) with glufosinate applied alone. Similarly, contrasts between 2,4-D vs. dicamba were also significant ($P \leq 0.01$), while all other contrast statements were non-significant ($P > 0.05$) (**Table 4**). In 2014, however, the

TABLE 3 | Glyphosate-resistant giant ragweed density, aboveground biomass reduction, and corn yield affected by 2,4-D, dicamba, and glufosinate applied alone or in tank-mixtures in glyphosate plus glufosinate-resistant maize in a field experiment conducted in 2013 and 2014 in Nebraska, United States.[a]

Herbicide	Rate	Giant ragweed[b]		Maize yield[b]	
		Density	Aboveground biomass reduction	2013	2014
	g ae or ai ha^{-1}	No. m^{-2}	%	——————kg ha^{1}——————	
Non-treated Control	–	20 a	0	0	0
2,4-D	280	11 b	38 e	5,23 d	6,280 g
2,4-D	560	4 c	88 abc	6,715 abc	10,205 abc
Dicamba	280	3 c	89 abc	6,211 abc	11,018 ab
Dicamba	560	0	100 a	9,924 abc	11,554 ab
Glufosinate	450	4 c	81 abcd	6,785 abc	7,246 dc
Glufosinate	590	4 c	74 dc	5,903 abc	10,143 abc
Glufosinate + 2,4-D	450 + 280	3 c	78 bcd	5,035 dc	11,018 ab
Glufosinate + 2,4-D	450 + 560	3 c	91 abc	6,519 abc	10,845 ab
Glufosinate + 2,4-D	590 + 280	3 c	84 abcd	5,066 bcd	8,821 bcd
Glufosinate + 2,4-D	590 + 560	4 c	68 d	5,745 abcd	11,143 ab
Glufosinate + dicamba	450 + 280	2 c	91 abc	9,057 abc	9,200 bcd
Glufosinate + dicamba	450 + 560	2 c	95 ab	10,783 a	12,416 a
Glufosinate + dicamba	590 + 280	2 c	86 abcd	8,808 abc	10,730 ab
Glufosinate + dicamba	590 + 560	3 c	95 ab	10,347 ab	11,030 ab
Dicamba + 2,4-D	280 + 140	2 c	92 abc	8,424 abc	11,014 ab
Glufosinate + dicamba + 2,4-D	450 + 280 + 140	4 c	85 abcd	7,196 abc	9,861 abc
Glufosinate + dicamba + 2,4-D	590 + 280 + 140	2 c	82 abdc	7,129 abc	10,425 abc
p-value		<0.0001	<0.0001	<0.0696	<0.0001

[a]*Data from non-treated control plots were excluded from the analysis.* [b]*Means followed by the same letter within a column are not statistically different according to Fisher's protected LSD test at P ≤ 0.05.*

TABLE 4 | Contrast statements to compare herbicide programs for density and aboveground biomass of glyphosate-resistant giant ragweed and crop yield in glyphosate plus glufosinate-resistant maize in a field experiment conducted in 2013 and 2014 in Nebraska, United States.[a]

Treatment	Giant ragweed[b]		Maize yield[b]	
	Density (# m^{-1})	Biomass (g m^{-1})	2013 (kg ha^{-1})	2014 (kg ha^{-1})
2,4-D vs. dicamba	8 vs. 2*	63 vs. 89**	3,619 vs. 8,068**	8,243 vs. 11,286**
Glufosinate + 2,4-D vs. 2,4-D alone	3 vs. 8*	80 vs. 63**	5,591 vs. 3,619 NS	10,457 vs. 8,243**
Glufosinate + 2,4-D vs. glufosinate alone	3 vs. 4 NS	80 vs. 78 NS	5,591 vs. 6,344 NS	10,457 vs. 8,695*
Glufosinate + dicamba vs. dicamba alone	2 vs. 2 NS	92 vs. 95 NS	9,749 vs. 8,068 NS	10,844 vs. 11,286 NS
Glufosinate + dicamba vs. glufosinate alone	2 vs. 4 NS	92 vs. 78*	9,749 vs. 6,344*	10,844 vs. 8,695**
Glufosinate + 2,4-D + dicamba vs. glufosinate + 2,4-D	3 vs. 3 NS	84 vs. 80 NS	7,163 vs. 5,591 NS	10,143 vs. 10,457 NS
Glufosinate + 2,4-D + dicamba vs. glufosinate + dicamba	3 vs. 2 NS	84 vs. 92 NS	7,163 vs. 9,749 NS	10,143 vs. 10,844 NS

[a]*Preplanned single degree of freedom contrast statements were used to compare treatments with herbicides used alone (2,4-D, dicamba or glufosinate) versus treatment combinations of 2,4-D and/or dicamba plus glufosinate, and three-way versus two-way combinations of 2,4-D, dicamba and glufosinate.* [b]**Significant with P ≤ 0.05; ***significant with P ≤ 0.01, NS, non-significant or P ≥ 0.05.*

contrast statements, including 2,4-D vs. dicamba, glufosinate plus 2,4-D vs. 2,4-D or glufosinate, and glufosinate plus dicamba vs. glufosinate, were significant ($P \leq 0.05$) (**Table 4**).

DISCUSSION

Giant ragweed control with 2,4-D or dicamba was in consensus with previous researches. For example, Barnett and Steckel (2013) reported 47 and 64% control of GR giant ragweed at 10 DAT with 2,4-D applied at 560 and 1,120 g ae ha^{-1}, respectively. In contrast, Kaur et al. (2014) reported 98% control of GR giant ragweed with 2,4-D at 560 g ae ha^{-1} applied 21 days before planting soybean when giant ragweed was ≤6 cm tall, compared with this study, where giant ragweed was 8 to 12 cm tall at the time of POST herbicide application. Similarly, Barnett and Steckel (2013) reported 62 to 67% control of GR giant ragweed at 10 DAT with dicamba applied at 280 or 560 g ae ha^{-1}. Irrespective of the application rate, dicamba provided a better giant ragweed control compared to 2,4-D. This might be because giant ragweed is more sensitive to dicamba compared to 2,4-D, though this might not be the case for other weed species. For example, Meyer et al. (2015) reported no differences between 2,4-D- and dicamba-based programs for the control of Palmer amaranth and common waterhemp. Glufosinate applied alone at 590 g ai ha^{-1} or tank-mixed with 2,4-D and/or dicamba provided ≥80% giant ragweed control. Similarly, Norsworthy et al. (2010) reported ≥90% control of GR or GS giant ragweed with glufosinate at 590 g ai ha^{-1} irrespective of growth stage at the time of application. Likewise, Craigmyle et al. (2013b) reported that tank-mixing 2,4-D amine at 560, 840, or 1,120 g ae ha^{-1} with glufosinate improved common waterhemp control to ≥95% compared with 75 to 92% or 78 to 98% control following 2,4-D or glufosinate, respectively.

Increasing 2,4-D rate from 280 to 560 g ae ha^{-1} improved giant ragweed control from ≤30% to 56 to 73%. Previous research also reported that increasing 2,4-D rate improved broadleaf weed control (Everitt and Keeling, 2007; Sarabi et al., 2011). For instance, 2,4-D provided less than 80% control of redroot pigweed (*Amaranthus retroflexus* L.) when applied at 400 g ae ha^{-1} compared to 100% control at 600 to 1,000 g ae ha^{-1} (Sarabi et al., 2011). However, dicamba or glufosinate resulted in comparable giant ragweed control throughout the season regardless of the rate of application. Soltani et al. (2011) reported 90% giant ragweed control at 56 DAT with dicamba at 600 g ae ha^{-1}. Nevertheless, Craigmyle et al. (2013b) reported that control of 20 to 25 cm tall common waterhemp improved from 84 to 90% with increasing glufosinate application rate from 450 to 730 g ai ha^{-1}. The results of this study indicated an additive interaction between glufosinate plus 2,4-D and/or dicamba. However, Joseph (2014) reported synergistic interaction between glufosinate plus dicamba for control of sicklepod [*Senna obtusifolia* (L.) Irwin and Barneby]. Steckel et al. (2006) reported at least 90% horseweed [*Conyza canadensis* (L.) Cronq.] control with application of glufosinate (470 g ai ha^{-1}) plus 2,4-D (530 g ae ha^{-1}) or dicamba (280 g ae ha^{-1}) at 14 and 56 DAT.

As with the results of giant ragweed control, the results of giant ragweed density and biomass reduction were in agreement with previous studies. Barnett and Steckel (2013) reported 5.8 and 7.3 giant ragweed plants m^{-2} with glufosinate and 2,4-D (560 g ai ha^{-1}). Chahal and Johnson (2012) reported comparable biomass reduction in horseweed and common lambsquarters (*Chenopodium album* L.) with glufosinate or glufosinate plus 2,4-D or dicamba. However, Barnett and Steckel (2013) reported a biomass of 19 and 23 g m^{-2} with glufosinate (590 g ai ha^{-1}) and 2,4-D (560 g ae ha^{-1}) compared to ≤12.5 g m^{-2} with 2,4-D applied at 1,120 g ae ha^{-1}, dicamba (280 or 560 g ae ha^{-1}), and glufosinate plus 2,4-D or dicamba irrespective of the application rate.

The results of this study revealed that 2,4-D (280 or 560 g ae ha^{-1}) resulted in ≤73% giant ragweed control throughout the season. Dicamba (280 or 560 g ae ha^{-1}) initially provided ≤83% control, but the control improved to ≥95% by 60 DAT or at harvest. The improvement in the efficacy of dicamba occurred most likely due to its systemic nature. In contrast, glufosinate initially resulted in 85 to 92% giant ragweed control, but control declined to 70 to 79% and 83 to 87% with 450 and 590 g ae ha^{-1} of glufosinate, respectively, at 60 DAT or at harvest. Similarly, Jhala et al. (2013) reported reduced control of Brazil pusley (*Richardia brasiliensis* Moq.), puncture vine (*Tribulus terrestris* L.), and eclipta (*Eclipta prostrata* L.) with glufosinate at 30 DAT compared to 15 DAT. Reduction in control of giant ragweed following glufosinate at 60 DAT compared to 14 or 28 DAT may be attributed to its contact nature and lack of residual activity (Anonymous, 2016). Glufosinate plus 2,4-D or dicamba provided 91 to 97% giant ragweed control at 14 and 28 DAT; nevertheless, control at 60 DAT and at harvest ranged from 80 to 92% with glufosinate plus 2,4-D in contrast to 89 to 98% control with glufosinate plus dicamba. Likewise, glufosinate plus dicamba plus 2,4-D provided more than 90% giant ragweed control throughout the season (**Table 1**). The herbicide mixtures showed an additive interaction at the rates used in this study, suggesting that mixtures including glufosinate plus 2,4-D or dicamba resulted in greater or mostly comparable giant ragweed control and reduction in density or aboveground biomass compared to when applied alone (**Table 1**). Similarly, Barnett and Steckel (2013) and Craigmyle et al. (2013a,b) reported an improved efficacy of glufosinate for control of giant ragweed and common waterhemp, respectively, when tank-mixed with synthetic auxins (2,4-D or dicamba) compared to glufosinate applied alone. Studies have also reported that glufosinate plus dicamba applied as PRE, early-post (EPOST), or mid-post (MPOST) improved control (79 to 100%) of Palmer amaranth compared to glufosinate alone (72 to 90%) (Cahoon et al., 2015). However, the interactions between the herbicides in a mixture may vary with the active ingredient, the weed species, and even the rate of the respective herbicides in a mixture. For example, synergistic interactions have been reported with 2,4-D plus halosulfuron for common lambsquarters control (Isaacs et al., 2006), and mesotrione plus glufosinate for common ragweed (*Ambrosia artemisiifolia* L.) and giant foxtail (*Setaria faberi* Herrm.) control (Armel et al., 2008). Conversely, Burke et al. (2005) reported that glufosinate at 290

or 410 g ai ha^{-1} antagonized clethodim, resulting in a reduction of goosegrass [*Eleusine indica* (L) Gaertn] control from ≥90% to ≤40%. Similarly, Koger et al. (2007) reported antagonistic effects of monosodium methanearsonate (MSMA) on glufosinate efficacy in browntop millet, hemp sesbania, ivyleaf morningglory [*Ipomoea hederacea* (L.) Jacq.], johnsongrass [*Sorghum halepense* (L.) Pers.], Palmer amaranth, pitted morningglory (*Ipomoea lacunosa* L.), prickly sida (*Sida spinosa* L.), redroot pigweed, and velvetleaf (*Abutilon theophrasti* Medik.). Therefore, though additive interactions between glufosinate and 2,4-D or dicamba were observed in GR giant ragweed, those interactions may vary with other weed species or tank-mix partners, including differing rates of 2,4-D or dicamba with glufosinate not tested in this study.

CONCLUSION

Results of this study indicated that tank-mixing glufosinate with 2,4-D or dicamba showed an additive interaction and provided an effective POST option for the control of GR giant ragweed in maize and secured optimum yield. Although results of this study reported excellent control of giant ragweed with 2,4-D/dicamba tank-mixed with glufosinate, a diverse weed management program should be adopted by growers, because relying on these herbicides, particularly applied alone, may result in the evolution of resistant weeds. For example, 2,4-D-resistant common waterhemp in eastern Nebraska (Bernards et al., 2012) and dicamba-resistant kochia in western Nebraska (Crespo et al., 2014) have been confirmed. Similarly, glufosinate resistance has been reported in few weed species including goosegrass, Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot], and perennial ryegrass (*Lolium perenne* L.) (Jalaludin et al., 2017). Thus, over-reliance on 2,4-D, glufosinate, or dicamba should be avoided and a diversity of herbicide chemistries must be maintained by using herbicide tank-mixtures with multiple effective modes of action, along with non-chemical weed control methods including crop rotation, tillage, competitive cultivars, weed seed destruction, and cover crops, among others (Norsworthy et al., 2012).

The rapid evolution of GR weeds has emphasized the importance of diverse weed management approaches, including PRE followed by POST herbicide programs along with non-chemical methods (Norsworthy et al., 2012; Riley and Bradley, 2014). Therefore, to avoid overdependence on these herbicide mixtures and ensure an effective use of multiple-resistant crop technology without enhancing the evolution of multiple herbicide-resistant weeds, an integrated weed management approach for GR giant ragweed or other weed species should be implemented. Recently, integrated weed management approaches involving preplant tillage followed by PRE and/or POST herbicides with multiple modes of action have been developed for the effective management of GR giant ragweed in maize and soybean (Ganie et al., 2016, 2017). Future studies should consider the evaluation of these herbicide mixtures for the control of other prominent GR weed species including common ragweed, common waterhemp, horseweed, kochia (*Kochia scoparia* L.), and Palmer amaranth in Nebraska.

AUTHOR CONTRIBUTIONS

ZG conducted the experiments, analyzed the data and wrote the manuscript and AJ conceptualized and designed the research and edited manuscript.

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Control of Glyphosate-Resistant Common Ragweed (*Ambrosia artemisiifolia* L.) in Glufosinate-Resistant Soybean [*Glycine max* (L.) Merr]

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Common ragweed emerges early in the season in Nebraska, USA and is competitive with soybean; therefore, preplant herbicides are important for effective control. Glyphosate has been used as a preplant control option; however, confirmation of glyphosate-resistant (GR) common ragweed in Nebraska necessitates evaluating other herbicide options. The objectives of this study were to (1) evaluate the efficacy of preplant (PP) herbicides followed by (fb) glufosinate alone or in tank-mixture with imazethapyr, acetochlor, or *S*-metolachlor applied post-emergence (POST) for control of GR common ragweed in glufosinate-resistant soybean; (2) their effect on common ragweed density, biomass, and soybean yield; and (3) the partial economics of herbicide programs. A field experiment was conducted in a grower's field infested with GR common ragweed in Gage County, Nebraska, USA in 2015 and 2016. Preplant herbicide programs containing glufosinate, paraquat, 2,4-D, dimethenamid-P, cloransulam-methyl, or high rates of flumioxazin plus chlorimuron-ethyl provided 90–99% control of common ragweed at 21 d after treatment (DAT). The aforementioned PP herbicides fb a POST application of glufosinate alone or in tank-mixture with imazethapyr, acetochlor, or *S*-metolachlor controlled GR common ragweed 84–98% at soybean harvest, reduced common ragweed density (≤20 plants m^{-2}) and biomass by ≥93%, and secured soybean yield 1,819–2,158 kg ha^{-1}. The PP fb POST herbicide programs resulted in the highest gross profit margins (US$373–US$506) compared to PP alone (US$91) or PRE fb POST programs (US$158). The results of this study conclude that effective and economical control of GR common ragweed in glufosinate-resistant soybean is achievable with PP fb POST herbicide programs.

Keywords: gross profit margin, herbicide efficacy, residual herbicides, tank-mixture, weed resistance

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INTRODUCTION

Common ragweed (*Ambrosia artemisiifolia* L.) is a native, herbaceous, annual weed that belongs to the Asteracea family and is commonly found throughout temperate North America (Dickerson and Sweet, 1971; Coble et al., 1981). Common ragweed typically emerges early in the season in Nebraska, USA (Werle et al., 2014; Barnes et al., in press) and is a competitive weed in several

agronomic crops, including soybean. Coble et al. (1981) reported that four common ragweed plants 10 m^{-1} row reduced 8% soybean yield. Similarly, Shurtleff and Coble (1985) and Weaver (2001) reported that 1.6 common ragweed plants m^{-1} row reduced soybean yield by 12 and 11%, respectively. Common ragweed is a monoecious species that has the potential to produce several thousand seeds per plant. A large (2.4 kg fresh weight) common ragweed plant can produce up to 62,000 seeds (Dickerson and Sweet, 1971) and can grow up to 2 m in height (Bassett and Crompton, 1975; Clewis et al., 2001). Allowing common ragweed seeds to enter the seed bank can lead to long term concern as seeds can remain viable in the soil for 39 years (Bassett and Crompton, 1975).

Glyphosate is a broad-spectrum, systemic, post-emergence (POST) herbicide (Duke and Powles, 2008) first marketed in 1974 (Franz et al., 1997). In 1996, GR soybean was first commercialized in the United States (Wiesbrook et al., 2001), and as of 2016, commercially grown GR crops include alfalfa (*Medicago sativa* L.), canola (*Brassica napus* L.), corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L.), and sugarbeet (*Beta vulgaris* L.) (Duke and Powles, 2009). With the commercialization of GR crops, POST application of glyphosate increased dramatically (Dill, 2005), resulting in the evolution of GR weeds. As of 2016, glyphosate resistance has been reported in 37 weed species globally, including 16 species in the United States (Heap, 2017). Missouri was the first state to confirm GR common ragweed in 2004 (Pollard, 2007; Heap, 2017), and since then, GR common ragweed has been confirmed in 15 states in the United States and in Ontario, Canada (Heap, 2017). GR common ragweed has been recently confirmed as the sixth GR weed in Nebraska, USA (Ganie and Jhala, 2017). In response to widespread adoption of GR corn and soybean, and the effective, broad-spectrum, and affordable weed control with glyphosate, no-tillage, and reduced tillage production systems increased as the use of glyphosate replaced pre-plant tillage (Givens et al., 2009). No-till soybean production reduces soil erosion and operating cost while providing comparable yields to conventional tillage systems (Stougaard et al., 1984).

Glufosinate blocks the glutamine synthetase enzyme, which leads to buildup of ammonium in plant tissue (Logusch et al., 1991). Glufosinate is a broad-spectrum, contact herbicide (Haas and Muller, 1987). Glufosinate-resistant soybean was first commercialized in 1999 (Wiesbrook et al., 2001). It can be applied up to 1,329 g ai ha^{-1} per growing season in glufosinate-resistant soybean in either single or sequential (>5 d apart) application up to but not including the bloom soybean growth stage (Anonymous, 2016). Glufosinate has no plant-back interval for corn or soybean and can be applied in a range of 593–736 g ai ha^{-1} in a single application depending on weed pressure (Anonymous, 2016). Glufosinate is an alternative herbicide option for control of GR weeds in glufosinate-resistant soybean if applied as per label direction (Jhala et al., 2014; Kaur et al., 2014).

Management of GR weeds is a challenge for soybean producers in Nebraska. Widespread occurrence of GR weeds in several states in the Midwestern United States, including Nebraska, requires alternate weed management programs. Planting of glufosinate-resistant soybean is increasing in several states, specifically for control of GR weeds. A survey conducted in 2011 in Arkansas reported that 12% of the soybean acreage was seeded to glufosinate-resistant cultivars (Riar et al., 2013), a number that had increased to 35% by 2016 (JK Norsworthy, personal communication). Similarly, the use of glufosinate-resistant soybean cultivars has increased in recent years in the Midwest (Jhala et al., 2017).

Preplant application of 2,4-D, flumioxazin, glufosinate, paraquat, saflufenacil, or sulfentrazone followed by (fb) a POST application of glufosinate alone or in tank-mixtures effectively controlled GR giant ragweed (*Ambrosia trifida* L.), a closely related species of common ragweed, in Nebraska (Kaur et al., 2014). Aulakh and Jhala (2015) reported that sulfentrazone plus metribuzin applied PRE fb a POST application of glufosinate tank-mixed with acetochlor, pyroxasulfone, or *S*-metolachlor controlled common lambsquarters (*Chenopodium album* L.), common waterhemp (*Amaranthus rudis* Sauer), eastern black nightshade (*Solanum ptychanthum* Dunal), velvetleaf (*Abutilon theophrasti* Medik.), large crabgrass (*Digitaria sanguinalis* L.), and green foxtail (*Setaria viridis* L.) ≥90% in glufosinate-resistant soybean. Van Wely et al. (2014, 2015) concluded that neither a single PP nor a single POST herbicide application provided full season control of GR common ragweed in GR soybean in Ontario and that PP fb POST programs would need to be considered. Common ragweed's early emergence reduces the likelihood of controlling it with a PRE application as most common ragweed have already emerged. The control of summer emerging weeds such as common waterhemp or Palmer amaranth (*Amaranthus palmeri S. Wats.*) requires the use of a residual PRE herbicide for control (Oliveira et al., 2017; Sarangi et al., 2017).

There has been no study published in the scientific literature about control of GR common ragweed in glufosinate-resistant soybean. The objectives of this study were to evaluate the efficacy of PP herbicides fb glufosinate alone or in tank-mixture with acetochlor, imazethapyr, or *S*-metolachlor for control of GR common ragweed in glufosinate-resistant soybean, their effect on soybean injury and yield, and the economics of herbicide programs. The hypothesis for this study was that a PP application of an effective herbicide fb glufosinate will provide effective control of GR common ragweed in glufosinate-resistant soybean.

MATERIALS AND METHODS

Field experiments were conducted in Gage County, Nebraska, USA in 2015 and 2016 in a field with confirmed GR common ragweed infestation (Ganie and Jhala, 2017). The field was non-irrigated and in a corn-soybean rotation which was planted to corn in 2014 and soybean in 2015. The research site consisted of a Wymore silty clay loam (37.6% silt, 37.6% clay, and 24.8% sand) with 2.5% organic matter and a pH of 6.0. The experimental design was a randomized complete block with 14 treatments (**Table 1**) and four replications. The plot size was 3 m wide (4 soybean rows spaced 0.75 m apart) by 9 m in length. Glufosinate-resistant soybean (5290LL, NuPride Genetics Network P.O. Box 830911 Lincoln, NE 68583) was planted under no-tillage conditions on May 19, 2015 and May 26, 2016

TABLE 1 | Herbicide treatments, application timing and rate, and products used in a field experiment conducted in Gage County, NE in 2015 and 2016.

Herbicide program	Timing[a]	Rate	Trade name	Manufacturer[b]	Adjuvant[c]
		g ai ha^{-1}			
Glufosinate	PP	594	Liberty 280	Bayer	AMS
Saflufenacil + Imazethapyr +Dimethenamid-P fb Glufosinate	PP EPOST	95 + 1,100 740	Optill + Outlook fb Liberty 280	BASF + BASF fb Bayer	MSO + AMS AMS
Sulfentrazone + Cloransulam-methyl fb Glufosinate	PP EPOST	314 740	Authority First fb Liberty 280	FMC fb Bayer	COC + AMS AMS
Flumioxazin + Chlorimuron-ethyl fb Glufosinate	PP EPOST	140 740	Valor XLT fb Liberty 280	Valent fb Bayer	COC + AMS AMS
S-metolachlor + Metribuzin fb Glufosinate	PP EPOST	2,050 740	Boundary fb Liberty 280	Syngenta fb Bayer	COC + AMS AMS
Chlorimuron-ethyl + Flumioxazin +Thifensulfuron-methyl fb Glufosinate	PP EPOST	94 740	Envive fb Liberty 280	Dupont fb Bayer	COC + AMS AMS
2,4-D fb Glufosinate + Imazethapyr	PP EPOST	1,180 740 + 70	2,4-D Amine fb Liberty 280 + Pursuit	Winfield fb Bayer + BASF	NIS + AMS NIS + AMS
Paraquat fb Glufosinate + Chlorimuron-ethyl + Acetochlor	PP EPOST	1,120 740 + 13.1 + 1,680	Gramoxone Inteon fb Liberty 280 + Classic + Warrant	Syngenta fb Bayer + Dupont + Monsanto	COC NIS + AMS
Saflufenacil fb Glufosinate + Acetochlor	PP EPOST	150 740 + 1,680	Sharpen fb Liberty 280 + Warrant	BASF fb Bayer + Monsanto	MSO + AMS AMS
Saflufenacil + 2,4-D fb Glufosinate + Acetochlor	PP EPOST	150 + 1,180 740 + 1,680	Sharpen + 2,4-D Amine fb Liberty 280 + Warrant	BASF + Winfield fb Bayer + Monsanto	MSO + AMS AMS
Flumioxazin + Chlorimuron-ethyl fb Glufosinate + S-metolachlor fb Glufosinate + Acetochlor	PP EPOST LPOST	112 594 + 1,480 594 + 1,260	Valor XLT fb Liberty 280 fb Warrant	Valent fb Bayer fb Monsanto	COC + AMS NIS + AMS AMS
2,4-D fb Sulfentrazone + Metribuzin fb Glufosinate	PP PRE LPOST	1,180 5.7 740	2,4-D Amine fb Authority MTZ fb Liberty 280	Winfield fb FMC fb Bayer	NIS + AMS COC AMS
Sulfentrazone + Metribuzin fb Glufosinate	PRE LPOST	6.3 740	Authority MTZ fb Liberty 280	FMC fb Bayer	COC AMS

[a] *AMS, ammonium sulfate (DSM Chemicals orth America Inc., Augusta, GA); COC, crop oil concentrate (Agridex, Helena Chemical Co., Collierville, TN); PP, Preplant; EPOST, early POST; LPOST, late POST; fb, followed by; MSO, methylated seed oil (Southern Ag Inc., Suwanee, GA); NIS, nonionic surfactant (Induce, Helena Chemical Co., Collierville, TN).*

[b] *Bayer CropScience, Research Triangle Park, NC 27709; Valent U.S.A. Corporation, Walnut Creek, CA 94596; FMC Corporation, Philadelphia, PA 19103; BASF Corporation, Research Triangle Park, NC 27709; Syngenta Crop Protection, Greensboro, NC 27419; Monsanto Company, St. Louis, MO 63167; DuPont Crop Protection, P.O. Box 80705, Wilmington, DE 19880; Winfield Solutions, LLC, P.O. Box 64589, St. Paul, MN 55164-0589.*

[c] *AMS at 2% (wt/v), COC or MSO at 1% (v/v), and NIS at 0.25% (v/v) were mixed with herbicides.*

at a population of 300,000 seeds ha^{-1} to a depth of 3 cm. The experiments included 13 herbicide programs comprised of four application timings: preplant (PP), pre-emergence (PRE), early POST (EPOST), and late POST (LPOST) (**Table 1**). The field experiments were conducted at grower's field infested with glyphosate-resistant common ragweed. The grower's field had limited space available to conduct research projects. Therefore, the treatment list was restricted and a weed-free control was not included. POST applications were scheduled based on soybean growth stage with EPOST applied around the third soybean trifoliate and LPOST applied before soybean began flowering. For comparison, a non-treated control was included. The labeled rate of each herbicide was used for all treatments.

Herbicides were applied with a CO_2 pressurized backpack sprayer and a boom equipped with four TT 110015 flat-fan nozzles (TeeJet, Spraying Systems Co., P.O. Box 7900, Wheaton, IL 60189) spaced 60 cm apart. Treatments were applied as PP (May 1, 2015 and May 5, 2016), PRE (May 21, 2015 and May 26, 2016), EPOST (June 16, 2015 and June 16, 2016), and LPOST (July 17, 2015 and June 30, 2016). Common ragweed ranged from 1–8 cm tall at the time of PP, 4–16 cm at PRE, 16–45 cm at EPOST, and 36–60 cm at the time of LPOST. Common ragweed control was assessed visually on a scale of 0–100%, with 0% representing no control and 100% representing complete control, at 21 d after PP and PRE, 14 DAEPOST and LPOST, and at soybean harvest. Soybean injury was assessed on a scale of 0–100%, with 0% representing no injury and 100% representing plant death, at 21 DAPRE, and 14 DAEPOST and LPOST. Common ragweed densities were assessed from two randomly placed 0.25 m^2 quadrats in each plot at 7 DAPRE, 14 DAEPOST, and 14 DALPOST. Common ragweed aboveground biomass was assessed from two randomly placed 0.25 m^2 quadrat in each plot at 70 DALPOST. Surviving common ragweed plants were cut near the soil surface, dried in paper bags at 50 C for 10 d, and their biomass was recorded. Percent biomass reduction compared with the non-treated control was calculated using the

equation (Wortman, 2014):

$$\% \text{ Biomass reduction} = [(C - B)/C] \times 100 \quad (1)$$

where C represents the common ragweed biomass from the non-treated control plot in the corresponding replication block and B represents the biomass of the treatment plots. Soybean was harvested with a plot combine and the yields were adjusted to 13% moisture content. Gross profit margin was calculated as gross revenue minus herbicide and application costs (Norsworthy and Oliver, 2001). Average herbicide prices from three independent commercial sources (Cargill, Country Partners Cooperative, Crop Production Services) in Nebraska were used to calculate herbicide cost ha^{-1}. Herbicide program cost was calculated by summing the herbicide cost ha^{-1} for each treatment and adding a custom application cost of US$18.11 ha^{-1} application^{-1}, the average of the three aforementioned independent sources in Nebraska. Gross revenue was calculated from the average yield for each treatment based on the average price received in Nebraska during harvest time in 2015 and 2016 (US$0.33 kg^{-1}; USDA, 2016).

Statistical Analysis

Data were subjected to ANOVA using PROC GLIMMIX procedure in SAS version 9.3 (SAS Institute Inc., Cary, NC). Years and treatments were considered fixed effects and replications nested within year were considered random effects in the model. Data were tested for normality using PROC UNIVARIATE before analysis. An arcsine square-root transformation was performed on common ragweed control estimates and biomass reduction data before analysis; however, data were back-transformed for presentation of results. Treatment means were separated at $P \leq 0.05$ using Fisher's protected least significant difference test. Orthogonal contrasts were conducted to compare PP fb POST treatments vs. PP alone, PRE fb LPOST, or PP fb PRE fb LPOST treatments.

RESULTS

Year-by-treatment interactions for GR common ragweed control, density, biomass, and soybean yield were not significant; therefore, data were combined. Average daily temperatures during the study were similar to the 30-year average (**Table 2**). May and June of 2015 received higher precipitation (36.2 cm) than the 30-year average (18.6 cm); however, the 2016 growing season received similar precipitation to the 30-year average (16.3 cm in May and June; **Table 2**).

TABLE 2 | Average monthly temperature and precipitation in a field experiment conducted in Gage County, NE in 2015 and 2016[a,b].

	Temperature (C)			Precipitation (cm)		
	2015	2016	30 yr avg	2015	2016	30 yr avg
May	15.5	15.6	16.1	11.7	7.0	6.3
June	21.6	23.4	21.7	24.5	9.3	12.3
July	23.6	22.4	24.3	13.2	6.4	11.0
August	21.5	23.0	23.6	9.2	9.1	10.2
September	21.6	20.3	18.8	6.0	12.2	9.0

[a] *30 yr avg, 30 year average (1981–2010).*

[b] *Monthly weather data acquired from the nearest High Plains Regional Climate Center station near Virginia, NE.*

Common Ragweed Control

Most of the PP herbicides controlled GR common ragweed ≥90% at 21 DAPP (**Table 3**). For example, herbicide programs containing glufosinate, paraquat, 2,4-D, imazethapyr, cloransulam-methyl, and flumioxazin provided 90–99% control of common ragweed at 21 DAPP (**Table 3**). A premix of flumioxazin and chlorimuron-ethyl provided 93–96% control at 14 DAPP in this study. Saflufenacil controlled common ragweed 75% at 21 DAPP; however, tank-mixing with imazethapyr plus dimethenamid-P as well as with 2,4-D provided 97 and 99% control, respectively (**Table 3**). Among PP herbicide programs, chlorimuron-ethyl plus flumioxazin plus thifensulfuron-methyl resulted in the lowest (52%) common ragweed control at 21 DAPP.

A PRE application of sulfentrazone plus metribuzin following a PP application of 2,4-D controlled GR common ragweed 97% at 21 DAPRE, comparable with several other treatments with only PP application; however, when sulfentrazone plus metribuzin was applied PRE even at a higher rate (6.3 g ai ha^{-1}) without a PP herbicide application, it resulted in 18% control of common ragweed at 21 DAPRE (**Table 3**). Moreover, the contrast statement confirmed that PP applications controlled 80% of common ragweed compared to a PRE application that resulted in only 18% control at 21 DAPRE (**Table 3**).

The PP herbicides fb glufosinate EPOST, alone or in tank-mixtures, controlled common ragweed 91–99% at 21 DAEPOST (**Table 3**). A LPOST application of glufosinate following a PP application of 2,4-D and a PRE application of sulfentrazone plus metribuzin controlled GR common ragweed 99% at 14 DALPOST. Glufosinate LPOST following sulfentrazone plus metribuzin PRE controlled GR common ragweed 92%. Glufosinate plus acetochlor applied LPOST following an EPOST application of glufosinate plus *S*-metolachlor and a PP application of flumioxazin plus chlorimuron-ethyl controlled GR common ragweed 99% at 14 DALPOST, comparable with several PP fb EPOST programs.

Most of herbicide programs that included both a PP and POST herbicide application provided season-long control (≥87%) of common ragweed at harvest (**Table 3**). Herbicide programs including chlorimuron-ethyl plus flumioxazin plus thifensulfuron-methyl or saflufenacil applied PP fb glufosinate EPOST, alone or tank-mixed with acetochlor, controlled GR common ragweed 62–64% at harvest. A single PP application of glufosinate controlled GR common ragweed 0% at harvest, suggesting that an in-crop application is needed for season-long common ragweed control. Sulfentrazone plus metribuzin applied PRE fb glufosinate applied LPOST controlled GR common ragweed 88% at harvest; however, when a PP application of 2,4-D was added to the program, the control increased to 99%. Orthogonal contrasts conclude that PP and PP fb EPOST herbicide programs controlled GR common ragweed 0 and 86%

TABLE 3 | Orthogonal contrasts for comparison of herbicide programs and control of glyphosate-resistant common ragweed in glufosinate-resistant soybean at 21 DAPP, 21 DAPRE, 14 DAEPOST, 14 DALPOST, and at harvest in a field experiment conducted in Gage County, NE in 2015 and 2016[a].

Herbicide program	Timing	Rate	Common ragweed control[b,c]				
			21 DA PP(%)	21 DA PRE(%)	14 DA EPOST(%)	14 DA LPOST(%)	At Harvest(%)
		g ai ha^{-1}					
Glufosinate	PP	594	92 ab	81 bc	40 d	0 f	0 e
Saflufenacil + Imazethapyr +Dimethenamid-P fb Glufosinate	PP EPOST	95 + 1,100 740	97 a	94 ab	97 abc	97 ab	97 abc
Sulfentrazone + Cloransulam-methyl fb Glufosinate	PP EPOST	314 740	96 a	91 ab	97 abc	97 ab	96 abc
Flumioxazin + Chlorimuron-ethyl fb Glufosinate	PP EPOST	140 740	93 a	84 abc	96 abc	96 abc	84 bc
S-metolachlor + Metribuzin fb Glufosinate	PP EPOST	2,050 740	76 b	70 cd	92 bc	91 de	87 abc
Chlorimuron-ethyl + Flumioxazin + Thifensulfuron-methyl fb Glufosinate	PP EPOST	94 740	52 c	45 e	91 c	88 e	62 d
2,4-D fb Glufosinate + Imazethapyr	PP EPOST	1,180 740 + 70	90 ab	88 abc	98 ab	97 ab	96 abc
Paraquat fb Glufosinate + Chlorimuron-ethyl + Acetochlor	PP EPOST	1,120 740 + 13.1 + 1,680	96 a	80 bc	96 abc	95 bcd	94 abc
Saflufenacil fb Glufosinate + Acetochlor	PP EPOST	150 740 + 1,680	75 b	55 de	93 bc	91 cde	64 d
Saflufenacil + 2,4-D fb Glufosinate + Acetochlor	PP EPOST	150 + 1,180 740 + 1,680	99 a	98 a	99 a	99 a	98 ab
Flumioxazin + Chlorimuron-ethyl fb Glufosinate + S-metolachlor fb Glufosinate + Acetochlor	PP EPOST LPOST	112 594 + 1,480 594 + 1,260	96 a	92 ab	99 a	99 a	99 a
2,4-D fb Sulfentrazone + Metribuzin fb Glufosinate	PP PRE LPOST	1,180 5.7 740	95 a	97 a	93 bc	99 a	99 ab
Sulfentrazone + Metribuzin fb Glufosinate	PRE LPOST	6.3 740	0 c	18 f	9 e	92 e	88 c
P-value			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
ORTHOGONAL CONTRASTS[d]							
PP vs. PRE			–	80 vs. 18 ****	–	–	–
PP fb EPOST vs. PP only			–	–	95 vs. 40 ****	95 vs. 0 ****	86 vs. 0 ****
PP fb EPOST vs. PRE fb LPOST			–	–	95 vs. 9 ****	95 vs. 92 ns	86 vs. 88 ns
PP fb EPOST vs. PP fb PRE fb LPOST			–	–	95 vs. 93 ns	95 vs. 99 **	86 vs. 99 *
PP fb EPOST vs. PP fb EPOST fb LPOST			–	–	–	95 vs. 99 **	86 vs. 99 *

[a] *DA, days after; EPOST, early POST; fb, followed by; LPOST, late POST; PP, Preplant.*

[b] *Year by treatment interaction was not significant; therefore, data from both years were combined. Data were arc-sine square-root transformed before analysis; however, back transformed values are presented based on the interpretation from the transformed data.*

[c] *Means presented within the same column with no common letter(s) are significantly different according to Fisher's Protected LSD where $\alpha = 0.05$.*

[d] *Significance levels: ns, non-significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.*

at harvest, respectively, and PP fb PRE fb LPOST program controlled GR common ragweed 99% at harvest (**Table 3**).

Common Ragweed Density and Biomass

Common ragweed density for the non-treated control was 1,337 and 1,159 plants m^{-2} at 7 DAPRE and 14 DAEPOST, respectively, compared with the average of herbicide treatments (305 and 177 plants m^{-2}, respectively; **Table 4**). Preplant herbicides resulted in common ragweed densities of 0 to 366 plants m^{-2}, except saflufenacil (844 plants m^{-2}), and chlorimuron-ethyl plus flumioxazin plus thifensulfuron-methyl (1,180 plants m^{-2}; **Table 4**). The PP application of 2,4-D fb sulfentrazone plus

TABLE 4 | Orthogonal contrasts for comparison of herbicide programs and effect of herbicide programs on glyphosate-resistant common ragweed density at 7 DAPRE, 14 DAEPOST, and 14 DALPOST, common ragweed biomass reduction, and soybean yield in a field experiment conducted in Gage County, NE in 2015 and 2016[a].

Herbicide	Timing	Rate	Common ragweed density[b]			Biomass reduction[b,c]	Soybean yield[b]
			7 DA PRE	14 DA EPOST	14 DA LPOST		
		g ai ha^{-1}		Plants m^{-2}		%	kg ha^{-1}
Nontreated control			1,337 a	1,159 a	1,145[d]	0 e	32 c
Glufosinate	PP	594	159 c	120 c	101 a	14 e	474 c
Saflufenacil + Imazethapyr +Dimethenamid-P fb Glufosinate	PP EPOST	95 + 1,100 740	11 c	0 c	0 d	98 abcd	2,158 a
Sulfentrazone + Cloransulam-methyl fb Glufosinate	PP EPOST	314 740	67 c	17 c	15 cd	100 ab	1,922 a
Flumioxazin + Chlorimuron-ethyl fb Glufosinate	PP EPOST	140 740	221 c	18 c	20 bcd	93 abcd	1,897 a
S-metolachlor + Metribuzin fb Glufosinate	PP EPOST	2,050 740	366 c	57 c	56 abc	86 abcd	1,819 a
Chlorimuron-ethyl + Flumioxazin + Thifensulfuron-methyl fb Glufosinate	PP EPOST	94 740	1,180 ab	108 c	102 a	82 d	1,860 a
2,4-D fb Glufosinate + Imazethapyr	PP EPOST	1,180 740 + 70	88 c	6 c	8 cd	99 abc	1,899 a
Paraquat fb Glufosinate + Chlorimuron-ethyl + Acetochlor	PP EPOST	1,120 740 + 13.1 + 1,680	59 c	3 c	0 d	93 abcd	1,859 a
Saflufenacil fb Glufosinate + Acetochlor	PP EPOST	150 740 + 1,680	844 b	62 c	68 ab	81 cd	1,819 a
Saflufenacil + 2,4-D fb Glufosinate + Acetochlor	PP EPOST	150 + 1,180 740 + 1,680	0 c	0 c	0 d	100 a	2,115 a
Flumioxazin + Chlorimuron-ethyl fb Glufosinate + S-metolachlor fb Glufosinate + Acetochlor	PP EPOST LPOST	112 594 + 1,480 594 + 1,260	51 c	0 c	0 d	100 a	2,003 a
2,4-D fb Sulfentrazone + Metribuzin fb Glufosinate	PP PRE LPOST	1,180 5.7 740	8 c	7 c	0 d	100 a	2,060 a
Sulfentrazone + Metribuzin fb Glufosinate	PRE LPOST	6.3 740	908 ab	744 b	93 a	83 bcd	1,014 b
P-value			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
ORTHOGONAL CONTRASTS[e]							
PP vs. PRE			277 vs. 908 ***	–	–	–	–
PP fb EPOST vs. PP only			–	30 vs. 120 ns	30 vs. 101 ***	92 vs. 14 ****	1,928 vs. 474 ****
PP fb EPOST vs. PRE fb LPOST			–	30 vs. 744 ****	30 vs. 93 **	92 vs. 83 ns	1,928 vs. 1,014 ****
PP fb EPOST vs. PP fb PRE fb LPOST			–	30 vs. 7 ns	30 vs. 0 ns	92 vs. 100 ns	1,928 vs. 2,060 ns
PP fb EPOST vs. PP fb EPOST fb LPOST			–	–	30 vs. 0 ns	92 vs. 100 ns	1,928 vs. 2,003 ns

[a] *DA, days after; EPOST, early POST; fb, followed by; LPOST, late POST; PP, preplant; vs., versus.*

[b] *Means presented within the same column with no common letter(s) are significantly different according to Fisher's Protected LSD where* $\alpha = 0.05$.

[c] *Year by treatment interaction was not significant; therefore, data from both years were combined. Data were arc-sine square-root transformed before analysis; however, back transformed values are presented based on the interpretation from the transformed data.*

[d] *Nontreated control was excluded from analysis as an outlier.*

[e] *Significance levels: ns, non-significant;* $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

metribuzin applied PRE reduced common ragweed density to eight plants m^{-2} at 7 DAPRE compared with sulfentrazone plus metribuzin applied PRE without PP herbicide application (908 plants m^{-2}; **Table 4**).

Based on orthogonal contrasts, PP and PRE herbicide programs on average resulted in 277 and 908 plants m^{-2} at 7 DAPRE, respectively (**Table 4**). The PP fb PRE fb LPOST or PP fb EPOST fb LPOST did not reduce common ragweed

densities compared to PP fb EPOST programs. Averaged across treatments, PP fb EPOST program had lower common ragweed density (30 plants m^{-2}) compared with PP (101 plants m^{-2}) or PRE fb LPOST (93 plants m^{-2}) program at 14 DALPOST (**Table 4**). Most herbicide programs with PP application resulted in 81–100% common ragweed biomass reduction. Averaged across treatments, PP, PP fb EPOST, and PRE fb LPOST reduced GR common ragweed biomass 14, 92, and 83%, respectively.

Soybean Yield

The lowest soybean yield was obtained in the non-treated control (32 kg ha^{-1}) and with glufosinate applied PP (474 kg ha^{-1}). Herbicide programs that included an effective PP fb glufosinate applied POST, alone or in tank-mixture, resulted in soybean yields 1,819–2,158 kg ha^{-1} with no difference among them (**Table 4**). Averaged across treatments, PP fb EPOST programs resulted in higher yields (1,928 kg ha^{-1}) compared with PP alone (474 kg ha^{-1}) or PRE fb LPOST (1,014 kg ha^{-1}) herbicide programs (**Table 4**). Averaged across treatments, PP fb EPOST programs resulted in similar yields (1,928 kg ha^{-1}) compared with PP fb PRE fb EPOST (2,060 kg ha^{-1}) or PP fb EPOST fb LPOST (2,003 kg ha^{-1}); therefore, if common ragweed is the major weed in a soybean field, a PP fb EPOST program can provide full season control and PP fb PRE fb POST programs are not needed to achieve optimum soybean yield (**Table 4**).

Economics

The cost of PP fb POST herbicide programs ranged from US$131.30 to $257.87 ha^{-1} and provided maximum gross profit margins (**Table 5**). The PP application of saflufenacil plus imazethapyr plus dimethenamid-P fb glufosinate EPOST cost $197.37 ha^{-1} and resulted in the highest gross profit margin of $505.96 ha^{-1} (**Table 5**). Glufosinate applied PP alone had the lowest cost ($63.25 ha^{-1}); but resulted in a gross profit margin of only $91.23 ha^{-1} due to poor control of common ragweed that resulted in low soybean yield (**Table 5**). The PRE fb LPOST program resulted in a gross profit margin of $158.23 ha^{-1} (**Table 5**). Although the PP fb PRE fb POST program resulted in 99% common ragweed control and 2,060 kg ha^{-1} soybean yield, gross profit margin was $471.14 compared with $372.79 to $505.96 for PP fb POST programs due to additional cost of PRE herbicide and application.

DISCUSSION

Six GR weeds, including common ragweed, have been confirmed in Nebraska and their management is a challenge for crop producers. This is the first report describing control of GR common ragweed in glufosinate-resistant soybean. Common ragweed is an early emerging weed in Nebraska. It has been reported that common ragweed start emerging in March reaching 10% emergence around 259 growing degree day (GDD) and 90% of emergence is achieved by the first or second week of May or 757 GDD calculated with a base temperature of 3 C (Barnes et al., in press). Shrestha et al. (1999) reported the base temperature for common ragweed to be 3.6 C. Therefore, as observed in this study, preplant application of herbicide is critical for control of GR common ragweed. This agrees with the findings of Kaur et al. (2014) and Jhala et al. (2014) reporting that PP application of herbicide is important for control of GR giant ragweed in Nebraska. Similarly, Ganie et al. (2016) reported that GR giant ragweed control was reduced to <83% at 21 DAPRE and ≤78% at harvest when PP herbicides were not included in the program.

Results of this study reported that a number of herbicide options such as saflufenacil plus imazethapyr plus dimethenamid-P, suflentrazone plus cloransulam-methyl, paraquat, safluefenacil plus 2,4-D, 2,4-D, and flumioxazin plus chlorimuron-ethyl are available for common ragweed control. Control of 1–8 cm tall common ragweed with PP herbicides in this study was similar to that which was reported in the literature. For example, Corbett et al. (2004) reported ≥99% control of 2–10 cm tall common ragweed at 14 and 20 DAT with glufosinate. Additionally, 83–85% common ragweed control was reported at 14 DAT with cloransulam-methyl (Taylor et al., 2002). Wilson and Worsham (1988) reported 83 and 64% common ragweed control at 28 DAT from paraquat and 2,4-D, respectively, with half the rates used in this study. Niekamp et al. (1999) reported 98% common ragweed control at 7 DAT with flumioxazin (90 g ai ha^{-1}) and chlorimuron-ethyl (70 g ai ha^{-1}). Kaur et al. (2014) further reported 96% control of giant ragweed with saflufenacil applied alone and 99% control when tank-mixed with 2,4-D at 14 DAT.

Preplant herbicide was followed by PRE and/or POST herbicide application for season-long control of GR common ragweed. A follow up application after PP was needed to avoid poor control and potential yield reduction. Glufosinate applied POST alone or in a tank-mixture with imazethapyr, acetochlor, or *S*-metolachlor controlled GR common ragweed 84–98%. Tharp and Kells (2002) reported that PRE herbicide followed by glufosinate controlled common ragweed, redroot pigweed (*Ameranthus retroflexus* L.), and common lambsquarters ≥92% at 28 DAT. Tharp and Kells (2002) also reported that glufosinate tank-mixed with *S*-metolachlor or acetochlor controlled common ragweed ≥99% at 28 DAT. Although a PP application fb sequential POST applications provided 99% control at 14 DALPOST, statistically it was comparable to PP fb single POST programs indicating that an effective PP herbicide fb a single POST application of glufosinate controlled GR common ragweed >90% and that a second POST application is not needed.

Preplant fb POST herbicide programs on average resulted in less common ragweed density (30 plants m^{-2}) and greater biomass reductions (92%) than single applications which is consistent with the literature. For instance, Aulakh and Jhala (2015) reported ≤4 plants m^{-2} for common lambsquarters, common waterhemp, eastern black nightshade, and velvetleaf; and ≤2 plants m^{-2} for green foxtail and large crabgrass at harvest with the use of PRE fb POST programs in glufosinate-resistant soybean. Moreover, programs including three herbicide applications (PP fb PRE fb POST) did not result in fewer common ragweed or more biomass reduction, suggesting that PP fb POST effectively reduces common ragweed density. Aulakh and Jhala (2015) reported the greatest biomass reduction of broadleaf and grass weeds in glufosinate-resistant soybean with PRE fb POST compared to single or sequential POST programs.

TABLE 5 | Cost of herbicide programs for controlling glyphosate-resistant common ragweed in glufosinate-resistant soybean, income from soybean yield, and gross profit margin in a field experiment conducted in Gage County, NE in 2015 and 2016.[a]

Herbicide	Timing	Rate	Program cost[b]	Gross revenue from soybean yield[c]	Gross profit margin[d]
		g ai ha^{-1}		\$ ha^{-1}	
Nontreated control	–	–	0.00	10.43	10.43
Glufosinate	PP	594	63.25	154.48	91.23
Saflufenacil + Imazethapyr + Dimethenamid-P fb Glufosinate	PP EPOST	95 + 1,100 740	197.37	703.33	505.96
Sulfentrazone + Cloransulam-methyl fb Glufosinate	PP EPOST	314 740	180.21	626.41	446.20
Flumioxazin + Chlorimuron-ethyl fb Glufosinate	PP EPOST	140 740	168.33	618.26	449.94
S-metolachlor + Metribuzin fb Glufosinate	PP EPOST	2,050 740	155.21	592.84	437.63
Chlorimuron-ethyl + Flumioxazin + Thifensulfuron-methyl fb Glufosinate	PP EPOST	94 740	131.30	606.20	474.91
2,4-D fb Glufosinate + Imazethapyr	PP EPOST	1,180 740 + 70	146.20	618.92	472.71
Paraquat fb Glufosinate + Chlorimuron-ethyl + Acetochlor	PP EPOST	1,120 740 + 13.1 + 1,680	176.30	605.88	429.57
Saflufenacil fb Glufosinate + Acetochlor	PP EPOST	150 740 + 1,680	220.05	592.84	372.79
Saflufenacil + 2,4-D fb Glufosinate + Acetochlor	PP EPOST	150 + 1,180 740 + 1,680	230.01	689.31	459.30
Flumioxazin + Chlorimuron-ethyl fb Glufosinate + S-metolachlor fb Glufosinate + Acetochlor	PP EPOST LPOST	112 594 + 1,480 594 + 1,260	257.87	652.81	394.94
2,4-D fb Sulfentrazone + Metribuzin fb Glufosinate	PP PRE LPOST	1,180 5.7 740	200.25	671.39	471.14
Sulfentrazone + Metribuzin fb Glufosinate	PRE LPOST	6.3 740	172.25	330.48	158.23

[a] *Herbicide costs were averaged from three independent sources in Nebraska.*
[b] *Program cost includes an average cost of application (\$18.11 ha^{-1} $application^{-1}$) from three independent sources in Nebraska.*
[c] *Gross Revenue from soybean yield was based on an average price received in Nebraska on the harvest month.*
[d] *Gross profit margins were calculated as gross revenue from soybean yield minus program cost.*

Similarly, Kaur et al. (2014) reported that herbicide programs with PP applications of 2,4-D, flumioxazin plus chlorimuron-ethyl, sulfentrazone plus cloransulam-methyl, or paraquat fb EPOST of glufosinate, alone or in tank-mixture, resulted in $\leq$14 plants m^{-2} and $\geq$88% biomass reduction of giant ragweed. The benefits of reducing GR common ragweed biomass and density extend into the following years as fewer GR common ragweed seeds can potentially enter the seed bank. The long survivability of common ragweed in the seed bank necessitates control measures that reduce the number of seeds returning to the seed bank each year.

Single application of PP herbicides in glufosinate-resistant soybean were unable to protect soybean yield potential, indicating that common ragweed can be extremely competitive in soybean fields if not controlled, or if controlled with only PP herbicide application without additional follow up treatments. Kaur et al. (2014) reported 100% soybean yield reduction when GR giant ragweed was not controlled. Similarly, Jhala et al. (2014) reported that a PP alone treatment resulted in 100% soybean yield reduction due to giant ragweed competition later in the season compared with PP fb POST programs. Furthermore, Aulakh and Jhala (2015) reported that a single POST herbicide application was ineffective in protecting soybean yield potential.

Gross profit margins were maximized with PP fb POST herbicide programs. Single applications resulted in low gross profit margin because of the inability of a single application to provide season-long control of common ragweed, thus allowing common ragweed to compete with soybean. Additionally, herbicide programs that included three applications protected soybean yield potential but the cost of herbicide and additional application cost reduced gross profit margins substantially. PP fb POST herbicide programs protected soybean yield potential and reduced the cost of application over other herbicide programs tested in this study.

CONCLUSION

Results of this study conclude that PP herbicide options are available for early season control of GR common ragweed; however, a follow-up POST application of glufosinate alone or in tank-mixture is needed to achieve season-long control. Most of the PP herbicides tested in this study provided effective control (>95%) of common ragweed during soybean emergence and establishment. Furthermore, glufosinate can be used as an effective POST herbicide for control of GR common ragweed and can be tank-mixed with other herbicides such as *S*-metolachlor or acetochlor depending on the weed species present in the field. A recent update to the glufosinate (Liberty) label in the USA allows a maximum cumulative rate of 1,783 g ai ha^{-1} per growing season. Two applications, each of 656–881 g ai ha^{-1}, could be made POST in glufosinate-resistant soybean before flowering (Anonymous, 2017). Soybean yields were reduced when a PP application was not made compared to PP fb POST programs, primarily due to early season common ragweed competition. PP fb PRE fb POST programs did not decrease density, improve biomass reduction, or increase soybean yield compared to PP fb POST programs, suggesting that three time herbicide application is not necessary for controlling GR common ragweed or protecting soybean yield.

The continued use of glufosinate can result in the evolution of glufosinate-resistant weeds through increased selection pressure; for example, Italian ryegrass (*Lolium perenne* L. ssp. *multiflorum* Lam. Husnot), perennial ryegrass (*Lomium perenne* L.), and goosegrass (*Galium indica* L. Gaertn.) have been documented resistant to glufosinate due to continuous use (Avila-Garcia and Mallory-Smith, 2011; Ghanizadeh et al., 2015; Jalaludin et al., 2015). Although, results of this study reported that herbicide options exist for control of common ragweed, an integrated weed management approach should be adopted for control of herbicide-resistant weeds that can include rotation of herbicides, the use of herbicides with multiple effective sites of action, the reintegration of tillage, and crop rotation.

AUTHOR CONTRIBUTIONS

EB and AJ initiated research hypothesis and developed protocol; SK, PS, and JL Provided inout in the protocol; EB conducted research, analyzed data, and written manuscript. All coauthors provided input in the manuscript.

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Vacuolar Sequestration of Paraquat Is Involved in the Resistance Mechanism in *Lolium perenne* L. spp. *multiflorum*

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Lolium perenne L. spp. *multiflorum* (Lam.) Husnot (LOLMU) is a winter annual weed, common to row crops, orchards and roadsides. Glyphosate-resistant populations of LOLMU are widespread in California. In many situations, growers have switched to paraquat or other postemergence herbicides to manage glyphosate-resistant LOLMU populations. Recently, poor control of LOLMU with paraquat was reported in a prune orchard in California where paraquat has been used several times. We hypothesize that the low efficacy observed is due to the selection of a paraquat-resistant biotype of LOLMU. Greenhouse dose-response experiments conducted with a susceptible (S) and the putative paraquat-resistant biotype (PRHC) confirmed paraquat resistance in PRHC. Herbicide absorption studies indicated that paraquat is absorbed faster in S than PRHC, although the maximum absorption estimates were similar for the two biotypes. Conversely, translocation of ^{14}C-paraquat under light-manipulated conditions was restricted to the treated leaf of PRHC, whereas herbicide translocation out of the treated leaf was nearly 20 times greater in S. To determine whether paraquat was active within the plant cells, the photosynthetic performance was assessed after paraquat application using the parameter maximum quantum yield of photosystem II (F_V/F_m). Paraquat reaches the chloroplasts of PRHC, since there was a transitory inhibition of photosynthetic activity in PRHC leaves. However, PRHC F_V/F_m recovered to initial levels by 48 h after paraquat treatment. No paraquat metabolites were found, indicating that resistance is not due to paraquat degradation. LOLMU leaf segments were exposed to paraquat following pretreatments with inhibitors of plasma membrane- and tonoplast-localized transporter systems to selectively block paraquat intracellular movement. Subsequent evaluation of membrane integrity indicated that pre-exposure to putrescine resulted in the resistant biotype responding to paraquat similarly to S. These results strongly indicate that vacuolar sequestration is involved in the resistance to paraquat in this population of LOLMU.

Keywords: chlorophyll fluorescence, dose-response, herbicide absorption, herbicide metabolism, herbicide translocation, polyamines, putrescine

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INTRODUCTION

Lolium perenne L. spp. *multiflorum* (Lam.) Husnot (LOLMU) is a problem weed around the world and causes yield losses in a variety of cropping systems due to its rapid initial development, high biomass production, and plasticity (Hill et al., 1985). Herbicide resistance in LOLMU has been reported in several countries around the world to a variety of modes of action (Heap, 2017). It has an obligate outcrossing, self-incompatible breeding system, which facilitates the dispersal of herbicide resistance traits within and among populations (Loureiro et al., 2016) and, in some cases, results in the accumulation of herbicide resistance traits (Mahmood et al., 2016).

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) was first discovered in the mid-1950's, and has been widely used for weed control due to its broad postemergence spectrum of weed control, non-selectivity and soil-inactivity (Hawkes, 2014). Paraquat has a redox potential of −0.466 mV (Homer et al., 1960), acting as a preferential electron acceptor from ferredoxin (Em, −0.430) in the photosystem I complex (PSI). Upon reduction, the paraquat di-cation becomes paraquat mono-cation radical, which in turn transfer an electron to molecular oxygen, producing reactive oxygen species (ROS) (Summers, 1980). Because paraquat returns to its original di-cation state upon electron transfer to ROS, catalytic concentrations of the herbicide in the chloroplasts are sufficient to cause lipid peroxidation and tissue necrosis (Summers, 1980).

Foliar absorption studies have shown that the plant cuticle is not an impediment to paraquat absorption (Bishop et al., 1987). Uptake is generally rapid and maximum absorption often can reach 90% or greater (Soar et al., 2003). Paraquat translocation, conversely, is strongly influenced by light conditions after application. Plants placed immediately under light conditions after paraquat application exhibit restricted paraquat movement. In the dark, however, paraquat is more mobile due to the relatively slower impacts of this light-dependent herbicide on conducting elements and other plant tissues (Preston et al., 2005). Restricted translocation has been recognized as being involved in the mechanism of resistance to paraquat (Yu et al., 2004) as well as to glyphosate (Preston and Wakelin, 2008; Brunharo et al., 2016).

Polyamines are small, polycationic molecules essential to all eukaryotes and, in plants, are associated with growth, responses to stress and other external environmental stimuli, and other crucial physiological processes (Groppa and Benavides, 2008). Cellular uptake of paraquat into plant cells is believed to be primarily mediated by polyamine transport systems (Hart et al., 1992), because of the structural similarity with the natural substrate of the transporters (Fujita and Shinozaki, 2014). More recently, an *Arabidopsis* L-type amino acid (LAT) transporter bound to the plasma membrane and an ATP-binding cassette (ABC) transporter were reported to be involved in paraquat uptake (Fujita et al., 2012; Xi et al., 2012). Once inside the cytoplasm, paraquat has to reach the chloroplasts where its site of action is located, although it is not clear whether paraquat diffuses or is actively transported to the chloroplast stroma (Li et al., 2013). Knockdown of the gene *PAR1*, which encodes a Golgi apparatus localized LAT transporter, reduced paraquat accumulation in chloroplasts, suggesting that LAT transporters are involved, at least partially, in the intracellular trafficking of paraquat (Li et al., 2013).

Polyamines are primarily stored in vacuoles and, because these molecules are involved in several important physiological and biochemical cellular processes, a highly regulated influx/efflux transport system is present in the tonoplast membrane (Kusano and Suzuki, 2015). Transport of paraquat into vacuoles has been suggested to be due to the structural similarities of the herbicide and polyamines, in particular the distance between positively charged nitrogen atoms on both molecules at physiological pH. Non-specific transport of paraquat into and out of the vacuole has been proposed as a mechanism of paraquat resistance in *Lolium rigidum* (Yu et al., 2010).

Because of the widespread occurrence of glyphosate-resistant LOLMU in California (Jasieniuk et al., 2008), many growers use paraquat instead of or in addition to glyphosate in orchards and vineyards for broad spectrum weed control. Recently, poor control of LOLMU with paraquat was reported in a prune (*Prunus domestica*) orchard in California after several paraquat applications (Brunharo and Hanson, 2016), raising the possibility of multiple resistance in this population. The objectives of this research were to confirm paraquat resistance in LOLMU, study the mobility of paraquat under light-manipulated conditions, and evaluate the stability of paraquat and its fate in the plant. The understanding of the mechanism of herbicide resistance in weeds may help elucidate biochemical processes and the fundamental mechanisms by which plants adapt and evolve.

MATERIALS AND METHODS

Source of Plant Material

Seeds from putative multiple resistant (PRHC) LOLMU plants were collected in May 2015 from a prune orchard near Hamilton City (39°45′08″ N, 122°00′58″ W), California, 1 week after a paraquat application was made by the orchard manager. Seeds were germinated in petri dishes after the seed dormancy was overcome by alternating 5°C in darkness with 25°C in light. Seedlings were then transplanted to pots filled with Ron's Mix soil[1] and kept in greenhouse until plants reached the BBCH-23 stage (Hess et al., 1997). Plants were treated with 840 g active ingredient (a.i.) ha^{-1} of paraquat to eliminate susceptible individuals from the field-collected seed. Surviving individuals were grown to maturity, bulked and allowed to produce seeds; this generation was also grown to maturity and treated with paraquat. Plants from the resulting F_2 generation (biotype PRHC) and a previously characterized susceptible LOLMU (S) (Jasieniuk et al., 2008) population from California were used in this research.

Whole-Plant Dose-Response

PRHC and S seeds were germinated and single plants were transplanted to potting mix as described in the previous section.

[1]http://greenhouse.ucdavis.edu/research/materials/mediafert.html

At BBCH-23 stage, plants were treated with formulated paraquat (240 g L^{-1}, Gramoxone SL 2.0, Syngenta Crop Protection, LLC, Greensboro, NC, United States) at rates ranging from 105 to 6720 g a.i. ha^{-1}, in addition to a non-treated control treatment, using a spray chamber equipped with an even flat spray nozzle and calibrated to deliver 200 L ha^{-1}. A non-ionic surfactant (90% a.i., Activator 90, Loveland Products, Inc, Greeley, CO, United States) was added at a concentration of 0.25% v/v, following manufacturer's recommendations. Pots were positioned in a completely randomized design with four replications per treatment per biotype and kept in greenhouse with daily maximum temperature of 24°C and minimum of 18°C throughout the experiment. Visual injury was evaluated 7, 14, 21, and 28 days after treatment (DAT) using a scale 0–100%, where 0% represents absence of visual injury and 100% represents complete mortality. At 28 DAT, above ground biomass was collected, dried, and weighed. Log-logistic regression was used to obtain growth reduction by 50% for both biotypes (GR_{50}) and the resistance index (RI) (Knezevic et al., 2007). The experiment was repeated and a Levene's ANOVA test for homoscedasticity of variance was performed before data were pooled across experiments.

Absorption and Translocation of ^{14}C-paraquat

PRHC and S plants were grown under controlled conditions. When they reached the BBCH-13 stage, plants were transplanted to a hydroponic system comprised of 40 mL vials with PTFE/silicon septa filled with a dilute nutrient solution (Moretti and Hanson, 2017). Three days after transplanting, plants were treated with 1.5 kBq of ^{14}C-paraquat (specific activity of 32 mCi $mmol^{-1}$, American Radiolabeled Chemicals, Inc, Saint Louis, MO, United States). Radiolabeled herbicide was mixed with a solution containing commercial paraquat (Gramoxone 2.0 SL) and non-ionic surfactant (Triton X-100, 95% purity, Fisher Scientific, Fair Lawn, NJ, United States), to yield a final concentration approximating a spray solution of 105 g a.i. ha^{-1} and 0.25% v/v, respectively. A 1-μL droplet of the solution was placed on the adaxial leaf surface of the youngest fully expanded leaf, 2 cm away from the leaf ligule towards the leaf blade apex, using a blunt-edged syringe (Nandula and Vencill, 2015). Plants were incubated in the dark for 6 h and then treatments were applied under dim light conditions [photosynthetically active radiation (PAR) equal zero]. Paraquat is a fast acting, light-dependent herbicide and, to ensure that the patterns of translocation were maintained after the application of the herbicide, plants were kept in the dark for 16 h after treatment and then were exposed to saturating photosynthetically active radiation (800 μmol m^{-2} s^{-1} PAR) for an additional 14-h period (Preston et al., 2005). Plants were kept in a 24°C growth chamber throughout the experiment and arranged in a completely randomized design. A subset of plants was destructively harvested at 0, 1, 3, 6, 12, and 16 HAT (dark conditions) and at 20, 24, and 30 HAT (light conditions). At each harvest, plants were split into treated leaf, non-treated leaves, and roots; the treated leaves were also rinsed with a leaf-washing solution (Moretti and Hanson, 2017) to quantify non-absorbed ^{14}C-paraquat and calculate percentage of absorption. Additionally, 1 mL of solution was collected from each vial to monitor root exudation of paraquat.

Non-absorbed ^{14}C-paraquat was quantified with the addition of a scintillation cocktail (Ultima Gold, Perkin Elmer, Walthan, MA, United States) and ^{14}C-carbon disintegration measured with a liquid scintillation spectrophotometer (LS 6500, Beckman Coulter, Fullerton, CA, United States). Treated leaves, shoot and roots were oven-dried and then combusted in a sample oxidizer (307 Sample Oxidizer, Perkin Elmer, Waltham, MA, United States) where $^{14}CO_2$ was trapped in a specific CO_2 trapping solution (Carbo-Sorb E, Perkin Elmer, Waltham, MA, United States), mixed with the appropriate scintillation cocktail (Permafluor E+, Perkin Elmer, Waltham, MA, United States), and ^{14}C decay quantified with liquid scintillation techniques. Each treatment by harvest combination was replicated four times and the experiment was conducted twice. Data were pooled following the same criteria as the whole-plant dose-response experiment. Absorption of ^{14}C-paraquat was calculated as percentage of applied and translocation as percentage of absorbed. Absorption data were subjected to non-linear regressions (Kniss et al., 2011) and translocation data were fit to polynomial models.

Metabolism of ^{14}C-paraquat

PRHC and S were grown, dark- and light-incubated, and treated as described in the absorption and translocation section. In this experiment, 16.6 kBq of ^{14}C-paraquat was applied to the youngest fully expanded leaf and plants were harvested at 0, 24, and 48 HAT, where a 32-h light period followed the 16-h dark-incubation period. Liquid nitrogen, a mortar and pestle was used to thoroughly grind whole-plants. Entire samples were transferred to a 50-ml falcon tube prior to the addition of 10 mL of an extraction solution composed of methanol/HCl 0.5 M (6:4). Falcon tubes were sonicated for 30 min at 65°C, centrifuged at 3800 *g* for 45 min, and a 1-mL aliquot was collected from the supernatant phase. To eliminate particulate matter in the 1-mL aliquot, samples were filtered with a 0.45 μm PVDF syringe filter (Millex-HV, EMD Millipore, Tullagreen, Co, Cork, Ireland) prior to being transferred to 2-mL injection vials. An HPLC (1200 Infinity LC, Agilent, Santa Clara, CA, United States) equipped with a mixed-mode column (100 mm × 3 mm ×3 μm, Acclaim Trinity Q1, ThermoFisher Scientific, San Jose, CA, United States) in line with a flow-through radioactivity detector (FlowStar LB 513, Berthold Technologies, Bad Wildbad, Germany) was used to quantify the parent compound and observe any potential metabolites. The mobile phase was composed of 25% ammonium acetate (100 mM, pH = 5, purity > 98%, Sigma–Aldrich, St. Louis, MO, United States) and 75% acetonitrile (99.9% purity, Fisher Scientific, Fair Lawn, NJ, United States), the column oven temperature was set to 30°C and flow rate 0.6 ml min^{-1}. The experiment was conducted using a completely randomized design with four replications per biotype at each time point and the experiment was repeated.

Maximum Quantum Yield of Photosystem II

PRHC and S plants were grown as described in the whole-plant dose-response section. When plants reach the BBCH-23 stage, the youngest fully expanded leaf of each experimental unit was marked and commercial paraquat (Gramoxone 2.0 SL), along with 0.25% v/v non-ionic surfactant (Activator 90), was applied at 105, 420, 840, and 3360 g a.i. ha^{-1}. Plants were kept in a growth chamber set at 24°C, 14/10 h day/night, and 800 μmol m^{-2} s^{-1} PAR. To assess the plant photosynthetic performance after exposure to paraquat, the maximum quantum yield of photosystem II (F_v/F_m) was measured by dark-adapting the marked leaves with dark-adaption clips (FL-DC, Opti-Sciences, Hudson, NH, United States) for 20 min prior to taking chlorophyll *a* fluorescence measurements with a chlorophyll fluorometer (OS5p+, Opti-Sciences, Hudson, NH, United States) (Maxwell and Johnson, 2000). F_v/F_m measurements were carried out before paraquat application and at 0.5, 1, 2, 5, 24, and 48 HAT and data were expressed as percentage of the initial control values. The experiment was arranged in a completely randomized design with four replications and was repeated. Data were pooled using criteria previously explained.

Behavior of Paraquat in the Plant Cell

An electrolyte leakage technique (Dayan and Watson, 2011) was adopted with modifications to assess the action of paraquat in PRHC and S after pre-exposure of plant tissue to selective transporter inhibitors. Youngest fully expanded leaves were harvested from PRHC and S plants at BBCH stage 23 by excising whole leaf blades and then sectioning each leaf into 2-cm leaf segments. Leaf segments were rinsed with deionized water to remove electrolytes present on the surfaces and incubated in dark with solutions containing one of four selective transporter inhibitor treatments. Inhibitor treatments included: (1) 100 μM putrescine (98.5% purity, Sigma–Aldrich, St. Louis, MO, United States), (2) 100 μM sodium-orthovanadate (99.8% purity, Sigma–Aldrich, St. Louis, MO, United States), (3) 50 μM verapamil (99% purity, Sigma–Aldrich, St. Louis, MO, United States) and (4) 100 μM potassium nitrate (99% purity, Sigma–Aldrich, St. Louis, MO, United States)]. Solutions also contained 2% sucrose (w/w, 95% purity, Fisher Scientific, Fair Lawn, NJ, United States), 1 mM 2-(*N*-morpholino)ethanesulfonic acid pH 6.5 (MES, Boston Bioproducts, Ashland, MA, United States) and 0.1% Triton X-100.

After 3 h of incubation in the inhibitor solutions, leaf segments were rinsed and transferred to glass scintillation vials containing 5 mL of solution [2% sucrose (w/w) + 1 mM MES] with or without paraquat [25 μM paraquat] and incubated for 14 h. Vials were arranged in a completely randomized design in a growth chamber set at 24°C throughout the experiment. Treatments containing paraquat were used to assess the role of the transporters in the resistance phenotype and treatments without the herbicide were to correct for background effects. After dark incubation, 800 μmol m^{-2} s^{-1} PAR was applied for 12 h to allow paraquat action.

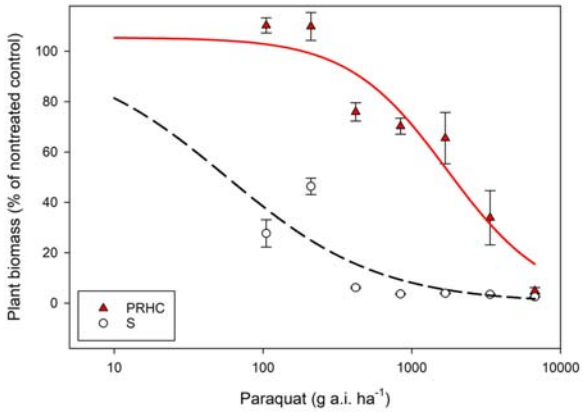

FIGURE 1 | Dose-response analysis of paraquat-resistant (PRHC) and -susceptible (S) *Lolium perenne* L. spp. *multiflorum*. Data points represent plant biomass of PRHC (solid, red triangles) and S (open, black circles) 28 days after paraquat treatment compared to a non-treated control. Bars on data points represent standard errors ($N = 8$). Lines represent three-parameters log-logistic regression of PRHC (red, solid lines) and S (black, dashed lines). $Y = d\ /1 + exp\ [b\ (log\ x - log\ e)]\}$, where *b* denotes the relative slope around *e*, *d* is the upper limit, and *e* is the amount of paraquat required to reduce biomass by 50% (in g a.i. ha^{-1}).

Conductivity measurements were carried out with a conductivity meter and probe (Seven Compact, Mettler Toledo, Columbus, OH, United States and InLab 751-4mm, Mettler Toledo, Columbus, OH, United States, respectively). An initial measurement was taken when leaf segments were transferred to solutions (0 HAT) to use as background conductivity. Measurements were also taken at 11 (dark), 14 (dark), 19 (light), 22 (light) and 26 HAT (light). Each measurement was standardized as a percentage of the maximum conductivity of the sample, obtained by exposing samples to 2000 μmol m^{-2} s^{-1} PAR for 24 h followed by four freeze-thaw cycles (−20°C freezer until solutions froze, followed thawing in a 70°C oven for 30 min). The experiment was repeated and data were pooled using criteria previously stated. Data were analyzed as a 6 by 6 factorial, with treatments as the main factors and incubation time as the subfactor.

RESULTS

Whole-Plant Dose-Response

Paraquat damage was observed in S at all herbicide rates as early as the first visual assessment (7 HAT). Conversely, damage to PRHC leaves was only visible at rates higher than 210 g a.i. ha^{-1} (data not shown). Lower paraquat rates (105 and 210 g a.i. ha^{-1}) did not reduce PRHC biomass, whereas these rates reduced S biomass by more than 50% compared to the non-treated control (**Figure 1**). Half of the recommended field rate (240 g a.i. ha^{-1}) reduced the biomass of S to near 0%, whereas eight times the recommended rate (6720 g a.i. ha^{-1}) was required to induce a comparable response in PRHC. Three-parameter log-logistic regressions were the best fit for the dose-response

TABLE 1 | Dose-response analysis of paraquat-resistant (PRHC) and –susceptible (S) *Lolium perenne* L. spp. *multiflorum*.

Log-logistic regression estimates[a]				
Biotype[b]	***b***	***d***	***e***	**RI[c]**
PRHC	1.3 ± 0.2	0.6 ± 0.0	1780.4 ± 332.4	30.1 ± 12.7
S	1.6 ± 0.7	0.8 ± 0.0	59.0 ± 22.3	

[a]*Equation: Y = d /1 + exp [b (log x – log e)]}, where b denotes the relative slope around e, d is the upper limit, and e is the amount of paraquat required to reduce biomass by 50% (in g a.i. ha^{-1}).* [b]*Values are means ± SE;* [c]*RI = Resistance Index [e(PRHC)/e(S)].*

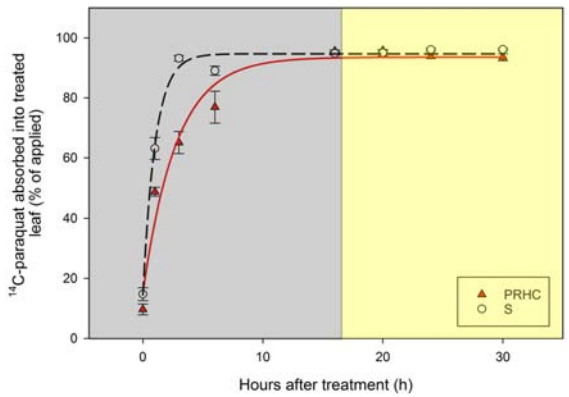

FIGURE 2 | Absorption of paraquat into treated leaf of paraquat-resistant (PRHC, solid, red triangles) and -susceptible (S, open, black circles) *Lolium perenne* L. spp. *multiflorum*. Data points are means and bars represent standard errors. Lines represent asymptotic regressions of PRHC (solid, red line) and S (dashed, black line) after 0, 1, 3, 6, 16, 20, 24, and 30 h after treatment. $Y = (A_{max}\ x\ t) / [(10/\theta)\ x\ t_\theta + t]$, where Y is the absorption (as percentage of applied), A_{max} is the maximum percentage of absorption at large values of t, t is time, and θ is an arbitrary percentage of t. Dark-shaded area represent timepoints harvested during dark-incubation. Light-shaded area represent timepoints harvested during light-incubation.

data. Large standard errors were observed when S data were modeled, particularly the estimated GR_{50}, presumably due to the high susceptibility of S to paraquat even at low herbicide rates (**Table 1**). The regression estimate *e* (GR_{50}) was 59 g a.i. ha^{-1} for S and 1780 g a.i. ha^{-1} for PRHC, resulting in a 30-fold RI.

Absorption and Translocation of ^{14}C-paraquat

Absorption of ^{14}C-paraquat into the treated leaf over time reached the upper limit by 16 HAT, within the dark-incubation period, and was best described by rectangular hyperbole models (**Figure 2**). Based on the regression estimate $t_{\theta\ =\ 90}$ (time required to 90% of the maximum absorption to be achieved), absorption into S treated leaves was faster ($P < 0.001$) compared to PRHC (**Table 2**). Conversely, A_{max}, which represents the maximum absorption percentage of the ^{14}C-paraquat applied, was similar for both biotypes.

Preliminary studies indicated that, when plants are light-incubated after treated with ^{14}C-paraquat, the herbicide movement out of the treated leaf is limited in both biotypes (data not shown). For this reason, light conditions before and during the absorption and translocation experiment were manipulated to allow ^{14}C-paraquat to translocate before its activity resulted in tissue damage. Translocation of ^{14}C-paraquat out of S leaves increased in an exponential fashion (**Figure 3**), reaching 56% by 30 HAT (i.e., 44% remained in treated leaves), whereas movement out of PRHC leaves exhibited a linear response and less than 3% of ^{14}C-paraquat was detected in plant parts other than the treated leaves. Paraquat exudation into the hydroponic solution was negligible (data not shown). The methodology adopted to evaluate the absorption and translocation of ^{14}C-paraquat yielded total recovery of 97.3 ± 2.9% (sum of radioactivity recovered in all plant parts over total radioactivity applied; data not shown).

TABLE 2 | ^{14}C-paraquat absorption regression analysis in paraquat-resistant (PRHC) and -susceptible (S) *Lolium perenne* L. spp. *multiflorum*.

Rectangular hyperbole[a] regression estimates[b]			
	Biotype		***P*-value[c]**
	PRHC	**S**	
A_{max}[d]	98.9 ± 1.8	98.5 ± 1.5	$>0.05^{ns}$
t_{90}[e]	11.4 ± 1.3	4.3 ± 0.6	<0.001

[a]*Equation: Y = (A_{max} x t)/[(10/θ) x t_θ + t], where Y is the absorption (as percentage of applied), A_{max} is the maximum percentage of absorption at large values of t, t is time, and θ is an arbitrary percentage of t.* [b]*Values are means ± SE;* [c]*ns: not significant;* [d]*A_{max}: maximum percentage of herbicide absorbed;* [e]*t_{90}: time (hours) for the maximum absorption to be achieved.*

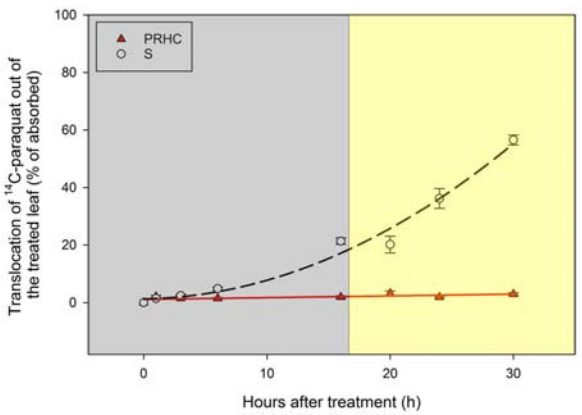

FIGURE 3 | Translocation of ^{14}C-paraquat out of treated leaf in paraquat-resistant (PRHC, solid, red triangles) and -susceptible (S, open, black circles) *Lolium perenne* L. spp. *multiflorum*. Data points are means ($N = 8$) and bars represent standard errors. Lines represent polynomial regressions of PRHC (solid red line, $Y = ax + b$) and S (dashed black line, $Y = ax^2 + bx + c$) after 0, 1, 3, 6, 16, 20, 24, and 30 h after treatment. Dark-shaded area represent timepoints harvested during dark-incubation. Light-shaded area represent timepoints harvested during light-incubation.

Metabolism of ^{14}C-paraquat

The extraction procedure recovered >98% of the applied ^{14}C-paraquat. A linear response was obtained with the in-line

radioactivity detector ($R^2 = 0.99$) over the range of ^{14}C-paraquat concentrations of 0.8 to 33.3 Bq μL^{-1}, with limits of detection lower than 0.8 Bq μL^{-1} (based on signal-to-noise ratio of 3:1 criteria). Elution of ^{14}C-paraquat occurred at 2.37 min after sample injection, and no other ^{14}C peaks were observed in samples from PRHC and S (data not shown) suggesting a lack of paraquat metabolism.

Maximum Quantum Yield of Photosystem II (F_v/F_m)

Plants exposed to biotic and abiotic stresses exhibit decreases in F_v/F_m values as a consequence of oxidative damage and loss of photosystem II reaction centers. The lowest rate of paraquat (105 g a.i. ha^{-1}) did not reduce PRHC F_v/F_m, whereas F_v/F_m in S plants was reduced to less than 10% of the non-treated control up to 48 HAT (**Figure 4**). Half (420 g a.i. ha^{-1}) and full (840 g a.i. ha^{-1}) of the recommended paraquat field rates transiently reduced PRHC F_v/F_m up to 5 HAT, but the photosynthetic performance recovered by 48 HAT, whereas F_v/F_m values in S plants dropped to zero by 48 HAT. The highest rate of paraquat (3360 g a.i. ha^{-1}) reduced F_v/F_m in both biotypes to 0% compared to the initial values.

Behavior of Paraquat in the Plant Cell

The technique employed to assess the behavior of paraquat in cells of LOLMU pre-treated with inhibitors produced consistent and reproducible results (**Figure 5**). PRHC leaf segments treated with only paraquat exhibited the lowest electrolyte leakage on average; values were statistically similar to leaf segments pre-treated with verapamil. Sodium-orthovanadate and potassium nitrate increased susceptibility of PRHC leaf segments to paraquat in comparison with paraquat-only treatments. Lastly, pre-treatment with putrescine, a polyamine transport inhibitor, followed by paraquat increased electrolyte leakage of PRHC leaf segments to levels similar to S leaf segments treated with paraquat-only, essentially reversing resistance to paraquat.

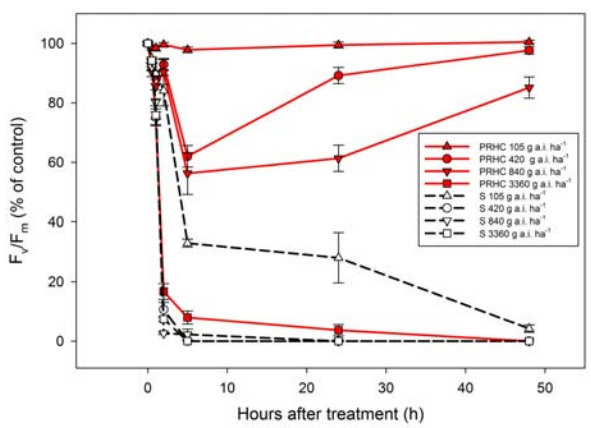

FIGURE 4 | Maximum quantum yield of photosystem II (F_v/F_m) after application of paraquat at 105 (triangle up), 420 (circle), 840 (triangle down) and 3360 g a.i. ha^{-1} (square) to paraquat-resistant (PRHC, solid, red lines) and -susceptible (S, dashed, black lines) *Lolium perenne* L. spp. *multiflorum*. Data points are means ($N = 8$) and bars represent standard errors.

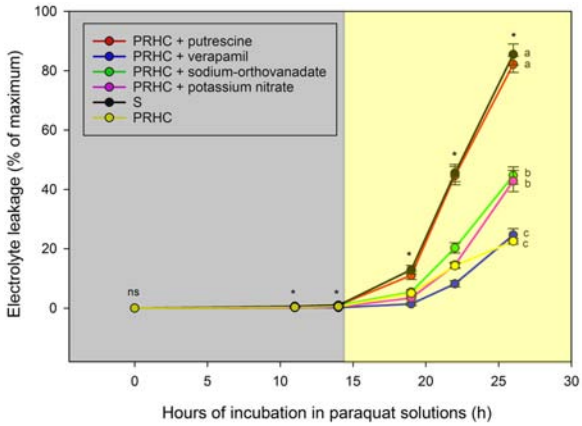

FIGURE 5 | Electrolyte leakage of paraquat-resistant *Lolium perenne* L. spp. *multiflorum* (PRHC) incubated in paraquat solutions for 26 h following pre-treatment with putrescine (red line, red circle), verapamil (blue line, blue circle), sodium-orthovanadate (green line, green circle), potassium nitrate (pink line, pink circle), and no inhibitor (yellow line, yellow circle) and -susceptible with no inhibitor (black line, black circle). Data points are means ($N = 10$), bars represent standard errors, and asterisks means significantly different ($P < 0.001$). Dark-shaded area represent timepoints harvested during dark-incubation. Light-shaded area represent timepoints harvested during light-incubation.

DISCUSSION

Whole-plant does-response confirmed paraquat resistance in biotype PRHC based on a RI of 30. These results corroborated grower experience and preliminary research conducted in the prune orchard in Hamilton City, CA, United States. To date, 32 paraquat-resistant species have been reported around the world (Heap, 2017); however, PRHC is the first reported paraquat-resistant LOLMU. Studies with model plants suggests that resistance to paraquat may be caused by mutations that reduce paraquat uptake (Fujita et al., 2012) and/or enhanced stress tolerance by means of increased expression of enzymes that protect the cell against reactive oxygen species (Murgia et al., 2004; Chen et al., 2009). However, these mechanisms confer only marginal tolerance to paraquat (RI < 4-fold) compared to field-selected weed biotypes (Hawkes, 2014) that exhibit RI as high as 352-fold (Moretti et al., 2016).

In tree and vine crops in California, recommended paraquat rates ranges from 700–1120 g a.i. ha^{-1}; these rates would be insufficient for full control of PRHC. The obligate-outcrossing self-incompatible nature of LOLMU facilitates the dispersal of herbicide resistance genes within and among populations (Loureiro et al., 2016), and the poor control of PRHC with paraquat allows the spread of paraquat resistance genes to areas where -resistant populations are absent.

The slower ^{14}C-paraquat absorption in PRHC compared to S suggests that differential absorption is not a primary cause of

resistance in this biotype. Conversely, restricted ^{14}C-paraquat mobility seems to be involved in the mechanism of resistance to paraquat, considering that virtually all the herbicide remained in the PRHC treated leaf while more than 50% translocated to other tissues in S plants (**Figure 3**). Non-target-site mechanisms of resistance are extensively reported in the literature, and particular attention to these types of mechanisms is given when paraquat-resistant biotypes are studied (Hawkes, 2014). The facts that paraquat was absorbed, remained in the treated leaf, and symptoms were not observed on treated leaves in PRHC suggests that paraquat is either excluded from the cytoplasm (i.e., away from its site of action) or is absorbed but maintained away from the chloroplasts. In fact, paraquat exclusion to the apoplast has been suggested to be the mechanism of resistance in *Hordeum glaucum* (Preston et al., 1992), whereas sequestration into the vacuole has been proposed to confer resistance in several other paraquat-resistant weed species (DiTomaso et al., 1993; Lasat et al., 1997; Yu et al., 2010).

Paraquat absorption through the plant cuticle does not seem to be light-dependent, since t_{90} for S and PRHC are reached within the dark-incubation period. Similar conclusions could be drawn about the translocation out of the treated leaf in S. However, basipetal paraquat movement is primarily due to reverse xylem flow driven by the disruption in water relations caused by paraquat damage to leaf tissue (Smith and Sagar, 1966), damage that did not occur in S during the 16 h dark-incubation period. Symplastic movement of ^{14}C-paraquat might explain, to a certain extent, the observed translocation of the herbicide out of the undamaged treated leaf during the dark-incubation period, if it is considered that the youngest fully expanded leaf received the treatments; these tissues are generally characterized as source organs. This hypothesis is supported by the fact that polyamines (putrescine and spermidine) are translocated in plants by long-distance transport systems (Friedman et al., 1986). A 40% increase in ^{14}C translocation out of the treated leaf was observed in S from the end of the dark-incubation to the end of the light-incubation period.

Paraquat degradation may be driven by biological and physical processes. The former involves an initial demethylation step, followed by ring cleavage of one of the heterocyclic ring (Funderburk and Bozarth, 1967), whereas the latter is given by the formation of 1-methyl-4-carboxypyridinium ion, followed by the formation of methylamine hydrochloride (Slade, 1965). With the observation that paraquat movement was restricted in PRHC, it was hypothesized that, if paraquat transport in plants relies on polyamine transport systems, then paraquat metabolites would no longer be recognized by the transporters, restricting the radiolabeled compounds to the treated leaf. This hypothesis was not supported, however, since paraquat metabolites were not detected in PRHC or S at any timepoint up to 48 HAT. This result is not unexpected because metabolism of paraquat in plants has not been previously reported (Hawkes, 2014), although soil microorganisms may be able to metabolize this quaternary ammonium compound (Funderburk and Bozarth, 1967).

The measurement of F_v/F_m from intact LOLMU leaves indicates that there is a dose-dependent mechanism of resistance acting in PRHC because the highest paraquat dose decreased F_v/F_m to near zero, whereas lower rates did not elicit a comparable response. Similar mechanisms are absent in S, since all doses used led to an irreversible drop in F_v/F_m early in the course of the experiment. Intermediate doses of paraquat (210 and 420 g a.i. ha^{-1}) transiently reduced F_v/F_m, in PRHC but the photosynthetic apparatus recovered by 48 HAT, suggesting not only that paraquat reaches PRHC chloroplasts, but also that the mechanism of resistance to paraquat does not involve herbicide exclusion from the plant cell as suggested for *Hordeum leporinum* (Preston et al., 2005). However, it seems that the mechanism of resistance to paraquat may be rate-limited to a certain extent because of the dose-dependent response in the resistant biotype. Similar transient, dose-dependent paraquat action was also observed in paraquat-resistant *Conyza canadensis* with more pronounced F_v/F_m recovery when plants were exposed to 500 PAR compared to lower light intensities (Varadi et al., 2000).

Studies with the sub-cellular compartmentation of paraquat in paraquat-susceptible *Zea mays* roots revealed that paraquat is slowly sequestered via a diamine carrier system, whereas the rate of paraquat efflux from the vacuole to the cytoplasm is saturable (Hart et al., 1992; DiTomaso et al., 1993). If it is assumed that LOLMU has an analogous paraquat vacuolar loading systems as *Z. mays*, then two mechanisms of resistance may be supported by our results. Because of the linear rate of paraquat loading into the vacuole (as observed in *Z. mays*), the time in which paraquat is in the cytoplasm is similar in PRHC and S, potentially with a paraquat exclusion mechanism in the chloroplasts preventing paraquat from reaching its site of action. This chloroplast exclusion mechanism might not be sufficiently expressed to eliminate damage from high paraquat doses but may be sufficient at lower doses, explaining the results obtained with the maximum quantum yield of PSII measurements. Reduction of paraquat accumulation in *Arabidopsis* chloroplasts with the gene *PAR1*, which encodes a Golgi-localized LAT transporter, has been shown to confer tolerance to the herbicide (Li et al., 2013). However, the authors also showed that inhibition of the vesicle trafficking only partially alleviated paraquat damage, suggesting that an unknown transporter (possibly a polyamine transporter) is involved in the transport of paraquat to the chloroplast. Another reasonable explanation to the results obtained with the F_v/F_m measurements might be the overproduction of the diamine carrier system that performs paraquat vacuolar loading, enhancing vacuolar sequestration of paraquat while (but not necessarily) maintaining the linear fashion of the natural vacuolar loading observed in *Z. mays* (DiTomaso et al., 1993).

Verapamil, which blocks Ca^{2+} channels (Huang et al., 1994) and inhibits multidrug ABC transporters (Kang et al., 2010) did not increase susceptibility of PRHC to paraquat compared to the paraquat-only control treatment, suggesting that Ca^{2+} channels and multidrug ABC transporters are not involved in the resistance to paraquat in this biotype. The inhibitor of plasma-membrane, tonoplast ATPases and plasma-membrane ABC transporters sodium-orthovanadate (Cocucci et al., 1980; Demichelis and Spanswick, 1986; Finbow and Harrison, 1997),

as well as the tonoplast H^+-ATPase pumps inhibitor potassium nitrate (Sze, 1984), led to intermediate paraquat damage to PRHC leaf segments, suggesting that the mechanism of resistance to paraquat requires energy supplied by the proton gradient across membranes, most likely across the tonoplast membrane. It may be pointed out that sodium-orthovanadate also inhibits plasma membrane ABC transporters (P-type ABC transporters), but since verapamil (all ABC transporters inhibitor) did not provide increased susceptibility of PRHC to paraquat, the involvement of P-type ABC transporters may be unlikely (Shitan et al., 2003).

The observation that pre-exposure of PRHC leaf segments to putrescine reverses resistance to paraquat strongly suggests that polyamine carrier systems are involved in the mechanism of resistance to paraquat in PRHC. Exposure of *Z. mays* roots to putrescine 20 min prior to paraquat incubation has been shown to inhibit polyamine transporter-mediated paraquat transport by up to 65% (Hart et al., 1992).

Polyamine transport can be accomplished by carriers bound to the plasma membrane (Hart et al., 1992) and tonoplast (Pistocchi et al., 1988). Because it was observed in this research that paraquat reaches PRHC chloroplasts, it may be inferred that plasma membrane-bound polyamine carriers do not have a major role in the mechanism of resistance in PRHC, eliminating the possibility of paraquat exclusion to the apoplast being involved in the resistance mechanism.

CONCLUSION

Poor weed management practices, particularly overreliance on a single/few herbicide modes of action, have frequently been associated with the selection of herbicide-resistant weed biotypes around the world. Paraquat used to control glyphosate-resistant LOLMU has selected for a multiple resistant biotype in a prune orchard in California; this biotype withstands paraquat at up to three times the maximum field rate that tree and vine growers are allowed to use in this region. The restricted translocation of ^{14}C-paraquat in PRHC observed seems to be primarily caused by the vacuolar sequestration of the herbicide mediated by tonoplast-bound polyamines transporters sensitive to the inhibitor putrescine. These findings are supported by the fact that PRHC photosynthetic apparatus is sensitive to paraquat and that paraquat is stable in the plants.

AUTHOR CONTRIBUTIONS

CB and BH conceived and designed the experiments. CB conducted the experiments and analyzed the data. CB and BH wrote the paper. All authors have read and approved this manuscript.

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Enhanced 2,4-D Metabolism in Two Resistant *Papaver rhoeas* Populations from Spain

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Corn poppy (*Papaver rhoeas*), the most problematic broadleaf weed in winter cereals in Southern Europe, has developed resistance to the widely-used herbicide, 2,4-D. The first reported resistance mechanism in this species to 2,4-D was reduced translocation from treated leaves to the rest of the plant. However, the presence of other non-target site resistance (NTSR) mechanisms has not been investigated up to date. Therefore, the main objective of this research was to reveal if enhanced 2,4-D metabolism is also present in two Spanish resistant (R) populations to synthetic auxins. With this aim, HPLC experiments at two 2,4-D rates (600 and 2,400 g ai ha^{-1}) were conducted to identify and quantify the metabolites produced and evaluate possible differences in 2,4-D degradation between resistant (R) and susceptible (S) plants. Secondarily, to determine the role of cytochrome P450 in the resistance response, dose-response experiments were performed using malathion as its inhibitor. Three populations were used: S, only 2,4-D R (R-703) and multiple R to 2,4-D and ALS inhibitors (R-213). HPLC studies indicated the presence of two hydroxy metabolites in these R populations in shoots and roots, which were not detected in S plants, at both rates. Therefore, enhanced metabolism becomes a new NTSR mechanism in these two *P. rhoeas* populations from Spain. Results from the dose-response experiments also showed that pre-treatment of R plants with the cytochrome P450 (P450) inhibitor malathion reversed the phenotype to 2,4-D from resistant to susceptible in both R populations. Therefore, it could be hypothesized that a malathion inhibited P450 is responsible of the formation of the hydroxy metabolites detected in the metabolism studies. This and previous research indicate that two resistant mechanisms to 2,4-D could be present in populations R-703 and R-213: reduced translocation and enhanced metabolism. Future experiments are required to confirm these hypotheses, understand the role of P450, and the relationship between both NTSR mechanisms. On this basis, selection pressure with synthetic auxins bears the risk of promoting the evolution enhanced metabolism in *Papaver rhoeas*.

Keywords: degradation, malathion, plant detoxification process, non-target site resistance, sugar conjugate, synthetic auxin

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INTRODUCTION

Synthetic auxins were the first herbicidal mode of action discovered, back into 1940 (Peterson et al., 2016). 2,4-Dichlorophenoxyacetic acid (2,4-D) was the first herbicide belonging to this group to be commercially developed and released worldwide in 1945 (Schulz and Segobye, 2016). 2,4-D provided very effective control to the majority of broadleaved weed species in cereals, revolutionizing crop protection, and for this reason it was rapidly adopted by farmers in all developed countries (Peterson, 1967). In 1957, the first resistance cases were reported in North-America for *Daucus carota* and *Commelina diffusa* (Heap, 2017). Nowadays, after more than 70 years, 31 weed species are reported to have developed resistance to synthetic auxins, excluding monocotyledonous weeds (three species) resistant to quinclorac (quinoline-carboxylic acids). In total, there are 51 different reported cases with resistance to synthetic auxins worldwide. Of those, there are 31 reported cases with resistance to fenoxy-carboxylic acids (16 to 2,4-D), seven cases to benzoic acids (dicamba), and 13 different cases to pyridine-carboxylic acids (i.e., clopiralid; Heap, 2017). The rarity in occurrence of auxinic herbicide resistance compared to the hundreds of weed species that have evolved resistance to other herbicide classes, such as PS II- or ALS-inhibiting herbicides (Heap, 2017), could be attributed to: proposed multiple sites of action of these compounds (Mithila et al., 2011), initial low frequencies of resistant alleles, low levels of resistance conferred by resistance mechanism(s), or reduction in plant fitness due to pleiotropic effects of auxinic herbicide resistant traits (Busi and Powles, 2017). Single dominant nuclear encoded genes are supposed to control auxinic resistance in different species (Riar et al., 2011; Busi and Powles, 2017). However, polygenic inheritance of resistance in some species (Weinberg et al., 2006), could also contribute to slow evolutionary rates of auxinic herbicide resistance.

Plant detoxification processes usually follow a four-phase schema, which can also affect herbicides (Yuan et al., 2007). In phase I, molecules are activated for phase II enzymes. Oxidation is a typical phase I reaction, which can be carried out by cytochrome P450 monooxygenases. Phase II reactions generally involve conjugation (i.e., with sugars) which enables the end product to be recognized by the phase III transporters (usually ABC family), moving the molecule into the vacuole or extracellular space by active transport (Klein et al., 2006). Previous researches have proposed that the selectivity of auxinic herbicides in monocots is because of either limited translocation and/or rapid degradation of exogenous auxin, altered vascular anatomy, or altered perception of auxin (Peterson et al., 2016). It seems that the primary metabolic pathway in grasses is ester hydrolysis followed by the formation of base-labile 2,4-D conjugates (Hamburg et al., 2001). On the contrary, dicotyledonous species further detoxify auxinic herbicides in a different metabolic route after ester hydrolysis, mainly by means of ring hydroxylation, as it was observed in potatoes by Hamburg et al. (2001), mediated by cytochrome P450 (Hatzios et al., 2005).

Resistance mechanisms to synthetic auxins in weeds and their molecular basis remain largely unknown for most species. The main reason is that the precise mode of action of synthetic auxins is not fully understood (Grossmann, 2010). Moreover, some studies point out that these herbicides would have more than one target protein (multi-target; Mithila et al., 2011), partially explaining the polygenic characteristic of the resistant traits (Busi and Powles, 2017). Nonetheless, new discoveries including nuclear auxin receptors (F-box proteins), influx (AUX/LAX family) and efflux carriers (ABC and PIN families) and plasma membrane bound receptors (ABP proteins) have provided basic clues as to the molecular mode of action of these herbicides (Song, 2014).

In view of the complicated mode of action of auxinic herbicides, the evolution of resistance in weeds is generally treated as a non-target-site-based phenomenon (Goggin et al., 2016). Only one study considered a possible Target-site resistant (TSR) mechanism in *Brassica kaber*, due to an altered binding of auxinic herbicides to an auxin-binding protein (ABP) receptor located in plasma membrane (Mithila and Hall, 2005). Most studies indicate that Non-Target-site resistant (NTSR) mechanisms are involved in the majority of weed species. The lack of TSR mechanisms for this mode of action is explained by the central role that synthetic auxins targets (nuclear and membrane receptors or influx and efflux carriers) play in the gene expression, physiology and development of plants (Grossmann, 2010). Among the NTSR mechanisms, different absorption, translocation patterns, or herbicide metabolism between susceptible plants and resistant plants have been described in the few studied species (Peterson et al., 2016). Reduced absorption has been reported only in *Glechoma hederacea* (Kohler et al., 2004); reduced translocation has been reported in *Galeopsis tetrahit* (Weinberg et al., 2006), *Centaurea solstitialis* (Fuerst et al., 1996), *Lactuca serriola* (Riar et al., 2011), and in *Raphanus raphanistrum*, involving ABCB transporters in this later species (Goggin et al., 2016); increased translocation to the roots only in a *R. raphanistrum* biotype (Jugulam et al., 2013); while enhanced metabolism in *G. tetrahit* (Weinberg et al., 2006) and *Stellaria media* (Coupland et al., 1990). For example, mecoprop degradation could be mediated by a cytochrome P450 in *S. media* (Coupland et al., 1990).

Papaver rhoeas L. is the only known species to have evolved resistance to synthetic auxins in Spain. Though it was already reported in the early 90s (Taberner et al., 1995), their resistance mechanisms have only been studied very recently (Rey-Caballero et al., 2016). This research suggests that reduced 2,4-D translocation is involved in the resistance mechanism to synthetic auxins, likely leading to less ethylene production and greater survival in R plants. However, the presence of other NTSR mechanisms cannot be excluded, such as enhanced herbicide metabolism, because one resistant mechanism does not exclude the presence of others (Yu and Powles, 2014). Therefore, NTSR mechanisms to synthetic auxins, particularly enhanced metabolism, should be also investigated in *P. rhoeas* because, if presenttheir implication for integrated weed management can be tremendous (Yu and Powles, 2014). Enhanced detoxification pose a great threat to agriculture because of the often unexpected multi-herbicide resistance and multi-gene involvement in the mechanisms (Yuan et al., 2007).

The main aim of this research was to study if herbicide detoxification is also present in two 2,4-D resistant *P. rhoeas* populations: one only resistant to 2,4-D and the second multiple resistant to 2,4-D and tribenuron-methyl (Rey-Caballero et al., 2016). To do so, a new methodology using HPLC was developed, with the advantage that no radio labeled herbicide is required. Afterwards, two types of experiments were carried out: (1) HPLC experiments to find out differences in 2,4-D degradation between resistant and susceptible plants and identify and quantify the metabolites produced, and (2) dose-response experiments with a detoxifying enzyme (cytochrome P450) inhibitor (malathion) to further validate its possible role in 2,4-D degradation.

MATERIALS AND METHODS

Plant Material

One susceptible (S) population (S-013) was included in this study, obtained from a seed dealer (Herbiseed, Twyford, UK) in 2008. The original field-evolved 2,4-D-resistant populations were collected from Almacelles (41°43′N, 0°27′E) in 2003 (population R-703) and Baldomar (41°54′N, 1°00′E) in 2013 (population R-213), both in North-eastern Spain; these populations displayed survival of ∼20% (Rey-Caballero et al., 2016), respectively, when sprayed with the recommended field rate (600 g active ingredient/ha) of formulated 2,4-D ester. Additionally, R-213 was also resistant to ALS inhibiting herbicides (Rey-Caballero et al., 2017). Seeds were sown in aluminum trays with peat and placed in a growth chamber at 20/10°C day/night, 16 h photoperiod under 350 μmol photosynthetic photon-flux density m^{-2} s^{-1}. After 14 days, seedlings were transplanted in 7 × 7 × 7 cm plastic pots filled with the following soil mixture: silty loam soil 40% (w/v), sand 30% (w/v), peat 30% (w/v). Pots were placed in a greenhouse in Lleida, north-eastern Spain (41°37′N, 0°38′E) and were watered regularly and fertilized as required.

2,4-D Metabolism Experiments

Seedlings from S and both R populations at six true leaves of development (5–6 cm) were treated at three different 2,4-D doses, 0, 600 g a.i.·ha^{-1} (field recommended rate, 1x) and 2,400 g a.i.·ha^{-1} (4x), as described below for the dose-response experiments. Six plants from each population and dose were harvested at 12, 24, 48, 96, and 168 h after treatment (HAT). Plants were separated into two parts: aerial part (leaves and shoots) and roots, each of which was rinsed using distilled water to remove unabsorbed herbicide. Each part was rapidly frozen in liquid nitrogen and then stored at −40°C until use.

To study the 2,4-D metabolism in *P. rhoeas*, some known methodologies were used (Chkanikov et al., 1977; Hamburg et al., 2001) to confirm the existence or not of its metabolites in the populations. These methodologies were adapted and modified to be able to work without radiolabelled herbicide, because it was not possible to obtain ^{14}C-2,4-D metabolites. The inability to obtain the ^{14}C-2,4-D-metabolites required identification and quantification by a chromatographic method. This method was based on that one by Hamburg et al. (2001), which also was used to identify the non-radiolabelled metabolites, according to the retention times. All details regarding how these methodologies were adapted and modified are provided below.

Reagents

Acetone (HPLC grade), acetic acid, chlorhydric acid (37%), 1-butanol, diethyl ether, ethanol (HPLC grade), petroleum ether, and 2,4-D herbicide standard were purchased from Sigma Aldrich (St Louis, MO, EE.UU.); acetonitrile, methanol (LC-MS grade) and ammonium hydroxide from Panreac AppliChem (Barcelona, Spain). Deionised water (18 MΩ·cm) was obtained with a purification system Millipore Milli-Q (Millipore, Bedford, MA, EE.UU.). Commercial 2,4-D herbicide was Esteron 60 (60% w/v, Dow AgroSciences).

Instruments and Apparatus

A magnetic stirrer with a temperature controller from Selecta (Barcelona, Spain) was used for some separation phase steps. Centrifugation of the extracts was carried out by a Coulter Avanti J-25 centrifuge with a temperature controller (Beckman, Fullerton, USA). A rotatory evaporator Mod. LABOROTA 4000 from Heidolph (Schwabach, Germany) was used for the organic solvent evaporation. The 20 × 20 cm silica gel TLC plates with inorganic fluorescent indicator F254 from Merck Millipore (Billerica, MA, USA) was used for separation steps. Nylon filters with a pore size of 20 μm and an inner diameter of 13 mm from Millipore (Billerica, MA, USA) were used to remove solid particles from the extracts before the LC analysis. A 15 Gold LC System from Beckman Coulter (Fullerton, USA) equipped with a 26 Gold DAD detector (wavelength range 190–600 nm) was used for individual separation and UV detection. The instrumental setup was controlled by the Karat 3.0.7 software, which also enabled data acquisition and processing. Chromatographic separation was carried out using a Kinetex® EVO C18 column (150 mm, 4.6 mm id, 2.6 μm particle size) from Phenomenex Inc. (Torrance, CA, USA), furnished with a 4.6 mm SecurityGuard™ ULTRA cartridges.

Sample Pre-treatment and First Partition

The extraction followed the methodology described by Chkanikov et al. (1977) with some modifications for the full extraction. Frozen samples were washed with 5 mL of 0.05 N of ammonium hydroxide. Each sample was placed in a porcelain mortar and flash-frozen using 20 mL liquid nitrogen and grinded to fine homogeneous powder using a porcelain pestle for 5 min. Then it was submerged in boiling water (three times) and the aqueous extracts were combined, cooled, and an equal volume of acetone was added. After 12 h the formed precipitate was removed using centrifugation at 4°C and 20,000 rpm. The precipitate was washing three times with 5 mL of ethanol and added to the acetone phase. The organic phase (ethanol and acetone) was removed at 40°C with a rotary evaporator. The aqueous residue was acidified to pH 2 with hydrochloric acid. It was treated three times with 5 mL of diethyl ether and evaporated this ether portion in the rotary evaporator at 40°C.

After the ether portion was evaporated, the residue resulting was dissolved in 90% acetone and an aliquot of this was developed by TLC in the solvent butanol-ammonium hydroxide-water

(5:1:4) (First partition). Unaltered 2,4-D (Rf 0.55) was separated from its "free" hydroxylated derivatives (Rf 0.2) and amino acid conjugates (Rf 0.1).

Second Partition

A second partition was realized using the acidified aqueous phase with1-butanol for the extraction, after the diethyl ether phase was removed. The 1-butanol was later evaporated in the rotary unit at 40°C. The residue was dissolved in 2 N hydrochloric acid and then hydrolyzed for 60 min in a boiling water bath. The metabolites were separated by TLC in the same solvents. As a result of hydrolysis l-O-(2,4-dichlorophenoxyacetyl)-ß-D-glucose broke down with the release of unaltered 2,4-D, while 4-O-ß-D-glucosides of 4-hydroxy-2,5-dichloro- and 4-hydroxy-2,3-dichlorophenoxyacetic acids released "free" 4-OH-2,5-D and 4-OH-2,3-D.

Third Partition

The third partition was realized using the extract with the 1-butanol totally evaporated, and hydrolyzed in 2 N hydrochloric acid at 100°C. The distillate was acidified with hydrochloric acid to pH 1 and was extracted with petroleum ether (40°C) in the rotatory unit. The substances were separated by TLC with the same solvents. A glycoside of 2,4-dichlorophenol was detected in the analysis of ring-labeled-herbicide-treated strawberry plants known for an extremely high rate of 2,4-D ether linkage breakdown. After TLC development, the TLC plates were exposed to a UV lamp at 256 nm for discover the metabolites and could be separated. The metabolites were scratched from the TLC plates (dark areas) and dissolved in 0.5 mL of acetone. The reconstituted sample was filtered through a nylon filter syringe before chromatographic analysis.

Chromatographic Method

The chromatographic method was the method used by Hamburg et al. (2001) with some modifications, which also was used to identify the metabolites according to the retention times. Fifty microliters of the reconstituted simple was injected in the HPLC system. 1% (v/v) acetic acid in water and acetonitrile as mobile phases A and B, respectively, were used. The elution program started by a linear gradient from 20% mobile phase B to 50% in 20 min (step 1), 50% mobile phase B to 100% in 5 min (step 2), and 100 to 10% acetonitrile in 10 min for equilibration (step 3). The constant flow rate and column temperature were 1.0 mL/min and 25°C, respectively. Quantification of 2,4-D and its metabolites was based on the calibration curve of 2,4-D, which is the unique commercially available standard. The results were expressed as μg of the analyte/g of plant.

Dose-Response Experiments

Five seedlings were sown per pot and after establishing, were thinned to four per pot. At the four to six leaf stage (4–5 cm), all populations were treated with either 0 or 2,000 g a.i./ha of the organophosphate insecticide malathion ([(dimethoxyphosphinothioyl)-thio] butanedioic acid diethyl ester). Preliminary tests showed that 2,000 g a.i./ha is around the maximum dose not affecting *P. rhoeas* survival or growth (data not shown). After approximately 1 h 30 min, 2,4-D (Esteron 60, Dow AgroSciences, 60%) was applied at 0, 300, 450, 600 (field dose), 900, 2,400, and 4,800 g a.i./ha to R populations and at 0, 225, 300, 450, 600, 900, and 2,400 g a.i./ha to S plants. Non-treated plants were used as controls. A total of four replicates (four plants per pot) were included at each dose. Herbicides were applied using a precision bench sprayer delivering 200 L/ha, at a pressure of 215 kPa. Four weeks after treatment, percentage of survival was estimated, and plants were harvested (above ground) and the dry weight (65°C for 48 h) was measured. The experiment was repeated twice.

Statistical Analysis

Data from dose-response experiments were analyzed using a nonlinear regression model (1). The herbicide rate required for 50% growth reduction of plants (GR50) was calculated with the use of a four parameter logistic curve of the type:

$$y = c + \frac{(d - c)}{1 + \mathrm{EXP}[b(\log(x) - \log(XR50)]}$$

where c = the lower limit set to 0, d = the upper limit set to 100, and b = the slope at the XR50 (SR50 for % of survival and GR50 for % of dry weight compared to untreated control). In this regression equation, the herbicide rate (g a.i./ha) was the independent variable (x) and the plants survival and the plants' dry weight expressed as percentage of the untreated control were the dependent variables (y). The resistance index (RI) was computed as GR50(R)/GR50(S). XR50 parameters were compared between susceptible and R populations (with and without malathion) with the Delta method at $P = 0.05$. Repetitions from the dose-response experiments were pooled due to lack of statistical differences between them. Data from 2,4-D metabolism experiment was subjected to analysis of variance (ANOVA). The requirement of homogeneity of variance was checked by visual inspection of the residual plots and residuals were analyzed using Shapiro–Wilk Test. When required, data were previously squared root transformed; in those few cases non-transformed values are presented for clarity. Where variances were not homogeneous, generalized linear models (GLM) were used. The binomial distribution (Logit-link) was used in all GLM, because this distribution resulted in normally distributed residues. Population means were compared using a *post-hoc* Tukey's pairwise procedure at $P = 0.05$.

All statistical analyses were carried out with the use of the R programming language (R Core Team, 2013), drc package (Knezevic et al., 2007) for the non-linear regression and multcom (Hothorn et al., 2008) for the *post-hoc* Tukey's test were employed.

RESULTS

2,4-D Metabolism Experiments

Qualitative assessment of TLC bands showed differences in the migration patterns between the studied populations (Supplementary Figure 1). In the S population (Supplementary Figure 1A), only parent 2,4-D migrating identically to the

standard was detected at all times from 0 to 168 HAT. On the other hand, from 24 HAT, migration patterns were different in both R populations. In the 2,4-D resistant population (Supplementary Figure 1B), compounds remaining close to the origin were already detected at 24 HAT in plants applied at 2,400 g a.i./ha (4x), while in the multiple resistant population (Supplementary Figure 1C) they were detected at 48 HAT at both doses. Interestingly, another compound appeared in the multiple resistant population at 96 and 168 HAT, even closer to the origin.

Quantification of the relative abundance of TLC bands of parent 2,4-D and its metabolites showed clear differences in 2,4-D metabolic capacity between the S and R *P. rhoeas* populations; so dots on TLC plates were due to compounds migrating differentially (less) to parent 2,4-D (**Table 1**). Up to 48 HAT, amounts of 2,4-D in the aerial part were similar between S and R plants at both rates. At 96 HAT significantly much less 2,4-D was detected in R populations, while at 168 HAT no parent 2,4-D was found. At 12 HAT the herbicide was already found in roots in all populations, but quantities were much higher in S plants in all assessment times. Ascribed 2,4-D metabolites (according to HPLC retention times) were only quantified in the R populations. In the R-703 population (2,4-D resistant), the first metabolite (2,3-D) was detected in aerial parts already 24 HAT and 48 HAT, at 4x and 1x rates, respectively, while in the R-213 population (multiple R), it was detected at 48 and 96 HAT, respectively. In roots of R-703 population, it was already detected 24 and 96 HAT, at 4x and 1x rates, respectively, while in R-213 it was detected at 48 and 168 HAT, respectively. The second metabolite (2,5-D), was first detected in the aerial parts at 48 HAT in both R populations at 4x, and at 96 HAT at 1x. Interestingly, this compound was only detected in roots of the multiple R population at 96 and 168 HAT at the highest dose. Finally, a third compound (a sugar conjugate) was only quantifiable in the roots of the 2,4-D R population at 96 and 168 HAT at the highest rate, while in the aerial only in the last assessment time.

Qualitative and quantitative differences were found between the populations in the HPLC profile of 2,4-D metabolism (**Figures 1A–C**). As expected, 2,4-D had a retention time on HPLC of ∼17 min in both S and R populations. *P. rhoeas* R populations produced a mixture of two polar metabolites (which remained near the origin on TLC), with HPLC retention times of 8 and 9 min, respectively, which were water soluble (**Figures 1B,C**). Additionally, very small amounts of another polar metabolite (smallest dots even closer to origin in TLC plates) had an HPLC retention time around 8.5 min, which was partitioned into the ether phase (**Figure 1D**). The two hydroxilated metabolites (2,3-D and 2,5-D) were detected at 168 HAT in the two R populations (2,4-D and multiple resistant), while in the sugar conjugate was only detected in the multiple R population. The amount of the 2,4-D found in S plants was higher (>10-folds) than in R plants (**Figure 1**), while the metabolites were not detected in S plants. With respect to the amount of the different metabolites between the populations, the first polar compound (2,3-D) was detected in similar quantities in both R populations, while the levels of the second compound (2,5-D) were 2.5-folds higher in the 2,4-D R population compared to the multiple R one.

Summarizing, the 2,4-D was rapidly degraded, through the hydroxylation of the phenyl ring generating 4-hydroxy-2,5-dichlorophenoxyacetic acid (2,5-D) and 4-hydroxy-2,3-dicholorophenoxyacetic acid (2,3-D), which were not present in S plants but were in the two R populations in significant amounts. The third metabolite, a sugar conjugate, might be a conjugation of OH-2,5-D with a carbohydrate. Sugar conjugates did not appear in R-703 (only 2,4-D R) and S plants (**Table 1**). Both 2,3-D and 2,5-D metabolites and the sugar conjugate are regarded as being far less phytotoxic than 2,4-D (Peterson et al., 2016). The three metabolites were ascribed to those previously appointed according to retentions times in the HPLC, but further identification with other methodologies, i.e., mass spectrometry, would be required.

Dose-Response Experiments

When malathion was applied alone at 2,000 g/ha, there was no effect on survival or growth of either the S or R populations. When 2,4-D was applied after malathion on the susceptible population, the behavior in terms of survival and biomass were similar without the presence of the insecticide (**Table 2**). In the presence of malathion, both R populations became susceptible to 2,4-D (**Figure 2**), and the RI for % of survival went done from 14 to 0.6 and from 8 to 1.4, for the 2,4-D resistant and the multiple resistant populations, respectively (**Table 2**). Similar results were obtained for the above-ground biomass (**Figure 2** and **Table 2**).

Visual inspection of treated plants of both R populations comparing both treatments, that is with or without previous application of malathion, clearly revealed that survival and growth were much reduced with previous applications of the insecticide (**Figure 3**).

DISCUSSION

In Spain, cereals (mainly rainfed winter barley and wheat) are most extended crops, with more than 6 million hectares in 2015. *P. rhoeas* is the most troublesome broadleaved weed in these crops due to the spread of multiple R populations to synthetic auxins and ALS inhibiting herbicides. This research is pioneer in studying the presence of enhanced 2,4-D metabolism in this species. For the first time, the presence of two 2,4-D hydroxy metabolites (2,3-D and 2,5-D) has been indicated in two R *P. rhoeas* populations, one only 2,4-D R and another one multiple R, while none were detected in S plants. Therefore, enhanced metabolism to synthetic auxins becomes a newly discovered resistance mechanism in this species. Other few reports of enhanced metabolism to phenoxyacetic acid herbicides included *Stellaria media*, *R. raphanistrum,* and *G. tetrahit* (Coupland et al., 1990; Weinberg et al., 2006; Goggin and Powles, 2014).

Results from the dose-response experiments showed that pre-treatment of R plants with the cytochrome P450 (P450) inhibitor malathion clearly reversed the phenotype to 2,4-D from resistant to susceptible in both R populations. These dramatically visual effects on survival and growth in R plants provided indirect evidence that differential activity of a P450 mono-oxygenase (inhibited by malathion) is required for the resistance response in *P. rhoeas*. Enhanced metabolism mediated by the cythochrome

TABLE 1 | Amount (μg/g plant) of 2,4-D and its metabolites of one susceptible (S) and two resistant (R-703 and R-213) *Papaver rhoeas* populations at 12, 24, 48, 96, and 168 HAT applied at two doses (1x for 600 g/ha; 4x for 2,400 g/ha).

Dose	Part	Product	Pop	12	24	48	96	168
1x	Aerial part	2,4-D	S	8.6a* (0.1)	9.2a (0.0)	10.7a (0.0)	10.7a (0.2)	9.3 (0.1)
			R-703	7.6b (0.0)	8.2c (0.0)	9.2b (0.0)	5.0c (0.1)	ND
			R-213	7.7b (0.1)	9.0b (0.0)	11.0a (0.2)	7.0b (0.2)	ND
		2,3-D	S	ND	ND	ND	ND	ND
			R-703	ND	ND	2.4 (0.0)	5.1a (0.1)	7.6a (0.1)
			R-213	ND	ND	ND	2.7b (0.1)	7.7a (0.3)
		2,5-D	S	ND	ND	ND	ND	ND
			R-703	ND	ND	ND	1.61a (0.05)	3.59a (0.05)
			R-213	ND	ND	ND	0.86b (0.03)	3.64a (0.13)
		Sugar conjugate	S	ND	ND	ND	ND	ND
			R-703	ND	ND	ND	ND	ND
			R-213	ND	ND	ND	ND	ND
	Root	2,4-D	S	0.2a (0.0)	0.3a (0.0)	0.7a (0.0)	0.9a (0.0)	2.4 (0.0)
			R-703	0.2b (0.0)	0.1b (0.0)	0.1c (0.0)	0.0c (0.0)	ND
			R-213	0.1b (0.0)	0.1b (0.0)	0.1b (0.0)	0.1b (0.0)	ND
		2,3-D	S	ND	ND	ND	ND	ND
			R-703	ND	ND	ND	0.02 (0.0)	0.03b (0.0)
			R-213	ND	ND	ND	ND	0.05a (0.0)
		2,5-D	S	ND	ND	ND	ND	ND
			R-703	ND	ND	ND	ND	ND
			R-213	ND	ND	ND	ND	ND
		Sugar conjugate	S	ND	ND	ND	ND	ND
			R-703	ND	ND	ND	ND	ND
			R-213	ND	ND	ND	ND	ND
4x	Aerial part	2,4-D	S	32.0a (0.0)	33.2b (0.1)	38.4a (0.1)	38.9a (0.1)	33.3a (0.5)
			R-703	31.7a (0.1)	33.1b (0.2)	35.2b (0.1)	13.3c (0.1)	0.0b (0.0)
			R-213	31.8a (0.2)	35.9a (0.1)	29.0c (0.9)	15.5b (0.1)	0.1b (0.0)
		2,3-D	S	ND	ND	ND	ND	ND
			R-703	ND	1.4 (0.1)	6.7b (0.0)	27.4b (0.1)	35.7a (0.1)
			R-213	ND	ND	10.4a (0.3)	22.8a (0.2)	34.5b (0.2)
		2,5-D	S	ND	ND	ND	ND	ND
			R-703	ND	ND	3.28b (0.01)	7.29a (0.03)	10.07a (0.03)
			R-213	ND	ND	5.15a (0.16)	6.06b (0.05)	9.99a (0.07)
		Sugar conjugate	S	ND	ND	ND	ND	ND
			R-703	ND	ND	ND	ND	ND
			R-213	ND	ND	ND	ND	0.91 (0.0)
	Root	2,4-D	S	0.7a (0.0)	1.2a (0.0)	2.7a (0.0)	3.4a (0.0)	8.9 (0.4)
			R-703	0.1c (0.0)	0.3b (0.0)	0.3c (0.0)	0.0c (0.0)	ND
			R-213	0.2b (0.0)	0.3b (0.0)	0.3b (0.0)	0.2b (0.0)	ND
		2,3-D	S	ND	ND	ND	ND	ND
			R-703	ND	ND	0.04b (0.0)	0.1b (0.0)	0.16b (0.01)
			R-213	ND	ND	0.17a (0.0)	0.3a (0.0)	0.47a (0.03)
		2,5-D	S	ND	ND	ND	ND	ND
			R-703	ND	ND	ND	ND	ND
			R-213	ND	ND	ND	0.09 (0.0)	0.14 (0.01)
		Sugar conjugate	S	ND	ND	ND	ND	ND
			R-703	ND	ND	ND	ND	ND
			R-213	ND	ND	ND	ND	0.01 (0.0)

**Means within a column, evaluation time, plant part and product followed by the same letter are not significantly different (P > 0.05).*

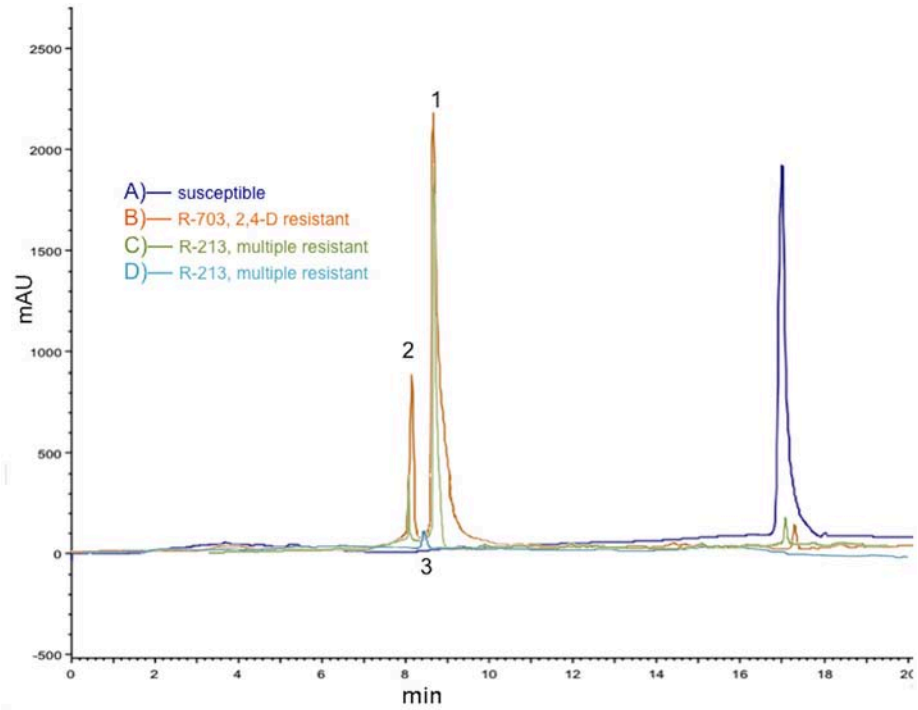

FIGURE 1 | Comparison of 2,4-D metabolism in three *Papaver rhoeas* populations in 2,4-D treated plants (2,400 g ai./ha after 168 HAT). **(A)** Representative HPLC chromatogram of extract (second partition) from S plants in purple; only 2,4-D was detected. **(B)** Representative HPLC chromatogram of extract (second partition) from only 2,4-D R plants (population R-703) in orange; 2,4-D and two metabolites, 2,3-D (1) and 2,5-D (2) were detected. **(C)** Representative HPLC chromatogram of extract (second partition) from multiple R plants (population R-213) in green; 2,4-D and two metabolites, 2,3-D (1) and 2,5-D (2) were detected. **(D)** Representative HPLC chromatogram of extract (third partition into ether phase) from multiple R plants in blue; a sugar conjugated compound (3) was detected. Representative HPLC chromatograms from three independent experiments are shown.

TABLE 2 | Equation parameters of the log-logistic models used to estimate dose-response regression curves (% Survival and % Dry weight of untreated control) in susceptible and auxin resistant populations (R-703 and R-213) for 2,4-D with (+ malathion) or without (− malathion) previous application of malathion at 2,000 g/ha.

Parameter	Population	Treatment	X50 ± SE (g a.i./ha)[a*]	B ± SE[b]	Res SS[c]	RI[d]
% Survival	Susceptible	− malathion	150 ± 53*a*	1.0 ± 0.4	242	1
		+ malathion	125 ± 45*a*	0.9 ± 0.3	253	0.8
	R-703	− malathion	2154 ± 283*b*	2.7 ± 0.8	13,906	14
		+ malathion	94 ± 84*a*	0.8 ± 0.4	52	0.6
	R-213	− malathion	1164 ± 170*b*	1.7 ± 0.3	1,463	8
		+ malathion	205 ± 85*a*	1.0 ± 0.4	190	1.4
Dry weight (% of untreated)	Susceptible	− malathion	352 ± 51*a*	1.7 ± 0.5	127	1
		+ malathion	301 ± 47*a*	1.6 ± 0.4	154	0.9
	R-703	− malathion	736 ± 52*b*	5.5 ± 1.5	5	2
		+ malathion	255 ± 74*a*	1.5 ± 0.6	156	0.7
	R-213	− malathion	687 ± 84*b*	2.4 ± 0.7	51	2
		+ malathion	274 ± 85*a*	1.3 ± 0.5	592	0.8

**Means within a column, evaluation time, plant part and product followed by the same letter are not significantly different (P > 0.05).*
[a]XR50, herbicide concentration for 50% reduction of corn poppy survival and dry weight.
[b]Slope at the XR50.
[c]Res SS, residual sum of squares.
[d]RI (resistance index) = GR 50(Population) ÷ GR50(susceptible).

P450 family was postulated in *S. media* for mecoprop (Yuan et al., 2007). Therefore, it is hypothesized that the 2,4-D hydroxy metabolites detected in the metabolism experiments as result of enhanced metabolism could be due to the enhanced activity of a P450. However, this interpretation should be investigated and confirmed in the future. For example, a characterization

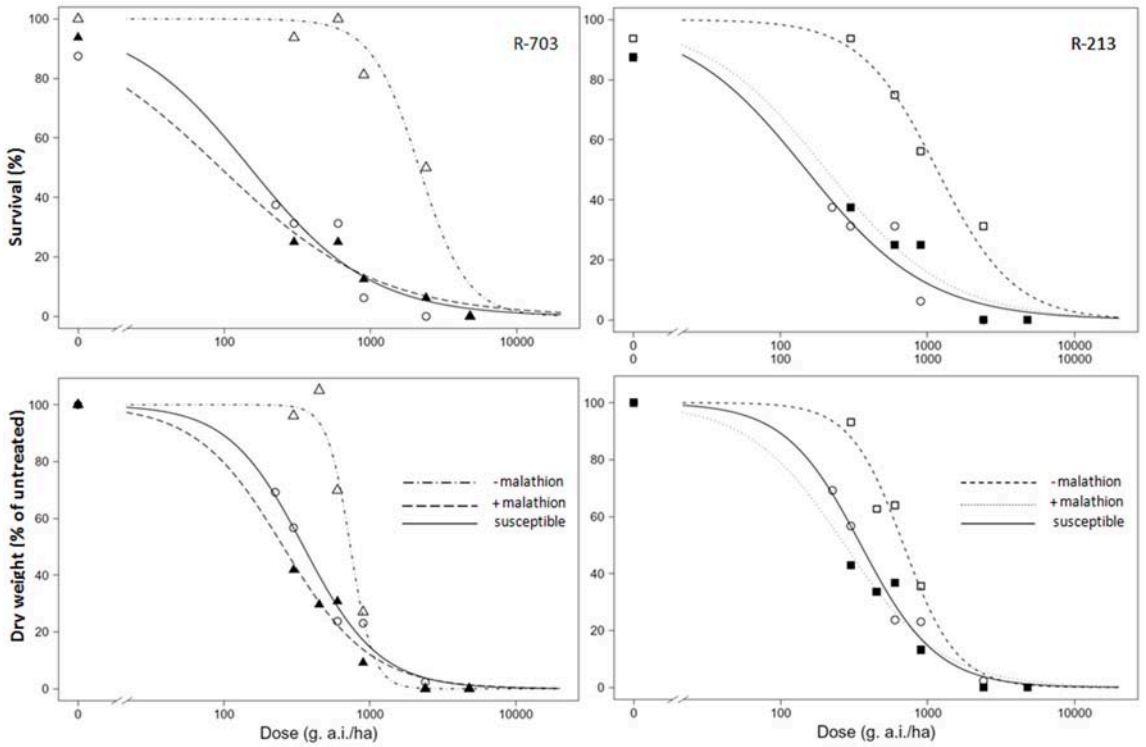

FIGURE 2 | Dose-response regression curves of 2,4-D on log scale without malathion or with addition of malathion at the dose 2,000 g/ha (+malathion) in susceptible and resistant populations R-703 (**left** side, 2,4-D resistant) and R-213 (**right** side, multiple resistant) of *Papaver rhoeas*. Up line, percentage of survival; Bottom line, percentage of the mean dry weight of untreated control plants. Dashed (R populations) and solid (susceptible) lines represent predicted values derived from the regression analysis.

of the possible P450 involved in the resistance response using different inhibitors (malathion, 1-aminobenzotriazole, piperonyl butoxide, or tetcyclasis) would be of value. Another issue to consider is whether this suspected enhanced metabolism in these two R *P. rhoeas* populations could detoxify or not herbicides from other modes of action, leading to cross-resistance or multiple resistance (Preston, 2004; Yu and Powles, 2014). In Spain, multiple resistant populations to ALS inhibitors and synthetic auxins were already reported back into the 90s (Rey-Caballero et al., 2017). But since then, cross-resistance cases to any other mode of action have not been reported in this species (Heap, 2017). Specificity of enzymes responsible of metabolic resistance to a given herbicide might explain the lack of cross resistances to other modes of action (Yu and Powles, 2014). A previous study reported ALS inhibitors enhanced metabolism in multiple R *P. rhoeas* from Spain (Rey-Caballero et al., 2017). Remains to be investigated if the detoxifying mechanisms in multiple R populations to 2,4-D and ALS inhibiting herbicides are linked or evolved independently.

Diverse NTSR mechanisms, including enhanced metabolism (Coupland et al., 1990) and decreased translocation (Jugulam et al., 2013; Goggin et al., 2016), have been reported in R weeds to auxinic herbicides. For example, *L. serriola* L. and *R. raphanistrum* resistant to 2,4-D displayed reduced uptake and translocation compared with S populations, but rates of 2,4-D metabolism were not different (Riar et al., 2011; Goggin et al., 2016). On the other hand, in MCPA-resistant *G. tetrahit* it was suggested that lower rate of MCPA translocation and a higher rate of MCPA metabolism in the roots were two different R mechanisms, as the inheritance of MCPA resistance was governed by at least two nuclear genes with additive effects (Weinberg et al., 2006). Interestingly, reduced translocation was also described in the same two *P. rhoeas* R populations used in this study (Rey-Caballero et al., 2016). So, it is hypothesized that two resistance mechanisms are present in these two populations, reduced translocation and herbicide degradation. How are they related and which is the primary mechanism remains unknown. One possibility is that 2,4-D metabolites could lead to decreased translocation due to less phloem mobility than parent compound (Han et al., 2013), or less likely due to permanent sequestration, i.e., in the vacuole via phase III ABC transporters (Riechers et al., 2010). More likely, it could be speculated that the impaired 2,4-D transport observed in previous studies (Rey-Caballero et al., 2016) is due to an alteration efflux ABCB transporters (auxin long-distance movement) preventing herbicide loading into phloem and its movement in resistant plants. The role of ABCB family in impaired transporter has been proposed for some species (Goggin et al., 2016; Kuepper et al., 2017). Afterwards,

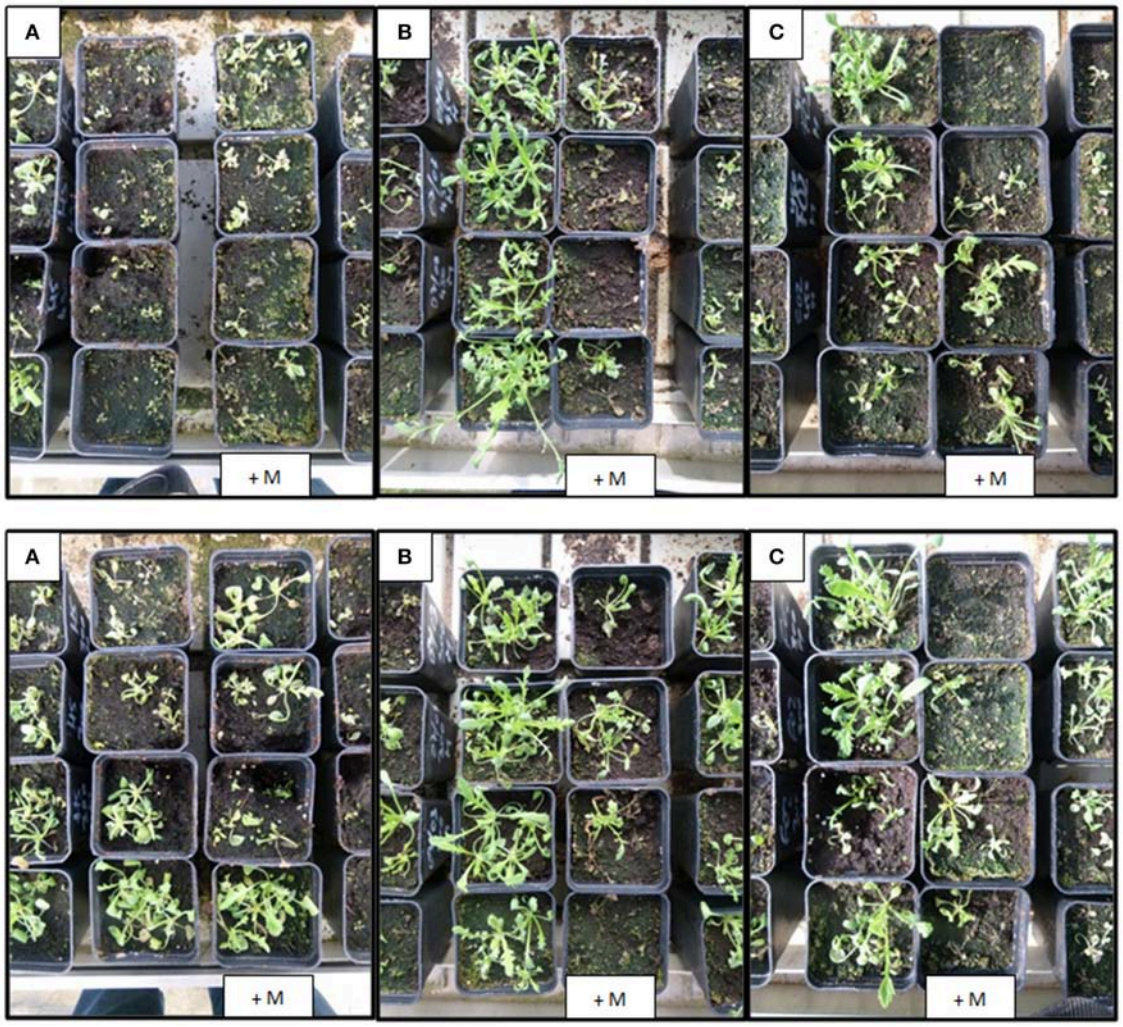

FIGURE 3 | Visual injury of three *Papaver rhoeas* populations 14 days after treatment with 2,4-D and with (+ M) or without previous malathion application at 2,000 g a.i./ha. Up line: 600 g a.i./ha; Bottom line: 450 g a.i./ha. Left **(A)**, susceptible population; Middle **(B)**, only 2,4-D R population (R-703); Right **(C)**, multiple R population (R-213).

while 2,4-D accumulation is occurring within cells cytoplasm, enhanced herbicide metabolism might start, a degrading route involving ring hydroxylation by means of a P450 in phase I. Again, new experiments are required to validate these statements and understand the relationship between these two resistance mechanisms.

A variety of metabolic degradation pathways for 2,4-D are known in plants and include side-chain degradation, side-chain lengthening, ring hydroxylation, conjugation, and ring cleavage (Riar et al., 2011). The presence of a sugar conjugate in the multiple R population (not in the 2,4-D R one) currently remains speculative, but it could be construed as an additional reaction in the plant detoxification processes. It is likely that the ascribed 2,4-D hydroxy metabolites were phase I products, that is, ring or methyl hydroxylates, whereas the third metabolite found in shoots and roots of the multiple resistant population (only at 168 HAT) could be a result of a second phase II reaction, that is, sugar conjugation, as ascribed afterwards by HPLC. This reaction might involve a glucosyl transferase enzyme (GT), which catalyzes a glucose conjugation and has been postulated as an enzyme implied in the enhanced metabolism observed in other resistant cases (Yu and Powles, 2014). This hypothesis could not indirectly be confirmed in this research, since to best of our knowledge, there are not known GT inhibitors to be used in dose-response experiments with whole plants. The sugar conjugate appeared to be mobile within *P. rhoeas* plants, since it was found both in shoots and, to a lesser amount, in roots. Considering that very small amounts of the sugar conjugate were detected only in the last assessment time, its presence should not be discharged in the only 2,4-D R population after longer evaluations times. An interesting report involving MCPA-resistant *R. raphanistrum* demonstrated increased MCPA

translocation to roots in the resistant population (in the absence of altered metabolism), which may have been related to extrusion of parent herbicide out of the roots into the soil (Jugulam et al., 2013). A root-localized ABC transporter (ABCB4) could play a role, as *Arabidopsis* abcb4 mutants were resistant to moderate concentrations of 2,4-D (Goggin et al., 2016).

In conclusion, this is the first study reporting enhanced 2,4-D metabolism in *P. rhoeas* in two R populations. According to the results presented in this research, we propose that the observed enhanced metabolism is mediated by a cytochrome P450. Resistance in this species is not only due to reduced translocation to target sites, as shown in a previous study with these same populations (Rey-Caballero et al., 2016), but also due to enhanced metabolism. So far, it is unknown which is the relative importance of each mechanism in the resistance response and how they are physiologically related. Future research, including inheritance studies and transcriptome analyses, should help elucidate the hypotheses stated in this research, the number of responsible genes and the potential risk of cross-resistance to other modes of action.

AUTHOR CONTRIBUTIONS

MS: Secured the funding; JT, AR-D, JR, MS, and RD: Idea and designed the experiments; JT, AR-D, JR, and AR-E: Performed the research; JT, AR-D, JR, AR-E, and RD: Interpretation and analysis of results (of raw data); JT, AR-D, JR, AR-E, MS, and RD: Wrote and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01584/full#supplementary-material

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ORIGINAL RESEARCH
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Mechanism of Resistance to Glyphosate in *Lolium perenne* from Argentina

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In Argentina, glyphosate resistance was reported in a *Lolium perenne* population after 12 years of successful herbicide use. The aim of the current paper was to put in evidence for the mechanism of glyphosate resistance of this weed. Susceptible leaves treated with different doses of glyphosate and incubated *in vitro* showed an accumulation of shikimic acid of around three to five times the basal level, while no changes were detected in leaves of glyphosate-resistant plants. The resistance mechanism prevents shikimate accumulation in leaves, even under such tissue-isolation conditions. The activity of the glyphosate target enzyme (EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase) was quantified at different herbicide concentrations. EPSPS from resistant plants showed no difference in glyphosate-sensitivity compared to EPSPS from susceptible plants, and, accordingly, no amino acid substitution causing mutations associated with resistance were found. While the glyphosate target enzymes were equally sensitive, the basal EPSPS activity in glyphosate resistant plants was approximately 3-fold higher than the EPSPS activity in susceptible plants. This increased EPSPS activity in glyphosate resistant plants was associated with a 15-fold higher expression of *EPSPS* compared with susceptible plants. Therefore, the over-expression of *EPSPS* appears to be the main mechanism responsible for resistance to glyphosate. This mechanism has a constitutive character and has important effects on plant fitness, as recently reported.

Keywords: glyphosate resistance, shikimate assay, EPSPS activity, *EPSPS* overexpression, perennial ryegrass

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INTRODUCTION

Glyphosate-resistant weeds put at risk the efficacy of glyphosate, an important herbicide used worldwide in current cropping systems. Since the first report of glyphosate resistance in 1998, the evolution of glyphosate resistant weed populations has rapidly escalated (Powles et al., 1998; Duke and Powles, 2008). At present, glyphosate resistance has been documented in 36 species (Heap, 2017). Herbicide-resistant weeds have several negative effects on farms and surrounding

regions. They would increase the costs and difficulties for weed control (Norsworthy et al., 2012). Accordingly, the evolution of glyphosate-resistant weeds threatens the long-term efficacy of the world's most important herbicide resource (Duke and Powles, 2008).

The target enzyme of glyphosate is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC. 2.5.1.19) that catalyses the reaction of shikimate-3-phosphate and phosphoenolpyruvate to yield 5-enolpyruvylshikimate-3-phosphate (Franz et al., 1997). This is the sixth enzyme on the shikimate pathway, which is essential for the synthesis of aromatic amino acids (Schönbrunn et al., 2001). The inhibition of EPSPS triggers the accumulation of shikimate, a substrate of the enzyme. Therefore, the effect of glyphosate on plants can be easily measured by monitoring the accumulation of this substrate of EPSPS (Dayan et al., 2015).

After EPSPS inhibition, several physiological processes are affected by glyphosate. The effect of the herbicide is not restricted to the inhibition of aromatic amino acid biosynthesis alone. It also disrupts the *de novo* biosynthesis of most non-aromatic amino acids, coupled with decreased protein synthesis (Maroli et al., 2016). In consequence, growth is inhibited (Pline et al., 2002; Orcaray et al., 2012), the transport of assimilates is reduced (Yanniccari et al., 2012a) and photosynthetic CO_2 assimilation decreases significantly (Olesen and Cedergreen, 2010). Several of these effects are limited or absent in glyphosate-resistant weed plants treated with the herbicide (Yanniccari et al., 2012a,b,c; Maroli et al., 2016).

Although more than 200 weed populations have shown glyphosate resistance in 26 countries (Heap, 2017), mechanism of plant survival was determined in a few cases. These mechanisms can be grouped into target-site and non-target-site based mechanisms (Powles and Yu, 2010). In the first category, the resistance can occur due to a gene mutation conferring an amino acid change that prevents herbicide binding. *EPSPS* target site mutations have been reported in six species, most frequently in the genus *Lolium* (Sammons and Gaines, 2014). Amino acid substitutions at Pro-106 have been the most common target site mutations in glyphosate-resistant *Lolium* spp. (Wakelin and Preston, 2006; Perez-Jones et al., 2007; Jasieniuk et al., 2008; Simarmata and Penner, 2008; Kaundun et al., 2011; Collavo and Sattin, 2012; Gonzalez-Torralva et al., 2012). However, Pro-106 substitutions confer only a modest degree of glyphosate resistance (Powles and Yu, 2010). Mutations in the *EPSPS* gene at Gly-101, Thr-102, Gly-144, and Ala-192 have been detected as sources of glyphosate resistance when expressed in *Escherichia coli*, but have not yet been reported in glyphosate-resistant weeds (Sammons and Gaines, 2014).

In crop fields under glyphosate selection, the evolution of a double amino acid substitution at Thr-102 and Pro-106 in the *EPSPS* in *Eleusine indica* conferring a high level of glyphosate resistance was recently communicated (Yu et al., 2015). In this species, other mechanisms of glyphosate resistance have also been found, associated with the over-expression of *EPSPS* (Chen et al., 2015). Target-gene duplication was previously reported in *Amaranthus palmeri* and *Lolium perenne* ssp. *multiflorum* (Gaines et al., 2010; Salas et al., 2012) and today it is a common and robust glyphosate resistance mechanism (Sammons and Gaines, 2014). On the other hand, non-target-site mechanisms of glyphosate resistance based on exclusion systems such as decreased herbicide retention and foliar uptake (Michitte et al., 2007), or reduced glyphosate translocation and vacuolar sequestration (Lorraine-Colwill et al., 2003; Perez-Jones et al., 2007; Ge et al., 2010; Vila-Aiub et al., 2012; Ghanizadeh et al., 2016) are well documented.

In the genus *Lolium*, allogamous species have demonstrated an unprecedented capacity to evolve resistance to multiple herbicides on five continents (Heap, 2017). In Argentina, glyphosate resistance was reported in a *L. perenne* population after 12 years of successful use (Yanniccari et al., 2012d). In this population, a 10.8-fold greater dose of glyphosate was necessary to match the control efficiency of a susceptible population (Yanniccari et al., 2012d). Nonetheless, the underlying mechanism of herbicide resistance has not been studied. The aim of the current paper was to determine the mechanism of glyphosate resistance in *L. perenne* from Argentina.

MATERIALS AND METHODS

Plant Material

Perennial ryegrass seeds from a glyphosate-resistant population were collected in a field in the south of Buenos Aires province (37°S, 62°W) in 2009. The fields had a history of 12 years of no-tillage agriculture, with weed control based on three applications of glyphosate per year, at doses ranging from 360 to 720 g ae ha^{-1} (Yanniccari et al., 2012d). One hundred field-collected seeds from 50 randomly chosen plants were germinated in Petri dishes containing filter paper with distilled water. The germination occurred in a growth chamber with 75 μmol m^{-2} s^{-1} of photosynthetically active radiation, in a regime of 12/12 h of light/darkness and temperatures of 25/15°C day/night. After 7 d, 25 seedlings taken at random were transferred to 250 cm^3 pots (contained in a plastic tray) filled with soil. The plants were grown in a greenhouse and the pots were randomized and subirrigated daily maintaining a water level of about 10 mm in the irrigation tray throughout the assay. Fertilizer (12:10:20, Nitrofoska, Compo Argentina) (2 g L^{-1}) was added every 15 d. Plants were grown for 8 weeks and vegetative clones of individual plants were propagated by tiller partition in order to obtain four ramets per plant. When individual ramets reached the BBCH 23–24 scale, each one was treated with glyphosate (isopropylamine salt of glyphosate, Roundup, 360 g L^{-1}, Monsanto Argentina) at 0, 500, or 2,000 g acid equivalent (ae) ha^{-1}. To this end, a backpack sprayer equipped with flat-fan nozzles (Teejet 11002) was used, delivering 150 L ha^{-1}. At 21 d post-application, plants were characterized as glyphosate susceptible (with no survivors at 500 g ae ha^{-1} and higher doses) or glyphosate resistant (surviving plants at 2,000 g ha^{-1} and lower doses).

Shikimate Accumulation Test

Six plants of each susceptible and glyphosate-resistant phenotype were employed to evaluate the shikimate accumulation in glyphosate-treated leaves incubated under *in vitro* conditions. Four last full-expanded leaves were cut at 0.5 cm above the ligule from tillers of each plant. Subsequently, the leaves of each

plant were randomly placed vertically on metal lab racks and treated with different doses of glyphosate (0, 500, 1,500 and 3,000 g ae ha^{-1}) using a manual sprayer calibrated to deliver 200 L ha^{-1}. After 10 min of application, each leaf was placed in a 20 cm^3 glass test tube, where the base of the leaf was immersed in with 1 mL of Hoagland nutrient solution diluted 1:3 with distilled water. The tubes were closed with flexible thermoplastic (Parafilm®) in which a 1 mm-diameter hole was made to promote gas exchange between the confined atmosphere and the exterior. Immediately, the tubes were incubated for 72 h under continuous light (300 μmol m^{-2} s^{-1} of photosynthetically active radiation) at 20°C (tubes were randomly placed every 24 h). After that, 0.05 g of fresh weight from the middle third of the each leaf was used for shikimic acid extraction, following the methodology of Singh and Shaner (1998) with the modifications of Perez-Jones et al. (2007). Shikimic acid was quantified with a double beam spectrophotometer (Shimadzu UV-160A, Shimadzu Corporation) at 382 ηm. The determination of the concentration of shikimic acid was based on a shikimate (3α,4α,5β-Trihydroxy-1-cyclohexene-1-carboxylic acid, 99%. Sigma Aldrich, Inc.) standard curve. The experiment was conducted as a completely randomized design and was repeated twice.

ANOVA was performed to evaluate the differences between experiment replicates, phenotypes, herbicide treatments, and interactions. Residual plots indicated that the variances were normally distributed and homogeneous. Means were compared using Fisher's LSD test ($p < 0.05$) when there were significant differences.

Enzyme Assay

The activity of EPSPS from glyphosate-susceptible and -resistant plants was evaluated under increasing glyphosate concentrations. The EPSPS extraction and assay protocols were modified from procedures described by Sammons et al. (2007). Forty grams of fresh unexpanded leaves were harvested from non-glyphosate treated clones of both phenotypes. Using liquid nitrogen, the samples were ground with 8 g of polyvinyl polypyrrolidone. Subsequently, 200 mL of extraction buffer (100 mM Tris-HCl, 5 mM EDTA, 10% (v/v) glycerol, 50 mM KCl, 10 mM 2-mercaptoethanol, 2 mM benzamidine, 0.5 mM phenymethysulphonyl fluoride and 10 μM leupeptine, pH 7.0) was added. Then, the crude extract was homogenized at 1,000 rpm for 5 min using a vertical axis micromotor with steel blades. After this, the extract was centrifuged at 800 × g for 45 min at 4°C and was subsequently filtered using a muslin cloth. Finally, the supernatant was fractionated with ammonium sulfate, and the 45–70% fraction was collected by centrifugation at 20,000 × g for 45 min. The pellet obtained was resuspended in a minimal volume of extraction buffer (2–3 mL) and desalted on a Sephadex G-25 column (PD-10, GE®) pre-equilibrated with extraction buffer according to the manufacturer's instructions. The eluate obtained was stored at −20°C until use in EPSPS activity determinations. For every fraction obtained, protein quantifications were carried out using the method of Bradford (1976) with bovine serum albumin as a reference standard.

EPSPS activity was assayed in 100 μL of 100 mM HEPES 100 mM, pH 7.0; 2 mM $(NH_4)_6Mo_7O_{24}$, 10 mM KF, 1 mM $MgCl_2$, 10% v/v glycerol, 0.5 mM shikimate-3-phosphate, 1.25 mM phosphoenolpyruvate (using ultrapure water (Milli-Q®) at 25°C by determining the amount of inorganic phosphate produced in the reaction following Eschenburg et al. (2002). EPSPS activity was expressed in enzyme unit (U) per milligram of protein (1 U stands for 1 ηmol of phosphate produced per minute in the assayed reaction). The activities of glyphosate-susceptible and -resistant plants were determined at 0, 1, 5, 25, 50, 500, 1,000, 10,000 μg L^{-1} glyphosate [96% N-(phosphomethyl) glycine, Sigma Aldrich] concentrations.

EPSPS activity data was used to build dose response curves with a non-linear log-logistic regression model of standard slope:

$$y = C + \frac{D - C}{1 + \frac{x}{I50}}$$

where y represents the EPSPS activity at the glyphosate concentration x; C is the lower asymptote, D is the upper asymptote and I50 is the herbicide concentration required to achieve 50% of the maximum response. To assess the accuracy of the model, an F-test for model significance, residual variance analysis and coefficient of determination (R^2) were calculated. Finally, the parameters from glyphosate-susceptible and -resistant models were contrasted using an F-test ($P < 0.05$).

EPSPS Partial Sequencing

Total RNA was extracted from leaf tissue of both glyphosate-susceptible and -resistant plants (S1, S2, S3 and R1, R2, R3; respectively) using the Trizol® reagent (Invitrogen) method according to the manufacturer's protocol. DNA in the RNA samples was degraded using DNase (RQ1 RNase-Free DNase kit, Promega) for 30 min at 37°C. Subsequently, the RNA was quantified spectrophotometrically at 260 ηm.

First strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA. Reverse transcription was carried out in a 25 μL reaction mixture containing 1 μg oligo $d(T)_{15}$ primer, M-MLV reverse transcriptase (200 U), RNasin (25 U), 8 mM dNTPs and M-MLV reverse transcriptase reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT) at 42°C for 1 h.

The resulting cDNA was used as a template for amplification of the *EPSPS* sequence. Forward primer (5′-AAAGGATGCCAAGGAGGAAGTAA-3′) and reverse primer (5′-GTACTTCTGTCCTCCTTTAATG-3′) were employed to amplify a highly conserved region in which point mutations conferring glyphosate resistance have been reported (Sammons and Gaines, 2014). A 466-bp fragment was obtained in PCR reactions (initial denaturation at 94°C for 2 min and 30 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min) containing: 250 ηg cDNA template, 0.4 μM of each primer, 0.2 mM dNTPs, 0.5 mM $MgCl_2$, 1X reaction buffer (Inbio Highway), and 0.5 U Taq polymerase (Inbio Highway) in a 25 μL reaction mix. PCR products were purified by precipitation at 20°C with three parts of absolute ethanol and 0.1 parts of sodium acetate (3 M) for each PCR

product. The samples were centrifuged at 32,000 g for 20 min at 4°C and then washed three times with 70% ethanol. Each DNA pellet was diluted in ultrapure water and sequenced from both ends. DNA sequences obtained were cleaned, aligned, translated and compared at the 101, 102, 106, 144, and 192 codons (numbers based on the plant EPSPS numbering system used by Padgette et al., 1996).

EPSPS Expression

The cDNAs obtained from three plants of both phenotypes were employed as a template for two step qPCR reactions using an StepOnePlus™ Real-Time PCR System (Life Technologies) and FastStart Universal SYBR Green Master (Roche). *Cinnamoyl-CoA reductase* (*CCR*, AY061888.1) was used for normalization (forward primer: 5′-AGCAGCCATACAAGATGT-3′ and reverse primer: 5′- AGCTAGGGTTTCCTTGTC-3′). Primers specific to *EPSPS* were designed (forward: 5′-CTTGAGTTCCTTGCTGATG-3′ and reverse: 5′-GTACTTCTGTCCTCCTTTAATG-3′) and the following program was used: one cycle at 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Real time fluorescence data were captured at the end of each cycle of 15 s. Melt-curve analysis was conducted by holding the samples at 95°C for 15 s, then reducing the temperature to 60°C for 1 min, and then increasing the temperature to 95°C for 15 s. During this change of temperature fluorescence was measured continuously. Negative controls consisting of primers with no template were included. Each measurement was performed in triplicate. Results were expressed as fold increases in *EPSPS* expression relative to *CCR* and data were analyzed using ANOVA. Residual plots corroborated that the variances were normally distributed and homogeneous. The means were compared using the LSD test ($P < 0.05$).

RESULTS

Shikimate Accumulation Test

Shikimate concentration in leaves was strongly affected by the herbicide treatment depending on the phenotype studied. The interaction between the phenotype and the treatment showed highly significant differences (**Table 1**). Both experiments carried out had similar results ($P > 0.05$, **Table 1**) and data from them were pooled.

Shikimic acid levels quantified in glyphosate treated leaves were significantly different between samples from glyphosate-susceptible and -resistant plants (**Figure 1**). Leaves from susceptible plants increased their shikimate concentration by around 3.5-fold in response to glyphosate application at 500 or 1,500 g ae ha^{-1} (**Figure 1**). When 3,000 g ae ha^{-1} was sprayed the susceptible leaves increased their shikimate levels 5-fold compared with controls without glyphosate (**Figure 1**). Neither of the glyphosate doses applied significantly affected the shikimic acid concentration of leaves from glyphosate-resistant plants (**Figure 1**).

TABLE 1 | Summary of ANOVA: mean square (MS), degrees of freedom (df), and probability values (P) for the effect of replication of the experiment comparing the effect of *Lolium perenne* phenotype (glyphosate-susceptible and -resistant phenotypes), treatment (dose of glyphosate: 0, 500, 1,500, and 3,000 g ae ha^{-1}) and their interaction on shikimate concentration.

Source of variation	*df*	MS	*P*
Experiment	1	194,002	0.7213
Phenotype	1	114,065,468	<0.0001
Treatment	3	25,795,044	<0.0001
Experiment*Phenotype	1	4,220	0.9580
Experiment*Treatment	3	495,236	0.8065
Phenotype*Treatment	3	15,145,488	<0.0001
Experiment*Phenotype*Treatment	3	235,714	0.9262
Error	144	1,518,769	
Total	159		

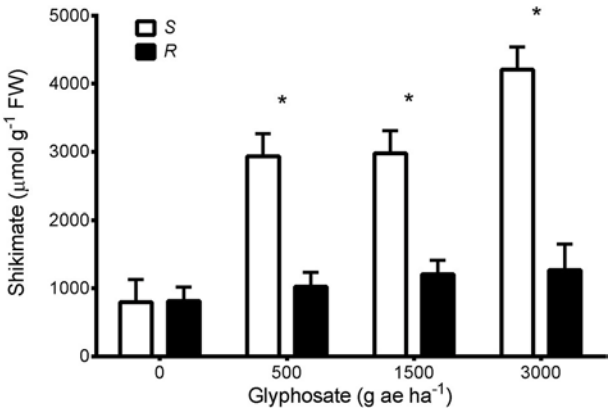

FIGURE 1 | Shikimate accumulation in foliar tissues of glyphosate-susceptible (S) and -resistant phenotypes (R) after glyphosate treatments. Vertical bars represent ±1 standard error of the mean. Asterisks indicate significant differences between phenotypes within each dose.

EPSPS Activity

Based on the EPSPS activity data, a model was fitted for each biotype ($P < 0.001$ in both cases) and the parameters of glyphosate-susceptible and -resistant models were contrasted (**Table 2**, **Figure 2**). Comparison of the lower asymptotes (*C*), i.e., the level of EPSPS activity at infinitely high concentrations of the inhibitor, showed no significant differences between phenotypes. The upper asymptote (*D*), which is associated with EPSPS activity without glyphosate in the reaction medium, was significantly different between phenotypes ($P = 0.0018$). In this sense, basal EPSPS activity from glyphosate-resistant plants was approximately 3-fold higher than the EPSPS activity from susceptible plants. The half-maximal inhibitory concentration (*I50*) was not significantly different between the glyphosate-susceptible and -resistant models ($P = 0.93$) (**Table 2**, **Figure 2**).

EPSPS Partial Sequencing

A cDNA fragment of the *EPSPS* gene was sequenced and no nucleotide differences were identified between the sequences

TABLE 2 | Non-linear regression parameters for the activity of EPSPS from glyphosate-susceptible (S) and -resistant (R) phenotypes and *P*-values for model accuracy.

Phenotype	D (U mg^{-1} protein)	C (U mg^{-1} protein)	I50 ($\mu g\ L^{-1}$ glyphosate)	*P*
S	3.2	−0.1	430.0	<0.001
R	8.7*	−0.1	462.8	<0.001

Asterisks indicate significant differences between phenotypes.

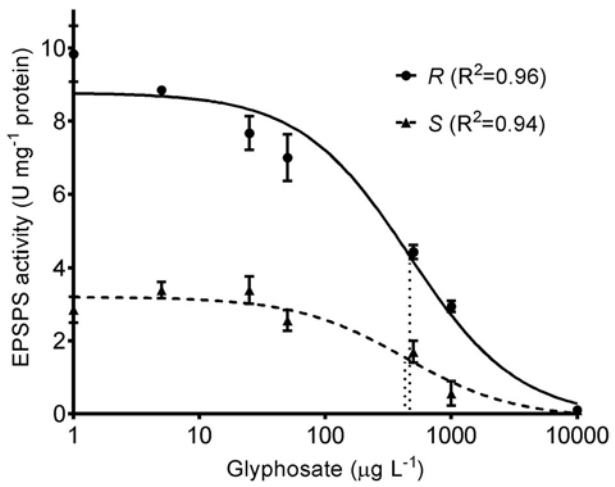

FIGURE 2 | EPSPS activity of glyphosate-susceptible (S) and -resistant phenotypes (R) subjected to different glyphosate concentrations. Symbols represent mean values and the vertical bars are ±1 standard error of the mean. The curves represent predicted responses and dotted lines indicate I50 for each model.

from glyphosate-resistant and -susceptible individuals. Therefore, no substitutions were recorded at Gly-101, Thr-102, Pro-106, Gly-144, or Ala-192 residues, which have been associated with a mechanism of resistance to glyphosate (Sammons and Gaines, 2014; **Figure 3**).

EPSPS Expression

EPSPS transcript abundance was measured relative to *cinnamoyl-CoA reductase*. The relative expression levels of *EPSPS* were significantly different between phenotypes ($P < 0.01$) wherein glyphosate-resistant plants showed around 15-fold higher expression than susceptible plants (**Figure 4**).

DISCUSSION

The quantification of shikimic acid in glyphosate-treated plants has been used as metabolic marker of glyphosate sensitivity (Dayan et al., 2015). The shikimate pathway consumes ≥30% of carbon fixed through the Calvin cycle (Maeda and Dudareva, 2012) and the inhibition of EPSPS causes a non-regulated carbon flux into the shikimate pathway through induction of the expression of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, the first enzyme of this metabolic pathway (Zabalza et al., 2017). These alterations are reflected in a massive carbon flow to shikimate-3-phosphate, which is converted into high levels of shikimate (Duke and Powles, 2008).

The shikimic acid concentration measured in susceptible *L. perenne* plants sprayed with glyphosate was approximately three to five times the basal level while no changes were detected in glyphosate-resistant counterparts (Yanniccari et al., 2012d). In the current results, susceptible leaves treated with different doses of glyphosate and incubated *in vitro* showed shikimic acid accumulation, but leaves from glyphosate-resistant plants showed no change in shikimate concentration. This could be considered as a semi-destructive method in order to evaluate the glyphosate sensitivity of plant materials, where susceptible genotypes could be tested without killing them.

While *in vitro* methods based on shikimate quantification have been proposed for identifying glyphosate-resistant plants, in most methods leaf fragments are immersed in a medium with glyphosate and thus incubated (Shaner et al., 2005). This methodology has been effective to quickly detect differences between glyphosate-susceptible and -resistant plants (Perez-Jones et al., 2005). However, the doses used in these methodologies cannot be compared to the doses sprayed under field conditions. Moreover, the process of herbicide absorption in a system where the leaves are immersed could be very different to the foliar uptake that occurs in treated plants under field conditions. In this regard, the shikimate accumulation test in the current work successfully addressed these limitations.

The glyphosate-resistant and -susceptible plants showed differential glyphosate sensitivity when the whole plant was sprayed with the herbicide (Yanniccari et al., 2012d), as when a sample of leaf was treated and shikimate concentration was recorded. In both cases, the mechanism of resistance put into play would prevent shikimate accumulation in glyphosate-resistant plants.

The activity of herbicide target enzymes at different herbicide concentrations has been used to determine if a target site mechanism provokes low sensitivity in resistant plants (Baerson et al., 2002; Burke et al., 2006; Yu et al., 2010; Cruz-Hipolito et al., 2011). Following this approach, glyphosate-susceptible and -resistant models of EPSPS activity showed no significant differences in half maximal inhibitory concentration (*I50*) (**Table 2**). As a consequence, EPSPS from glyphosate-resistant plants was not different in glyphosate sensitivity compared to EPSPS from susceptible plants. Accordingly, no changes in the nucleotide sequences associated with Gly-101, Thr-102, Pro-106, Gly-144, and Ala-192 were found in either case. The affinity between the glyphosate molecule and EPSPS from resistant plants was not affected, unlike in other cases of glyphosate resistance (Ng et al., 2004; Yu et al., 2007; Simarmata and Penner, 2008; Kaundun et al., 2011).

While the glyphosate target-site was equally sensitive, differential levels of EPSPS activity was detected when comparing the plant materials. At a glyphosate concentration of 1,000 µg L^{-1}, when the EPSPS activity of susceptible plants was inhibited

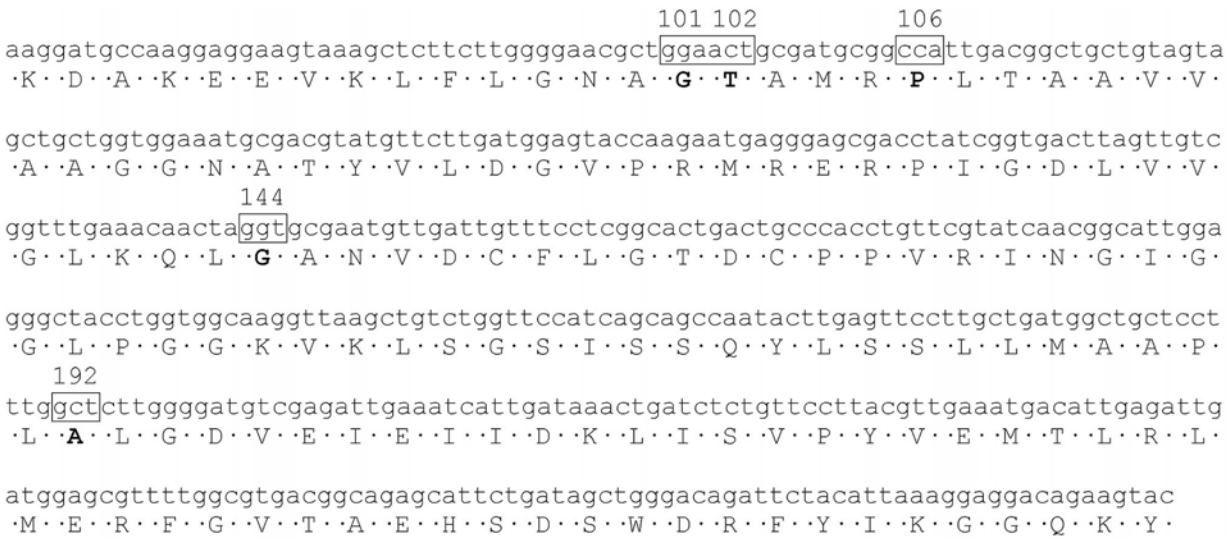

FIGURE 3 | Sequence of *EPSPS* cDNAs isolated from the glyphosate-resistant phenotype and conceptual translation of the amino acid sequence. Resistance conferring codons are show in boxes (numbers refer to amino acid positions in the EPSPS plant sequence according to Padgette et al. (1996). No mutations were detected in positions reported as sources of glyphosate resistance.

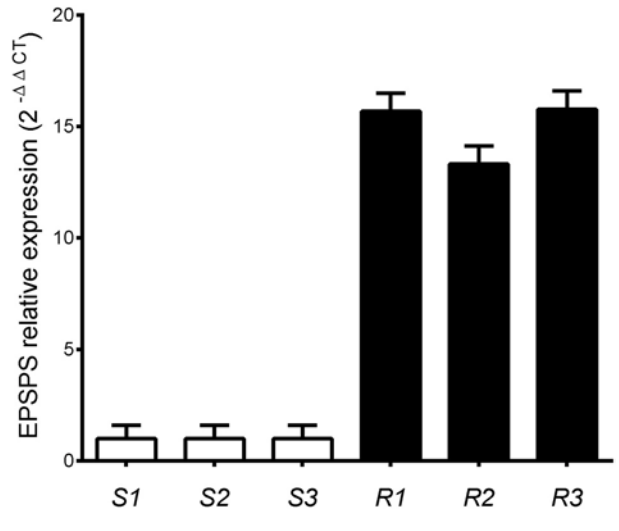

FIGURE 4 | Relative *EPSPS* expression in glyphosate-susceptible (S) and -resistant (R) plants of *Lolium perenne* plants, using the *cinnamoyl-CoA reductase* gene for normalization. Vertical bars represent ±1 standard error of the mean.

by ∼90%, the level of EPSPS activity measured in the crude extract from glyphosate-resistant plants was not different to the EPSPS activity of the susceptible material without glyphosate (**Figure 2**). This higher EPSPS activity likely ensures maintenance of a functional shikimate pathway after glyphosate treatment in the glyphosate-resistant plants.

The increased EPSPS activity may be due to increased transcription of the *EPSPS* gene or greater stability of the EPSPS enzyme at a similar transcription rate compared with susceptible plants. In the current work, the glyphosate-resistant plants showed 15-fold higher expression of *EPSPS* than the susceptible plants and this explains the differential glyphosate sensitivity between individuals. In this sense, the glyphosate effect on net carbon assimilation, chlorophyll fluorescence, and production and transportation of assimilated carbon would be prevented by the high activity of the target enzyme (Yanniccari et al., 2012a,b).

This mechanism of resistance is constitutive in character, i.e., it does not depend of a glyphosate treatment to be induced. In consequence, the high level of expression of *EPSPS* likely has important effects on plant fitness, as have recently been detected on the inhibition of growth and seed production (Yanniccari et al., 2016). Considering that molecules from the shikimate pathway can account for up to 60% of the total plant dry weight (Haslam, 1993), the over-expression of *EPSPS* likely affects the regulation of the shikimate pathway and would therefore provoke pleiotropic effects on several physiological processes associated with growth and seed production.

Although is not possible to rule out that other resistance mechanisms also operate, the over-expression of *EPSPS* appears to be the main mechanism responsible for resistance to glyphosate. This mechanism would be controlled by a single locus with incomplete dominance (Yanniccari et al., 2015) and would provoke low glyphosate sensitivity in plants treated in the field as observed for foliar tissues sprayed with glyphosate and incubated *in vitro*.

AUTHOR CONTRIBUTIONS

MY, CI, CN, DG, and AC conceived and designed the experiments; MY and MG performed the experiments; MY, DG, and AC analyzed the data and MY wrote the paper.

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Differential Resistance Mechanisms to Glyphosate Result in Fitness Cost for *Lolium perenne* and *L. multiflorum*

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Multiple mechanisms of resistance to glyphosate are exhibited by populations of *Lolium* spp. worldwide. Association of resistance with growth and reproductive fitness is an important predictor for long-term success of glyphosate-resistant (R) versus glyphosate-susceptible (S) biotypes. Numerous studies were conducted on R- and S-biotypes of Italian ryegrass (*Lolium multiflorum*) and perennial ryegrass (*L. perenne*) to characterize the underlying mechanism(s) of glyphosate resistance and associate this with growth and reproductive fitness. *L. perenne* expressed both altered uptake and translocation as well as a genetic change at 106-Pro to –Ser, This pattern for two resistance mechanisms is unique. *L. multiflorum* also exhibited altered uptake and translocation as well as duplication of EPSPS gene copies. Reduced plant biomass and height for R-versus S-biotypes of both species was evident over two growing seasons. This resulted in S- versus R- *L. multiflorum* producing up to 47 and 38% more seeds in 2014 and 2015, respectively. S- *L. perenne* produced up to 20 and 30% more seeds in 2014 and 2015, respectively. Both non-target site and target-site mechanisms of glyphosate resistance can render *Lolium* spp. at a competitive disadvantage. This has long-term implications for the success of glyphosate-resistant plants in the absence of selection pressure.

Keywords: *Lolium* spp., resistance, glyphosate, mechanisms, fitness cost

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INTRODUCTION

Over the past two decades, glyphosate (*N*-phosphonomethyl glycine) has been widely used worldwide for non-selective weed control in genetically modified crops, and for over four decades as a non-selective herbicide in crop and non-crop situations (Duke, 2017). Glyphosate (group G) inhibits the enzyme 5-enolpyruvlshikimate-3-phosphate synthase (EPSPS), which catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate to form 5-enolpyruvyl-shikimate-3-phosphate (EPSP) (Maeda and Dudareva, 2012). Inhibition of EPSPS specifically results in accumulation of shikimate in sensitive plants and measurement of shikimate levels is a common method to ascertain resistance in selected species (Singh and Shaner, 1998). The result is prevention of biosynthesis of aromatic amino acids, which is highly lethal to sensitive plants (Sammons and Gaines, 2014). Metabolism in plants is limited; symptoms in treated plants are slow to develop, but plant death is evident within 20 days following treatment.

Long-term use of glyphosate has contributed to selection of over 37 weed species world-wide that exhibit resistance (Heap, 2017). Herbicide resistance is an evolutionary phenomenon, allowing resistant weeds exposed to the labeled dose of an herbicide to sustain growth with little or no symptomology (Fernández-Moreno et al., 2017a). Factors important in the development of herbicide-resistant species include strict dependence on one herbicide mode of action and continuous use (within and over years) of this herbicide mode of action until control failures are observed (Fernández-Moreno et al., 2017b).

Initial reports of glyphosate-resistant (R) *Lolium* species occurred in Australia in 1996 (Pratley et al., 1996; Powles et al., 1998). Currently, three *Lolium* species, *L. rigidum* Gaud., *L. perenne* L., and *L. multiflorum* Lam. exhibit glyphosate resistance, with populations scattered across 14 countries (Heap, 2017). Similar to species evolving resistance to other herbicides, R *Lolium* spp. resulted following exclusive use of glyphosate multiple times within and over growing seasons (Nandula et al., 2008; Bracamonte et al., 2016). The level of resistance in *L. rigidum* and *L. multiflorum* differs widely among populations, varying from 2- to 100-fold (Preston et al., 2009).

A plethora of mechanisms underlying resistance to glyphosate have evolved, and can be categorized into two groups: non-target site resistance (NTSR), and target site resistance (TSR) (Sammons and Gaines, 2014). Expression of NTSR mechanisms are common in many weed species, and essentially *in vivo* limit glyphosate from reaching the target enzyme in sensitive plants (Ghanizadeh and Harrington, 2017). This mechanism consists of reduced uptake and translocation, increased vacuolar sequestration, and metabolism to non-toxic compounds, resulting in decreased levels of glyphosate interacting with EPSPS (Ge et al., 2014; Fernández-Moreno et al., 2017b; Kleinman and Rubin, 2017). Levels of resistance conferred by the NTSR mechanisms are variable and unpredictable between plant species (Ghanizadeh et al., 2015). However, TSR mechanisms have been studied and reported more often than NTSR mechanisms.

Target site resistance mechanisms have been widely studied in plant species for many herbicide modes of action, and often underlie resistance. One TSR mechanism involves one or more mutations in the DNA encoding the target protein of the herbicide, which leads to changes in amino acids or conformational changes in protein folding, ultimately resulting in high levels of resistance (Sammons and Gaines, 2014; Fernandez et al., 2015; Yu et al., 2015; Alcántara-de la Cruz et al., 2016a). Sammons and Gaines (2014) summarized the single mutations in the Pro-106 position of the EPSPS gene that have resulted in resistance for a number of species. In addition, a double mutation was found in the Thr-102-Ile position as well as Pro-106-Ser position, conferring resistance in *Eleusine indica* (Chen et al., 2015; Yu et al., 2015). Alternatively, overexpression of the herbicide's target protein confers a limited level of resistance. This mechanism is exemplified by amplification of genes encoding EPSPS protein (Gaines et al., 2010; Salas et al., 2012, 2015). Recently, glyphosate resistance involving two TSR mechanisms, Pro-106 mutation and EPSPS amplification, was discovered in a population of *E. indica* (Gherekhloo et al., 2017).

Evolved herbicide resistance and subsequent enrichment of resistant individuals by high selection pressure can result in large populations of resistant weeds (Vila-Aiub et al., 2009, 2011). In the continued presence of selection pressure, resistant weeds have an ecological advantage versus susceptible individuals of the same species. However, Vila-Aiub et al. (2014) theorized that in the absence of selection pressure, the cost of herbicide resistance may render plants at a competitive disadvantage.

The expression of reduced fitness associated with herbicide resistance varies with species and herbicide mode of action (Yanniccari et al., 2012a). Glyphosate resistance associated with fitness costs have periodically been identified (Yanniccari et al., 2016) for both NTSR and TSR mechanisms (Preston and Wakelin, 2008; Yu et al., 2015). Pedersen et al. (2007) reported that seed production of glyphosate-resistant (R) versus glyphosate-susceptible (S) *L. rigidum* was reduced, with TSR as the underlying mechanism of resistance. The objective of this research was to assess the level of glyphosate resistance in suspect resistant and susceptible biotypes of *L. multiflorum* and *L. perenne*. In addition, the mechanism(s) involved in both suspect *R*-glyphosate biotypes was assessed, as well as any associated growth and reproductive fitness cost.

MATERIALS AND METHODS

Plant Material

Mature seeds of suspect R Italian ryegrass (*L. multiflorum*) biotype were collected from a vineyard located in Peso da Régua, Portugal. This vineyard was treated with 1080 g ae ha^{-1} of glyphosate (3 L ha^{-1}) or higher for at least 15 consecutive years (Roundup®, 360 g ae L^{-1} as isopropylamine salt). Seeds of an S ryegrass biotype were collected from a nearby vineyard; glyphosate had never been used at that location. These biotypes will hereafter be termed R-Douro and S-Douro. In addition, seeds of R and S perennial ryegrass (*L. perenne*) biotypes were provided by the FITO® seed company (Barcelona, Spain). These biotypes will hereafter be termed R-Golf and S-Golf (R-Golf is targeted for production on golf courses).

Seeds were germinated in petri dishes with moistened filter paper (distilled water). Germinating seedlings were transplanted into pots (7 × 7 × 6 cm) containing sand and peat (1:2 v/v) and placed in a growth chamber under the following conditions: 28/18°C (day/night); 16 h photoperiod, light intensity of 850 μmol m^{-2} s^{-1} photosynthetic photon flux density; and 80% relative humidity.

Identification of *Lolium* Weed Species Using Molecular Markers

Following the methodology of Fernández-Moreno et al. (2017a), AFLP markers were used to characterize the genetic similarity of R and S biotypes for each *Lolium* species, enabling assessment if the populations belonged to different species. Plant material included two S biotypes (S-Golf and -Douro), and two R biotypes (R-Golf and -Douro). Twenty plants of each biotype were used for molecular analysis. Additionally, twelve reference S-plants (*L. multiflorum* and *L. perenne*) were included in the study.

DNA was extracted from the leaf tissue (50 mg) using the Speedtools DNA Extraction Plant kit (BIOTOOLS, Madrid, Spain). The quality and concentration of the DNA was evaluated by spectrophotometric analysis with light absorption at 260 and 280 nm. AFLP analysis was carried out using the fluorescent AFLP IRDye kit for Large Plant Genome Analysis (LI-COR Biosciences). Template preparation was performed following the protocol included in the kit, including digestions with EcoRI and MseI restriction enzymes (Invitrogen). Fernández-Moreno et al. (2017a) have described primers for selective amplification.

AFLP products were separated by polyacrylamide electrophoresis using an automated sequencer (LICOR 4300). Polymorphic AFLP markers and primers were identified and individuals were scored for the presence or absence of AFLP fragments using the computer package SAGAMX 2 GENERATION. UPGMA analysis was performed with AFLP marker data using the computer program NTSYSpc 2.2.

Whole Plant Dose-Response

Seedlings at the 3–4 leaf growth stage were treated with glyphosate using a laboratory chamber (SBS-060 De Vries Manufacturing, Hollandale, MN, United States) equipped with 8002 flat fan nozzles and delivering 200 L ha^{-1} at 250 KPa. The isopropylamine salt formulation of glyphosate (Roundup Energy SL, 450 g ae L^{-1}, Monsanto) was applied at: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 g ae ha^{-1}. The experiment was arranged using nine replications and was repeated. Plant mortality and dry mass were evaluated 21 days after treatment (DAT). Plant dry mass was measured for above ground tissue after drying at 60°C for 72 h.

Spray Retention Assay

At the 3–4 leaf growth stage, R- and S-plants of each biotype were sprayed with 300 g ha^{-1} of glyphosate and 100 mg L^{-1} Na-fluorescein using conditions as described above. Na-fluorescein was used as a labeling reagent to determine the amount of herbicide solution retained. Once the foliage had dried (30 min), shoot tissue was harvested and immersed in 50 mL of 5 mM NaOH for 30 s to remove spray solution. Fluorescein absorbance was determined using a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) with excitation wavelength of 490 nm and absorbance at 510 nm. Plant tissue was dried for 48 h at 60°C and recorded. The experiment was arranged in a completely randomized design with four replications per biotype and repeated. Spray retention was expressed as mL spraying solution per gram dry matter.

Shikimic Acid Accumulation

Fifty mg (4 mm leaf disks) were harvested from the youngest fully expanded leaf at the 3–4 tiller stage from 15 plants per biotype (Pedersen et al., 2007; Hanson et al., 2009). Five disks of fresh tissue were transferred to 2 mL Eppendorf tubes containing 1 mL of 1 mM $NH_4H_2PO_4$ (pH 4.4). At this point, 1 μL of glyphosate was added to each tube resulting in the following concentrations: 0, 0.1, 0.5, 1, 5, 10, 50, 100, 200, 400, 500, 600, and 1000 μM. Tubes were incubated in a growth chamber for 24 h under the above conditions. After 24 h, tubes were stored at −20°C until further analysis. For analysis, tubes were thawed at 60°C for 30 min. Thereafter, 250 μL of 1.25 N HCL was added to each Eppendorf. Tubes were incubated at 60°C for 15 min. A 125 μL aliquot from each Eppendorf was pipetted into a new 2 mL Eppendorf, and 500 μL of periodic acid and sodium metaperiodate (0.25 % [wt/v] each) was added. After incubation at room temperature for 90 min, 500 μL of 0.6 N sodium hydroxide and 0.22 M sodium sulfite were added. Finally, liquid in the tubes was transferred to glass vials. Within 30 min, light absorption at 380 nm was measured in a spectrophotometer. For each glyphosate concentration and biotype, five replications were used and the experiment repeated.

^{14}C-Glyphosate Uptake and Translocation

Experiments were carried out according to Fernández-Moreno et al. (2016). A solution of ^{14}C-glyphosate (American Radiolabeled Chemicals, Inc., Saint Louis, MO, United States) was prepared by adding radiolabeled glyphosate to commercially formulated glyphosate, with the final specific activity of 0.834 kBq μL^{-1}. This concentration corresponded to 300 g ae ha^{-1} and a volume of 200 L ha^{-1}. When R- and S-biotype plants reached the 3–4 leaf growth stage, 1 μL (0.834 KBq $plant^{-1}$) of solution was applied onto the adaxial surface of the second most mature leaf with a micropipette (LabMate Soft, HTL Lab Solutions, Warsaw, Poland).

Following 12, 24, 48, 72, and 96 h after treatment (HAT), the treated leaf was washed with 3 mL of water:acetone (1:1 v/v) solution to remove unabsorbed glyphosate. The rinsate was mixed with 2 mL of scintillation cocktail and radioactivity analyzed by liquid scintillation spectrometry (LSS) using a scintillation counter (Beckman LS 6500, Fullerton, CA, United States.). The remainder of the plant was carefully removed from the pot and roots gently washed with distilled water. Plant tissue was sectioned into treated leaf, remaining shoot tissue, and roots. Plant tissue was dried at 60°C for 96 h and combusted in a Packard Tri Carb 307 biological sample oxidizer (Packard Instruments, Meriden, CT, United States). Evolved $^{14}CO_2$ was trapped and counted by LSS in an 18-mL mixture of Carbo-Sorb E and Permafluor E++ (1:1v/v) (Perkin-Elmer, Packard Bioscience BV). The quantity of radiolabeled glyphosate deposited on plant leaves was assessed by washing a plant leaf of each biotype immediately after treatment. The experiment was arranged in a completely randomized design with five replications per biotype and was repeated. The percentage of absorbed herbicide was expressed as: [kBq in combusted tissue/(kBq in combusted tissue + kBq in leaf washes)] × 100.

^{14}C-Glyphosate Visualization

Distribution of ^{14}C-glyphosate throughout the R and S plants was visualized using a phosphor imager (Cyclone, Perkin-Elmer, Waltham, MA, United States). Plants were grown, treated, and tissue collected in the same way as described for the uptake and translocation experiments. Following 96 HAT, roots of intact plants were rinsed to remove soil and treated leaves were rinsed to remove unabsorbed glyphosate. Following gentle blotting on

absorbent tissue to remove water, plants were gently pressed and dried at room temperature. Dry tissue was placed adjacent to 25 × 12.5 cm phosphor storage film for 13 h and scanned for radiolabel distribution using a phosphor imager. The experiment was conducted once using three plants for each biotype.

Metabolism Study

R- and S-biotypes were grown to the 3–4 leaf growth stage, and then treated with glyphosate at 300 g ha^{-1} as described in the dose response section. At 96 HAT, the methodology of Rojano-Delgado et al. (2010) was followed to determine glyphosate and the primary metabolites, aminomethylphosphonic acid (AMPA), glyoxylate, sarcosine, and formaldehyde. Quantification of glyphosate and metabolites was determined by reversed polarity capillary electrophoresis using a 3D Capillary Electrophoresis Agilent G1600A instrument equipped with a diode array detector (DAD) at a wavelength of 190–600 nm. For calibration of instrumentation, purchased standards of glyphosate, AMPA, sarcosine, formaldehyde, and glyoxylate were used. Preparation of treated leaf tissue for analysis was as follows: leaf tissue was washed with distilled water, frozen in liquid nitrogen, and stored at −40 °C until use. The aqueous background electrolyte consisted of 10 mM potassium phthalate, 0.5 mM hexadecyltrimethylammonium bromide, and 10% acetonitrile at pH 7.5. The calibration equations were established from non-treated plants and known concentrations of glyphosate and associated metabolites, which were determined from their peak areas in the electropherogram. The average value for the content of glyoxylate, which is naturally produced by plants, was subtracted from the mean content of each biotype. The experiment was arranged in a completely randomized design with five replications per biotype. Experiments with each biotype were repeated.

EPSPS Enzyme Activity Assays

Samples of 5 g of leaf tissue (3–4 leaf growth stage) from each biotype were ground to a fine powder using a pestle and mortar. The methodology described by Sammons et al. (2007) was used for EPSPS extraction. The total content of protein in the extract was measured using a Kit for Protein Determination (Sigma–Aldrich, Madrid, Spain).

Specific EPSPS activity in plants from each biotype was estimated in the presence and absence (basal activity) of glyphosate. The EPSPS activity was determined using an EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, United States). The glyphosate concentrations used were: 0, 0.1, 1, 10, 100, and 1000 μM. Five replications at each glyphosate concentration were used, and the experiment was repeated. EPSPS enzyme activity was expressed as percentage of enzyme activity in presence of glyphosate with respect to the control (without glyphosate). The EPSPS activity was calculated to determine the amount of phosphate (μmol) released per μg of total soluble protein (TSP) min^{-1}.

EPSPS Gene Sequence

Samples of young leaf tissue (∼100 mg) from 10 individual plants within each biotype were harvested and stored at −80°C. Total RNA was isolated from leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. RNA was purified with TURBO DNase (RNase-Free; Ambion, Warrington, United Kingdom) and stored at −80°C. Synthesis of cDNA utilized 2 μg of total RNA following an M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (Invitrogen, Carlsbad, CA, United States) in combination with oligo $(dT)_{12-18}$ and random nonamers (Amersham Biosciences, Amersham, United Kingdom) according to the manufacturer's instructions. To amplify the EPSPS gene, known primers (forward: 5′ AGCTGTAGTCGTTGGCTGTG 3′; reverse: 5′ GCCAAGAAATAGCTCGCACT3 ′) were used and PCR reactions were carried out using cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 1× buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain) in a final volume of 25 μL. All PCR reactions were in duplicate and cycling conditions included: 94°C, 3 min; 35 cycles at 94°C, 30 s; 55°C, 30 s; 72°C, 1 min; and a final extension cycle of 72°C, 10 min. An aliquot of the PCR product was loaded on a 1% agarose gel to assess amplification of the correct band. The remaining PCR product was purified using ExoSAP-IT® (USB, Cleveland, OH, United States) as instructed. Ten purified PCR products per biotype were sequenced (STAB VIDA, Caparica, Portugal).

EPSPS Gene Expression

The cDNA of five sequenced individuals, corresponding to the previous section, was used for qPCR analysis. The primer pair EPSPS F2 (5′-CTGATGGCTGCTCCTTTAGCTC-3′) and EPSPSR2 (5′-CCCAGCTATCAGAATGCTCTGC-3′) designed by Salas et al. (2012), was used. Moreover, the primers LpCCR1 F2 (5′-GATGTCGAACCAGAAGCTCCA-3′) and LpCCR1 R2 (5′-GCAGCTAGGGTTTCCTTGTCC-3′) (McInnes et al., 2002), corresponding to cinnamoyl-CoA reductase (CCR), gene expressed as a single copy gene in perennial ryegrass, were used as an internal standard to normalize the samples for differences in the amounts of cDNA. The qPCR conditions described by Salas et al. (2012) was followed, and the PCR-reactions conducted using an ABI Prism 7500 sequence detection system (Applied Biosystems, United States). EPSPS gene expression analyzes were performed according to Alcántara-de la Cruz et al. (2016b).

EPSPS expression level was calculated for each qPCR reaction. The PCR efficiency of each sample and the stability of the CCR were determined using geNorm software according to Vandesompele et al. (2002). Two-three technical replications per plant were arranged in a completely randomized design.

Fitness Assessment

Progeny selection for both Douro and Golf biotypes was conducted by cloning plants following the methodology described by Yanniccari et al. (2012b). Plants were grown and vegetative clones of individual plants were propagated by tiller partition to obtain four ramets per plant. When ramets reached four leaves, plants were treated with 0, 360, 500, 720, 1000, and

2000 g ha^{-1} of glyphosate as described in the dose-response section. At 21 DAT, a visual assessment was made, with all plants not surviving 500 g ha^{-1} characterized as S, and those surviving 1000 g ha^{-1} or greater considered R. Cloned R- and S-plants were transferred to a greenhouse. Prior to flowering, cross pollination within R and S biotypes of each species was precluded by isolating plants in pollen-proof enclosures until maturity, and all seeds per plant were collected, enumerated, and stored at 4°C (seed did not exhibit a dormancy requirement). Cloning and selection was carried out in 2014 and again in 2015.

Seeds generated from the selection and isolation process described above were germinated in sand and peat medium and maintained in conditions described earlier. At 30, 60, 90, 120, and 150 days after planting (DAP), plant height (from soil level to flag sheet) and shoot weight (2 days at room temperature) were measured. In addition, seed germination (500 seeds per plant for each biotype) was estimated for seeds generated in both 2014 and 2015. The reduction in plant fitness associated with glyphosate resistance was estimated as: [1−(number of resistant seeds/number of susceptible seeds) × 100] (Yanniccari et al., 2016).

Statistical Analysis

Dose-response and EPSPS enzyme activity data were subjected to non-linear regression analysis using a log-logistic equation: $Y = c+\{(d-c)/[1+(x/g)b]\}$ (Ritz et al., 2015); where Y is the percentage of fresh weight, survival and/or EPSPS-inhibiting with respect to the control, c and d are the parameters corresponding to the lower and upper asymptotes, b is the slope of the curve at the inflection point, g is the herbicide rate at the inflection point (i.e., GR_{50}, LD_{50} or I_{50}), and × (independent variable) is the glyphosate dose. Using this equation, the amount of glyphosate needed to reduce the fresh weight (GR_{50}), mortality (LD_{50}), and to inhibit EPSPS activity (I_{50}) by 50% of each biotype were calculated. Regression analyses were conducted using the *drc* package with program R version 3.2.5 (R Core Team, 2015), and the data were plotted using SigmaPlot 11.0 (Systat Software, Inc., United States). Resistance indices (RI = R/S) were calculated as the ratio of R to- S GR_{50}, LD_{50}, or I_{50}.

An analysis of variance (ANOVA) was conducted to test for differences between R- and S-biotypes with respect to the spray retention assay, accumulation of shikimate, glyphosate metabolite levels, ^{14}C-glyphosate uptake and translocation, and basal enzyme activity, as well as plant growth and fecundity (fitness penalty). Differences between means were separated using the Tukey HSD test at $P < 0.05$. Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected. All ANOVAs were conducted using Statistix (version. 9.0) (Analytical Software, United States) software.

RESULTS

Identification of *Lolium* Weed Species Using Molecular Markers

In the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendogram, two main clusters converged at the 70% similarity level. The first cluster comprised a *L. perenne* control with R- and S-Golf biotypes. The second cluster was formed by *L. multiflorum* with R- and S-Douro biotypes. According to these results, the *Lolium* species used in this study clearly corresponded to *L. perenne* and *L. multiflorum* (**Figure 1**). Genetic characterization of the two species was necessary because *Lolium* species are obligate outcrossers; Salas et al. (2012) reported genetic variation for response to glyphosate from a collection of individual plants in the same location.

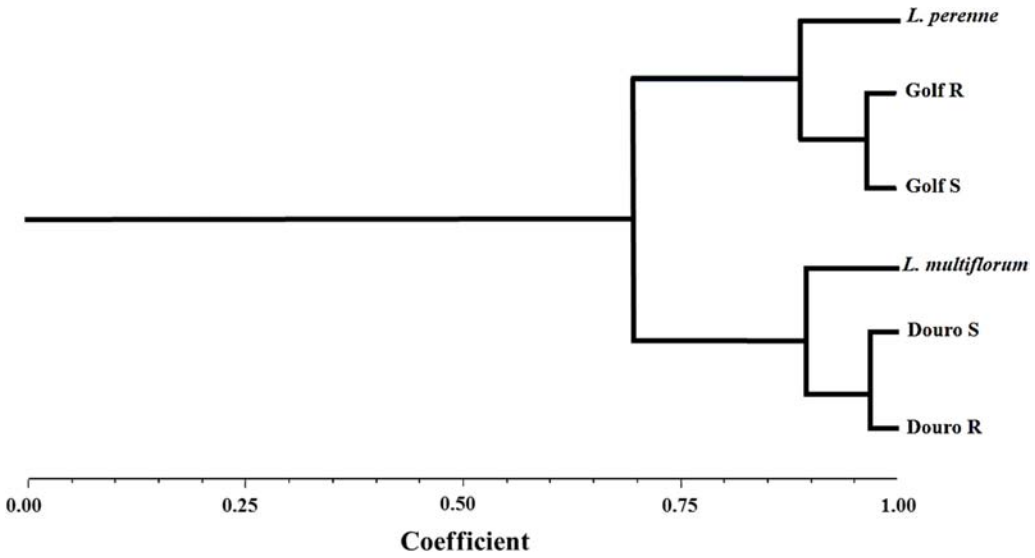

FIGURE 1 | Dendogram of the genetic similarities among *Lolium* species after UPGMA analysis performed with AFLP marker data.

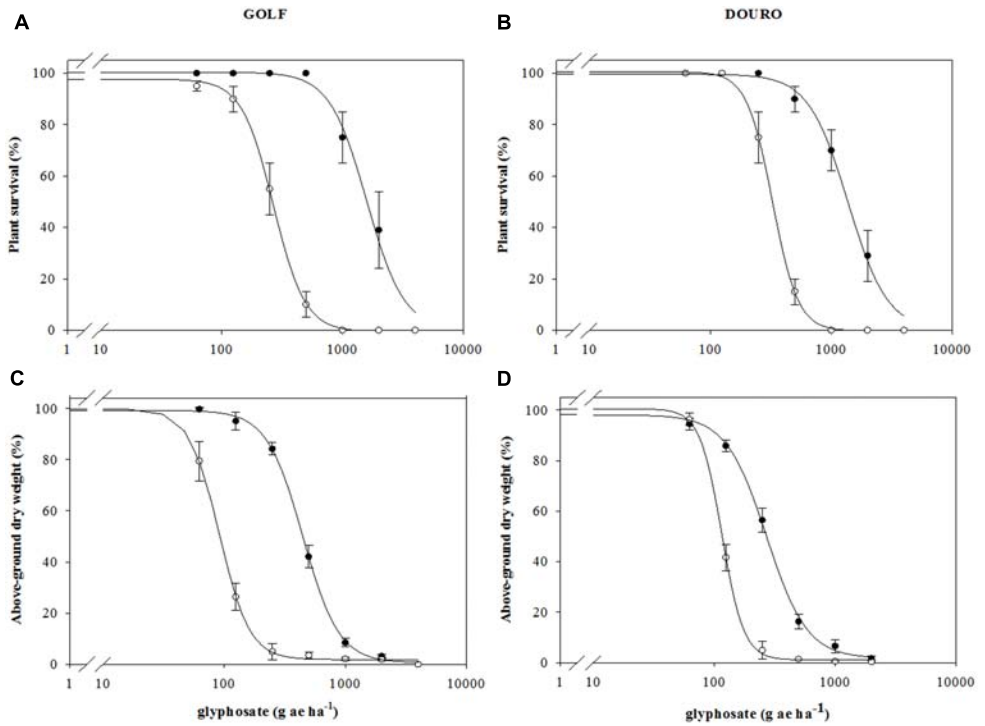

FIGURE 2 | Mean glyphosate dose-response on plant survival **(A,B)** and above-ground dry weight **(C,D)** expressed as percentage of untreated control of glyphosate-resistant (●) and -susceptible (○) Golf and Douro biotypes. Values represent mean ($n = 9$) and vertical bars represent ± standard errors.

TABLE 1 | Mortality (LD_{50}), dry weight reduction (GR_{50}), shikimic acid accumulation and inhibition of EPSPS enzyme activity (I_{50}) by 50% in glyphosate-resistant (R) and -susceptible (S) Golf (*Lolium perenne*) and Douro (*L. multiflorum*) biotypes.

Biotype		Mortality reduction[a]			Dry weight reduction[a]			Shikimic acid[b]	EPSPS activity[c]		
		LD_{50}	RI	*P*	GR_{50}	RI	*P*		I_{50}	RI	*P*
Golf	R	1590.6 ± 39.7	5.9	0.0001	443.8 ± 6.5	4.9	0.0001	36.5 ± 2.1	91.7 ± 6.9	28.7	0.0001
	S	267.0 ± 6.0			90.9 ± 1.8			214.9 ± 19.2	3.2 ± 0.3		
Douro	R	1375.2 ± 53.8	4.2	0.0001	271.5 ± 5.5	2.3	0.0001	45.4 ± 7.0	30.2 ± 4.3	10.8	0.0001
	S	326.5 ± 3.8			115.9 ± 1.4			193.6 ± 15.2	2.8 ± 0.2		

[a]*LD_{50} and GR_{50} values represent the glyphosate dose (g ae ha^{-1}) needed to reduce mortality and dry weight, respectively by 50%. ±Standard error of the mean (n = 9).* [b]*Shikimic acid accumulation (μg g^{-1} fresh weight) at 1000 μM glyphosate ± standard error of the mean (n = 9).* [c]*I_{50} values represent the glyphosate dose (μM) needed to reduce inhibition of EPSPS activity by 50%. ±Standard error of the mean (n = 5). RI, Resistance indices (RI = R/S) calculated as the ratio of R to- S GR_{50}, LD_{50} or I_{50} values. P, probability values.*

Whole Plant Dose-Response

Survival (LD_{50}) between R- and S-biotypes of Golf and Douro was different in the presence of glyphosate. Meanwhile the plants of the S-biotypes for each species died at 1000 g ha^{-1}, but 70 and 80% of plants of the R-biotypes survived (**Figures 2A,B**). R-Golf exhibited 5.9-fold resistance (RI; resistance index) relative to S-Golf. Additionally, the R-Douro biotype showed 4.2-fold resistance to glyphosate relative to S-Douro (**Table 1**). Accumulation of plant biomass in the presence of glyphosate was also different between R- and S-biotypes of each species. Although mortality differences for R- and S-biotypes of both Golf and Douro were exhibited at 2000 g ha^{-1}, dry weights were similar (**Figures 2C,D**). The RI for R-Golf and R-Douro was 4.9 and 2.3, respectively, in comparison to their respective S biotype (**Table 1**). Differences in both biomass accumulation and plant mortality confirm glyphosate resistance in R-Golf and R-Douro. The extent of resistance also suggests the potential underlying mechanism.

Spray Retention

Leaf surface characteristics and plant architecture contribute to variability in the amount of herbicide solution retained by treated plants. The spray solution retained (mL per g dry weight) for the R- and S-Golf biotypes was 0.41 ± 0.08 and 0.48 ± 0.07 mL, respectively, which was significant (P = 0.004). For R- and S-Douro, spray retention was 0.32 ± 0.09 and 0.47 ± 0.06 mL of sprayed solution retained per g dry weight, respectively. These results were also significantly different between biotypes

($P = 0.002$). Reduced retention of 17 and 47% were evident for R-Golf and R-Douro, respectively.

Shikimic Acid Accumulation

Shikimic acid levels in leaf segments of S-Golf and -Douro increased significantly by 24 h following exposure to 1000 μM glyphosate (**Table 1**). The R-Golf and -Douro biotypes accumulated 5.9- and 4.2-fold less shikimic acid, respectively than their respective S biotypes. Accumulation of shikimate in susceptible plants is a classic response to glyphosate; lack of shikimate accumulation in R-Golf and –Douro substantiates evidence for glyphosate resistance. The dynamics of shikimic acid accumulation were different between the S biotypes, showing that Golf was more sensitive to glyphosate than Douro. In both R biotypes, the accumulation of shikimic acid was limited and similar (**Table 1**).

^{14}C-Glyphosate Uptake, Translocation, and Visualization

Total recovery of ^{14}C-glyphosate in this research was 94.3, 95.1, 92.2, and 94.8% for R-Douro, S-Douro, R-Golf and S-Golf biotypes, respectively (data not shown). Little or no glyphosate was released by roots of treated plants from both Douro and Golf biotypes.

Uptake of ^{14}C-glyphosate appeared to be bi-phasic, with uptake most rapid from 12 to 48 HAT, and slowing from 48 to 96 HAT. For Golf, glyphosate uptake through 24 HAT was similar for R- and S-biotypes (∼25% of maximum). From 24 through 96 HAT, uptake was more rapid in S- (82.7%) than R-Golf (56.6%). For Douro, ^{14}C-glyphosate uptake was almost 2-fold higher for S- (50.7%) versus the R-biotype at 24 HAT. Differences in uptake continued through 96 HAT, with 91.2 and 50.7% total uptake for S- and R-Douro biotypes, respectively. In both R-biotypes, ^{14}C-glyphosate levels in treated leaves (as a percentage of uptake) declined linearly from 12 to 96 HAT. However, the rate of movement out of the treated leaf for S-biotypes was greater compared to R-biotypes; 30.7 and 33.9% for Golf and Douro, respectively. Corresponding accumulation of ^{14}C-glyphosate was measured in the remaining shoot tissue, although accumulation was 8.6 and 15.1% greater for S- compared to R-biotypes of Golf and Douro, respectively. Differences in ^{14}C-glyphosate translocation to roots between S- and R-biotypes of each species were most noticeable beyond 48 HAT. S- compared to R-biotypes resulted in 2.4- and 2.8-fold higher levels of ^{14}C-glyphosate in roots for Golf and Douro *Lolium,* respectively at 96 HAT (**Table 2**). The qualitative results obtained by phosphor imaging corroborated these differences in glyphosate distribution for S- versus R-biotypes of both species (**Figure 3**). These data reveal that restricting glyphosate uptake and translocation contributes to the survival of R-Golf and –Douro.

Metabolism Study

For glyphosate absorbed into Golf and Douro biotypes, much of the herbicide was unaltered in plants by 96 HAT (>87%). The mean levels of AMPA ranged from 8.2 to 10.4% and were not different between R- and S-biotypes (**Table 3**).

TABLE 2 | Uptake and translocation of ^{14}C-glyphosate from 12 to 96 h after treatment (HAT) in glyphosate-resistant (R) and -susceptible (S) Golf (*Lolium perenne*) and Douro (*L. multiflorum*) biotypes.

Biotype		HAT	Uptake (%)[a]	Translocation (%)[b]		
				Treated leaf	Rest of shoots	Roots
Golf	R	12	11.3 ± 2.8 G	95.9 ± 2.5 a	3.6 ± 0.7ef	0.5 ± 0.1f
		24	27.1 ± 3.9 F	89.2 ± 1.7 b	7.2 ± 1.6e	3.7 ± 0.9ef
		48	49.8 ± 3.4 E	76.0 ± 4.4 d	17.6 ± 2.5c	6.5 ± 1.4e
		72	54.3 ± 2.7 D	70.4 ± 3.4 e	18.2 ±2.4c	11.4 ± 2.6d
		96	56.5 ± 1.3 D	59.8 ± 4.7 f	24.5 ± 4.7b	15.7 ± 3.1c
	S	12	10.3 ± 1.9 G	96.1 ± 3.1 a	2.7 ± 0.4f	1.2 ± 0.6f
		24	25.3 ± 2.3 F	81.2 ± 5.0 c	12.7 ± 4.0d	6.1 ± 2.1e
		48	66.5 ± 4.0 C	62.6 ± 1.7 f	29.7 ± 2.8a	7.7 ± 1.7de
		72	75.0 ± 1.7 B	38.9 ± 3.7 g	32.1 ± 4.0a	29.0 ± 4.7b
		96	82.7 ± 2.5 A	29.0 ± 2.8 h	33.1 ± 2.1a	37.9 ± 4.0a
Douro	R	12	8.5 ± 3.9 I	94.9 ± 2.4 a	2.8 ± 0.4f	2.3 ± 0.9ef
		24	16.9 ± 3.4 H	88.0 ± 1.4 b	9.0 ± 1.7e	3.0 ± 1.1ef
		48	40.0 ± 2.8 F	81.8 ± 2.9 c	10.2 ± 2.8e	7.9 ± 2.7cd
		72	45.3 ± 3.9 E	77.2 ± 3.6 d	15.0 ± 1.7d	8.1 ± 2.0cd
		96	50.7 ± 2.9 D	70.8 ± 4.3 e	19.1 ± 2.1c	10.1 ± 2.4bc
	S	12	16.8 ± 2.8 H	97.3 ± 1.4 a	1.5 ± 0.3f	1.2 ± 0.2f
		24	31.2 ± 3.2 G	80.8 ± 4.4 c	13.9 ± 1.9d	5.3 ± 1.2de
		48	73.6 ± 3.2 C	65.1 ± 3.9 f	22.0 ± 2.8c	13.0 ± 2.3b
		72	83.5 ± 2.0 B	41.0 ± 4.2 g	30.8 ± 3.1b	28.3 ± 3.8a
		96	91.2 ± 3.4 A	36.8 ± 2.6 h	34.2 ± 3.8a	29.0 ± 3.2a

±Standard error of the mean (n = 6). Same letters in a column are not different at 95% by the Tukey's test. [a]Percent of applied label. [b]Percent of uptake label.

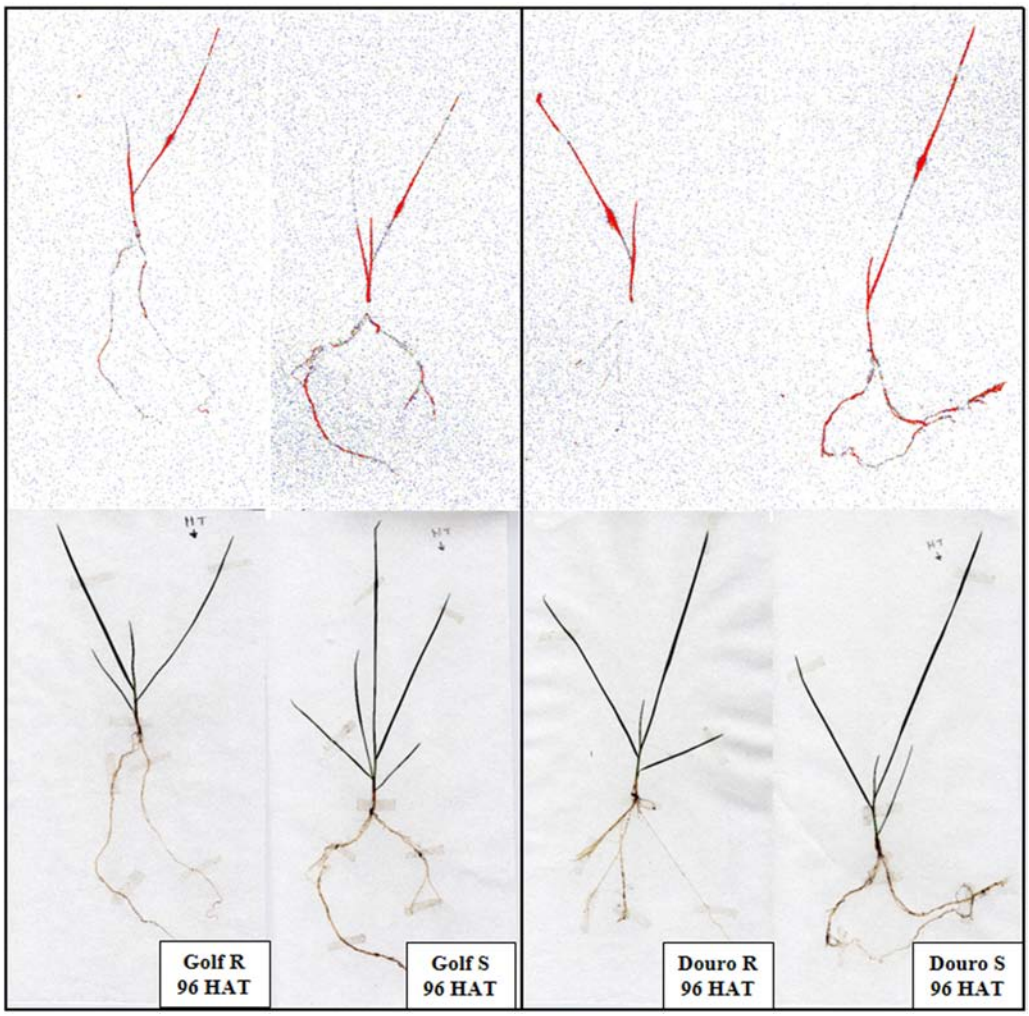

FIGURE 3 | Visible movement of ^{14}C-glyphosate in glyphosate-resistant and -susceptible Golf and Douro biotypes 96 h after an application to the treated leaf.

Glyoxylate levels ranged from 1.2 to 3.1%; R-Douro accumulated 65% more glyoxylate than S-Douro. Considering the small amount of glyoxylate formed, this difference is not likely biologically meaningful (**Table 3**). AMPA and glyoxylate are natural products of the degradation of glyphosate in some plants such as glyphosate-resistant soybean (Duke et al., 2003). The absence of differences in metabolite levels in R- and S-Golf and –Douro indicates this mechanism does not underlie resistance.

EPSPS Enzyme Activity Assays

Examination of constitutive EPSPS activity in the absence of glyphosate (basal enzyme activity) revealed a striking difference between R- and S-Golf versus R- and S-Douro. Activity in R- and S-Golf was similar, with 0.097 and 0.085 μmol μg protein^{-1} min^{-1}. However, basal enzyme activity of R-Douro was 5.2-fold higher compared to S-Douro (**Figure 4A**). Similar EPSPS activity in the absence of glyphosate for Golf biotypes is evidence that R-Golf does not exhibit overexpression of enzyme. However, an increase in basal EPSPS activity for R-Douro suggests EPSPS copy number may be elevated. Inhibition of EPSPS activity was achieved by challenging R- and S-biotypes of Golf and Douro with glyphosate (**Figure 4B**). The glyphosate rate necessary to inhibit EPSPS activity by 50% (I_{50}) was 28.7-fold higher for R-versus S-Golf and 10.8-fold higher for R-versus S-Douro, respectively (**Table 1**). Elevation in the dose of glyphosate needed to attain an I_{50} value for R-versus S-Golf suggests reduced sensitivity of EPSPS.

EPSPS Gene Sequence and Expression

The amplified fragment of the EPSPS gene included Thr-102 and Pro-106 positions, which frequently have been associated with conferring glyphosate resistance. In R- compared to S-Golf, a single codon change (from CCA to TCA) resulted in a conversion of the amino acid Pro to Ser at position 106. However, no nucleotide changes were observed for R-versus S-Douro. No additional codon changes in the Thr-102 position or others positions were observed for R-biotypes of either *Lolium* species

TABLE 3 | Metabolism of glyphosate at 96 h after treatment in glyphosate-resistant (R) and -susceptible (S) Golf (*Lolium perenne*) and Douro (*L. multiflorum*) biotypes.

Biotypes		Glyphosate	AMPA	Glyoxylate
Golf	R	87.5 ± 3.2	10.4 ± 1.3	2.1 ± 0.8
	S	90.1 ± 5.3	8.7 ± 2.9	1.2 ± 0.2
	P	0.1652	0.2073	0.1263
Douro	R	88.7 ± 4.3	8.2 ± 3.7	3.1 ± 0.4 a
	S	89.0 ± 3.9	9.1 ± 3.2	1.9 ± 0.7 b
	P	0.2788	0.0786	0.0214

±Standard error of the mean (n = 6). Different letters in a column are different at 95% by the Tukey's test. AMPA, aminomethylphosphonic acid. Plants treated with 300 g ae ha^{-1} glyphosate at the 3–4 leaf growth stage.

(**Figure 5**). Clearly, a mutation in the EPSPS gene contributes to glyphosate resistance in R-Golf but not R-Douro.

The EPSPS gene expression, relative to the *CCR* gene quantified from cDNA in *Lolium* sp. biotypes, showed no differences between R-Golf, S-Golf and S-Douro ranging from 0.95 to 1.06. However, the EPSPS gene expression of the R-Douro biotype ranged from 13.8 to 29.2-fold (average of 21.2-fold) higher compared to the other *Lolium* biotypes. The EPSPS expression level of these biotypes was positively correlated with the EPSPS basal activity (**Figure 6**).

Fitness Assessment

Distinct differences between R- and S-biotypes of each species were observed for a number of vegetative and reproductive parameters (**Figures 7**, **8**). For Golf, the ANOVA (**Table 4**) comparing the vegetative parameters plant height and weight were significant ($P < 0.05$), but numerically close in value. However, the ANOVA comparing plant height and weight variables for the Douro biotypes were highly significant and numerically distinct. At 30 DAP, seedlings of S- versus R-Douro were 17.3 and 4.5% taller in 2014 and 2015, respectively. Plant height differences increased for the duration of the experiment, with S-Douro 32.1 and 30.3% taller than R-Douro at 150 DAP in 2014 and 2015, respectively. For Golf, plant height for R- and S-Golf was similar at 30 DAP, however, S-plants were 12.6 and 8.1% taller than R-plants 150 DAP in 2014 and 2015, respectively. Golf biotypes were shorter in height than Douro biotypes, likely a reflection of Golf being used in the turf industry. Plant biomass differences between R- and S-biotypes of both species followed differences in plant height (**Figure 7**). Over time, the biomass of S-Douro increased faster than that of R-Douro. At 150 DAP, biomass of S- versus R-Douro was 32 and 36.1% greater in 2014 and 2015, respectively. For Golf, biomass was similar for S- and R-biotypes throughout the duration of the experiment. By 150 DAP, biomass of S- versus R-Golf was 3.3 and 6.2% greater in 2014 and 2015, respectively (**Figure 7**). Biomass results were similar for 2014 and 2015. Growth parameters for Golf were similar over years (biotype*year interaction); significance in the biotype*year interaction for plant height of Douro indicate some variability between biotypes for 2014 compared to 2015 (**Table 4**).

Greater plant height and biomass for S- compared to R-biotypes of Douro impacted seed production. For Douro, mean seed production of S- versus R-biotypes was 3,355 and 2,926 seeds higher in 2014 and 2015, respectively. S-Golf produced 351 and 528 more seeds than the R-plants in 2014 and 2015, respectively. Seed germination of Golf and Douro biotypes ranged from 61 to 87% in 2014 and 66 to 92% in 2015. Initial germination was observed 4 days after seeding, and reached an optimum 12 days after seeding. For both species, germinability of the S-biotype was higher compared to the R-biotype. In 2014 and 2015, germination of S- versus R-Golf was 28.8 and 24.4% higher, respectively. Small differences in germinability were also observed for Douro. Seed germination for S- versus R-Douro was 15.5 and 20.0% higher in 2014 and 2015, respectively (**Figure 8**).

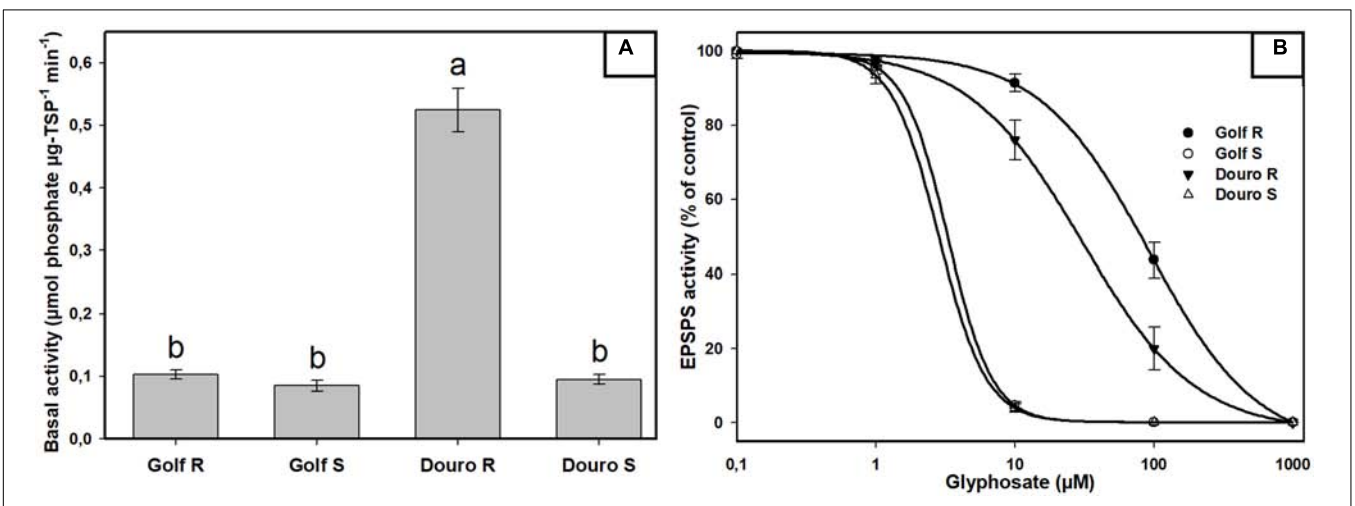

FIGURE 4 | EPSPS enzyme expressed as a percentage of the untreated control in leaf extracts of glyphosate-resistant and -susceptible Golf and Douro biotypes. **(A)** Basal EPSPS activity where histograms represent treatment means and vertical bars ± standard error ($n = 5$). **(B)** EPSPS enzyme activity expressed as a percentage of the untreated control; vertical bars represent ± standard error ($n = 5$).

Position	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114
Consensus sequence	TTG	GGG	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG	GCT	GCT	GTA	GTA	GCT	GCT
Amino acid	Leu	Gly	Asn	Ala	Gly	**Thr**	Ala	Met	Arg	**Pro**	Leu	Thr	Ala	Ala	Val	Val	Ala	Ala
Douro S	TTG	GGG	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG	GCT	GCT	GTA	GTA	GCT	GCT
Douro R	TTG	GGG	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG	GCT	GCT	GTA	GTA	GCT	GCT
Golf S	TTG	GGG	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG	GCT	GCT	GTA	GTA	GCT	GCT
Golf R	TTG	GGG	AAC	GCT	GGA	ACT	GCG	ATG	CGG	**TCA**	TTG	ACG	GCT	GCT	GTA	GTA	GCT	GCT

FIGURE 5 | Partial nucleotide sequence of 5-enolpyruvlshikimate-3-phosphate synthase (EPSPS) DNA isolated from the glyphosate-resistant (R) and –susceptible (S) Golf and Douro biotypes.

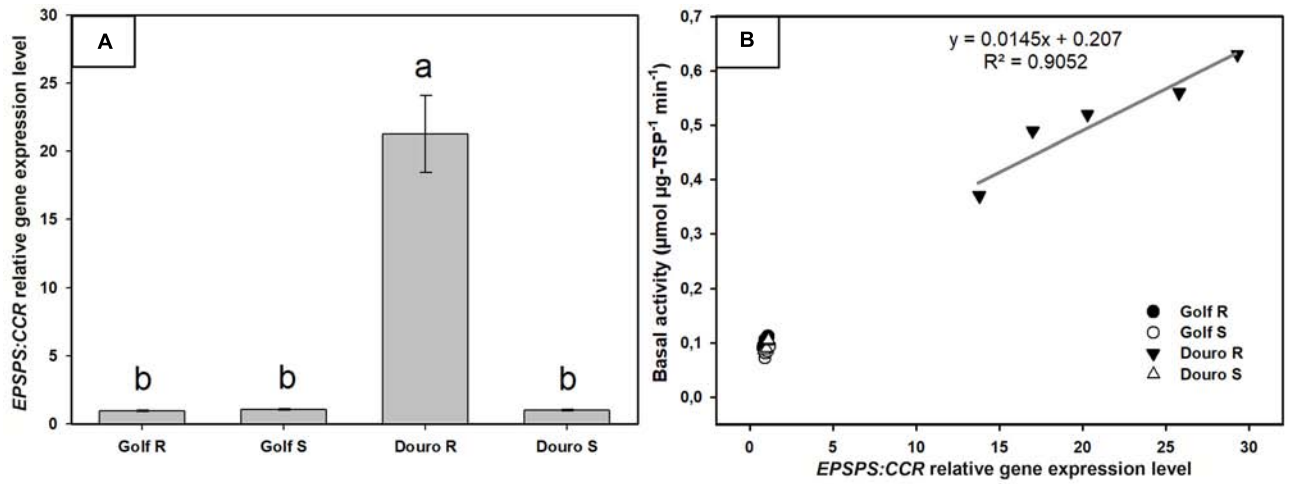

FIGURE 6 | EPSPS gene expression relative to cinnamoyl-CoA reductase (*CCR*) gene in glyphosate-resistant and -susceptible Golf and Douro biotypes. **(A)** EPSPS expression level for glyphosate-susceptible and -resistant plants of Golf and Douro biotypes. Histograms represent the treatment means and vertical bars ± standard error ($n = 6$). **(B)** Correlation between the EPSPS expression and EPSPS basal activity.

DISCUSSION

In this research, characterization of both NTSR and TSR mechanisms were explored, reflecting the most frequently expressed mechanisms in *Lolium* species. First, the *Lolium* species assayed by AFPL-markers were identified as *L. perenne* for the Golf biotypes and *L. multiflorum* for the Douro biotypes, revealing molecular-based relationships between three basic entities. The use of AFPL-markers allowed easy identification of species, but was not able to detect resistance to glyphosate between *Lolium* populations. AFLPs can produce biased results due to fragment-size homoplasy, caused by the lack of homology of fragments (Caballero et al., 2008; Chandi et al., 2013). Golf and Douro biotypes exhibit a high level of genetic similarity, showing minimal differences between R- and S-biotypes within each species (>0.95).

The occurrence of glyphosate resistance in *Lolium* spp. is not novel. Between 1996 and 2017, 14 countries documented R *Lolium* (Heap, 2017), the most occurring in *L. rigidum* and *L. multiflorum*. Levels of resistance in R-Golf (5.9) and R-Douro (4.2) (**Table 1**) were similar to that reported for other glyphosate-resistant biotypes (Perez-Jones et al., 2007; Jasieniuk et al., 2008; González-Torralva et al., 2012). Preston et al. (2009) summarized that R/S ratios for *L. multiflorum* biotypes with reduced translocation as the basis for resistance varied from 3 to 9. However, amino substitutions at Pro-106 to serine or alanine in 5 different biotypes resulted in R/S ratios from 5 to 15. In Argentina, *L. perenne* exhibited 10.8-fold resistance to glyphosate (Yanniccari et al., 2012b), with the mechanism later stated to be overexpression of EPSPS (Yanniccari et al., 2016).

Differences in retention of glyphosate on treated leaves has not been characterized as a resistance mechanism in selected weeds. The contact angle for R-versus S-biotypes of *L. multiflorum* was up to 30 degrees lower, resulting in 35% less glyphosate retained on leaves (Michitte et al., 2007). However, R-biotypes of *L. multiflorum* in Spain did not exhibit unique morphology compared to S-biotypes, and herbicide retention was similar (González-Torralva et al., 2012). A reduction in the amount of glyphosate retained on Golf and Douro plants would not preclude the herbicide's physiological effects. Because biotype differences in plant mortality and biomass accumulation ranged from 230 to 590%, small differences in retention could not alone underlie R-Golf and –Douro resistance.

Shikimic acid accumulation in both S-Golf and -Douro indicated herbicide interaction with the target enzyme (EPSPS), but accumulation of shikimate was distinctively higher in S plants (**Table 1**). Inhibition of EPSPS concomitantly results in accumulation of shikimate in glyphosate sensitive plants (Maeda and Dudareva, 2012; Sammons and Gaines, 2014), suggesting limited interaction of glyphosate with EPSPS of R plants. Higher

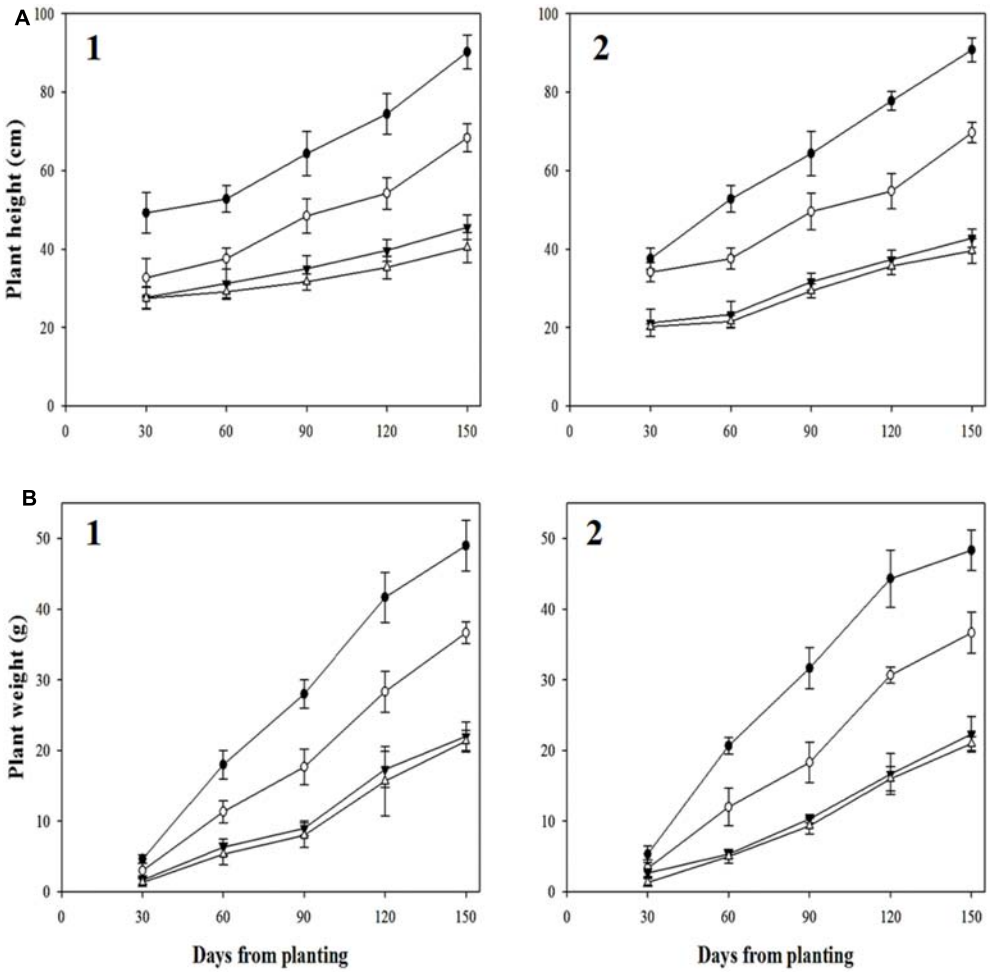

FIGURE 7 | Plant height **(A)** and weight **(B)** of glyphosate-resistant Golf (△) and Douro (○) as well as glyphosate-susceptible Golf (▼) and Douro (●) biotypes between 30 and 150 days after planting in 2014 (1) and 2015 (2). Values represent mean ($n = 9$) and vertical bars represent ± standard errors.

accumulation of shikimic acid in S- versus R-biotypes was similar to other *Lolium* populations (Michitte et al., 2007; Jasieniuk et al., 2008; Nandula et al., 2008), where shikimic acid levels 2- to 6-times higher in S- versus R-plants were observed. An S-biotype of *L. multiflorum* in Spain accumulated 7.3-fold more shikimic acid compared to the R-biotype (González-Torralva et al., 2012). For S- versus R-biotypes of *L. perenne* (Golf), also from Spain, the differences in accumulation of shikimic acid were less pronounced. S- versus R-plants of *L. perenne* reached levels approximately 3-fold higher by 72 h after glyphosate application (Yanniccari et al., 2012a). Therefore, limited increases in shikimic acid content in R-biotypes is an indicator of reduced sensitivity to glyphosate, but does not indicate the mechanism underlying resistance.

Reduced uptake and/or translocation is an underlying mechanism of glyphosate resistance and has been documented in a number of *Lolium* species (González-Torralva et al., 2012; Ghanizadeh et al., 2015; Salas et al., 2015). In *L. multiflorum* from Chile (Michitte et al., 2007) and Mississippi (Nandula et al., 2008), the limited uptake in R-biotypes corresponded with reduced movement out of labeled tissue or accumulation in tips of treated leaves. Greater translocation of ^{14}C-glyphosate in S- versus R-plants of *L. perenne* resulted in accumulation in pseudostem regions (Ghanizadeh et al., 2015) or roots (Lorraine-Colwill et al., 2003; González-Torralva et al., 2012). Uptake of glyphosate across leaf cuticles is facilitated by a carrier-mediated process (Sterling, 1994), with maximum absorption into plants reached within 74 HAT (Li et al., 2005). Reduced uptake in R-Golf and R-Douro may be related to changes in cuticular properties, and be independent from mechanisms contributing to restricted movement of glyphosate (**Table 2**). Reduced uptake into R *L. multiflorum* from Chile was due to cuticular properties on the abaxial leaf surface (Michitte et al., 2007).

R plants of the Douro and Golf biotypes showed reduced glyphosate translocation (**Table 2**). The reduction in translocation to active and sensitive sites, such as root and shoot meristems, has a negative impact on glyphosate efficacy (Preston and Wakelin, 2008), contributing to the loss of

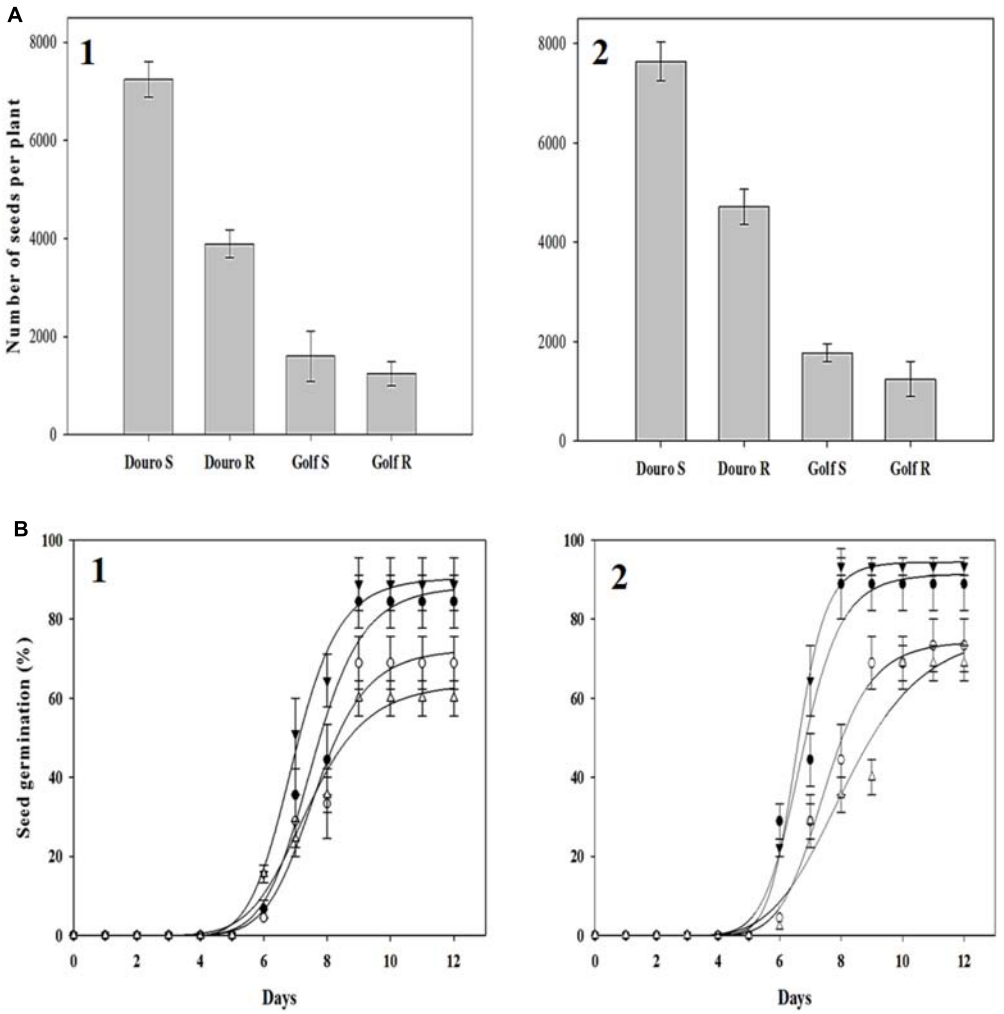

FIGURE 8 | Harvested seed **(A)** and seed germination **(B)** from glyphosate-resistant Golf (△) and Douro (○) as well as glyphosate-susceptible Golf (▼) and Douro (●) biotypes following seed harvest in 2014 (1) and 2015 (2). Harvested seed values represent mean ($n = 9$) and vertical bars represent ± standard errors. Germination values represent the mean ($n = 500$) at 12 days after seeding. Vertical bars represent ± standard errors.

herbicide sensitivity in R plants. The physiological mechanism that reduces glyphosate translocation in R-versus S-plants is not fully known (Lorraine-Colwill et al., 2003; Gherekhloo et al., 2017). However, it has been suggested that there is an unknown barrier in the phloem system or in the mesophyll cells (Yanniccari et al., 2012a). This barrier may alter the subcellular accumulation of glyphosate at the point where the herbicide is translocated (Kleinman and Rubin, 2017). The R plants of the Douro and Golf biotypes retained the herbicide applied mainly on the treated leaves. Similar translocation patterns were reported in R- and S-biotypes of *L. rigidum,* where 89 and 58%, respectively of applied ^{14}C-glyphosate was retained in the treated leaves at 96 HAT (Fernández-Moreno et al., 2017b). In our research, NTSR mechanisms involving restricted uptake and translocation of glyphosate contribute to the resistance exhibited in R-biotypes of both *L. multiflorum* (Douro) and *L. perenne* (Golf).

Glyphosate degradation to the major metabolites AMPA and glyoxylate has been investigated in *Lolium* (Salas et al., 2012; González-Torralva et al., 2012; Fernandez et al., 2015) and other species (Feng et al., 2004; Cruz-Hipolito et al., 2011; Fernández-Moreno et al., 2016), and is low compared to metabolism in glyphosate-resistant crop plants (Duke, 2011). Lorraine-Colwill et al. (2003) and González-Torralva et al. (2012) in *L. multiflorum*, and Fernández-Moreno et al. (2017b) in *L. rigidum* concluded that metabolism does not contribute to resistance. With greater than 85% of absorbed glyphosate remaining unaltered both in S- as well as R- Douro and Golf biotypes (*L. multiflorum* and *L. perenne*, respectively) (**Table 3**), metabolism cannot explain the level of glyphosate resistance observed.

Elevated basal activity of EPSPS has been reported in R biotypes of *Lolium* species (Salas et al., 2012; Fernández-Moreno et al., 2016). The R-Douro biotype also exhibited higher basal activity (**Figure 4**). Differences in basal activity

TABLE 4 | Probability values (*P*) to assess fitness of glyphosate-resistant and -susceptible Golf (*Lolium perenne*) and Douro (*L. multiflorum*) biotypes.

	Plant height	Plant weight	Seed number	Germination
Golf				
Biotype	$P < 0.0001$	$P = 0.0337$	$P = 0.0614$	$P < 0.0001$
Year	$P < 0.0001$	$P = 0.6394$	$P = 0.1167$	$P < 0.0001$
DAP	$P < 0.0001$	$P < 0.0001$		
Biotype*Year	$P = 0.1970$	$P = 1.000$	$P = 0.1193$	$P < 0.0001$
Biotype*DAP	$P = 0.0658$	$P = 0.9968$		
Year*DAP	$P < 0.0001$	$P = 0.6364$		
Biotype*Year*DAP	$P = 0.6804$	$P = 0.9361$		
Douro				
Biotype	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Year	$P = 0.548$	$P = 0.47$	$P = 0.0024$	$P < 0.0001$
DAP	$P < 0.0001$	$P < 0.0001$		
Biotype*Year	$P = 0.0288$	$P = 0.8504$	$P = 0.2028$	$P < 0.0001$
Biotype*DAP	$P < 0.0001$	$P = 0.0002$		
Year*DAP	$P = 0.0008$	$P = 0.5427$		
Biotype *Year*DAP	$P < 0.0001$	$P = 0.7349$		

Variables included biotype, year (2014 or 2015), days after planting (DAP; 30, 60, 90, 120, and 150). Plant characteristics included plant height and biomass, seed production, and germination of harvested seed.

are explained by duplication of EPSPS gene copy number (Salas et al., 2012; Gherekhloo et al., 2017). Gene amplification allows plants to carry on normal metabolic activities in the presence of once lethal concentrations of glyphosate (Powles, 2010); significantly higher doses of glyphosate are necessary for complete inhibition of EPSPS in R plants. This explains why the R-Douro biotype required greater amounts of glyphosate to inhibit EPSPS activity by 50% (**Figure 4**). There is generally a positive correlation between EPSPS gene copy numbers and EPSPS transcription (Gaines et al., 2013; Salas et al., 2015). Our results suggest that R-Douro had a ratio of ± 21-fold more EPSPS gene copy number than S-Douro. Higher basal EPSPS activity associated with additional EPSPS gene copies was reported in R-plants of *L. perenne* (Salas et al., 2012, 2015), but our results revealed this mechanism for the first time in *L. multiflorum*.

Nucleotide substitution resulting in amino acid changes in the EPSPS gene have frequently conferred glyphosate-resistance in biotypes of *Lolium* (Perez-Jones et al., 2007; Jasieniuk et al., 2008; Ghanizadeh et al., 2015; Fernandez et al., 2015). In this research, R-Golf exhibited a 106-Pro to –Ser substitution, altering the binding of glyphosate to the target site (**Figure 5**). Sammons and Gaines (2014) summarized that Pro-106-Ser mutation was reported in *L. multiflorum* (Perez-Jones et al., 2007; Jasieniuk et al., 2008; González-Torralva et al., 2012), *L. rigidum* (Simarmata and Penner, 2008; Bostaman et al., 2012; Fernandez et al., 2015) and *L. perenne* (Ghanizadeh et al., 2015). The 106-Pro-Thr (Wakelin and Preston, 2006; Bostaman et al., 2012), Pro-106-Leu (Kaundun et al., 2011), and Pro-106-Ala (Yu et al., 2007) mutations were identified in *L. rigidum*. Variable amino acid substitutions impact the efficiency of glyphosate, resulting in variable levels of resistance (Preston et al., 2009).

As *Lolium* are an obligatory outcrossing species (Terrell, 1968), it is not unexpected that R *Lolium* populations exhibiting different mechanisms could hybridize, resulting in a progeny expressing more than one resistance mechanism. Resistant *L. multiflorum* populations from Oregon (Perez-Jones et al., 2007) and Spain (González-Torralva et al., 2012) showed altered translocation and a Pro-106-Ser mutation underlying resistance. Two mechanisms were also expressed in R *L. perenne* from New Zealand (Ghanizadeh et al., 2015). In this research, both R biotypes exhibit two resistance mechanisms. R-Golf expressed both reduced uptake and translocation (NTSR) as well as a 106-Pro-Ser mutation. R-Douro also demonstrates reduced uptake and translocation, but also overexpresses EPSPS. Recently, *E. indica* from Mexico was found to exhibit two TSR mechanisms (Gherekhloo et al., 2017).

Expression of glyphosate resistance in weed species via one or multiple mechanisms may impact the fitness of resistant biotypes. Cumulative germination of R goosegrass (*E. indica* Gaertn.) in Malaysia was higher compared to an S-population collected 2 km away (Ismail et al., 2002). The life cycle of R-glyphosate goosegrass may be shorter and integrated management techniques could reduce the incidence of R plants in the soil seed bank (Ismail et al., 2002). *L. rigidum* phenotypes from a single population did not differ in vegetative growth or competitive ability with wheat (*Triticum aestivum* L.), where R-versus S-plants generated 28% greater seed yield per plant, although seed number per plant was 7.5% less (Pedersen et al., 2007). R versus S *L. perenne* were shorter with lower shoot biomass and reduced leaf area, resulting in 40% fewer seeds in R plants (Yanniccari et al., 2016). Leaf areas of R and S *L. multiflorum* and *L. perenne* were not compared, but if lower in R-versus S-biotypes, this could impact glyphosate retention.

Determination of the growth and reproductive fitness of R-plants depends upon minimizing genetic variation. Ideally, isogenic lines of each biotype should be used, but this is often not achievable. The genetic variability of *Lolium* is high, which complicates fitness assessments. High variability among accessions within different classes of herbicide-resistant *L. rigidum* precludes identification of distinct emergence and growth characteristics (Gill et al., 1996). Differences in growth and fecundity can result from environmental conditions as well as the genetic background, as revealed by the UPGMA analysis in this research (**Figure 1**). This suggests minimal differences for fitness characteristics between R- and S-biotypes within each species are likely the result of differences in sensitivity to glyphosate.

Resistance to glyphosate based upon altered uptake and translocation appears to exact some influence on the fitness of the R-biotypes. R- and S-populations of *L. rigidum* were similar in vegetative growth in the absence or presence of competing wheat, but R-plants produced 3–9% more seed when competing with wheat at densities of 200 plants m^{-2} or higher (Pedersen et al., 2007). Under field conditions in the absence of glyphosate, survival of R-plants declined by almost 50% after three generations (Preston et al., 2009). The R-versus S-biotype of *L. multiflorum* (Douro) exhibited differences in growth (plant height and biomass) as well as reproductive (seed production and

germination) characteristics (**Table 4** and **Figures 7**, **8**). In the absence of glyphosate use, the agricultural success of R-Golf and R-Douro is unknown. Certainly the fitness cost for a number of growth and reproductive parameters would collectively place R biotypes of each species at a competitive disadvantage compared to S biotypes.

Gene amplification of EPSPS may also be costly to growth and fecundity of R-plants. Height, biomass, leaf area and seed production of R versus S *L. perenne* from Argentina was significantly reduced (Yanniccari et al., 2016). The continued use of glyphosate was responsible for dominance of R-plants in the weed population. The level of fitness cost associated with R *L. perenne* from Argentina (Yanniccari et al., 2016) was similar to that reported in this research, although the basis of resistance was not due to EPSPS amplification. However, this mechanism was identified in *L. multiflorum* (R-Douro).

Fitness studies in plants with molecular changes in the EPSPS gene underlying glyphosate resistance have not been determined to be an ecological cost in R-plants. In *E. indica* clones from an R population in Malaysia, the RR TIPS mutants (carrying the Thr-102-Ile and Pro-106-Ser mutations) showed a slight fitness cost, but were out-performed over time by Rr TIPS mutants, which may suffer little if any fitness cost (Yu et al., 2015). R-plants of this species exhibited up to 74% greater emergence than S-plants, although subsequent growth characteristics were not studied (Ismail et al., 2002). In this research, R-glyphosate *L. perenne* (Golf) exhibits small differences in plant height as well as biomass compared to the *S*-glyphosate (**Figure 7**). Reduction in cumulative germination of R-*E. indica* (Ismail et al., 2002), suggest that non-glyphosate based management could reduce the incidence of the R-*Lolium* biotypes.

A common theme contributing to selection for glyphosate resistant weed populations is continuous use of the same herbicide mode of action over an extended period of time. If R- and S-biotypes exhibit similar traits for growth and fecundity, eliminating the use of glyphosate will not favor a shift in the balance of the endemic weed population for the S-biotype. However, association of a fitness penalty with TSR or NTSR mechanisms provides an opportunity to exploit the particular penalty and reduce the frequency of the R biotype. Using cloned plants of genetically similar *L. multiflorum* and *L. perenne*, there does appear to be a moderate to severe fitness penalty on the growth and fecundity of R-plants. This research is the first study examining fitness penalties for R-biotypes of *L. perenne* and *L. multiflorum* expressing two mechanisms of resistance to glyphosate. Additional studies are necessary under field conditions to demonstrate that reduced fitness of glyphosate-resistant plants can translate into meaningful reductions in the incidence of R-populations.

AUTHOR CONTRIBUTIONS

PF-M, RAC, RS, and RDP designed all experiments performed and data analysis; and PF-M, RAC, RS, and RDP wrote the paper.

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Identifying *Chloris* Species from Cuban Citrus Orchards and Determining Their Glyphosate-Resistance Status

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The *Chloris* genus is a C_4 photosynthetic species mainly distributed in tropical and subtropical regions. Populations of three *Chloris* species occurring in citrus orchards from central Cuba, under long history glyphosate-based weed management, were studied for glyphosate-resistant status by characterizing their herbicide resistance/tolerance mechanisms. Morphological and molecular analyses allowed these species to be identified as *C. ciliata* Sw., *Chloris elata* Desv., and *Chloris barbata* Sw. Based on the glyphosate rate that causes 50% mortality of the treated plants, glyphosate resistance (R) was confirmed only in *C. elata*, The R population was 6.1-fold more resistant compared to the susceptible (S) population. In addition, R plants of *C. elata* accumulated 4.6-fold less shikimate after glyphosate application than S plants. Meanwhile, populations of *C. barbata* and *C. ciliata* with or without glyphosate application histories showed similar LD_{50} values and shikimic acid accumulation rates, demonstrating that resistance to glyphosate have not evolved in these species. Plants of R and S populations of *C. elata* differed in ^{14}C-glyphosate absorption and translocation. The R population exhibited 27.3-fold greater 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) activity than the S population due to a target site mutation corresponding to a Pro-106-Ser substitution found in the EPSPS gene. These reports show the innate tolerance to glyphosate of *C. barbata* and *C. ciliata,* and confirm the resistance of *C. elata* to this herbicide, showing that both non-target site and target-site mechanisms are involved in its resistance to glyphosate. This is the first case of herbicide resistance in Cuba.

Keywords: 5-enolpyruvyl shikimate-3-phosphate synthase, glyphosate translocation, herbicide resistance mechanisms, Pro-106 mutation, tall windmill grass

INTRODUCTION

The use of herbicides is the most common weed control method (Délye, 2013; Fernández-Moreno et al., 2017b). However, herbicide resistance has caused this method to be quickly undermined. This scenario is the result of evolutionary adaptations in a target weed to herbicide applications (Powles and Yu, 2010; Beckie and Harker, 2017). Glyphosate [(N-phosphonomethyl)-glycine]

is one of the most widely used herbicides, although it is also an herbicide with many cases of resistance (37 glyphosate-resistant species; Shaner et al., 2012; Bracamonte et al., 2016; Heap, 2017). This herbicide is systemic, non-selective and is used post-emergence, and it inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene (EC 2.5.1.19), triggering the catalysis of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to form 5-enolpyruvyl-3-phosphate, an important step in the biosynthesis of aromatic amino acids in plants (Schönbrunn et al., 2001).

The mechanisms conferring glyphosate resistance are grouped into two major groups (Sammons and Gaines, 2014). Target-site resistance (TSR) can involve EPSPS gene mutations or EPSPS gene amplification. TSR was revealed to be caused by point mutations in the EPSPS gene with substitutions at Thr-102 and Pro-106 (Yu et al., 2015; Alcántara-de la Cruz et al., 2016b). Pro-106 substitutions have been found in several weeds (González-Torralva et al., 2012; Alarcón-Reverte et al., 2015; Fernandez et al., 2015), conferring low resistance levels to glyphosate on the order of 2- to 5-fold, while a double mutation (Thr-102 and Pro-106) increases resistance levels. Gene amplification is an adaptation also confers resistance to glyphosate (Gaines et al., 2013). The additional EPSPS produced from the amplified gene copies enables plants to survive higher glyphosate doses (Gaines et al., 2013; Chen et al., 2015; Yu et al., 2015). Non-target-site resistance (NTSR) mechanism results from reduced absorption and/or translocation, increased vacuolar sequestration, and metabolism to non-toxic compounds, causing less glyphosate transport to the EPSPS via the xylem and phloem (Délye, 2013). NTSR has being described as the most common mechanism of resistance to glyphosate and can confer unpredictable resistance (Powles and Yu, 2010). Similar to TSR, NTSR has been found to be a mechanism involved in resistance in many weeds (de Carvalho et al., 2012; Ge et al., 2012; Rojano-Delgado et al., 2012; Vila-Aiub et al., 2012).

The genus *Chloris* Sw. (Poaceae: Chloridoidae) is a C_4 photosynthetic species distributed in tropical and subtropical regions (Molina and Agrasar, 2004). It has also been found in semi-arid areas inhabiting semi-natural grasslands and rural habitats such as roads and barren places (Cerros-Tlatilpa et al., 2015). The genus comprises 50–60 species in both hemispheres (Molina and Agrasar, 2004; Barkworth, 2007). Species of this genus have great economic and ecological importance worldwide because they are a source of forage, resist drought, increase soil fertility, require low inversion, and can be used as plant cover to protect soil from rain-driven erosion (Michael et al., 2012). However, some of them could be considered invasive weed species (Cerros-Tlatilpa et al., 2015).

Studies of herbicide resistant weeds in Cuba are scarce because of lack of knowledge of the issue. This situation is similar to the Dominican Republic, in which studies have already begun to be carried out (Bracamonte et al., 2016). Unfortunately, growers have already started to have many weeds in their citrus groves that are not controlled at the recommended field dose of glyphosate (720 g ae ha^{-1}). Given that weed control strategies in larger commercial fields are absolutely the focus of glyphosate applications at the post-emergence growth stage, scientific confirmation is necessary. In this way, this study could define the species that are evolving toward to glyphosate resistance or even tolerant species to this herbicide.

An accurate assessment of taxonomic identity is a prerequisite to addressing population and individual plant-based functional studies. This is particularly true in the case of highly diverse genera, for which taxonomic and nomenclatural complexities generally arise, as is the case in *Chloris* (Molina and Agrasar, 2004).

This work aimed to characterize suspicious glyphosate-resistant populations of three different *Chloris* species in Cuba. Studies were conducted to (1) establish their taxonomical identity based on morphological and molecular analyses, (2) evaluate their resistance/tolerance levels, and (3) determine the mechanisms involved.

MATERIAL AND METHODS

Plant Material and Experimental Conditions

In 2014, our research group (Dr. Rafael De Prado) together with the Weed Science group of the Ministry of Agriculture of Cuba (Dr. Jorge Cueto), prospected for *Chloris* species in citrus orchards in central Cuba. These fields had been repeatedly treated with glyphosate (5 L ha^{-1}, 36% w/v) continuously for over 10 years, and sometimes received more than one application per year (Cueto, personal communication).

Mature seeds of three suspicious glyphosate-resistant populations of *Clhoris* species (treated = T) were harvested separately in in citrus orchards from Arimao and Ceiba, in Cienfuegos Province, from at least 20 plants that had been survived to the last glyphosate treatment. Seeds from a population of each species from nearby locations with no known records of exposure to glyphosate were also collected (non-treated = NT).

The seeds were germinated in containers using a substrate of sand/peat (1:2 v/v), covered with parafilm, and placed in a growth chamber at temperatures of 28/18°C (day/night), with a 16 h photoperiod (850 μmol m^{-2} s^{-1}) and 80% humidity. Subsequently, the seedlings from each population of the different *Chloris* species were transplanted individually into pots (1 plant per pot) containing the same substrate and placed in a growth chamber under the conditions described above. Furthermore, 20–30 plants from each population were placed in a greenhouse until flowering and fruiting.

Morphometric Study and Taxonomic Identity

Different taxonomically relevant morphological traits of inflorescences and caryopses were measured in greenhouse-grown plants of each species of the *Chloris* populations. The examined traits of inflorescences were the number of racemes, raceme length and spikelet density (number of spikelets per cm of raceme). For the spikelets, we examined the length and width

of the lower and upper glumes, the number of sterile florets, the length of hairs surrounding the callus, the length and width of the lemma of the fertile floret (fertile lemma), the presence and length of hairs on or adjacent to the keel and on the margins of fertile lemma, the length of the palea of the fertile floret, the lemma length and width of the basal sterile floret, the lemma length of any additional sterile floret, and the presence and length of awns on lemmas. The characteristics of the caryopses were length, width, thickness, shape, and length of the embryo mark. The shape of caryopses was quantified as the variance in their three dimensions, each relative to length (Thompson et al., 1993). This dimensionless shape index varies between 0 for a perfect sphere and 0.22 for a disk- or needle-shaped item. Based on the above morphological characters, the three pairs of study populations were identified to the species level according to Molina and Agrasar (2004), and the nomenclature followed IPNI (2017).

Molecular Characterization of the *Chloris* Species by AFLP Primer Analysis

Twenty-four accessions from the *Chloris* spp. were employed as the study material. Genomic DNA was extracted from fresh young leaves of eight individual plants per species (four T and four NT), using the Speedtools Plant DNA Extraction kit (Biotools). The DNA concentration was measured using a NanoDrop ND 1000 spectophotometer. DNA was diluted to a final concentration of 10 ng/μL.

Twelve AFLP primer pairs were used [E36-M48 (E-ACC M-CAC); E36-M60 (E-ACC M-CTC); E37-M49 (E-ACG M-CAG); E38-M50 (E-ACT M-CAT); E40-M61 (E-AGC M-CTG); E35-M49 (E-ACA M-CAG); E36-M49 (E-ACC M-CAG); E35-M61 (E-ACA M-CTG); E40-M62 (E-AGC M-CTT); E32-M60 (E-AAC M-CTC); E33-M50 (E-AAG M-CAT)]. The reaction mix contained 10 ng template DNA, 2.5 U Taq DNA polymerase, 40 pmol primer, 200 μM dNTPs, 2.5 mM $MgCl_2$, and 10 mM Tris-HCl all in a volume of 20 μl. The optimized thermal cycling conditions were 2 min at 94°C, followed by 40 cycles of 94°C for 25 s, 56°C for 25 s, 72°C for 25 s and a final extension at 72°C for 7 min. AFLP fragments were resolved in 25-cm gels (0.25 mm spacer thickness). Electrophoresis and detection were performed on a two-dye, model 4300 LICOR automated DNA Sequencer. Digital AFLP gel images were scored to obtain binary (band presence/absence) data using the SAGA GENERATION 2 software program.

Data clustering was conducted for AFLPs with the NTSYS-pc-2.2 software (Rohlf, 2000) using Jaccard's coefficients to define unweighted pair-group (UPGMA) dendograms. A principal coordinate analysis (PCA) was also performed with the NTSYS-pc program.

Dose-Response Assays

Plants of each *Chloris* population were sprayed at the 3–4 leaf growth stage. Glyphosate applications were applied with a laboratory chamber (SBS-060 De Vries Manufactering, Hollandale, MN) equipped with 8002 flat fan nozzle delivering 200 L ha^{-1} at 250 KPa at the height of 50 cm. The following glyphosate (Roundup®, 360 g ae L^{-1} as isopropylamine salt) rates were used: 0, 62.5, 125, 250, 500, 1,000, 2,000, 3,000, and 4,000 g ae ha^{-1}. The experiment was design using nine replications per rate and was repeated twice. Plants were cut down at the soil surface 21 days after application (DAT).

Shikimic Accumulation Assay

Fifty mg of fresh tissue (4 mm leaf disks) were harvested from the youngest fully expanded leaf at the 3–4 leaf growth stage from 15 plants per population. Shikimic acid accumulation was determined according to Hanson et al. (2009). The glyphosate concentrations used were: 0, 500, and 1,000 μM. Sample absorbance was measured in a Beckman DU-640 spectrophotometer at 380 nm. The test was performed in triplicate on five treated and non-treated plants of each biotype in a completely random design. Results were expressed in mg of shikimic acid g^{-1} fresh tissue.

Absorption and Translocation

This study was carried out in the two *C. elata* populations. ^{14}C-glyphosate (American Radiolabeled Chemicals, Inc., USA) was added to the commercial herbicide to prepare a solution with a specific activity of 0.834 kBq μL^{-1}. The final glyphosate concentration corresponded to 360 g ae ha^{-1} in 200 L ha^{-1}. Plants were harvested at 24, 48, 48, 72, and 96 h after ^{14}C-glyphosate treatment (0.834 kBq/plant). Five plants per populations at each time evaluated in a completely random design were handled according to Fernández-Moreno et al. (2017b). Radioactivity was analyzed by liquid scintillation spectrometry (LSS) in a Beckman LS 6500 scintillation counter (Beckman Coulter Inc. Fullerton, USA) during 10 min per sample. Percentage of ^{14}C-glyphosate absorbed was expressed as [kBq in combusted tissue/(kBq in combusted tissue + kBq in leaf washes)] × 100.

Translocation of ^{14}C-glyphosate in plants of the two *C. elata* populations was visualized using a phosphor imager (Cyclone, Perkin-Elmer, Waltham, MA, USA).

Glyphosate Metabolism

Six plants by each *C. elata* population at 3–4 leaf growth stage, were treated with 300 g ae ha^{-1} of glyphosate (as described in the dose-response assays) in a completely randomized design. Untreated plants were used as controls. Leaf tissues were washed with distilled water at 96 HAT, flash-frozen in liquid nitrogen, and stored at −40°C until use. Following the methodology described by Rojano-Delgado et al. (2010), glyphosate and its metabolites [aminomethyl phosphonate (AMPA), glyoxylate, sarcosine, and formaldehyde] were determined by reversed polarity capillary electrophoresis using a 3D Capillary Electrophoresis Agilent G1600A instrument equipped with a diode array detector (DAD, wavelength range 190–600 nm). Standard compounds used (glyphosate, AMPA, sarcosine, formaldehyde, and glyoxylate), were provided by Sigma-Aldrich, Spain. Glyoxylate naturally produced (untreated plants) was subtracted from the average of glyoxylate produced from glyphosate metabolism (treated plants) for each population.

EPSPS Enzyme Activity Assays

Leaf tissue of the *C. elata* populations (three samples of 5 g each) were ground to fine powder in liquid nitrogen a chilled mortar. The enzyme activity was extracted according to the protocol described by Sammons et al. (2007). The basal EPSPS activity in the extract was measured using a Modified Lowry Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain) in accordance with the manufacturer's instructions. The specific EPSPS activity was determined using the EnzCheck Phosphate Assay Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, to determine the inorganic phosphate release. The glyphosate concentrations used were: 0, 0.1, 1, 10, 100, and 1,000 μM. The EPSPS activity was measured during 10 min at 360 nm in a spectrophotometer (Beckman DU-640) to determine the amount of phosphate (μmol) released μg of total soluble protein $(TSP)^{-1}$ min^{-1} and expressed as a percentage with respect to the control (without glyphosate). The experiment was repeated three times for each samples.

EPSPS Gene Sequence

Young tissue (100–200 mg) was collected from 10 plants of each *C. elata* population and stored at −80°C for RNA extraction. Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was then treated with TURBO DNase (RNase-Free; Ambion, Warrington, UK) to eliminate any DNA contamination. cDNA synthesis was carried out using 2 μg of total RNA and M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in combination with oligo $(dT)_{12-18}$ and random nonamers (Amersham Biosciences, Amersham, UK) according to the manufacturer's instructions. To amplify the EPSPS gene, primers previously designed by Perez-Jones et al. (2007) (forward: 5′ A GCTGTAGTCGTTGGCTGTG 3′; reverse: 5′ GCCAAGAAAT AGCTCGCACT 3′), and de Carvalho et al. (2012) (forward: 5′ TAGTACAGCCAAAAGGGCAGTC-3′; reverse: 5′ GCCGT TGCTGGAGGAAATTC 3′) were used. These primers expand a 120-bp fragment of the EPSPS gene that contains the mutation site described as conferring resistance to glyphosate. The PCR reactions were carried out using cDNA from 50 ng of total RNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 1× buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) enzymes (BIOTOOLS, Madrid, Spain) in a final volume of 25 μL. All PCR reactions were completed in duplicate, and the cycling conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with a final extension cycle of 72°C for 10 min. An aliquot of the PCR product was loaded onto a 1% agarose gel to confirm the correct band amplification. The remainder of the PCR product was then purified using ExoSAP-IT® for PCR Product Clean-Up (USB, Ohio, USA) as indicated by the manufacturers. Five purified PCR products per population were sequenced (STAB VIDA, Caparica, Portugal).

Statistical Analysis

Dose-response and EPSPS enzyme activity data were subjected to non-linear regression analysis to determine the amount of glyphosate needed to reduce the fresh weight (GR_{50}), increase the mortality (LD_{50}), and inhibit the EPSPS activity (I_{50}) by 50% in each *Chloris* population using the three-parameter log-logistic function: $y = ([(d) / 1+(x/g)^b])$ where y is, depending on the analysis, the above ground fresh weight, survival, or EPSPS-activity expressed as the percentage of the non-treated control, d is the parameter corresponding to the upper asymptote, b is the slope, g is the GR_{50}, LD_{50}, or I_{50}, and x (independent variable) is the glyphosate rate. Regression analyses were conducted using the *drc* package in the R program version 3.2.5 (Ritz et al., 2015). Resistance indexes (RI = R/S) were computed as R-to-S GR_{50}, LD_{50}, or I_{50} ratios.

An analysis of variance (ANOVA) was conducted to test for differences between populations in the different assays. When needed, differences between means were separated using the Tukey HSD test at $P < 0.05$. Model assumptions of the normal distribution of errors and homogeneous variance were graphically inspected. The ANOVAs were conducted using the Statistix (version 9.0) (Analytical Software, USA) software.

RESULTS

Morphometric Study and Taxonomic Identity

Based on the examined morphological traits, the populations studied were identified as *C. ciliata* Sw., *C. elata* Desv., and *Chloris barbata* Sw. Only the last species listed is an annual species, and it can be easily separated from the other two species by its long-awned lemmas, the glabrous keel of the fertile lemma and the presence of hairs flanking the keel. In addition, caryopses of *C. barbata* were clearly more elongated in shape than those of the remaining species, as indicated by the higher values of the seed shape index. Distinctive traits of *C. ciliata* include a low number of racemes in the inflorescences, more than two sterile florets per spikelet, long keel hairs and relatively short awns. Compared to the other two species, racemes of *C. elata* plants were consistently longer and their fertile lemmas were shorter in length, showing much longer marginal hairs. In addition, the embryo mark in caryopses was shorter in this species (**Table 1**).

Molecular Characterization of the Genus *Chloris*

A cluster analysis using UPGMA methods classified the *Chloris* populations into two major groups (I and II), thus providing complementary information to the morphological analysis. Group I contained all samples of *C. ciliata* whereas Group II consisted of two subgroups, II-1 including *C. elata*, and II-2 including *C. barbata*. It is noting that the cluster analysis did not separate T and NT populations of *C. ciliata* or *C. barbata*, while the T and NT populations of *C. elata* were clearly separated (**Figure 1**).

TABLE 1 | Comparison of morphological traits from inflorescences and caryopses for the three pairs of studied *Chloris* populations, and their taxonomic identity at the species level.

Species		*C. ciliata* T	*C. ciliata* NT	*C. elata* T	*C. elata* NT	*C. barbata* T	*C. barbata* NT
Number of racemes composing inflorescences		5 (5–7)	5 (4–5)	17–28	11–27	13 (11-15)	12–18
Racemes length		54.6 ± 6.8	76.0 ± 12.0	117.0 ± 20.9	96.0 ± 16.9	71.2 ± 8.5	73.0 ± 9.2
Number of spikelet per cm raceme		14 (14–14)	14 (12–14)	11 (9–11)	14 (12–15)	12 (12–13)	13 (12–13)
Number of sterile florets in spikelets		3 (3–4)	4 (3–4)	2 (2–2)	2 (2–2)	2 (2–2)	2 (2–2)
Length of hairs surrounding spikelet callus		0.66 ± 0.21	0.79 ± 0.12	0.18 ± 0.03	0.40 ± 0.06	0.95 ± 0.08	0.97 ± 0.12
Fertile floret, lemma	Length	2.68 ± 0.19	2.65 ± 0.18	1.98 ± 0.11	1.87 ± 0.10	2.63 ± 0.12	2.54 ± 0.10
	Max width	2.14 ± 0.19	2.18 ± 0.20	1.36 ± 0.09	1.56 ± 0.10	1.18 ± 0.17	1.13 ± 0.14
	Hairy (H)/glabrous (G) keel	H	H	H	H	G	G
	Length of keel hairs	1.20 ± 0.15	1.30 ± 0.15	0.67 ± 0.05	0.70 ± 0.06	–	–
	Length of hairs flanking keel	–	–	–	–	0.57 ± 0.08	0.58 ± 0.14
	Length of marginal hairs	1.13 ± 0.15	1.42 ± 0.26	2.22 ± 0.16	2.09 ± 0.10	1.40 ± 0.13	1.40 ± 0.06
	Awn length	1.63 ± 0.26	1.71 ± 0.35	3.14 ± 0.36	2.53 ± 0.27	6.97 ± 0.60	5.53 ± 1.08
Fertile floret, palea	Length	2.44 ± 0.14	2.55 ± 0.20	1.82 ± 0.09	1.58 ± 0.09	2.45 ± 0.14	2.35 ± 0.23
Basal (first) sterile floret, lemma	Length	1.66 ± 0.14	1.73 ± 0.18	1.09 ± 0.08	1.05 ± 0.05	1.38 ± 0.12	1.37 ± 0.05
	Max width	2.15 ± 0.21	2.23 ± 0.23	0.92 ± 0.10	0.97 ± 0.08	1.27 ± 0.28	1.33 ± 0.08
	Awn length	1.58 ± 0.26	1.72 ± 0.23	2.83 ± 0.15	2.22 ± 0.29	6.37 ± 1.23	5.33 ± 1.89
Second sterile floret, lemma	Length	1.14 ± 0.17	1.08 ± 0.15	0.50 ± 0.05	0.53 ± 0.04	1.29 ± 0.09	1.33 ± 0.08
	Awn length	A	A	A	A	5.22 ± 0.81	3.82 ± 0.88
Glumes	Upper, length	2.57 ± 0.17	2.78 ± 0.07	3.10 ± 0.06	3.25 ± 0.20	2.82 ± 0.08	2.78 ± 0.12
	Upper, width	1.00 ± 0.19	0.92 ± 0.13	0.53 ± 0.05	0.45 ± 0.14	0.60 ± 0.06	0.55 ± 0.05
	Lower, length	1.75 ± 0.15	1.73 ± 0.08	2.07 ± 0.13	2.03 ± 0.14	1.78 ± 0.08	1.80 ± 0.06
	Lower, width	0.83 ± 0.10	0.78 ± 0.04	0.73 ± 0.08	0.80 ± 0.06	0.58 ± 0.08	0.45 ± 0.05
Caryopsis	Length	1.43 ± 0.08	1.35 ± 0.12	1.12 ± 0.04	1.03 ± 0.05	1.45 ± 0.05	1.40 ± 0.00
	Width	0.70 ± 0.04	0.58 ± 0.09	0.57 ± 0.04	0.60 ± 0.00	0.45 ± 0.03	0.43 ± 0.03
	Thickness	0.58 ± 0.04	0.53 ± 0.07	0.45 ± 0.03	0.47 ± 0.03	0.33 ± 0.03	0.31 ± 0.02
	Length of the embryo mark	0.83 ± 0.08	0.77 ± 0.09	0.59 ± 0.07	0.62 ± 0.13	0.93 ± 0.03	0.90 ± 0.05
	Seed shape index[a]	0.069 ± 0.008	0.078 ± 0.001	0.068 ± 0.005	0.055 ± 0.008	0.121 ± 0.004	0.122 ± 0.005

[a] *Variance of the three dimensions, each relative to length.*

Means and standard deviations are given for quantitative traits, and modal (if any), minimum and maximum values for qualitative traits. Sample size n = 6. Linear dimensions are given in mm. A, absent.

Dose-Response Assays

The fresh weight reduction by 50% (GR_{50}) for the NT and T populations of *C. elata* was achieved at 88.3 and 542.1 g ae ha^{-1}, respectively, i.e., T populations were 6.1-fold more resistance to glyphosate than the NT population. The LD_{50} values of the T populations exhibited 15-fold resistance than the NT population of *C. elata*. In contrast, GR_{50} and LD_{50} values in both *C. barbata* and *C. ciliata* species were not different between T and NT populations (**Table 2,Figure 2**).

Shikimic Acid Accumulation

No differences were found between the exposure of leaf disks to 500 or 1,000 μM of glyphosate. At 1000 μM, the NT population of *C. elata* presented 4.6-fold more shikimic than the T population. However, the T populations of *C. barbata* and *C. ciliata* showed no differences, accumulating only 1.13 and 1.07-fold more shikimic acid, respectively, than their respective NT populations (**Figure 3**).

It was determined that *C. barbata* and *C. ciliata* exhibited natural tolerance to glyphosate, and the T and NT populations of *C. elata* were renamed resistant (R) and susceptible (S) to glyphosate, respectively. In the following assays, we focused in *C. elata.*

Absorption and Translocation

Total ^{14}C-glyphosate recovery was 93.2 and 94.3% for the R and S populations of *C. elata*, respectively (data not shown). ^{14}C-glyphosate absorption increased slowly in the first 72 HAT. At this time, population S had absorbed 30% of glyphosate, while population R had only absorbed 22%. The maximum glyphosate absorption rate was observed between 72 and 96 HAT, which was the two-fold higher in the S population (64%) than in the R population (33%; **Figure 4A**).

In both populations, ^{14}C-glyphosate levels in treated leaves of *C. eleta* declined from 24 to 96 HAT, with the rate of movement out of the treated leaf greater and faster in the S population than in R population. The initial amount quantified of 73% at 24 HAT in the treated leaf decreased to 38% at 96 HAT in S population. Conversely, the ^{14}C-glyphosate was retained mainly in the leaf treated in the R population, dropping from 81 to 62% at 24 and

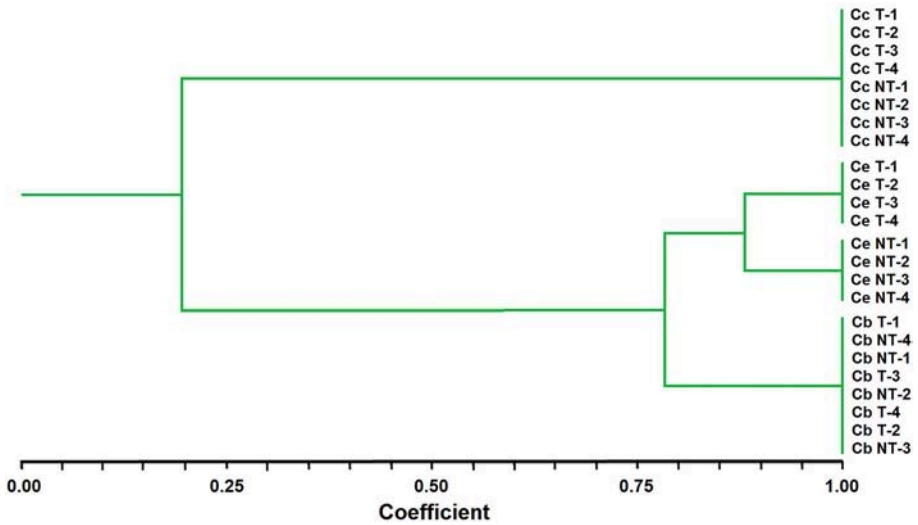

FIGURE 1 | Genetic similarities among *Chloris* species after UPGMA analysis performed with AFLP marker data. Ce, *Chloris elata*; Cb, C. *barbata*; and Cc, *C. Ciliata;* T, plants with glyphosate history applications; NT, non-treated plants.

TABLE 2 | Glyphosate rates required for 50 % reduction fresh weight (GR_{50}) and survival (LD_{50}) expressed as percentage of the mean untreated control of *Chloris* species.

Species	Status[a]	GR_{50} (g ae ha^{-1})	RI[b]	*P*	LD_{50} (g ae ha^{-1})	RI[b]	*P*
C. elata	T	542.1 ± 31.3	6.1	0.001	2277.7 ± 245.1	15.0	0.001
	NT	88.3 ± 4.8			151.6 ± 24.8		
C. barbata	T	217.2 ± 19.6	1.1	0.241	889.4 ± 71.5	1.1	0.382
	NT	198.9 ± 21.9			820.2 ± 79.0		
C. ciliata	T	263.1 ± 19.2	1.1	0.159	912.7 ± 68.6	1.1	0.297
	NT	231.3 ± 37.4			875.2 ± 59.3		

±Standard error (n = 10). [a]Status: T, populations with glyphosate history applications; and NT, non-treated populations with glyphosate. [b]RI, Resistance index (R/S) calculated using the corresponding ED_{50}, or LD_{50} values of the resistant populations respect to the susceptible one.

96 HAT, respectively. An average of 32 and 30% of the glyphosate translocated reached the remaining shoot tissue and roots at 96 HAT in the S population, whereas in R population it was only of 20 and 18%, respectively (**Figures 4B,C**).

The ^{14}C-glyphosate visualization by phosphor imaging revealed differences in the distribution between the S and R populations of *C. elata*. There was a difference in the translocation of glyphosate from treated leaves to shoots and roots, and the S population translocated higher amounts of ^{14}C-glyphosate compared to the R population (**Figure 5**).

Glyphosate Metabolism

For glyphosate absorbed into the R and S *C. elata* populations, much of the herbicide was unaltered in plants by 96 HAT. At this time, 89.4 and 91.0% of the applied herbicide remained as glyphosate in plants of the R and S populations, respectively. The levels of AMPA were 7.2 and 6.4%, while glyoxylate levels reached 3.4 and 2.6% in the R and S plants, respectively. For both AMPA and glyoxylate, these differences between the R and S populations were non-significant ($P = 0.8741$ for AMPA, and $P = 0.6318$ for glyoxylate).

EPSPS Enzyme Activity Assays

The R population was 27.3-fold more resistant than the S population. The basal enzyme activity showed no differences between populations with 0.0987 to 0.0937 mmol mg^{-1} TPS^{-1} min^{-1} for the R and S populations, respectively (**Figure 6**).

Sequencing of the EPSPS Gene

A total of 120 bp of the EPSPS gene of the R and S *C. elata* populations was sequenced. The fragments were aligned and numbered based on a published EPSPS sequence of *Leptochloa virgata* (GenBank: KX425854) (Alcántara-de la Cruz et al., 2016c). Protein alignment of the predicted EPSPS fragments from R and S populations of *C. elata* showed 91.6 and 92.5% protein similarity, respectively, to that of *L. virgata*. The R population of *C. elata* showed an amino acid substitution at position 106 consisting of a proline to serine change. The substitution resulted in a TCG (serine) codon instead of a CCG (proline) codon (**Figure 7**).

DISCUSSION

The three *Chloris* populations from citrus orchards in Cuba were identified as *C. barbata, C. ciliata,* and *C. elata*. The number of species of *Chloris* recognized by different authors in Mexico and in the nearby Caribbean Islands is variable (Barkworth, 2007; Cerros-Tlatilpa et al., 2015). In these regions, the most frequent species are *C. ciliata, C. elata, C. barbata,* and *C. virgata* (Cerros-Tlatilpa et al., 2015). In Cuba, 12 species of the *Chloris* genus have been found (Catasús-Guerra, 2002).

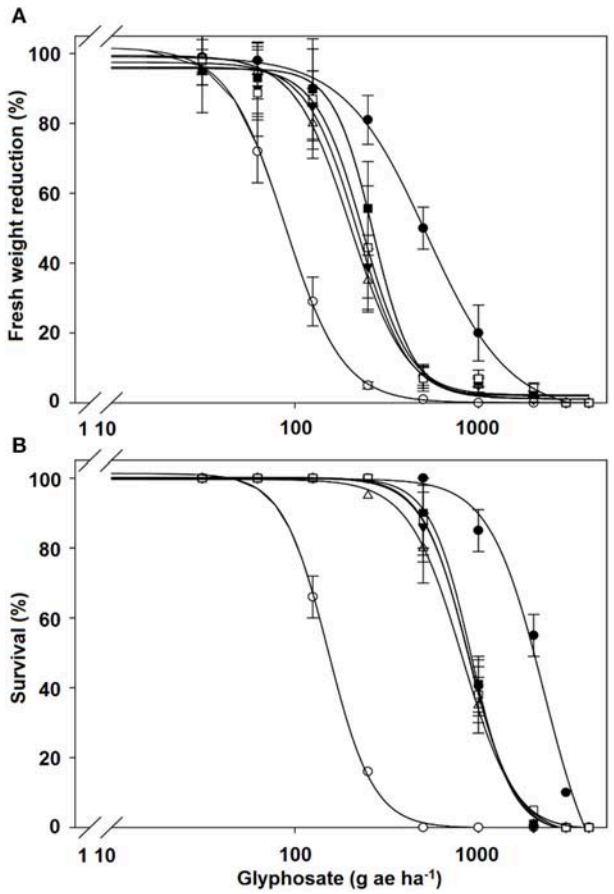

FIGURE 2 | Glyphosate **(A)** dose-response on fresh weight reduction and **(B)** % survival expressed as percentage respect to untreated control of the treated (T) (•) and non-treated (NT) (○) populations of *Chloris elata*; T (▼) and NT (△) populations of *C. barbata*; and T (■) and NT (□) populations of *C. ciliata*. Vertical bars are ± standard errors ($n = 9$).

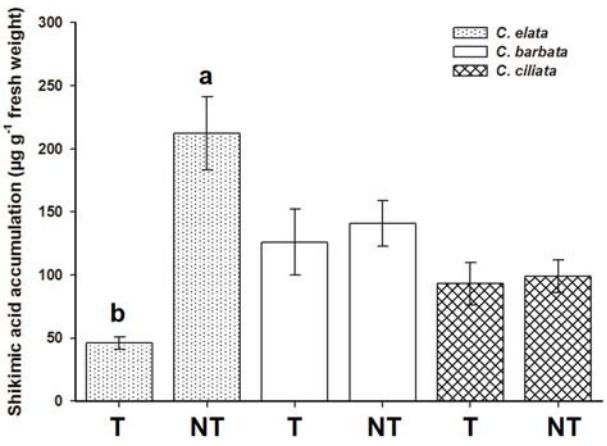

FIGURE 3 | Shikimic acid accumulation in *Chloris* spp. plants at 1,000 µM glyphosate concentration. T = plants with glyphosate history applications; and NT = non-treated plants. Vertical bars are ± standard errors ($n = 5$). Different letters shown differences at 95% by the Tukey's test.

The AFLP-based classification of *Chloris* populations revealed molecular-based relationships between three basic entities, which closely matched the morphology-based identification of three different species. The selected AFLP markers can be useful candidates in the pursuit of disentangling phylogenetic relationships among *Chloris* species. However, although these markers allowed to separate the T and NT populations of *C. elata,* they cannot be used to detect glyphosate resistance, because could produce biased results due to fragment-size homoplasy (Caballero et al., 2008).

To characterize the glyphosate susceptibility in *Chloris* species, it is important to consider the innate tolerance to this herbicide that has been observed in some species of the genus. Depending on the species studied, the LD_{50} (% survival plant) values can vary between 515 and 703 g ae ha^{-1} (Ngo et al., 2017a,b). These values are lower than those we have found for *C. barbata* and *C. ciliata*, including the populations never exposed to this herbicide, demonstrating an innate tolerance to glyphosate in these two species. Similar results were described for *Chloris polydactyla* from Brazil, where even some accessions with no history of applications presented lower susceptibility to glyphosate than those accessions with a history of glyphosate applications (Barroso et al., 2014). Innate tolerance has been well studied in grass weeds (Fernández-Moreno et al., 2016), and leguminous species (Cruz-Hipolito et al., 2009, 2011; Rojano-Delgado et al., 2012; Alcántara-de la Cruz et al., 2016a; Mao et al., 2016). The mechanism proposed is a lack of ^{14}C-glyphosate absorption and/or translocation in tolerant plants compared to susceptible ones.

C. elata has a different profile than *C. barbata* and *C. ciliata*, and its GR_{50} and LD_{50} values demonstrate a clear quantitative difference between those plants harvested from T fields compared to those plants from NT fields. The lower shikimic accumulation by the T *C. elata* population (4.9 times) compared to the NT population showed the greatest resistance level of this species, similar to other *Chloris* species, such as *C. elata* (5.4) from Brazil (Brunharo et al., 2016) and *C. virgata* (2.0–9.7) and *Chloris truncata* (2.4–8.7) from Australia (Ngo et al., 2017a,b). When glyphosate is applied via foliar application, the EPSPS enzyme is inhibited, and there is a rapid accumulation of shikimate (Shaner et al., 2005). The amount of glyphosate in the NT population of *C. elata* determined the inhibition of EPSPS and rapid shikimate accumulation demonstrating the high susceptibility (S) of this population. Therefore, the T population of *C. elata* was characterized as resistant (R) to glyphosate, and the populations T and NT of *C. barbata* and *C. ciliata* are tolerant to this herbicide. These results are reflected in those obtained in dose response assays. For this reason, we continued to study the glyphosate resistance mechanisms only in the case of *C. elata*.

To date, few cases of reduced glyphosate absorption and/or translocation have been studied as a mechanism of resistance in the genus *Chloris,* and the results are contradictory. ^{14}C-glyphosate studies on *C. virgata* and *C. truncata* do not

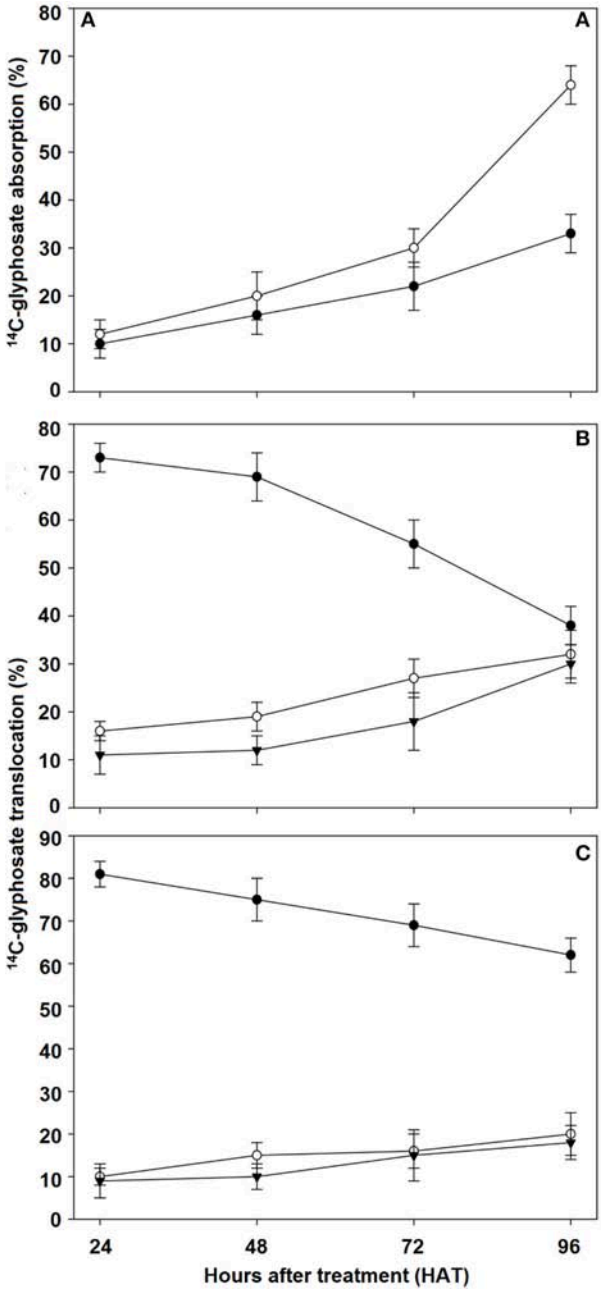

FIGURE 4 | ^{14}C-glyphosate absorption and translocation in susceptible (S) and resistant (R) *Chloris elata* populations from 24 to 96 h after glyphosate treatment. **(A)** ^{14}C-glyphosate absorption in S (○) and R (•) plants. ^{14}C-glyphosate translocation in S **(B)** and R **(C)** plants. Treated leaf (•), remaining shoot tissue (○), and roots (▼). Vertical bars are ± standard errors ($n = 5$).

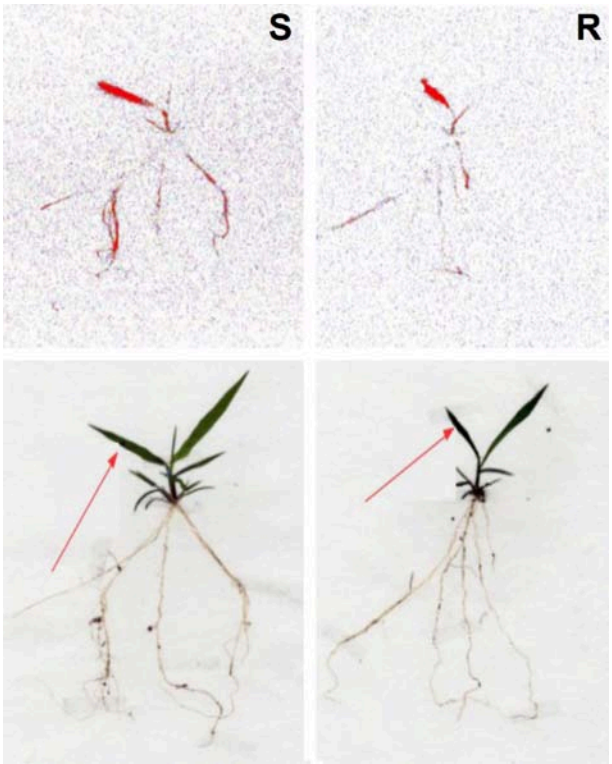

FIGURE 5 | Translocation of ^{14}C-glyphosate in susceptible **(S)** and resistant **(R)** *Chloris elata* plants at 96 h after application. The highest concentration of ^{14}C-glyphosate is highlighted in red. Arrows indicate the treated leaf.

show significant differences in the absorption and subsequent translocation of the herbicide, and the resistance was determined by mechanisms within the target site (Ngo et al., 2017a,b). However, another study on *C. elata* shows that lower glyphosate absorption and translocation in the R population are the only mechanisms involved in its glyphosate resistance (Brunharo et al., 2016). In our case, the R *C. elata* population collected in Cuba shows a resistance mechanism similar to that previously found for the species. Thus, ^{14}C-absorption and translocation are higher in the S population than in the R population. These results suggest that less absorption and translocation contributed to the resistance to glyphosate in R *C. elata* plants.

Glyphosate metabolism has thus far not been identified as a major mechanism of resistance in plants, but is likely the result of plants not succumbing to glyphosate because of the expression of another resistance mechanism (Sammons and Gaines, 2014; Fernández-Moreno et al., 2017a). Only in a few cases has metabolism been demonstrated to be a secondary mechanism in glyphosate resistance, because in these cases, other major mechanisms are involved (Bracamonte et al., 2016). Our research substantiates that of other studies, with <90% of the absorbed glyphosate remaining unaltered in R and S plants of *C. elata*. It is likely that the ability of grass weeds to metabolize glyphosate is diminished once EPSPS is inhibited (González-Torralva et al., 2012; Fernandez et al., 2015). Considering the small extent of glyphosate metabolism, the significance of this result is unlikely biologically meaningful in the resistance to glyphosate in *C. elata*.

The I_{50} values were significantly different between the *C. elata* populations. The R population exhibited a high resistance level compared to the S population. The results with high I_{50} values and low shikimic acid values, as has already been explained and demonstrated in other studies, are associated with alterations

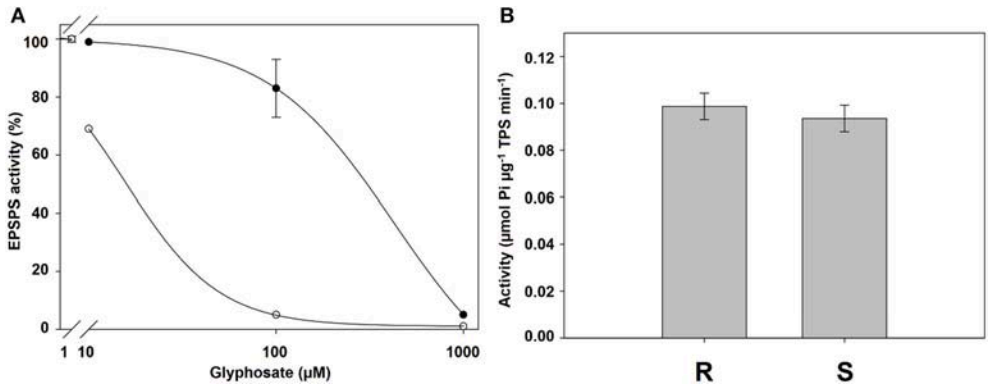

FIGURE 6 | (A) EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from glyphosate-susceptible (○) and -resistant (•) *Chloris elata* plants. **(B)** Basal EPSPS activity for *C. elata* populations. Vertical bars are ± standard errors ($n = 3$).

Position	99	100	101	**102**	103	104	105	**106**	107	108	109
Consensus sequence	AAT	GCA	GGA	ACC	GCT	ATG	CGC	**CCG**	TTG	ACC	GCT
L. virgata (KX425854)	AAC	GCT	GGA	ACT	GCG	ATG	CGC	**CCA**	TTG	ACG	GCT
Amino acid translation	Asn	Ala	Gly	Thr	Ala	Met	Arg	**Pro**	Leu	Thr	Ala
C. elata S	Asn	Ala	Gly	Thr	Ala	Met	Arg	**Pro**	Leu	Thr	Ala
C. elata R	Asn	Ala	Gly	Thr	Ala	Met	Arg	**Ser**	Leu	Thr	Ala

FIGURE 7 | Partial alignment of nucleotides and amino acid sequences of EPSPS genes between glyphosate-susceptible and -resistant *Chloris elata* populations and *Leptochloa virgata* (GenBank: KX425854). The yellow color indicates nucleotide changes among species. The green color indicates an amino acid substitution at 106 position from Proline (CCG) to Serine (TCG). Box includes the 102 (blue) and 106 (orange) positions (amino acid number based on the start codon (ATG) of *Arabidopsis thaliana*, corresponding to point mutations associated for conferring glyphosate resistance.

in the gene encoding the herbicide target protein (Sammons and Gaines, 2014; Yu et al., 2015). Then, the TSR mechanism could be involved in the resistance to this species. Similar results have been shown in other weed species, including *L. virgata* (Alcántara-de la Cruz et al., 2016c), *Lolium multiflorum* (Salas et al., 2015), and *L. rigidum* (Fernandez et al., 2015). In these cases, higher I_{50} values, as well as the higher basal activity of EPSPS, were found in the resistant populations compared to the susceptible populations. It was thought that an overexpression of EPSPS played a role as a resistance mechanism (Ngo et al., 2017a). However, there were no significant differences in the basal activity of EPSPS between R and S populations of *C. elata*, precluding the involvement of such a mechanism.

The EPSPS sequence alignment showed only a mutation point at position Pro-106-Ser in the R *C. elata* population. Four substitutions in this genomic EPSPS position (Pro-106-Ser, Pro-106-Thr, Pro-106-Ala, and Pro-106-Leu), have been reported in mono- and dicotyledonous weeds, endowing resistance to glyphosate (Sammons and Gaines, 2014). A mutation to a different amino acid at this point causes a structural change in the target site, shifting the other amino acids toward the inhibitor by reducing the available space (Healy-Fried et al., 2007). These explain the resistance of the R population of *C. elata* at a molecular level. Some grassweed species which have shown a mutation at Pro-106 position are: *C. virgata* (Ngo et al., 2017a), *Echonoclhoa colona* (Alarcón-Reverte et al., 2015), *L. virgata* (Alcántara-de la Cruz et al., 2016c), *L. rigidum* (Fernandez et al., 2015), and *Poa annua* (Cross et al., 2015), among others.

CONCLUSIONS

Morphological- and molecular-based analysis allowed the identification of the three *Chloris* species collected in citrus orchards from central Cuba. *C. barbata* and *C. ciliata* were characterized as being innately tolerant to glyphosate, and *C. elata* was identified as resistant to this herbicide. The last species had non-target site (reduced absorption and translocation) and target site (Pro-106-Ser mutation) resistance mechanisms to glyphosate.

These results confirm the first case of herbicide resistance in Cuba and strongly suggest that species of the *Chloris* genus can be either resistant or tolerant to glyphosate, supporting the previous reports of both glyphosate statuses in this genus.

AUTHOR CONTRIBUTIONS

EB and RDP: Idea and designed the experiments; EB, PF-M, MO, FB, HC-H, and RA-dlC: Performed the research. PF-M, FB, MO, RA-dlC, and RDP; Analyzed the results. All authors contributed to write and approve the manuscript.

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Effects of EPSPS Copy Number Variation (CNV) and Glyphosate Application on the Aromatic and Branched Chain Amino Acid Synthesis Pathways in *Amaranthus palmeri*

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A key enzyme of the shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), is the known target of the widely used herbicide glyphosate. Glyphosate resistance in *Amaranthus palmeri*, one of the most troublesome weeds in agriculture, has evolved through increased *EPSPS* gene copy number. The aim of this work was to study the pleiotropic effects of (*i*) *EPSPS* increased transcript abundance due to gene copy number variation (CNV) and of (*ii*) glyphosate application on the aromatic amino acid (AAA) and branched chain amino acid (BCAA) synthesis pathways. Hydroponically grown glyphosate sensitive (GS) and glyphosate resistant (GR) plants were treated with glyphosate 3 days after treatment. In absence of glyphosate treatment, high *EPSPS* gene copy number had only a subtle effect on transcriptional regulation of AAA and BCAA pathway genes. In contrast, glyphosate treatment provoked a general accumulation of the transcripts corresponding to genes of the AAA pathway leading to synthesis of chorismate in both GS and GR. After chorismate, anthranilate synthase transcript abundance was higher while chorismate mutase transcription showed a small decrease in GR and remained stable in GS, suggesting a regulatory branch point in the pathway that favors synthesis toward tryptophan over phenylalanine and tyrosine after glyphosate treatment. This was confirmed by studying enzyme activities *in vitro* and amino acid analysis. Importantly, this upregulation was glyphosate dose dependent and was observed similarly in both GS and GR populations. Glyphosate treatment also had a slight effect on the expression of BCAA genes but no general effect on the pathway could be observed. Taken together, our observations suggest that the high CNV of *EPSPS* in *A. palmeri* GR populations has no major pleiotropic effect on the expression of AAA biosynthetic genes, even in response to glyphosate treatment. This finding supports the idea that the fitness cost associated with *EPSPS* CNV in *A. palmeri* may be limited.

Keywords: glyphosate, aromatic amino acid pathway, branched chain amino acid pathway, mRNA relative expression, EPSPS, CM, AS, *Amaranthus palmeri*

INTRODUCTION

The shikimate pathway uses carbon from primary metabolism to form chorismate, a precursor of the essential aromatic amino acids (AAAs) phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) (Tzin and Galili, 2010). These AAAs are not only essential components of protein synthesis but also serve as precursors for a wide range of secondary metabolites with multiple biological functions in plants, including plant stress tolerance (Dyer et al., 1989; Keith et al., 1991; Gorlach et al., 1995; Janzik et al., 2005; Maeda and Dudareva, 2012). The AAA synthesis pathway can be subdivided into two steps: (i) the pre-chorismate (shikimate) pathway which provides the precursor chorismate used for synthesis of all AAAs and (ii) the post-chorismate pathway which can lead to either synthesis of Phe and Tyr, or Trp, via different routes (**Figure 1**) (Maeda and Dudareva, 2012). Synthesis of chorismate is catalyzed by seven enzymes acting sequentially (**Figure 1**): D-arabino-heptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQSD), shikimate kinase (SK), 5-enolypyruvylshikimate 3-phosphate synthase (EPSPS), and chorismate synthase (CS). After formation of chorismate, synthesis of Trp is catalyzed by anthranilate synthase (AS) while synthesis of Phe and Tyr is catalyzed by chorismate mutase (CM) (Tohge et al., 2013).

Due to its importance for plant biology, the synthesis of AAA is a tightly regulated process controlled by many inputs (Bentley and Haslam, 1990; Tzin and Galili, 2010; Tohge et al., 2013; Galili et al., 2016). Four points appear as checkpoints: the entrance of the pathway with the enzyme DAHPS (Sato et al., 2006), an exit of major importance with the phenylalanine ammonia lyase (PAL) (Hahlbrock and Scheel, 1989), the branch point in the post-chorismate pathway (Maeda and Dudareva, 2012) and the enzyme EPSPS. The enzyme EPSPS is the target of the herbicide glyphosate (Steinrücken and Amrhein, 1980) and therefore a key step in the shikimate pathway.

The intensive and continuous use of glyphosate has led to the emergence of glyphosate resistant (GR) weed populations (Powles, 2008). The global issue of herbicide resistance for weed management is a serious challenge for global food security (Délye et al., 2013). One of the most damaging glyphosate-resistant weed species is *Amaranthus palmeri* S. Wats (Culpepper et al., 2006; Powles and Yu, 2010). Glyphosate resistance is conferred by gene amplification of *EPSPS*, which leads to a massive production of the enzyme EPSPS (Gaines et al., 2010). The recommended field dose is not sufficient to inhibit EPSPS activity, and plants survive. Copy number variation (CNV) of *EPSPS* is now reported to confer glyphosate resistance in several weed species including *Lolium multiflorum* (Salas et al., 2012) and *Kochia scoparia* (Wiersma et al., 2015) and particularly in *Amaranthus* species such as *Amaranthus tuberculatus* (Lorentz et al., 2014) and *Amaranthus spinosus* (Nandula et al., 2014).

To date, how the AAA pathway is regulated and how glyphosate may affect this regulation is not clearly understood. In particular, it is unknown whether there are pleiotropic effects associated with *EPSPS* CNV, particularly at the AAA synthesis pathway. Notably, no fitness cost has been associated with massive increase of EPSPS activity in GR populations (Giacomini et al., 2014; Vila-Aiub et al., 2014). However, the gene amplification resistance mechanism found in *A. palmeri* offers us the opportunity to study the regulation of the shikimate pathway, the effect of *EPSPS* overexpression due to extra *EPSPS* gene copies, and the effect of glyphosate application. In addition to the feedback regulation of AAA biosynthetic pathway, the hypothesis of the existence of cross-regulation of amino acid metabolic pathways at the transcriptional level has been revised (Pratelli and Pilot, 2014). A close correlation between AAA and branched chain amino acids (BCAAs) has been found (Noctor et al., 2002).

In this study, the main objective was to evaluate the impact of *EPSPS* overexpression by gene amplification and of glyphosate treatment on the regulation of the AAA pathway and free AAA content. To this aim, the response of glyphosate sensitive (GS) and GR populations of *A. palmeri* to glyphosate were evaluated at the molecular and biochemical levels. Additionally, *m*RNA relative expression of the main enzymes from the BCAA pathway was developed to test whether there is any variation in their levels because of the overexpression of *EPSPS* or glyphosate treatment.

MATERIALS AND METHODS

Plant Material and Herbicide Application

Seeds of *A. palmeri* GS and GR biotypes were originally collected from North Carolina (United States) (Chandi et al., 2012; Fernández-Escalada et al., 2016). The resistance mechanism of the GR biotype has been described to be *EPSPS* gene amplification (Chandi et al., 2012), with 47.5 more gene copies in GR than in GS plants (Fernández-Escalada et al., 2016).

Plants were germinated and grown in aerated hydroponic culture under controlled conditions according to procedures described in Fernández-Escalada et al. (2016). Three week-old plants [after reaching the growth stage defined as BBCH 14 (Hess et al., 1997)] were treated with glyphosate (commercial formula, Glyfos, 360 g a.e. L^{-1}, isopropylamine salt, BayerGarden, Valencia, Spain) at both recommended field rate ($1 \times = 0.84$ kg ha^{-1}) and three times that rate ($3 \times = 2.52$ kg ha^{-1}), according to Culpepper et al. (2006). Glyphosate treatment was performed using an aerograph (Junior Start model; Definik; Sagola). Control plants were treated with water. At 3 days after treatment, leaves were collected, frozen, and ground to a fine power as previously described (Fernández-Escalada et al., 2016). The experiment was conducted twice.

Quantitative Reverse Transcription-PCR

RNA was extracted from leaf tissues using the Machery-Nagel NucleoSpin® RNA Plant kit following manufacturer's instructions. Total RNA concentration was measured with Gen 5.1.11 (Biotek Instruments, Inc., United States) and RNA quality was assessed using RNA gel electrophoresis. The gels were visualized using a Gel Doc 2000 system (BIORAD Laboratories, Inc., Hercules, CA, United States).

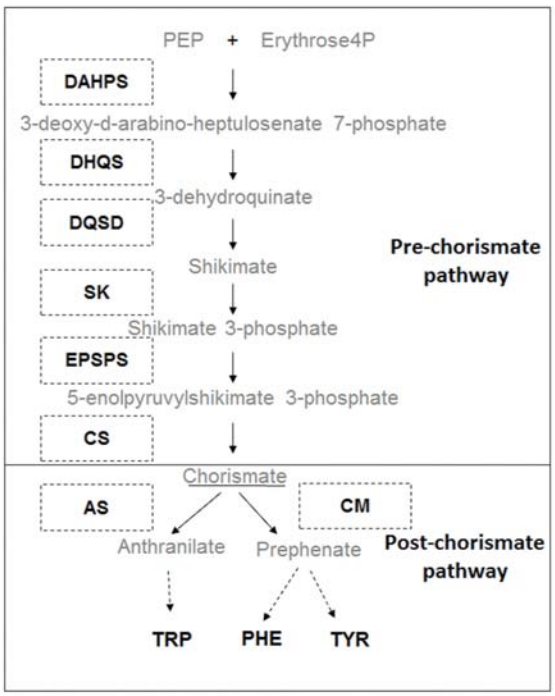

FIGURE 1 | Biosynthetic pathway of aromatic amino acids (AAAs). Consecutive enzymatic steps of pre-chorismate pathway: D-arabino-heptulosonate 7-phosphate synthase (DAHPS), dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQSD), shikimate kinase (SK), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), and chorismate synthase (CS); and post-chorismate pathway: anthranilate synthase (AS) chorismate mutase (CM) leading to the synthesis of tyrosine (TYR), phenylalanine (PHE), and tryptophan (TRP).

cDNA synthesis was performed using BIORAD iScript^{TM}cDNA Synthesis Kit with 1 μg of total RNA following manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR) was performed using a Thermocycler BIORAD CFX Connect TM Real-Time System. The reaction kit used for qPCR was PerfeCTa SYBR® Green SuperMix (Quantabio, Beverly, MA, United States). Each reaction was performed using 1 μL of cDNA template. The following thermal profile was used for all PCRs: denaturation at 95°C for 2 min, 40 cycles of 95°C for 15 s and 52–61°C for annealing and extension for 20 s. Optimal annealing temperature for each primer was determined using gradient PCR. All primers and annealing temperatures are listed in Supplementary Table 1. *EPSPS* primer was modified from Gaines et al. (2010). Melting curve analysis was conducted to verify amplification of single PCR products. Gene expression was monitored in five biological replicates. Primer efficiency (E) for each primer is presented in Supplementary Table 1 and was calculated according to E = 10 [−1/slope] (Pfaffl, 2001). Relative transcript level was calculated as $E_{GOI}^{CP_{GOI\ control} - CP_{GOI\ treated}} / E_{REF}^{CP_{REF\ control} - CP_{REF\ treated}}$ (Pfaffl, 2001), where GOI = gene of interest, REF = reference gene (beta tubulin was used as normalization gene), and CP = crossing point, the cycle at which fluorescence from amplification exceeded the background fluorescence. Relative transcript level was calculated for all genes of the AAA synthesis pathway, corresponding to eight enzymes and four genes of the BCAA synthesis pathway.

EPSPS, DAHPS, and PAL Immunoblotting

Protein extraction was performed using 0.1 g of ground leaf tissue in 0.2 mL of extraction buffer (MOPS 100 mM, EDTA 5 mM, Triton-X 100 1%, glycerin 10%, KCl 50 mM, benzamidine 1 mM, iodoacetamide 100 μM, PVP 5% and PMSF 1 mM). Proteins were separated by 12.5% SDS-PAGE and immunoblots were produced according to standard techniques. The protein amount loaded per well for each antibody used is specified in the figure legends. EPSPS and DAHPS antibody dilutions were 1:2000 (Fernández-Escalada et al., 2016) and 1:1000 (Orcaray et al., 2011), respectively. PAL antibody was produced by a custom peptide facility (Biogenes, Berlin, Germany) using a short, conjugated peptide as an antigen (C-QFAKPR-SDSFEEKN). The antibody was raised in rabbits using standard protocols from the manufacturer, and the primary antibody dilution was 1:500. An anti-rabbit AP conjugated antibody (Sigma Chemical, Co., St. Louis, MO, United States) was used as a secondary antibody at a dilution of 1:20000. Bands were identified using a BCIP/NBT kit which was Amplified alkaline phosphatase immunoblot assay kit (BIORAD 170, BIORAD Laboratories, Inc., Hercules, CA, United States). Immunoblots were scanned using a GS-800 densitometer, and protein bands were quantified using Quantity One software (BIORAD Laboratories, Inc., Hercules, CA, United States). In the case of EPSPS protein, membrane signals were normalized according to total soluble protein loading quantity. In the case of DAHPS and PAL, absolute signals were used.

Enzymatic Activities

5-Enolpyruvylshikimate-3-phosphate synthase activity was performed using the procedure described in Gaines et al. (2010). PAL activity was carried out according to Orcaray et al. (2011) with the following modifications. Samples were immediately centrifuged after extraction (12,000 *g*, 5 min) The reaction was started by the addition of 25 mM L-phenylalanine (Maroli et al., 2015). Controls (without L-phenylalanine) were prepared to determine endogenous levels of transcinnamic acid (t-CA). Incubation was performed for 1 h at 37°C (Sarma et al., 1998; Wang et al., 2007).

Protein extraction for CM and AS activity assays was developed as described in Singh and Widholm (1974) with addition of 1 mM PMSF (Goers and Jensen, 1984). Samples were desalted using PD-10 columns (Ishimoto et al., 2010). CM enzymatic activity was measured as described in Goers and Jensen (1984). Control for each sample was carried out using enzymatic extracts previously inactivated with 1 N HCl. AS activity was quantified as described in Ishimoto et al. (2010). Controls were performed using boiled enzymatic extract (Matsukawa et al., 2002).

Shikimate Determination

For shikimate content determination, three leaf disks (4 mm diameter) were excised from the youngest leaf of each plant. Leaf disks were placed in a screw-top 2 mL Eppendorf tube, frozen, and stored at −80°C until analysis. Shikimate was extracted as described in Koger et al. (2005). After addition of 100 μL of 0.25 N HCl per disk to each vial, samples were incubated at 22°C for 1.5 h and mixed by vortexing. Shikimate content was quantified spectrophotometrically (Cromartie and Polge, 2000).

Aromatic Amino Acid Content Determination

Ground leaf (0.1 g) was homogenized in 1 M HCl for amino acid extraction. Protein precipitation was performed after incubation on ice and centrifugation (Orcaray et al., 2010). After derivatization with fluorescein isothiocyanate, AAA content was measured by capillary electrophoresis coupled to a laser-induced fluorescence detector, as described in Zulet et al. (2013b). Analyses were performed at 20°C and at a voltage of +30 kV. For tryptophan determination, the voltage was reduced to +20 kV in order to improve separation.

Statistical Analysis

Transcript level analyses were performed using five biological replicates. For immunoblot, enzyme activity, shikimate and AAA quantification, four biological replicates were used. One-way ANOVA with a multiple-comparison adjustment for least significant difference (LSD) at $p \leq 0.05$ was used. Statistical analyses were performed using SPSS Statistics 24.0 (IBM, Corp., Armonk, NY, United States).

RESULTS

The number of *EPSPS* copies in the studied GR biotype was reported to be 47.5 fold when compared to the corresponding GS biotype (Fernández-Escalada et al., 2016). In the absence of glyphosate, protein level was increased by 25 fold (**Figures 2A,B**) and EPSPS activity was 26 fold higher (**Figure 2C**). In response to glyphosate, only a mild increase of the abundance of EPSPS protein was observed in the GR biotype at the highest glyphosate dose (**Figures 2A,B**). EPSPS activity was not affected by glyphosate in the GR biotype, regardless of the dose, while it was slightly decreased in the GS biotype with the highest dose applied (**Figure 2C**). While shikimate content was almost negligible in untreated plants of both populations, it accumulated after glyphosate treatment in GS and in GR only at the highest glyphosate dose. Shikimate accumulated significantly more in GS than in GR at each glyphosate dose (**Figure 2D**), confirming the inhibition of EPSPS by glyphosate observed in GS (**Figure 2C**).

To study the impact of the high *EPSPS* copy number on the regulation of the AAA biosynthetic pathway, transcript levels for seven enzymes were analyzed by qRT-PCR. In absence of glyphosate treatment, *EPSPS* transcript level was increased by 55 fold in GR (**Figure 3A**), confirming the results of Fernández-Escalada et al. (2016). For other enzymes, particularly *CS* and

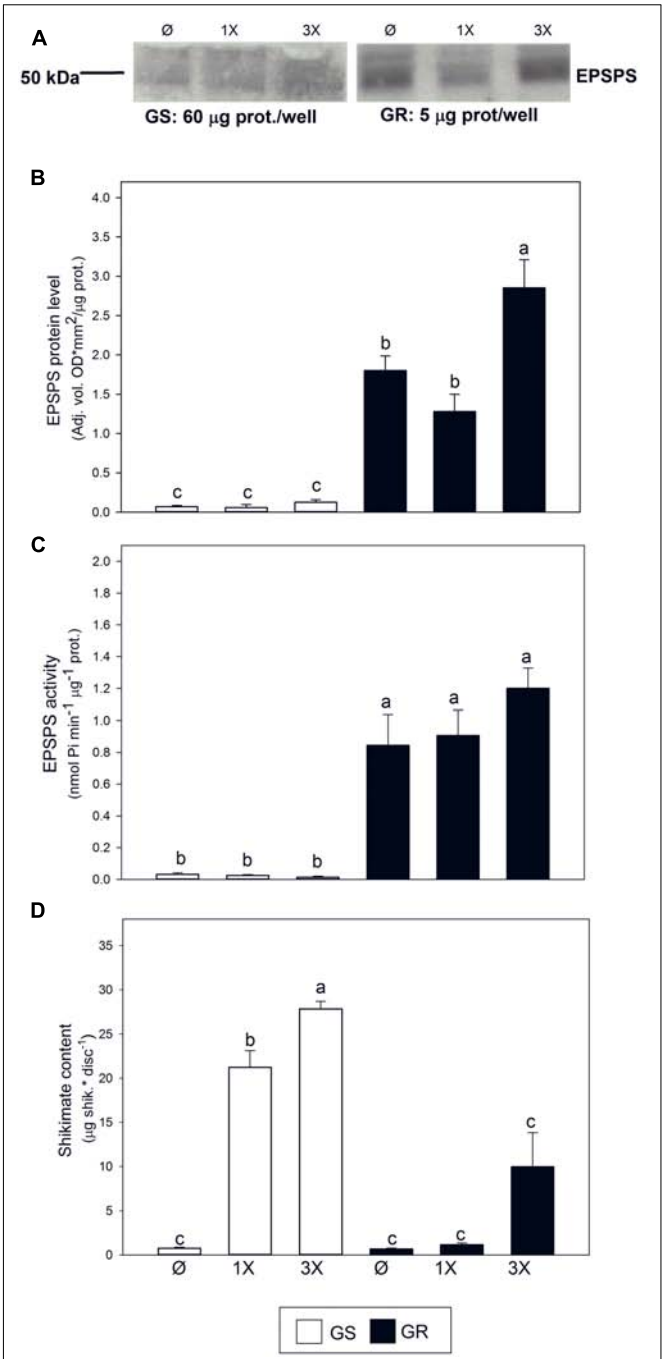

FIGURE 2 | Characterization of resistance in *Amaranthus palmeri* populations. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or treated with glyphosate and measured 3 days after treatment with one (1X) or three times (3X) field dose. **(A)** Representative immunoblots for EPSPS. Total soluble proteins (60 μg for GS or 5 μg for GR) were fractioned by 12.5% SDS-PAGE and blotted. **(B)** Normalization of the intensity of the EPSPS bands expressed as optical density for unit of area per μg of protein (Mean ± SE; $n = 3$). **(C)** EPSPS *in vitro* enzymatic activity measured spectrophotometrically in semicrude leaf extracts (Mean ± SE; $n = 4$). **(D)** Shikimate content was measured spectrophotometrically after extraction from leaf disks of treated plants (Mean ± SE; $n = 4$). Different letters indicate significant differences between treatments and/or populations (p-value ≤ 0.05, LSD test).

A

Enzyme	DAHPS	DHQS	DQSD	SK	EPSPS	CS	CM	AS
Relative expression in untreated plants R/S	0.86 ± 0.19	0.92 ± 0.12	1.48 ± 0.68	1.32 ± 0.19	55.02 ± 11.18	1.68 ± 0.17	2.33 ± 0.10	1.01 ± 0.21

B

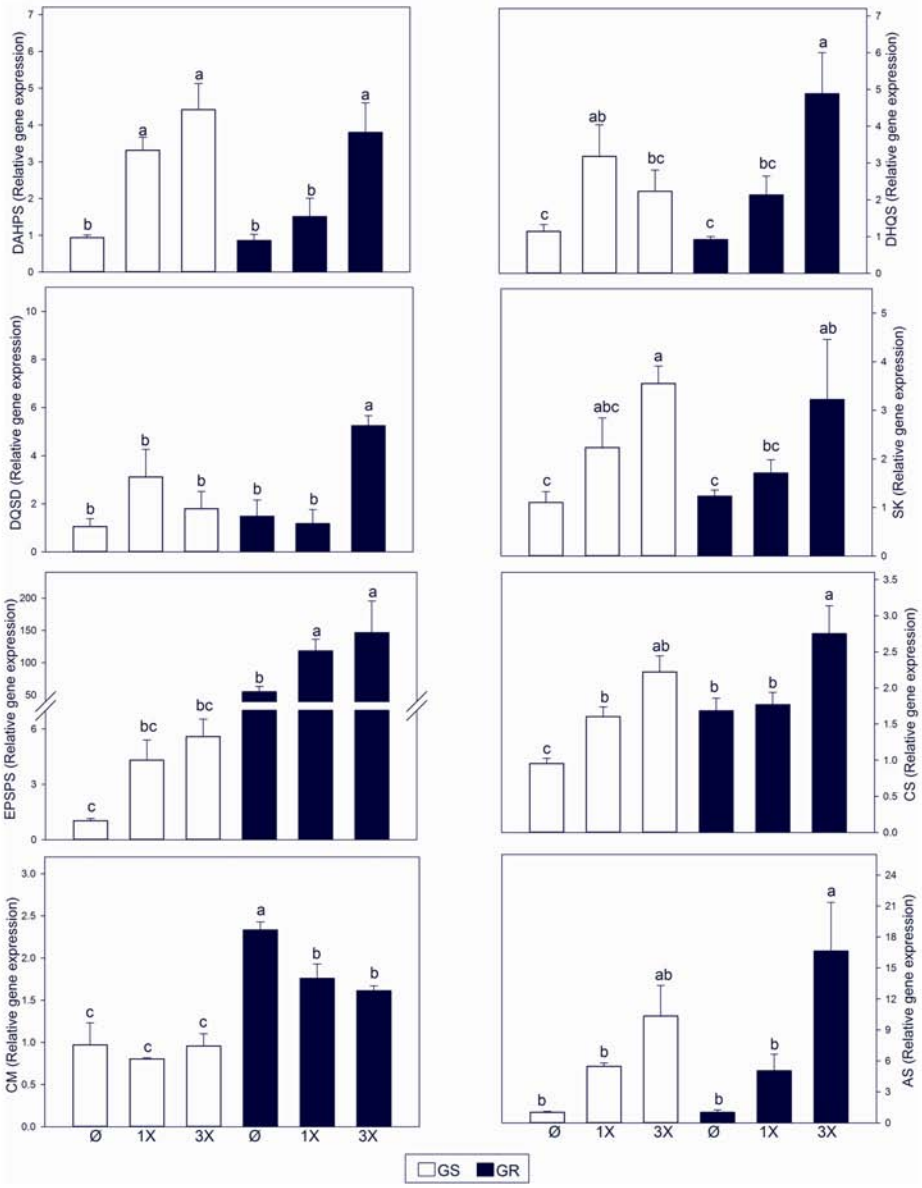

FIGURE 3 | Transcript abundance of genes in the aromatic amino acid (AAA) biosynthetic pathway. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or 3 days after treatment with glyphosate at one (1X) or three times (3X) field dose. Enzyme abbreviations as described in **Figure 1**. **(A)** Ratio of GR to GS relative transcript abundance measured with qRT-PCR normalized using the normalization gene *beta tubulin*. **(B)** Relative transcript abundance normalized using the normalization gene *beta tubulin*, relative to GS untreated plants (Mean ± SE; $n = 5$). Different letters indicate significant differences between treatments and/or populations (p-value ≤ 0.05, LSD test).

CM, only marginal changes were observed (1.68 and 2.33 fold, respectively) (**Figure 3A**).

Glyphosate provoked an induction of the expression of all the genes of the shikimate pathway, with the exception of *CM* (**Figure 3B**). The change in gene expression was dose dependent. The same effect was observed in both GS and GR populations. *CM* showed the opposite behavior, with no change (GS) or a slight decrease (GR) in *CM* transcript accumulation after treatment with glyphosate (**Figure 3B**). The most responsive gene was *AS* with upregulation over 15 fold in GR with the highest dose

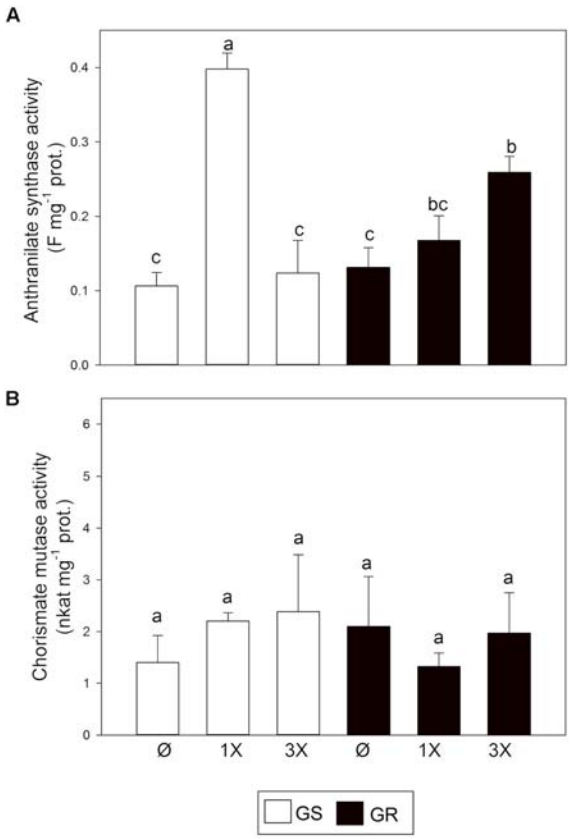

FIGURE 4 | Anthranilate synthase (AS) and chorismate mutase (CM) enzymatic activities. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or 3 days after treatment with glyphosate at one (1X) or three times (3X) field dose. **(A)** AS was measured in desalted leaf extracts by measuring the fluorescence of the produced anthranilate. **(B)** CM was measured in desalted leaf extracts by measuring prephenate production spectrophotometrically (Mean ± SE; $n = 4$). Different letters indicate significant differences between treatments and/or populations (p-value ≤ 0.05, LSD test).

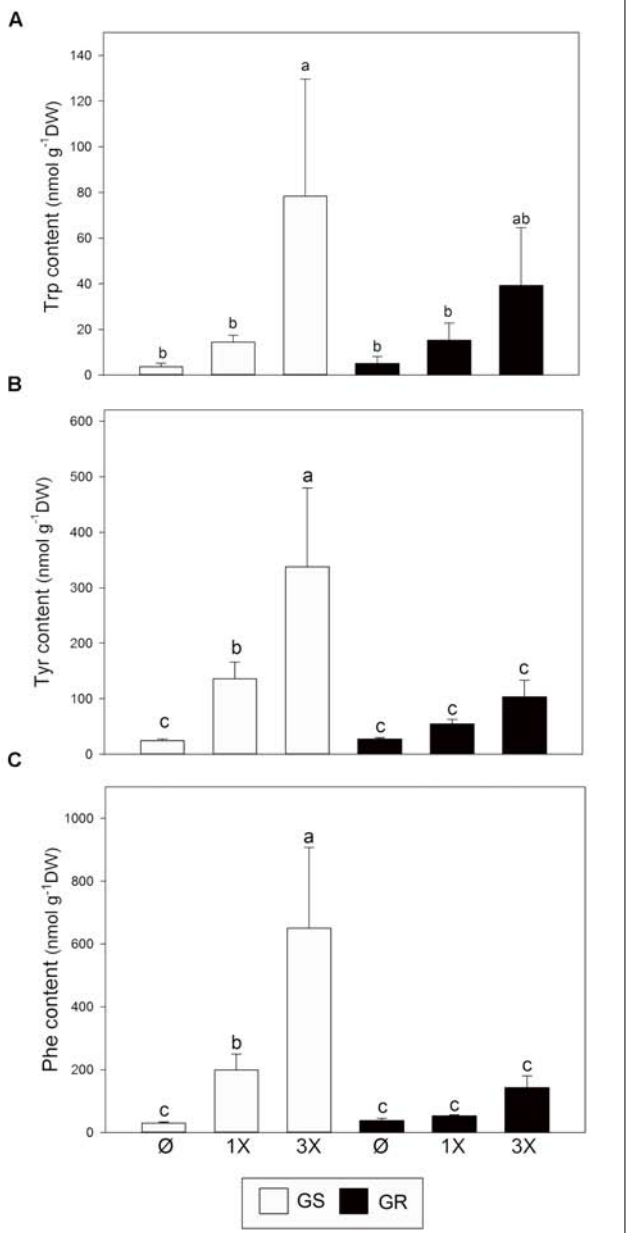

FIGURE 5 | Aromatic amino acid content. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or 3 days after treatment with glyphosate at one (1X) or three times (3X) field dose. Tryptophan (Trp; **A**), tyrosine (Tyr; **B**), and phenylalanine (Phe; **C**) were measured by capillary electrophoresis in leaf acidic extracts (Mean ± SE; $n = 4$). Different letters indicate significant differences between treatments and/or populations (p-value ≤ 0.05, LSD test).

(**Figure 3B**). This may suggest a preferential flux to the Trp biosynthesis branch rather than to the Phe and Tyr branch in response to glyphosate treatment.

To pursue this hypothesis, the activity of CM and AS enzymes was studied. In the absence of glyphosate, AS (**Figure 4A**) and CM (**Figure 4B**) activities were similar in both biotypes. Changes in the activity of AS and CM confirmed the trend observed at the transcript level, suggesting a preferential synthesis toward Trp after glyphosate treatment. *AS* expression induction was concomitant with an increase in the enzyme activity while CM activity was unchanged.

Next, AAA levels were measured (**Figure 5**). Before treatment with glyphosate, levels of Trp (**Figure 5A**), Tyr (**Figure 5B**), and Phe (**Figure 5C**) were similar in both GS and GR biotypes. This result confirms that the striking change in *EPSPS* expression due to CNV does not have a major effect on AAA levels. After glyphosate treatment, the level of all AAA increased (**Figures 5A–C**). However, significant changes were detected only in GS. In GR, the highest increase was detected for Trp.

Previous studies with the same populations and the same time of study and concentration of glyphosate provoked a threefold increase of total free amino acid content and a 12 fold increase of BCAA content (Fernández-Escalada et al., 2016). The higher

effect of glyphosate on BCAA content than on other amino acid types suggests a possible effect of the herbicide on the BCAA biosynthetic pathway. Based on this, the expression pattern of four enzymes of BCAA biosynthetic pathway was also measured: acetohydroxyacid synthase (AHAS), ketol-acid reductoisomerase (AHAIR), dihydroxyacid dehydratase (DHAD) and branched-chain amino acid transaminase (TA) (**Figure 6**). Transcript abundance of the BCAA biosynthetic pathway was not different between the untreated plants of both populations, suggesting that *EPSPS* overexpression does not affect BCAA pathway expression. After glyphosate treatment, *AHAS*, *DHAD*, and *TA* showed no change at either dose in GS or in GR. *AHAIR* transcript abundance was increased in GS at the highest glyphosate dose, while it did not change in GR after glyphosate treatment.

DISCUSSION

Characterization of Resistance in *A. palmeri* Populations

In the GR population of *A. palmeri* an *EPSPS* gene amplification (Fernández-Escalada et al., 2016) results in a massive increase of the accumulation of corresponding transcript (**Figure 3A**) and of the protein level and activity (**Figures 2B,C**). Our data validate results previously reported in other populations of *A. palmeri* (Gaines et al., 2010, 2011; Ribeiro et al., 2014), and other weedy plant species such as *A. tuberculatus* (Lorentz et al., 2014; Chatham et al., 2015), *Lolium perenne* ssp. *multiflorum* (Salas et al., 2012), *Eleusine indica* (Chen et al., 2015), and *Kochia scoparia* (Wiersma et al., 2015). Additionally our data confirmed the accumulation of shikimate following treatment with glyphosate, mostly in the GS population (**Figure 2D**). Shikimate is a known stress marker which accumulates following EPSPS inhibition in GS populations (Dyer et al., 1988; Baerson et al., 2002; Zhu et al., 2008; Whitaker et al., 2013; Doğramacı et al., 2015; Fernández-Escalada et al., 2016; Dillon et al., 2017).

Gene Amplification of *EPSPS* in *A. palmeri* GR Populations Has No Major Pleiotropic Effect on the Expression of AAA Biosynthetic Genes

Despite all these traits that characterize a GR population at molecular and biochemical levels, our work revealed that gene amplification of *EPSPS* had no major effect on the overall AAA pathway (**Figures 2**–**5**). In particular, in untreated plants, the level of free AAA content was similar in GR and GS populations (**Figure 5**). Similar AAA content in glyphosate resistant/sensitive biotypes has been previously described (Maroli et al., 2015). This is consistent with previous reports suggesting that the overexpression of *EPSPS* may have no fitness cost in *A. palmeri* (Giacomini et al., 2014; Vila-Aiub et al., 2014).

The entrance of the primary metabolism to AAA pathway is through DAHPS enzyme (Tohge et al., 2013). Plants control the carbon flux into the pathway by controlling *DAHPS* transcription and protein abundance (Herrmann and Weaver, 1999). However, it was previously unknown whether GR

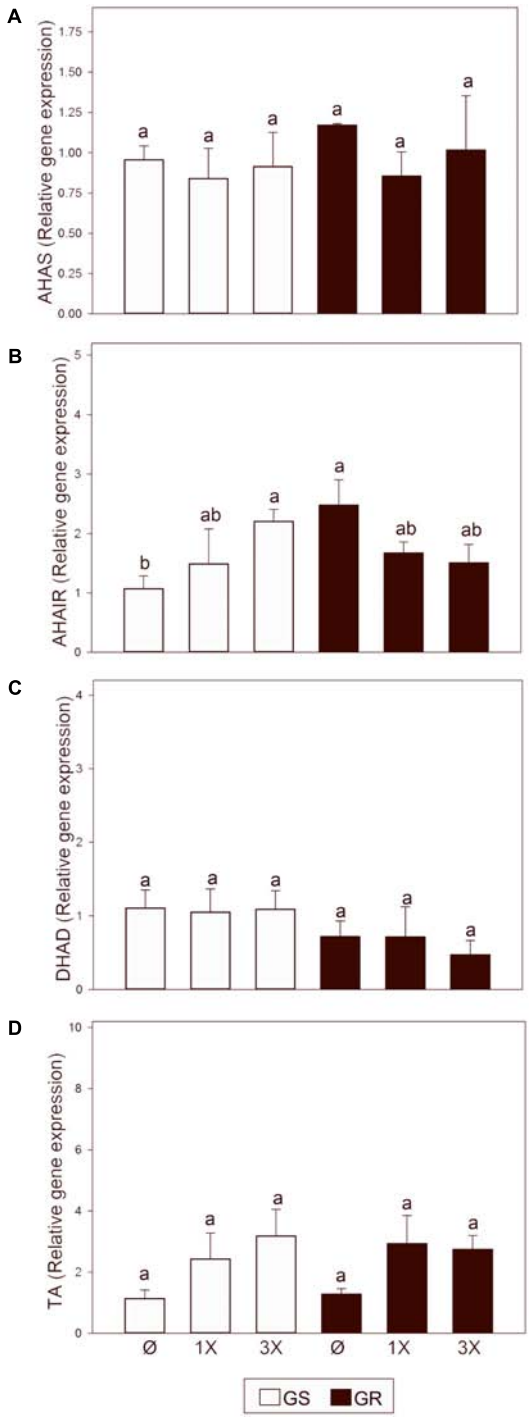

FIGURE 6 | Transcript abundance of genes in the branched chain amino acid (BCAA) biosynthetic pathway. Glyphosate sensitive (white bars; GS) and glyphosate resistant (black bars; GR) populations were untreated (Ø) or 3 days after treatment with glyphosate at one (1X) or three times (3X) field dose. Relative expression of acetohydroxyacid synthase (AHAS; **A**) ketol-acid reductoisomerase (AHAIR; **B**), dihydroxyacid dehydratase (DHAD; **C**) and branched-chain amino acid transaminase (TA; **D**) normalized with the normalization gene *beta tubulin*, and relative to untreated GS plants (Mean ± SE; $n = 5$). Different letters indicate significant differences between treatments and/or populations (p-value ≤ 0.05, LSD test).

populations with increased EPSPS expression would have altered *DAHPS* regulation. Higher levels of DAHPS activity were described in GR populations compared to sensitive populations in *Nicotiana tabacum L.* (Dyer et al., 1988) and *Convolvulus arvensis* (Westwood and Weller, 1997). In *Lolium rigidum GR* populations with higher *EPSPS* expression, levels of *DAHPS* transcripts were similar to sensitive population (Baerson et al., 2002). In this study, while *DAHPS* mRNA relative expression was similar in both populations (**Figure 3B**), the DAHPS protein level in GR was more than twofold higher than in GS (Supplementary Figures 1A,B). It could implicate a translational regulation (or at least post-transcriptional mechanism) that controls DAHPS, and this may be related to *EPSPS* gene overexpression.

In Sensitive and Resistant Plants Glyphosate Treatment Provokes Increased Transcript Abundance Leading to Synthesis of Chorismate, and after This Regulatory Point, Tryptophan

Our study shows that glyphosate treatment provoked an accumulation of the transcripts encoding virtually all the enzymes of the shikimate pathway, including *EPSPS*, in a dose-dependent manner (**Figure 3B**). This trend seems to be specific for enzymes of the AAA pathway and was not observed for the enzymes of the BCAA pathway (**Figure 6**). Although increases in some enzymes of the shikimate pathway such as EPSPS (Baerson et al., 2002; Yuan et al., 2002; Chen et al., 2015; Mao et al., 2016) and DAHPS (Baerson et al., 2002) have been previously described, this is the first report suggesting a potential coordinated transcriptional regulation of the shikimate pathway after glyphosate treatment. Because this regulation is observed in both GS and GR populations (**Figure 3B**), it suggests that this gene upregulation does not occur in response to the level of inhibition of EPSPS activity. Instead, it can be hypothesized that glyphosate itself, or indirectly, may affect plant amino acid metabolism, in addition to its known impact on EPSPS. Future research is needed to determine if glyphosate has unreported effects on plants and what signal causes this general gene induction of the pre-chorismate pathway.

This general upregulation of the expression of genes participating in the pre-chorismate pathway is accompanied with an increase of the accumulation of free AAAs, which is more pronounced in the GS population (**Figure 5**). Although already reported (Vivancos et al., 2011; Maroli et al., 2015; Fernández-Escalada et al., 2016), this might appear counter-intuitive at first glance because glyphosate is inhibiting the entry of carbon in this biosynthetic pathway, and therefore is expected to prevent synthesis of AAA. It is possible that the accumulation of free AAA comes from an increase in protein turnover in the plant following glyphosate treatment (Zabalza et al., 2006; Zulet et al., 2013a; Fernández-Escalada et al., 2016). Isotopic studies in *A. palmeri* revealed that both *de novo* synthesis of amino acids and protein turnover contribute to AAA accumulation in response to glyphosate (Maroli et al., 2016). While gene expression induction after glyphosate was similar in GR and GS populations (**Figure 3B**), the accumulation of AAA was mainly observed in GS plants (**Figure 5**). That observation may suggest that AAA accumulation following glyphosate treatment is rather related to the level of stress experienced by the plant.

After chorismate, *AS* increase in transcript abundance was higher than any other enzyme in the pathway in response to glyphosate treatment (**Figure 3B**). *AS* expression was induced while *CM* expression was repressed, suggesting a regulatory branch point in the pathway (**Figure 1**) for a preferential flux of carbon toward Trp biosynthesis over Phe and Tyr biosynthesis. This potential stream toward Trp was confirmed by studying AS and CM enzyme activities *in vitro* (**Figure 4**). Data obtained in *Arabidopsis thaliana* (Sasaki-Sekimoto et al., 2005) and other plant species (Galili et al., 2016) also support this hypothesis. However, measurements of free AAA in treated plants did not reveal any specific accumulation of Trp. Instead all three AAA were accumulated to a similar extent in GS plants (**Figure 5**). Yet, a slight difference was detected in the GR plants, which may suggest that under "mild" stress (3x dose in GR), synthesis of Trp is prioritized over the synthesis of Phe and Tyr. It is possible that this regulation is related to the inhibition of DAHPS by arogenate (Siehl, 1997), an intermediate product of the CM pathway. DAHPS may be key to the regulation of shikimate synthesis because it represents the entry point in this pathway (Maeda and Dudareva, 2012). Interestingly, *DAHPS* gene expression was induced by glyphosate in both populations (**Figure 3B**) while the increase in DAHPS protein was only detected in GS population (Supplementary Figures 1A,B). This might indicate that other layers of regulation (post-transcriptional) might fine-tune the regulation of this pathway. PAL protein level and enzyme activity have also been studied, because it represents the most important output from the AAA pathway (Hahlbrock and Scheel, 1989). No differences were found between populations for PAL protein abundance or activity level in untreated and treated plants (Supplementary Figures 1C–E). While other studies with other species show important effects of glyphosate on PAL (Hoagland et al., 1979; Zabalza et al., 2017), our results show that PAL abundance and enzyme activity are not affected in *A. palmeri*.

The results obtained after glyphosate treatment suggest that a stress-induced response to glyphosate increases the enzyme expression in the AAA pathway, which may require a substantial increase in energy consumption (Benevenuto et al., 2017). Trying to increase the carbon flux, which could further increase shikimate accumulation upon glyphosate treatment, could lead to the loss of feedback control in the pathway (Marchiosi et al., 2009). Reduction in AAA levels does not appear to elicit the increased expression of AAA pathway genes, because the AAA concentrations increase with glyphosate dose. Further research is needed to understand the signal(s) that upregulates the AAA pathway following glyphosate treatment.

No Cross Regulation between AAA and BCAA Pathway Was Detected

In general, the free amino acid pool increases after glyphosate treatment (Orcaray et al., 2010; Vivancos et al., 2011; Zulet et al., 2013a, 2015; Liu et al., 2015) but the higher relative increase

is in BCAA levels (Orcaray et al., 2010). The higher effect of glyphosate on BCAA than on other amino acid types suggests a possible effect of the herbicide on the BCAA biosynthetic pathway. The expression pattern of the BCAA biosynthetic pathway was measured (**Figure 6**) and no clear patterns for expression changes of the BCAA enzymes in plants treated with glyphosate were identified (**Figure 6**), while an induction of expression of AAA enzymes was detected (**Figure 3B**). Although some authors (Guyer et al., 1995; Noctor et al., 2002; Pratelli and Pilot, 2014) have proposed cross-regulation between the levels of AAA and BCAA, and close correlation was observed between the AAA pathway and the BCAA pathway (Noctor et al., 2002), no cross-regulation at the transcriptional level was found in this study.

CONCLUSION

No differences were found (other than *EPSPS*) in transcriptional regulation of the shikimate pathway between *A. palmeri* GR and GS untreated plant, which implies that pleiotropic effects due to shikimate pathway perturbation are not apparent. Transcriptional induction of the AAA pathway was detected following glyphosate treatment in both GR and GS plants, suggesting a potential coordinated transcriptional regulation. AAA content was not the signal causing this response, because AAA accumulation was detected only in GS plants and further research will be needed to determine the signal. Glyphosate treatment resulted in an upregulation of the Trp biosynthesis branch instead of the Phe and Tyr branch, indicating that this branch point may be a regulatory point in the pathway. With respect to cross-regulation between the AAA and BCAA pathways, no differences in BCAA transcriptional regulation were found due to either EPSPS gene amplification or to glyphosate treatment.

AUTHOR CONTRIBUTIONS

MR and AZ conceived and designed the experiments, performed by MF-E; KR and MG-M contributed with analysis tools; MF-E, AZ-G, and MG-M prepared figures; MF-E, AZ, KR, TG, and MR analyzed, discussed results and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.01970/full#supplementary-material

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A New Ala-122-Asn Amino Acid Change Confers Decreased Fitness to ALS-Resistant *Echinochloa crus-galli*

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Gene mutations conferring herbicide resistance may cause pleiotropic effects on plant fitness. Knowledge of these effects is important for managing the evolution of herbicide-resistant weeds. An *Echinochloa crus-galli* population resistant to acetolactate synthase (ALS) herbicides was collected in a maize field in north-eastern Italy and the cross-resistance pattern, resistance mechanism and fitness costs associated to mutant-resistant plants under field conditions in the presence or absence of intra-specific competition were determined. The study reports for the first time the Ala-122-Asn amino-acid change in the ALS gene that confers high levels of cross-resistance to all ALS inhibitors tested. Results of 3-year growth analysis showed that mutant resistant *E. crus-galli* plants had a delayed development in comparison with susceptible plants and this was registered in both competitive (3, 7, and 20 plants m^{-2}) and non-competitive (spaced plants) situations. The number of panicles produced by resistant plants was also lower (about 40% fewer panicles) than susceptible plants under no-intraspecific competition. Instead, with the increasing competition level, the difference in panicle production at harvest time decreased until it became negligible at 20 plants m^{-2}. Evaluation of total dry biomass as well as biomass allocation in vegetative parts did not highlight any difference between resistant and susceptible plants. Instead, panicle dry weight was higher in susceptible plants indicating that they allocated more biomass than resistant ones to the reproductive organs, especially in no-competition and in competition situations at lower plant densities. The different fitness between resistant and susceptible phenotypes suggests that keeping the infestation density as low as possible can increase the reproduction success of the susceptible phenotype and therefore contribute to lowering the ratio between resistant and susceptible alleles. If adequately embedded in a medium or long-term integrated weed management strategy, the presence of R plants with a fitness penalty provides an opportunity to minimize or reverse herbicide resistance evolution through the implementation of integrated weed management, i.e., all possible control tools available.

Keywords: barnyardgrass, phenology, biomass allocation, target-site resistance, Ala-122-Asn mutation, seed production, herbicide resistance management

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INTRODUCTION

Weed resistance to herbicides is a severe threat to the sustainability of intensive cropping systems. Herbicides applied to large weed populations impose a strong selection pressure that has led to the evolution of many resistant populations worldwide (Heap, 2017). Weeds can withstand herbicide effects because of the presence of mutated resistant alleles (Powles and Yu, 2010). Target site resistance mechanism (TSR) is determined by mutations causing structural changes at the herbicide binding site, therefore limiting the herbicide impact. Instead non-target site resistance (NTSR) includes all mechanisms able to reduce the quantity of herbicide that reaches the target-site. These mechanisms can be determined by modifications at the active site of a metabolic enzyme or at a transporter protein that enhances these proteins' activity in herbicide degradation or compartmentation/sequestration away from its site of action (Yuan et al., 2007; Powles and Yu, 2010).

Theory predicts that any mutation endowing herbicide resistance may be associated with negative pleiotropic effects on fitness, also known as adaptation cost (Purrington, 2000; Vila-Aiub et al., 2009) and defined as the reduction of plant fitness in a herbicide-free environment caused by negative pleiotropic effects of resistance alleles (Vila-Aiub et al., 2015).

Plant relative fitness, which according to Primack and Kang (1989) is defined as the average success in producing offspring contributing to the next generation by a particular phenotype relative to another phenotype (i.e., resistant vs. susceptible in the case of resistance costs), plays an important role in evolutionary terms. Fitness is a phenotypic response that depends on the evolved life-history traits, and therefore, it is greatly affected by environmental conditions and genetic variation (Primack and Kang, 1989). In particular, resistance costs expression has been shown to depend on several factors: resistance mechanism involved (Vila-Aiub et al., 2005a), specific mutant resistance allele (Menchari et al., 2008), dominance of the resistance cost (Roux et al., 2004), pleiotropic effects on the kinetics of herbicide target proteins (Yu et al., 2010), genetic background (Paris et al., 2008), and environmental conditions (Gassmann, 2005).

The resistance cost is estimated by determining the difference in fitness between a herbicide-resistant (R) and herbicide-susceptible (S) genotype. In this context, it is important that individuals share a similar genetic background, except for the alleles endowing herbicide resistance (Vila-Aiub et al., 2009). In this way, the genetic variability between R and S genotypes is limited and only fitness costs associated to those genes that endow resistance are assessed (Vila-Aiub et al., 2011).

A better understanding of the cost/benefit trade-offs of resistant and susceptible alleles is crucial for predicting the herbicide resistance evolution, and more generally the dynamics of weed populations (Menalled et al., 2016). In the last three decades, the widespread and rapid evolution of herbicide resistance produces evidence that resistance mutations confer large fitness benefits in the presence of herbicides, however, several studies suggest that resistance costs when herbicides are not applied range from moderate to relatively small (Vila-Aiub et al., 2009). In an agricultural environment, where herbicides may be rotated or mixed and/or used less frequently, resistance will evolve where the fitness advantage in the presence of certain herbicides (resistance benefit) is greater than the resistance cost (Neve et al., 2014). In other words, resistance costs will affect resistance evolution in environments when the selecting agent(s), and possibly other actives with the same site of action, are less or no longer used. From a management perspective, a better insight into the life history trade-offs associated with resistance may have practical implications in devising strategies which manipulate fitness costs that can result in selection against resistance alleles.

Echinochloa crus-galli (L.) P. Beauv. (barnyardgrass) is a polyploid, therophyte, predominantly self-pollinating, summer annual weed that grows well in organic soils. It has a C_4 photosynthetic cycle that contributes to making it a very successful competitor and one of the most problematic weeds in maize, rice, and other summer crops. It is a prolific seed producer, which often results in considerable soil seed banks (Holm et al., 1977). A healthy plant can yield up to more than 400,000 seeds (Norris, 1992), making it very problematic for crop production if not adequately controlled. *E. crus-galli* is ranked second globally as a weed that evolved resistance to numerous herbicides with different sites of action (Heap, 2017). Since 2000, resistance to herbicides inhibiting acetolactate synthase (ALS; also referred to as acetohydroxyacid synthase, AHAS; EC 2.2.1.6), a key enzyme in the synthesis of branched chain amino acids, has been reported in several European countries, i.e., Italy, Spain, Austria, France, and Germany (Heap, 2017).

Acetolactate synthase inhibitors are the herbicides most prone to select for resistance in weeds (Beckie and Tardif, 2012; Yu and Powles, 2014). In many cases, evolved resistance is target-site-based, caused by amino acid substitutions in one of the conserved regions of the ALS target enzyme. Over the past 27 years, 29 resistance-endowing amino acid substitutions at eight positions of the AHAS gene in 64 weed species have been identified (reviewed by Tranel et al., 2017).

Resistance costs associated with ALS resistance have been studied in a limited number of cases and the costs often were incorrectly determined because of inappropriate plant material, lack of identification of the resistance mechanism involved or inappropriate methodology to measure fitness (Vila-Aiub et al., 2015). Fitness consequences have been assessed for the Pro-197-His substitution in *Lactuca serriola* L., where a reduction in vegetative biomass of resistant compared with susceptible plants growing under competitive conditions has been demonstrated (Alcocer-Ruthling et al., 1992). Substantial pleiotropic effects on plant morphology and anatomy, causing a fitness cost, have been described in field evolved ALS-resistant *Amaranthus powellii* S. Watson with the Trp-574-Leu ALS mutation (Tardif et al., 2006). More recent studies, where ALS resistance alleles were identified and genetic background of resistant and susceptible plants was adequately considered, have assessed the subtle impact of ALS resistance on plant growth and fitness. In homozygous *Lolium rigidum* Gaudin plants with an ALS allele endowing resistance (Ala-197, Arg-197, Glu-197, Ser-197, or Leu-574), no pleiotropic effects on vegetative growth were found (Yu et al., 2010). Similarly, it was demonstrated that the ALS alleles Tyr-122,

Ser-197, Glu-376, and Leu-574 do not cause negative pleiotropic effects on *Raphanus raphanistrum* L. vegetative growth (Li et al., 2013). All these findings highlight that the impact of resistance-endowing ALS gene mutations on plant fitness is dependent on weed species and mutant resistant ALS allele considered. Therefore, generalization is impossible.

In this research the so-called 'single-population' approach (Vila-Aiub et al., 2005b) was used: two seed stocks, S and R, were preliminarily selected from plants harvested in the same site highly infested by *E. crus-galli* resistant to ALS inhibitors. The aims of the research were: (1) to confirm the resistance to ALS-inhibiting herbicides and determine the cross-resistance pattern of the R sub-population; (2) to determine the resistance mechanism(s) involved; (3) to assess the fitness costs associated to mutant ALS-resistant *E. crus-galli* plants under field conditions, in the presence or absence of intra-specific competition.

MATERIALS AND METHODS

Plant Material

Experimental Maize Field

The experimental field site was located in Cona (Venice province, Veneto region, NE Italy, N 45° 09' 27.2", E 12° 09' 12.7", −1.6 m below sea level) where continuous silage maize had been cultivated since the end of the 1950s. No sprinkler irrigation is used in the area and soil moisture is maintained by keeping a high level of water in the ditches. The soil is highly organic (around 27% C), not allowing the application of pre-emergence herbicides and so chemical weed control is only done in post-emergence, mainly with ALS inhibitors. Since 2002 the control of *E. crus-galli* by ALS inhibitors has become progressively unsatisfactory and in 2005, seeds were collected from about 40 plants that survived the sulfonylurea treatments (i.e., two treatments with nicosulfuron 60 g a.i. ha^{-1}) (population 05-31).

Greenhouse bioassay conducted by the Italian Herbicide Resistance Working Group (GIRE, 2017) confirmed that population 05-31 was highly resistant to the ALS inhibitor nicosulfuron, while it was controlled by all other herbicides with a different site of action (i.e., terbuthylazine+mesotrione, fluazifop and *S*-metolachlor). A population is defined as highly resistant to a herbicide when plant survival is >20% at the recommended field dose (1x) as well as >10% at dose 3x (Panozzo et al., 2015). For population 05-31, plant survival of nicosulfuron was not dose-dependent (69% ± 2.4 at dose 1x and 73% ± 15.8 at dose 3x) and the population was ascribed as highly resistant.

Selection of Controlled Genetic Background Sub-populations

In 2011, 100 *E. crus-galli* seedlings at two to three leaf stages were harvested in the field, transplanted into pots and grown to the stage of three to four tillers in the greenhouse. Two vegetative clones from each individual plant were then produced: two tillers with intact roots were excised from each parent plant, trimmed to 2 cm of shoot material and individually repotted. Five days later, one clone for each parent plant was sprayed with nicosulfuron (60 g a.i. ha^{-1}). Four weeks after treatment (WAT) resistant (R) and susceptible (S) plants were assessed and the phenotype of the respective untreated clone was identified. Then, 30 untreated R and S plants were placed in the greenhouse in separate compartments. Even if *E. crus-galli* is predominantly a self-pollinated species, S plants were kept separated from the R ones to avoid any possible cross pollination. Mature seeds were collected from senescent plants, cleaned and stored at 20°C. The bulk of S and R plants (hereafter called simply sub-populations S and R), were used to determine the cross-resistance pattern, the resistance mechanism(s) involved and ultimately for the in-field fitness experiments.

Resistance Status and Resistance Mechanisms Involved

Dose–Response Pot Experiment

An outdoor dose–response experiment was conducted to determine the level of resistance of the S and R sub-populations of *E. crus-galli* to nicosulfuron (sulfonylurea), penoxsulam (triazolopyrimidine), bispyribac-Na (pyrimidinylthiobenzoate), and imazamox (imidazolinone), four ALS herbicides belonging to different chemical families (**Table 1**). Seeds were chemically scarified in concentrated sulfuric acid (96%) for 20 min, rinsed with water and sown in plastic boxes (10 cm × 10 cm × 6 cm) containing peat. Boxes were then placed in a germination cabinet for 6 days at 15/25°C (night/day) and 12 h photoperiod with neon tubes providing a Photosynthetic Photon Flux Density (PPFD) of 15–30 $\mu mol\ m^{-2}\ s^{-1}$. Twenty seedlings, at one-leaf stage (BBCH 11 – Hess et al., 1997), were transplanted into plastic trays (325 mm × 265 mm 95 mm) with a standard potting mix (60% silty loam soil, 15% sand, 15% perlite, and 10% peat). The trays were placed in a greenhouse where temperature ranged from 15 to 19°C and from 26 to 33°C night/day, respectively, and watered daily to maintain the substrate at or near field capacity. When plants reached the two to three leaf stages (BBCH 12-13), herbicides were applied as commercial formulations (**Table 1**), with recommended surfactants, using a precision bench sprayer delivering 300 L ha^{-1}, at a pressure of 215 kPa and a speed of 0.75 m s^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (TeeJet®, 11002). Each sub-population was treated with seven doses of each herbicide calculated following a geometric progression and ranging from 1/16x to 2x (plus the dose 2/3x) and from 1/4x to 16x for S and R sub-populations, respectively. The experimental design was a complete randomized block with three replicates (i.e., one tray per replicate) per herbicide dose.

Plant survival and shoot fresh weight per tray were recorded four WAT and expressed as percentage of the untreated control. Dead plants or plants with dead leaves and no regrowth from the basal part of the plant were classified as susceptible. Plant survival and fresh weight was expressed as percentage of untreated control and standard error was calculated per each mean value.

Detection of Mutation in ALS Gene

To check for mutation(s) endowing herbicide resistance, 10 plants from sub-population S and 10 plants from sub-population R were analyzed. Total RNA was extracted from 100 mg of young leaf tissue (one leaf per plant) from

TABLE 1 | Details of herbicides used in the dose–response experiment.

Active ingredient (a.i.)	Commercial product	Company	Concentration (g a.i. L^{-1})	Field dose (1x) (L ha^{-1})
Nicosulfuron	Ghibli	Syngenta	40	1.5
Penoxsulam	Viper	Dow Agrosciences	22.5	2.7
Bispyribac-Na	Nominee*	Bayer CropScience	408	0.075
Imazamox	Altorex**	BASF	40	1

** Applied with Biopower 1 L ha^{-1}. ** Applied with Dash 0.5%.*

plants that survived a treatment with nicosulfuron at dose 60 g a.i. ha^{-1} using the Invitrap® spin plant RNA mini kit (Stratec Biomedical AG, Germany). Nucleic acid concentration was measured using a NanoDrop 2000c Spectrophotometer (NanoDrop Products, United States). cDNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega, United States) as follows: 1 μg of target RNA and 0.5 μg of oligo dT_{15} were mixed with nuclease free water to a final volume of 5 μL; samples were incubated for 5 min at 70°C and then quick-chilled for 5 min on ice; after this denaturation step, the reaction mix (2.25 mM $MgCl_2$, 0.5 mM dNTP mix, 1 μL of Improm-II™ Reverse Transcriptase, 1x concentration of supplied buffer and nuclease free water to a final volume of 15 μL) was added to each sample and samples were then placed in a T1 plus Thermocycler 96 (Biometra, Germany) at 25°C for 5 min, 1 h at 42°C, and 10 min at 70°C.

The pair of primers ECH-ALS-F (5′- TCGCAAGGGCGC GGACATCCTCGT -3′) and ECH-3R (5′- TCCTGCCATCAC CHTCCAKGA -3′) were used to amplify the full ALS gene sequence including all known conserved domains (Panozzo et al., 2013). PCR amplification was conducted using the Advantage® 2 PCR kit (Clontech) in a 50 μL mixture of 1x Advantage® 2 SA PCR Buffer, 1x dNTP mix, 0.2 μM of each primer, 5% DMSO, 1x Advantage® 2 Polymerase Mix and 100 ng cDNA. Amplification was conducted using the following program: 1 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 60°C, and 120 s at 68°C; 3 min at 68°C. PCR products were purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co., Germany), sequenced by BMR Genomics (Padova, Italy) and edited with FinchTV 1.4.0.

Once the full ALS length had been sequenced and mutation endowing resistance identified, only a 500 bp amplicon starting from the 5′-end part of the ALS gene was amplified for all R and S plants in order to individuate the amino-acid at position 122 of the ALS gene (position referred to the ALS sequence of *Arabidopsis thaliana*). The PCR amplification was conducted as described above with primer ECH-ALS-F and the reverse primer ECH-5RACE-1 (5′-GCCGCGACTCACCAACAAGA-3′) and with the following Thermocycler program: 1 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 58°C, and 40 s at 68°C; 3 min at 68°C.

ALS Enzyme *in Vitro* Bioassay

ALS enzyme bioassay was performed to measure the activity of the enzyme exposed to increasing doses of herbicides in the two sub-populations S and R.

Proteins were extracted from seedlings fresh leaf tissue as described by Wright et al. (1998) with slight modifications. Five grams of the youngest leaves, pooled from 40 to 50 plants, were homogenized with liquid nitrogen in a mortar and suspended in 35 mL of ice-cold homogenization buffer [100 mM potassium phosphate buffer (pH 7.2), 5 mM Na-pyruvate, 5 mM $MgCl_2$, 10 μM flavin adenine dinucleotide (FAD), 1 mM thiamine pyrophosphate (TPP), 1 mM dithiothreitol (DTT), 10% (V/V) glycerol] and 1% (wt/wt) polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through four layers of cheesecloth, and then centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant was decanted to a new centrifuge tube and an appropriate quantity of ammonium sulfate was added to create a 45% (wt/V) solution. The mixture was stirred gently for 1 h on ice, and then centrifuged at 20,000 × *g* for 20 min at 4°C. The resultant pellet was dissolved in 3 mL of re-suspension buffer [50 mM potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 1 mM TPP, and 10 μM FAD]. The resulting solution was desalted using Disposable PD-10 Desalting Columns (GE Healthcare). Protein concentration of the extract was determined using a NanoDrop 2000c Spectrophotometer.

ALS enzyme assays were performed as described by Schmitzer et al. (1993). Enzyme activity was determined based on the amount of acetoin formed from acetolactate using the method of Westerfeld (1945). Each reaction contained 50 μL of protein extract (2.0 μg μL^{-1}), 50 μL of the standard reaction buffer [50 mM potassium phosphate buffer (pH 7.0), 100 mM Na-pyruvate, 5 mM $MgCl_2$, 1 mM TPP, 10 μM FAD] and 50 μL of various concentrations of the technical grade of imazamox and nicosulfuron. A positive control without herbicide and a blank with sulfuric acid (see below) were included. The two sub-populations were assayed with two ALS inhibitors, the selecting agent nicosulfuron and imazamox, the ALS inhibitor that had given the highest RI in the whole-plant pot bioassay. The experiment was repeated twice, with three replicates per herbicide concentration. Herbicide concentrations ranged from 10^{-3} to 10^6 nM in 10-fold increments. The mixtures were incubated at 37°C for 90 min. The reactions were stopped by adding 25 μL of 3.5% (V/V) sulfuric acid, and then incubated at 60°C for 20 min. The amount of acetoin formed was determined by incubating the mixture with 150 μL of 0.55% (wt/V) creatine, 5.5% (wt/V) α-naphthol, and 1.375 N NaOH at 37°C for 40 min. Acetoin concentration was measured by spectrophotometry at a wavelength of 530 nm.

The R/S ratio was obtained as the ratio between the I_{50} of sub-populations R and S.

TABLE 2 | Parameters of the log-logistic equations for whole plant dose–response of sub-population S and R, for the four herbicides tested; LD_{50} and GR_{50} are the herbicide doses causing 50% reduction in plant survival and fresh weight, respectively; standard errors are given in brackets.

Herbicide	Sub-population	*b* (slope)	LD_{50} (g a.i. ha^{-1})	Residual sum of squares	RI[a]
Survival					
Nicosulfuron	S	4.7 (0.4)	20 (0.6)	505	
	R	21 (1.9)	383 (30)	306	19
Penoxsulam	S	3.6 (0.9)	12 (1.0)	4,459	
	R	–	>653	–	>56
Bispyribac-Na	S	6.5 (0.9)	5.6 (0.3)	731	
	R	–	>490	–	>42
Imazamox	S	4.4 (0.8)	7.5 (0.4)	1671	
	R	–	>640	–	>85
Herbicide	**Sub-population**	***b* (slope)**	**GR_{50} (g a.i. ha^{-1})**	**Residual sum of squares**	**RI[a]**
Fresh weight					
Nicosulfuron	S	6.6 (0.9)	21 (1.1)	871	
	R	5.4 (0.9)	361 (9.3)	361	17
Penoxsulam	S	2.9 (0.7)	5.9 (0.6)	4,150	
	R	1.6 (0.2)	330 (26)	1,022	56
Bispyribac-Na	S	1.6 (0.1)	1.7 (0.1)	307	
	R	1.1 (0.1)	56 (6.6)	2,167	32
Imazamox	S	2.8 (0.4)	4.9 (0.2)	445	
	R	–	>640	–	>132

[a]*RI, resistance indexes calculated as the ratio of LD_{50} R over LD_{50} S or GR_{50} R over GR_{50} S.*

Growth Analysis in Field Experiments in Different Competitive Situations

To detect if a fitness cost was associated with the resistant allele, a 3-year field comparative growth analysis was conducted in north-eastern Italy in 2012 (experiment 1), 2013 (experiment 2), and 2014 (experiment 3) on the farm where the original resistant population 05-31 had been collected. In each experiment *E. crus-galli* plants growth were followed from the beginning of May to mid/end of September (i.e., to maize harvest). R and S plants were grown in virtually non-competitive (hereafter called no-competition) and intraspecific competitive situations: well-spaced (plants were 2 m apart, no-competition) as well as at 3, 7, and 20 plants m^{-2} (competition). In competitive situations, each target plant was surrounded by 12 equally spaced plants of the other phenotype (i.e., the S target plant was surrounded by R plants and vice-versa) (Beruto et al., 1996). The experimental design was a completely randomized block with three replicates (see Supplementary Figure S1).

At the beginning of May, seeds of the two sub-populations S and R were chemically scarified in concentrated sulfuric acid (96%) for 20 min, carefully rinsed and conserved in a solution of KNO_3 at 2% (wt/V) until sowing. Sowing was done in the field by putting a few seeds in equidistant pockets (dibbling method). To make sure that the germinated plants were of the correct phenotype, some sterilized soil was put in a buried plastic tube (10 cm × 10 cm) and 15 seeds were sown inside and watered daily. Then seedlings were thinned to one plant and plastic tubes were removed to allow the plants to grow freely.

Phenology of the two phenotypes was monitored throughout the growing cycle: beginning of flowering was recorded for the target plants in all treatments; panicle numbers were counted weekly and covered with glassine bags (Glassine Bag Co. Global Polythene, Corringham, Essex, United Kingdom) just prior to the onset of seed shattering.

Temperature sum was calculated based on Growing-Degree Day equation (GDD):

$$\mathrm{GDD} = [(T_{\max} - T_{\min})/2] - T_{\mathrm{b}}$$

where $T_{\max}$ and $T_{\min}$ are the daily maximum and minimum temperatures, respectively, and T_{b} is the base temperature, estimated to be 10.5°C for *E. crus-galli* (Sartorato and Pignata, 2008). When $T_{\min}$ was $\leq T_{\mathrm{b}}$, the value of T_{b} was considered. Air temperature and rainfall data were recorded by the nearest ARPAV (Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto[1]) weather station (about 8 km away from the experimental field).

In 2012 and 2013 other three well-spaced plants for each phenotype were grown and panicles of these plants were covered with glassine bags in order to evaluate the total number of seeds produced (both retained and shattered). Seed loss (shattered seeds) was estimated as the average difference between the panicles' weight of the covered plants and those uncovered (i.e., the plants in no-competition situation described above). A correlation between panicles' weight and the number of panicles was also calculated. This correlation allowed the estimation of the panicles weight in 2014 through counting only the number of panicles.

[1]www.arpa.veneto.it

A destructive sampling was done concurrently with maize harvested in a nearby field: target plants were harvested, different plant parts (stem, leaves, and panicles) were separated and oven dried at 105°C for 36 h, and dry weights were then recorded. In the first year, seeds were separated from rachides and mean dry weight of 100 seeds (in three replicates) was also evaluated to (1) estimate the number of seeds produced by each plant in the different conditions, (2) calculate a correlation between panicle weight and seed weight in order to use the former as indication of reproduction effort (RE) of each phenotype in the different growth conditions (i.e., seed production was estimated using panicle dry weight). The intra-specific competitive ability was evaluated as discussed by Ross and Harper (1972). The space available for plants in no-competitive condition was calculated considering a cube of the radius circle of 1 m (as spaced plants were 2 m apart).

Statistical Analyses

The dose–response data and ALS enzyme assay data were analyzed using a non-linear regression analysis based on the log-logistic equation (Seefeldt et al., 1995):

$$Y = C + [(D - C)/[1 + (x/I_{50})^{b}]$$

where Y is plant survival or fresh weight, C and D are the lower and upper asymptotes at high and zero doses, respectively (note: in dose–response data, for biological reasons, and to improve the estimates of the parameters, the upper and lower asymptotes were forced to 100 and 0, respectively), I_{50} is the dose giving the 50% response, b is the slope and x the herbicide rate. For dose–response experiments, doses giving the 50% response, i.e., LD_{50} (based on survival data) and GR_{50} (based on fresh weight data), and relative standard errors, were calculated using the macro BIOASSAY97 developed by Onofri (2005) and running in Windows Excel environment. The resistance index (RI) for the different herbicides was calculated as the ratio between the LD_{50} (GR_{50}) of sub-population R and LD_{50} (GR_{50}) of sub-population S.

Growth analyses data were analyzed using the software STATISTICA 7 (Hilbe, 2007): linear regression was used to calculate the relation between panicle and seed dry weight and between panicle number and panicle dry weight in experiment 1 in order to estimate the panicle dry weight in experiment 3. To test differences in slopes among regression lines the ANCOVA was performed.

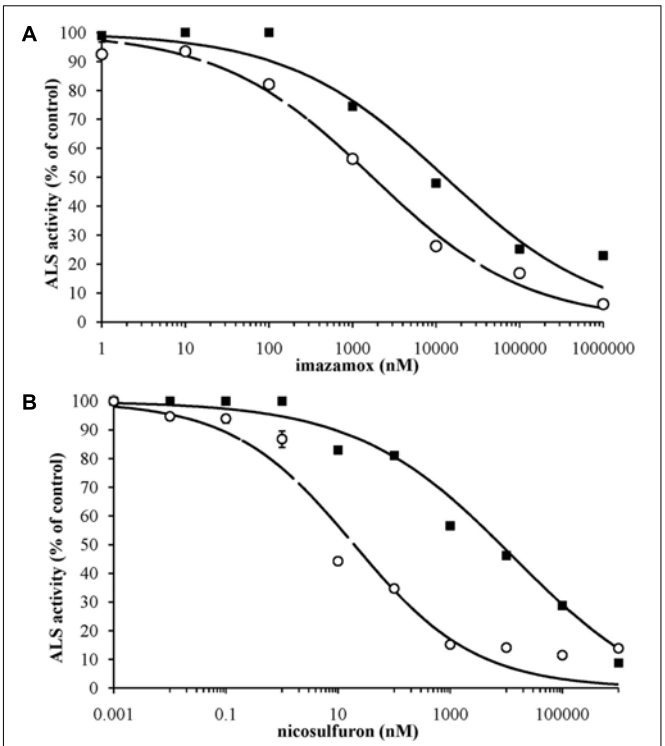

FIGURE 1 | Inhibition curves by imazamox **(A)** and nicosulfuron **(B)** of ALS enzyme extracted from S (– –○– –) and R (–■–) sub-populations. Curves represent the response predicted by non-linear regression; symbols represent percentage of mean ALS activity, based on the untreated controls. Standard errors are reported as vertical bars.

RESULTS

Cross-Resistance Pattern of the *E. crus-galli*

The results from the outdoor dose–response pot experiment confirmed the preliminary test conducted by GIRE on a seed sample collected in 2005 from plants that had survived a herbicide treatment with nicosulfuron on the same farm. Plant survival and fresh weight gave similar results (**Table 2**). It was confirmed that S phenotype was adequately controlled by all ALS inhibitors tested, whereas R phenotype was highly cross-resistant to all ALS inhibitors with high RI calculated considering both plant survival and fresh weight (**Table 2**). Apart from

TABLE 3 | Parameters of the log-logistic equations for ALS enzyme activity of sub-population S and R, for the two herbicides tested; I_{50} is the herbicide dose causing 50% reduction in ALS activity; standard errors are given in brackets.

Herbicide	Sub-population	*b* (slope)	I_{50} (nM)	Residual sum of squares	R/S ratio[a]
Nicosulfuron	S	0.4 (0.1)	19 (9.4)	555	
	R	0.3 (0.1)	7,980 (930)	146	413
Imazamox	S	0.5 (0.0)	1,735 (274)	68	
	R	0.5 (0.1)	12,830 (4,075)	263	7

[a]*R/S ratio, resistance indexes calculated as the ratio of I_{50} R over I_{50} S.*

TABLE 4 | Beginning of flowering of the S and R target plants in no-intraspecific competition and competition (3, 7, and 20 plants m^{-2}) in the three experiments.

		No competition	Competition (plants m^{-2})		
			3	7	20
2012	S	10-July	12-July	12-July	25-July
	R	17-July	20-July	30-July	30-July
	Δ (d)	7	8	8	5
	Δ (°C d)	99	110	252	78
2013	S	5-August	1-August	5-August	1-August
	R	13-August	8-August	13-August	13-August
	Δ (d)	8	7	8	12
	Δ (°C d)	124	123	124	194
2014	S	26-June	29-June	29-June	1-July
	R	29-June	1-July	9-July	9-July
	Δ (d)	3	2	10	8
	Δ (°C d)	36	56	116	97

The difference (Δ) between S and R phenotypes is reported in days (d) and temperature sum (°C d).

nicosulfuron, which controlled more than 90% of R plants when applied at 320 g a.i. ha^{-1} (i.e., 8x), for all other herbicides plant survival was always higher than 50% of the untreated control. Therefore it was impossible to fit the data with the log-logistic equation and LD_{50} values were considered as being higher than the maximum herbicide dose used, e.g., for penoxsulam it was >653 g a.i. ha^{-1} (**Table 2**). For fresh weight, it was not possible to calculate GR_{50} for imazamox: plants were not damaged even at the highest dose applied (640 g a.i. ha^{-1}, i.e., 16x). Instead, GR_{50} were calculated for bispyribac-Na and penoxsulam, indicating that plants were damaged, though alive, starting from dose 1x and 4x, respectively. These two herbicides were also more effective on the S phenotype and plants were already damaged at very low doses, therefore the RI resulted as very high (**Table 2**).

Resistance Mechanisms

Molecular analyses proved that a target-site resistance mechanism is involved. The PCR amplification with ECH-ALS-F and ECH-3R led to obtaining a 1,870 bp sequence including all the known conserved domains identified previously carrying mutations endowing resistance. In all the resistant plants a double nucleotide substitution GC-AA, giving an Ala-Asn change at amino acid position 122 of the ALS gene, was detected. All plants were heterozygous for the mutation at the ALS locus. In the susceptible plants not one known mutation was identified.

The *in vitro* measurements of ALS activity indicated a sigmoidal response to increasing doses of imazamox and nicosulfuron (**Figure 1**) and the data were well fitted by the log-logistic equation (**Table 3**). The results of the *in vitro* bioassay support the presence of a target-site mediated as main resistance mechanism. The R/S ratios calculated were 7.4 for imazamox and 413 for nicosulfuron. The large difference in I_{50} between R (I_{50} = 7980) and S (I_{50} = 19) sub-populations led to the exclusion of non-target-site mediated resistance mechanisms for the selecting agent of the resistant phenotype, i.e., nicosulfuron. This is in keeping with the results obtained from the molecular analyses.

Comparative Growth Analyses

All *E. crus-galli* seedlings used as target plants were analyzed to verify the presence or not of the mutant ALS allele. All resistant plants had the double substitution GC-AA, giving an Ala-122-Asn amino-acid change, whereas none of the susceptible plants had any nucleotide substitution.

The onset of flowering was delayed of about 1 month in 2013 in comparison to 2012 and 2014 (**Table 4**). In all years a later flowering in R plants with respect to S ones was recorded in both competitive and non-competitive situations (**Table 4**). Considering the cumulative number of panicles under no-competition in relation to the temperature sum (**Figure 2**), the same delay of about 1 month was recorded in 2013. It is worth mentioning that the temperature sum at the end of August 2013 was around 1,000°C d, whereas this value was reached at the beginning of August in 2012 and 2014. The production of panicles for R and S plants in 2013 was similar, whereas at harvest R plants produced 55% and 70% less panicles than S plants in 2012 and 2014, respectively (**Figure 2**). Considering the intraspecific competition, the larger differences between R and S plants were recorded in 2013 and 2014 at the lowest density (3 plants m^{-2}) and progressively disappeared at higher plant densities (**Figure 3**). No significant differences were detected in 2012, likely due to the higher variability in panicles produced by S plants.

Evaluation of the dry biomass, in particular the panicle dry weight produced by S and R plants, consolidated the data from the phenological analyses. The highly significant correlation (R^2 = 0.99) between seed dry weight (separated from rachides) and panicle dry weight in the first year of experiment allowed this latter variable to be used to estimate the seed production of S and R plants. In 2012 and 2013, panicle number and panicle dry weight were recorded for R and S plants and a linear regression analysis between these two parameters was obtained. In 2012,

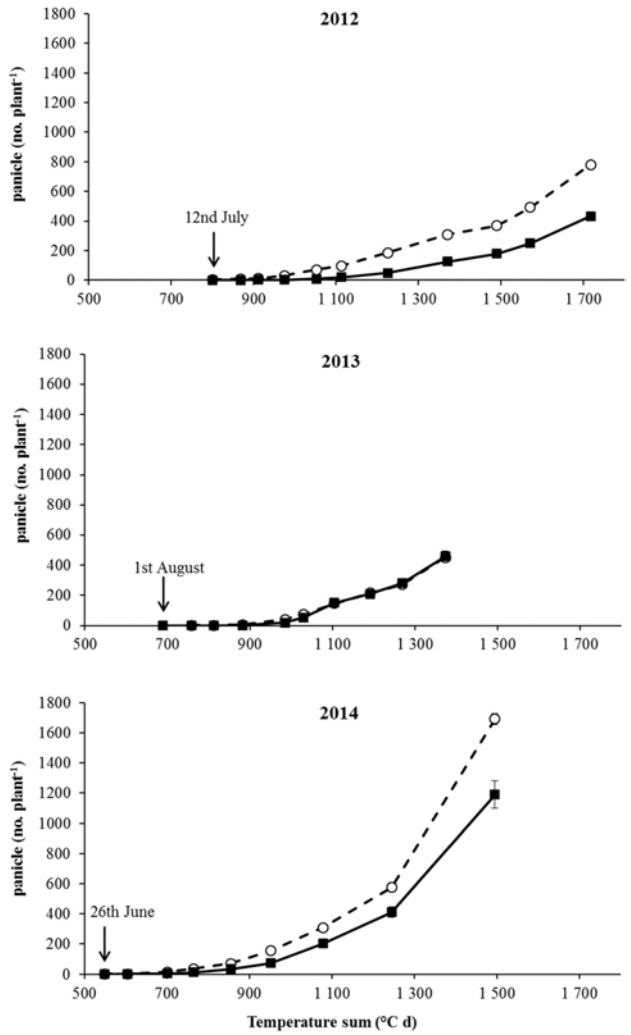

FIGURE 2 | Panicle number under no-intraspecific competition from the beginning of flowering to harvest for S (– –○– –) and R (–■–) plants in the three experiments (2012, 2013, and 2014). The arrows report the dates of the flowering start. Vertical bars represent standard errors.

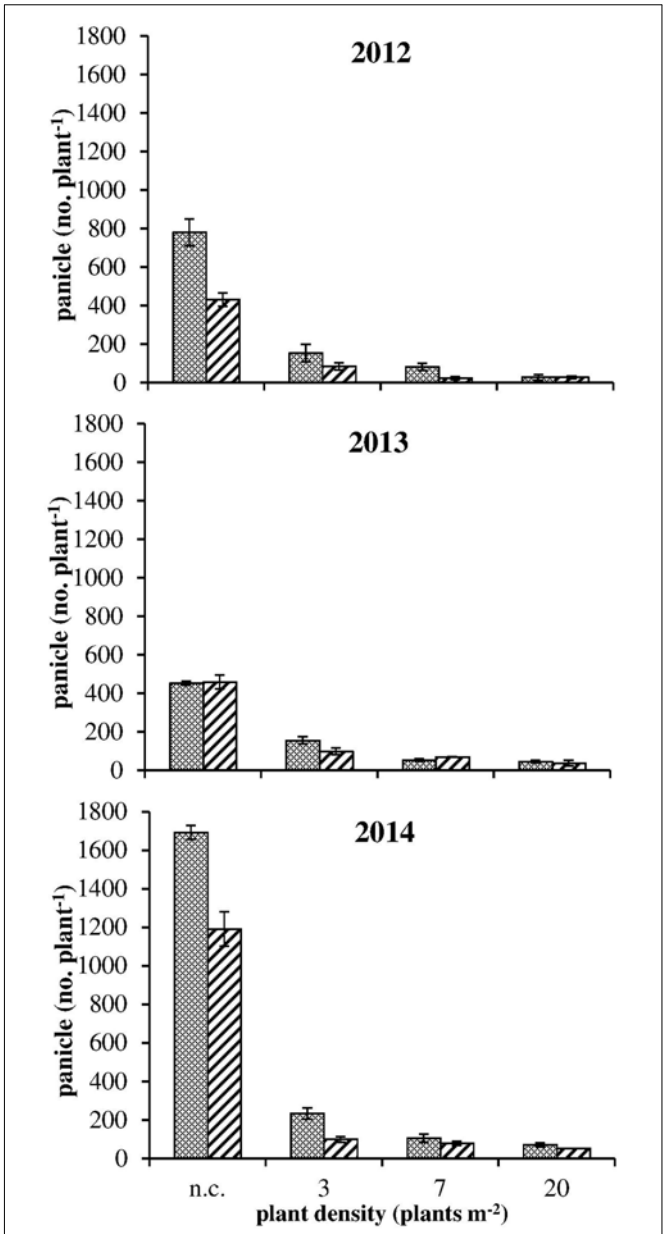

FIGURE 3 | Panicle number at harvest for S (▨) and R (▨) plants in competition (3, 7, and 20 plants m^{-2}) and in no-competition (n.c.) condition in the three experiments. Vertical bars represent standard errors.

the R^2 was 0.98 (linear equation: $y = 0.675x$), whereas in 2013 the R^2 was slightly lower (0.93). Bartlett's test for homogeneity of variance revealed that it was not possible to analyze all data together. Therefore, because of the higher R^2 only 2012 data were used for the estimation of panicle dry weight in the last experiment.

The comparison between different vegetative plant parts (stems and leaves) did not highlight any difference between R and S plants (data not shown). Considering the total dry weight (**Figure 4**, on the right): a difference was detected in only two cases (i.e., 2012 – 7 plants m^{-2} and 2013 – 3 plants m^{-2}). Instead, considering panicle dry weight, S plants allocated more biomass to the reproductive organs than R ones especially in no-competition and at lower plant densities (**Figure 4**, on the left). At the highest plant density (i.e., 20 plants m^{-2}) the competition among plants was high and panicle production very low, giving no differences in reproductive effort between S and R plants. Data from 2012 and 2014 were consistent, whereas in 2013 a slight difference (not significant) in panicle dry weight between S and R plants was detected only in the 3 plants m^{-2} situation (**Figure 4**). Other differences between S and R phenotypes were detected analyzing seed weight in 2012. Seed dry weight of S plants was about 30% higher than in R plants in no-competition situation (**Figure 5A**). Also in this case, the difference decreased enhancing the competition level. However, the mean weights of 100 seeds evaluated for the different situations have a different trend: mean seed weight increased with competition level for

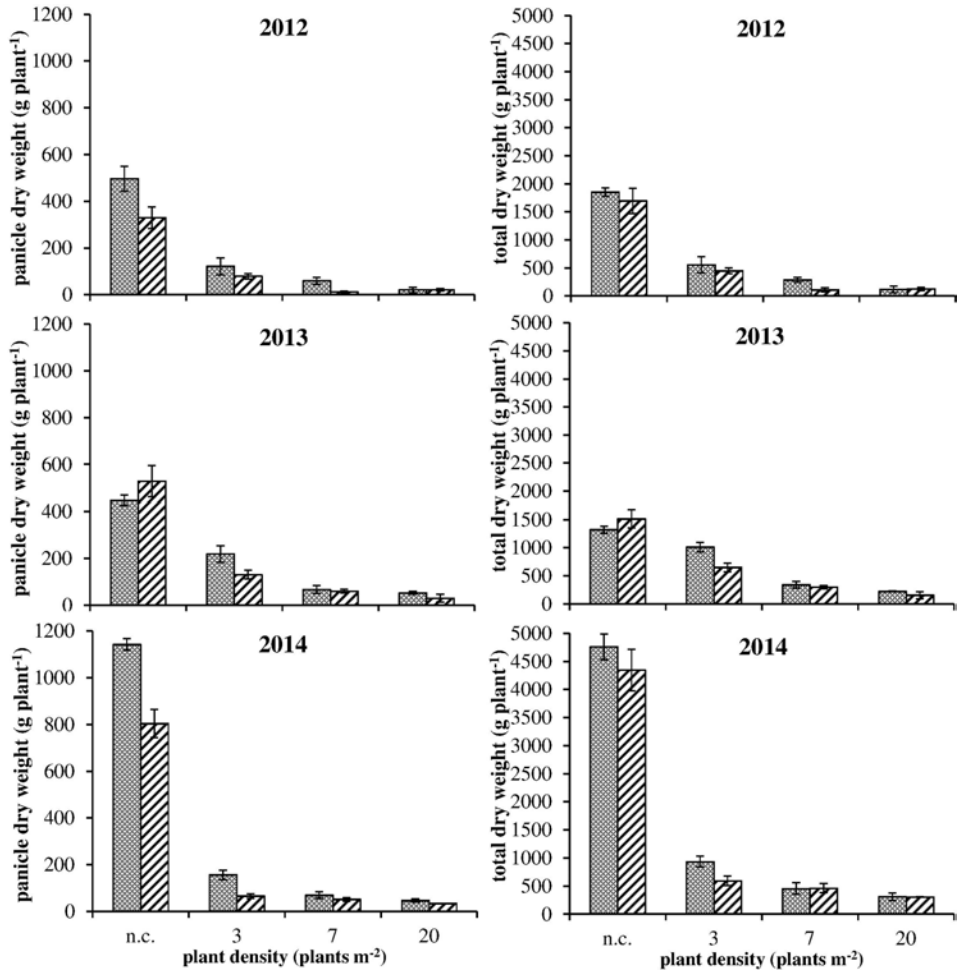

FIGURE 4 | Panicle dry weight (on the left) and total dry weight (on the right) for S (▨) and R (▨) plants under intraspecific competition (3, 7, and 20 plants m^{-2}) and no-competition (n.c.) in the three experiments. Vertical bars represent standard errors.

S plants, whereas it decreased for R ones (**Figure 5B**). In no-competition situation, the mean seed weight of S plants was lower than that of R ones, but S plants produced much more biomass. The resulting estimated number of seeds produced by R and S plants was significantly different (**Figure 5C**), i.e., the lower weight of a single seed in the S plant increases the difference in the number of seeds between S and R plants. The S plant produced 43% more seeds than the R one (**Figure 5C**). This difference disappeared at the highest plant density.

In order to compare the intra-specific competitive ability (i.e., between R and S plants), the relation between total dry weight (or panicle dry weight) and the three-dimensional space that plants have available, expressed as cube of radius of the circle, was calculated (**Figure 6**) (Ross and Harper, 1972). Considering the total dry weight, the slope of the regression lines between S and R plants was not significantly different in both 2012 and 2014 (t-value = 0.719, $P = 0.512$ in 2012; t-value = 1.02, $P = 0.366$ in 2014) (**Figure 6A**). Different behavior is observed for panicle dry weight (**Figure 6B**). The slopes of the regression lines between S and R plants were significantly different in both 2012 and 2014 (t-value 7.410, $P = 0.002$ in 2012; t-value 3.209, $P = 0.03$ in 2014). The more space available for plant growth the bigger the difference is in the biomass allocated to panicles (and therefore to seeds) between R and S plants. This implies that R plants have a lower potential for seed production, in other words they suffer from a fitness penalty in comparison with S plants.

DISCUSSION

ALS Gene Sequencing Revealed a Novel Ala-122-Asn Substitution

This study reports for the first time the Ala-122-Asn amino-acid change in the ALS gene that confers a high level of cross-resistance to ALS inhibitors worldwide. Resistance-endowing mutations at Ala-122 have so far been reported in seven weed species (Tranel et al., 2017). The Ala-122-Thr endowed resistance only to imidazolinones but no resistance to sulfonylureas and pyrimidinylthiobenzoates in *Solanum ptycanthum*, *Amaranthus*

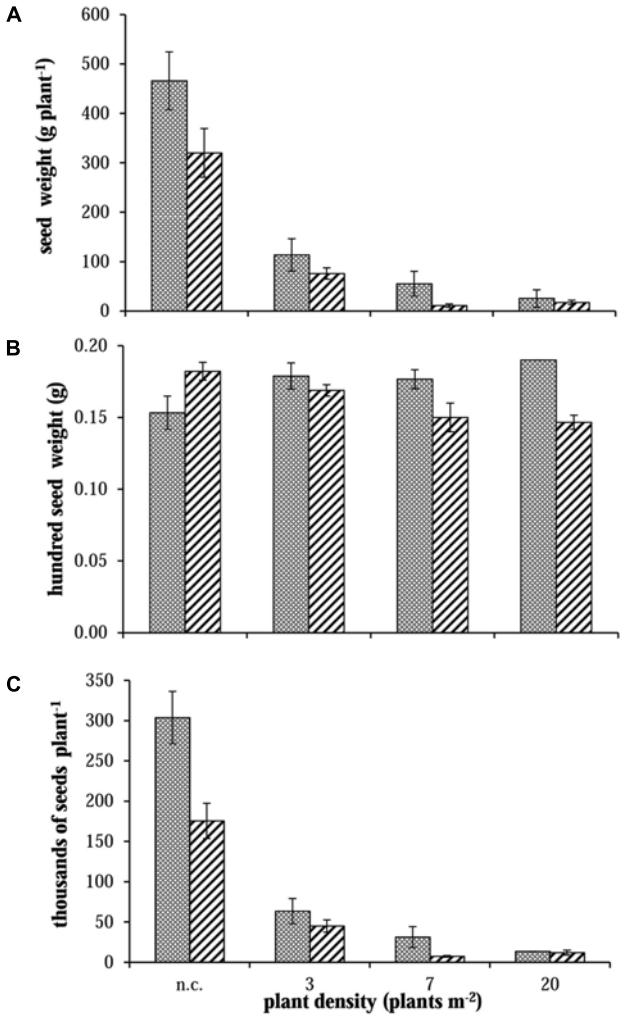

FIGURE 5 | Evaluation of **(A)** seed dry weight, **(B)** mean weight for 100 seeds and **(C)** seed number produced per plant for S (▨) and R (▨) phenotypes in 2012 in no-competition (n.c.) and competition (3, 7, and 20 plants m^{-2}). Vertical bars represent standard errors.

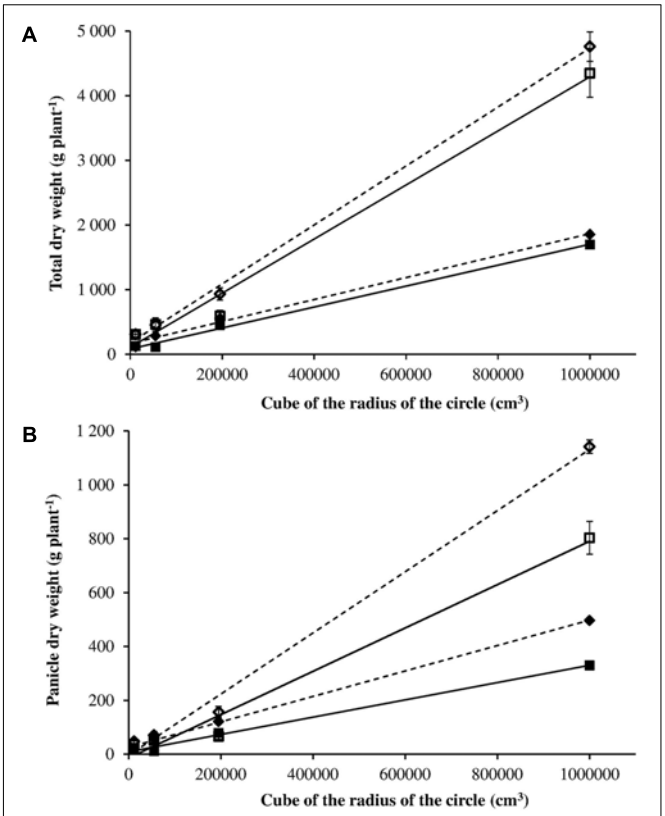

FIGURE 6 | Panicle dry weight **(A)** and total dry weight **(B)** in relation to the three-dimensional space that plants have available expressed as cube of radius of the circle for S (– – –) and R (–) phenotypes in 2012 (◆ ■) and 2014 (◇ □).

retroflexus, *A. powellii*, and *Apera spica-venti* (Tranel et al., 2017). In *E. crus-galli* from Arkansas, this amino acid change conferred resistance not only to imidazolinones but also to triazolopyrimidines (Riar et al., 2013). The Ala-122-Val did not confer a broad resistance to the ALS inhibitors either (Riar et al., 2013; Tranel et al., 2017). Instead, the double point mutation GCT-TAT that determines an Ala-Tyr amino-acid change, detected only in *R. raphanistrum* (Han et al., 2012) conferred high-level and broad-spectrum resistance to ALS-inhibiting herbicides (Han et al., 2012; Tranel et al., 2017). Similarly, in *E. crus-galli* populations analyzed herein, the Ala-122-Asn amino-acid change in the ALS gene confers a broad spectrum resistance. Overall, resistance-endowing mutations at Ala-122 of the ALS gene can determine a different resistance pattern according to weed species and amino acid change. This reinforces the idea that each resistance case needs a specific study.

Evidence of Fitness Costs in Ala-122-Mutated Plants

The resistance costs associated to specific ALS resistance-endowing mutations that have evolved in field weed populations have been examined in a limited number of cases. Here, resistant and susceptible plants of *E. crus-galli* with similar genetic background were selected and the detection of a target-site mediated resistance mechanism involved has allowed fitness costs associated with resistance to ALS herbicides to be estimated and interpreted.

Results of the 3-year growth analysis indicate that a fitness cost is present in plants bearing the Ala-122-Asn substitution. Differences were observed in growth and development: a slower development of R phenotype in relation to the S one was recorded (**Table 3**). Even if during the second year experiment, the date of beginning of flowering was delayed by 1 month with respect to 2012 and 2014, this 1 month delay was recorded throughout the life cycle, from appearance of the first panicle until harvest time (**Table 3**). As previously argued, the results of field experiments can hardly be treated quantitatively because many indeterminate factors, including the weather, may be of great importance (De Wit, 1960). However, our results highlight that the differences between R and S plants were consistent in different years and

climatic conditions. Furthermore, in no-competition condition, R plants produced about 40% fewer panicles than S plants (**Figure 2**) and the high panicle dry weights recorded for S plants indicate that they allocated more biomass to reproductive organs than the R ones. It can be inferred that the lack of differences between R and S in the number of panicles produced in 2013 can be attributed to the different climatic condition. During 2013, there was a rather rainy spring with respect to 2012 and 2014 that delayed seedling emergence and therefore the subsequent phenological growth phases.

Considering the competitiveness of R and S phenotypes, the results indicated that the higher the plant density the less the difference was between R and S phenotypes in terms of number of panicles produced. No differences were recorded at the highest plant density. Hence, at low plant densities the susceptible plants exhibited a potential fitness advantage. The only study of fitness costs on mutation at Ala-122 was conducted on *R. Raphanistrum* having the Ala-122-Tyr mutation and no evidence of pleiotropic effects on plant growth were recorded between R and S plants (Li et al., 2013).

Overall, it seems that fitness costs related to ALS resistance vary according to the species, the mutated allele conferring resistance, the genetic background, experimental conditions (glass-house, field) and environmental conditions involved. This study reveals that fitness costs associated to Ala-122-Asn mutant *E. crus*-galli plants are evident under good growth conditions, but progressively disappear in highly competitive conditions.

Management/Evolution of ALS-Resistant *E. crus-galli*

Experiments conducted under field conditions allow fitness to be compared in a real situation where resistance evolves and has to be managed. This study proves that the evolved resistance to ALS inhibitors in *E. crus-galli* due to the Ala-122-Asn amino-acid change is associated with a fitness cost. However, the impact of this penalty in the absence of ALS inhibitors may be influenced by many factors, including environmental conditions (Gassmann, 2005). Different climatic conditions during 2013 influenced the relative plant growth, reducing the disadvantages of R plants toward the S plants.

The differential population dynamics observed between R and S *E. crus-galli* phenotypes can be a good starting point for devising a resistance management strategy. Differences in life history traits can be manipulated in fields to minimize or reverse the resistance evolution (Vila-Aiub et al., 2011). In our case the lack of a fitness cost at high plant densities suggests that keeping the infestation density as low as possible can increase the reproductive success of the S phenotype and therefore contribute to lowering the ratio between R and S alleles. In a situation where ALS-resistance is well established, a longer-term management based on crop rotation, lack of selection pressure from ALS-inhibiting herbicides and the use of any other available control tool should help to induce some change in the R/S plant ratio.

The different weather conditions encountered in 2013 may suggest other management options. The frequent and relatively abundant rainfall events in spring delayed maize sowing and weed control operations for about 1 month in relation to the normal schedule. As a consequence, the emergence of *E. crus-galli*, usually characterized by two main fluxes of emergences regulated by rainfall and temperature, was instead only one large and longer flux. In this case, the delay in sowing helped the control of weeds.

Another experiment was conducted in the same maize field where the conventional weed control strategy (with ALS inhibitors) adopted by the farm was compared with another one that did not include these herbicides for 4 years. The results showed that the simple measure of ceasing to use the selecting agent does not significantly modify the ratio between R and S plants when the barnyardgrass seed bank initially had more than 95% of "R seeds" (Scarabel et al., unpublished data). It is clear that the long-lived seed bank of *E. crus-galli* (Holm et al., 1977) imposes long-term resistance management strategies when the seed bank contains predominantly "R seeds."

It is widely accepted that herbicides are not silver bullets (e.g., Mortensen et al., 2000) for the management of resistance, but rather diversified control tools should be used as "little hammers" (Barzman et al., 2015). If adequately embedded in a medium or long-term integrated weed management strategy, the presence of R plants with a fitness penalty provides an opportunity to minimize or reverse herbicide resistance evolution.

AUTHOR CONTRIBUTIONS

SP, MS, and LS designed the experiments. SP and VR carried out the field and laboratory experiments. SP, LS, and MS analyzed the data and wrote the manuscript. MS supervised this study. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02042/full#supplementary-material

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No Vegetative and Fecundity Fitness Cost Associated with Acetyl-Coenzyme A Carboxylase Non-target-site Resistance in a Black-Grass (*Alopecurus myosuroides* Huds) Population

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Attention should be devoted to weeds evolving herbicide resistance with non-target-site resistance (NTSR) mechanism due to their unpredictable resistance patterns. Quantification of fitness cost can be used in NTSR management strategies to determine the long-term fate of resistant plants in weed populations. To our knowledge, this is the first report evaluating potential fecundity and vegetative fitness of a NTSR black-grass (*Alopecurus myosuroides* Huds), the most important herbicide resistant weed in Europe, with controlled genetic background. The susceptible (S) and NTSR sub-populations were identified and isolated from a fenoxaprop-*P*-ethyl resistant population by a plant cloning technique. Using a target-neighborhood design, competitive responses of S and NTSR black-grass sub-populations to increasing density of winter wheat were quantified for 2 years in greenhouse and 1 year in field. Fitness traits including potential seed production, vegetative biomass and tiller number of both sub-populations significantly decreased with increasing density of winter wheat. More importantly, no statistically significant differences were found in fitness traits between S and NTSR sub-populations either grown alone (no competition) or in competition with winter wheat. According to the results, the NTSR black-grass is probably to persist in field even in the cessation of fenoxaprop-*P*-ethyl. So, effective herbicide resistant management strategies are strongly suggested to prevent and stop the spread of the NTSR black-grass, otherwise NTSR loci conferring resistance to a range of herbicides in black-grass will persist in the gene pool even in the absence of herbicide application. Consequently, herbicide as an effective tool for control of black-grass will gradually be lost in fields infested by NTSR black-grass.

Keywords: ACCase, biomass, black-grass, fitness penalty, NTSR, potential seed production

INTRODUCTION

Herbicides are the most reliable, applicable and cost-effective method of weed control (Moss, 2010; Keshtkar, 2015; Owen, 2016). However, the continuous use of herbicides for many years have led to widespread problems with herbicide resistance. Resistance to herbicides is a major threat to the sustainability of agricultural industry with now 251 herbicide resistance weed species being reported worldwide (Heap, 2017).

Black-grass (*Alopecurus myosuroides* Huds), a common winter-annual grass weed species, can reduce crops yield substantially (Lutman et al., 2013). Black-grass has been reported in 37 countries and it is the most important weed species in European cereals especially it is a major weed in the United Kingdom, France, and Germany (Holm et al., 1997; Chauvel et al., 2002). It is also the most important herbicide resistant weed species in Europe (Moss et al., 2007; Lutman et al., 2013). Black-grass has evolved resistance to seven different sites of action including group A (1), B (2), C1 (5), C2 (7), K1 (3), K3 (15), and N (8) (Heap, 2017) and both target-site resistance (TSR) and non-target-site resistance (NTSR) mechanisms were detected among black-grass populations (Délye, 2005). TSR mechanism to group A herbicides (ACCase inhibitors) were reported in black-grass ACCase gene at five codons including Ile-1781, Trp-2027, Ile-2041, Asp-2078 and Gly-2096 (Kaundun, 2014). Target-site mutation conferring resistance to group B herbicides (ALS inhibitors) were observed in black-grass ALS gene at position Pro-197 and Trp-574 (Hull et al., 2008; Tranel et al., 2017). As one of several mechanisms endowing NTSR, enhanced herbicide metabolism mediated by cytochrome P 450 monooxygenases (P-450) and glutathione *S*-transferase (GST) was reported in black-grass populations evolving resistance to different group of herbicides (Hall et al., 1995; Cummins et al., 1999; Preston, 2004; Délye, 2005). It was also confirmed that higher activity of *O*-glucosyltransferases (OGTs) endows NTSR (Brazier et al., 2002). As our literature review revealed, other NTSR mechanisms such as reduced penetration and translocation have not been reported in black-grass (Hall et al., 1995; Menendez and De Prado, 1996), meaning that enhanced metabolism is the main mechanism endowing NTSR in black-grass.

Herbicide resistance strategies should aim to prevent or delay the evolution of herbicide resistance and whenever resistant weed species appear, management strategies should aim to reduce the number and frequency of resistant plants within populations. According with general eco-evolutionary theories resistant (R) plants are expected to be less fit than susceptible plants in the absence of selection pressure (no herbicide) (Vila-Aiub et al., 2009b; Vila-Aiub et al., 2011). This hypothesis holds true for weed biotypes carrying the *Gly264 psbA* gene endowing resistance to triazine herbicides (Gronwald, 1994). It can, however, not be generalized to other cases of resistance. In some cases no resistance costs were reported as for glyphosate-resistant palmer amaranth (*Amaranthus palmeri*) (Giacomini et al., 2014; Vila-Aiub et al., 2014), while in other cases, surprisingly, a higher fitness was reported, e.g., for a sethoxydim-resistant green foxtail (*Setaria italic*) (Wang et al., 2010). Thus, it is necessary to evaluate fitness costs of each case of resistance individually (Lehnhoff et al., 2013) to develop robust resistance management programs (Park et al., 2004; Vila-Aiub et al., 2011). For instance, Gly-2078 ACCase allele conferring resistance to fenoxaprop-*P*-ethyl and clodinafop-propargyl in a black-grass population was associated with a fitness cost, hence using competitive crops like alfalfa in crop rotation programs can strongly hamper the development and seed production of plants carrying the R allele compared with wild type plants (Menchari et al., 2008).

It has been stressed by several researchers that factors like the genetic background, environmental condition, and resource competition should be considered in fitness studies (Holt, 1990; Menchari et al., 2008; Vila-Aiub et al., 2009b). Among these, control of genetic background of S and R biotypes is one of the main obstacles assessing fitness cost. Vila-Aiub et al. (2009b) found that the genetic background was only controlled in 25% of the published fitness studies. In many studies S and R populations originating from different geographical locations with different genetic background were used and results from such studies might be inconclusive as different populations may differ genetically at a number of loci other than resistant locus or loci (Keshtkar et al., 2017).

Fitness can be defined as reproductive success of a plant in a given environment (Holt, 1990), i.e., the number of offspring sent to next generations by an individual compared to another (Vila-Aiub et al., 2009b). Therefore, seed production is a crucial parameter determining fitness (Menchari et al., 2008; Vila-Aiub et al., 2009b). The seed production of black-grass correlated to the number of tillers (Maréchal et al., 2012). Around 50% of produced caryopsis (i.e., seeds) can be viable (Pye, 2000) and viability of seeds is affected by the time of seed shedding (Moss, 1983). In a life cycle model of black-grass, it was also assumed that viability of the produced seeds are 55%, while half of the viable seeds are lost due to predation, seed decay in soil and germination before cultivation (Cavan et al., 2000). Cropping systems can also affect seed production and density of black-grass (Chauvel et al., 2001), especially spring crops such as spring wheat reducing up to 88% of black-grass population (Lutman et al., 2013).

In contrast to many studies that only evaluated vegetative output of plants in non-competition condition, in this study we monitored the whole life cycle of the R and S plants (i.e., both vegetative and reproductive potential) under both competitive and non-competitive conditions. Studies on resistance costs of NTSR are sparse (Vila-Aiub et al., 2005a). In a previous study, we evaluated the costs of NTSR in a black-grass population on germinability and seedling emergence under contrasting temperature regimes (optimal and low) and different sowing depths. In comparison to the S sub-population, around 24 h delay was observed in seedling emergence of the R sub-population, where the seeds were grown at suboptimal conditions. Importantly, the seedling emergence of the R sub-population was around four-fold lower than the corresponding S sub-population. These results suggested that manipulation of agronomy practices such as delayed drilling of winter wheat and avoiding no-tillage can cause unfavorable conditions for the R sub-population (Keshtkar et al., 2017). In that and the present

study, we followed the experimental protocol proposed by Vila-Aiub et al. (2011) previously described by Vila-Aiub et al. (2005b) and Pedersen et al. (2007) to control genetic background of plant material through selection of R and S phenotypes within a single population. The objective of present study was to assess the vegetative and reproductive capacity under non-competitive and competitive condition of the same NTSR black-grass sub-population. The response of the S and NTSR sub-populations were evaluated in a target-neighborhood design at increasing densities of winter wheat in greenhouse and field conditions. To our knowledge, this is the first study comparing vegetative and reproductive output of S and NTSR black-grass sub-populations sharing random homogeneous genetic background.

MATERIALS AND METHODS

Plant Material

A known fenoxaprop-*P*-ethyl NTSR black-grass population (population ID914) originating from a field near Odense on the island of Funen in Denmark (54° 57′ 21.93″ N 10° 36′ 39.53″ E) was used in this study. Enhanced herbicide metabolism assumed to be the mechanism endowing NTSR in the studied population ID914, as it is common in black-grass populations (Hall et al., 1995; Cummins et al., 1999; Preston, 2004; Délye, 2005) and was also expected in 75% of ACCase (i.e., fenoxaprop-*P*-ethyl and clodinafop-propargyl)-resistant black-grass populations in France (Délye et al., 2007). Recently, it was also demonstrated that enhanced metabolism plays an important role in developing resistant black-grass populations (Kaiser and Gerhards, 2015). Also, other NTSR mechanisms including reduced penetration and translocation have not been reported in black-grass (Hall et al., 1995; Menendez and De Prado, 1996). The population ID914 was highly resistant to fenoxaprop-*P*-ethyl [resistance index (RI) > 64], moderately resistant to pendimethalin and flupyrsulfuron-methyl-sodium (RI > 10), and slightly resistant to prosulfocarb (RI > 2.5) (Keshtkar et al., 2015).

To select susceptible and NTSR phenotypes within the population ID914 a plant cloning technique, the so-called "Single Population Protocol" described by Vila-Aiub et al. (2005b) and Pedersen et al. (2007), was used as in a previous study (Keshtkar et al., 2017). Briefly, the seedlings of populations ID914 at the 2–3 tillering stage were propagated asexually by a dividing method. The parent plants and their corresponding clones were numbered for identification. The clones were sprayed with fenoxaprop-*P*-ethyl at the 3–4 leaf stage and classified as S (dead plants), partly resistant (PR, alive but no regrowth) and R (alive and vigorously regrowth). The corresponding parent plants were then identified according to their number. The PR parent plants were discarded, while the S and R parent plants were grown in separate tables in a cold greenhouse for vernalization. The plants surrounded by a polyethylene pollen-proof enclosure to prevent cross-pollination between the S and NTSR parent plants until seed production in mid-July 2012. Seeds were harvested and to break primary seed dormancy they were placed at high temperature (35 ± 2°C) for 6 weeks as suggested by Moss (1999) and then stored at a constant temperature of 4°C until the onset of the experiments. To confirm resistance status of the R and S sub-populations selected within the mother population (i.e., population ID914), a dose-response experiment was carried out using different dose of fenoxaprop-*P*-ethyl. Results of the dose-response experiment confirmed that the plant cloning technique selected the R and S sub-populations very well as the R sub-population was highly resistant to fenoxaprop-*P*-ethyl, while the S one was sensitive (Keshtkar et al., 2017). Molecular assays showed that none of the known mutations conferring target site resistance to ALS and ACCase inhibiting herbicides were present in either the original parent population, i.e., the population ID914 (Keshtkar et al., 2015) or in the S and R sub-populations, suggesting that the mechanism of resistance was NTSR (Keshtkar et al., 2017). Also, herbicides with four different sites of action failed to control the parent population ID914 (Keshtkar et al., 2015), providing more evidence that the mechanism of resistance was NTSR (Keshtkar et al., 2017). As it was described above, it is expected that NTSR mechanism is due to enhanced herbicide metabolism.

Greenhouse Experiment

A target-neighborhood design was used to compare the vegetative and reproductive ability of the S and R sub-populations (target plant), in response to increasing densities of winter wheat (neighbor plant) where winter wheat was either at the 2-leaf or the 3–4 leaf stage.

Five winter wheat (cv. Herford) densities of 0, 96, 192, 385, and 770 plants m^{-2} were established by sowing 0, 4, 8, 16, and 32 plants in pots (23 cm diameter × 30 cm height). The pots were filled with a potting mixture consisting of soil, peat, and sand (2:1:1 w/w/w) containing all necessary micro and macro nutrients. A template was used to achieve the same distance between each neighbor plant and the target plant and a uniform distance among neighbor plants (**Figure 1**). Additional winter wheat plants were grown in plastic boxes and transplanted at the 2-leaf growth stage to the pots where winter wheat seeds failed to germinate or germinated late.

Uniform sized non-dormant seeds of the R and S black-grass sub-populations were pre-germinated in 9-cm Petri-dishes containing three cellulose filter papers (Whatman No. 1) covered by one glass-fiber filter paper (Whatman GF/A). Seven ml of distilled water was added to each Petri-dish and the dishes were placed at 17/10°C day/night with 14/10 h light/darkness photoperiods with a Photosynthetic Photon Flux Density of 175 μmol m^{-2} s^{-1}. The seedlings with approximately 1 cm length were transplanted into small Jiffy pots and grown till they reached the 2-leaf stage. Uniform 2-leaf stage black-grass seedlings were re-transplanted into the center of each pot when winter wheat was either at the 2-leaf (**Figure 1A**) or the 3–4-leaf growth stage (**Figure 1B**).The 3–4-leaf growth stage treatment was included in the study as we wanted to introduce an additional stress on black-grass by exposing the plants to competition from the more competitive wheat plants, i.e., the 3–4 leaf growth. The 2-leaf growth stages of both wheat and black-grass mimics the scenario where black-grass seeds germinate at the same time as winter wheat, while the 3–4 leaf growth stage of winter wheat reflects a scenario where black-grass seeds germinate later than winter wheat which is not uncommon in black-grass germinates

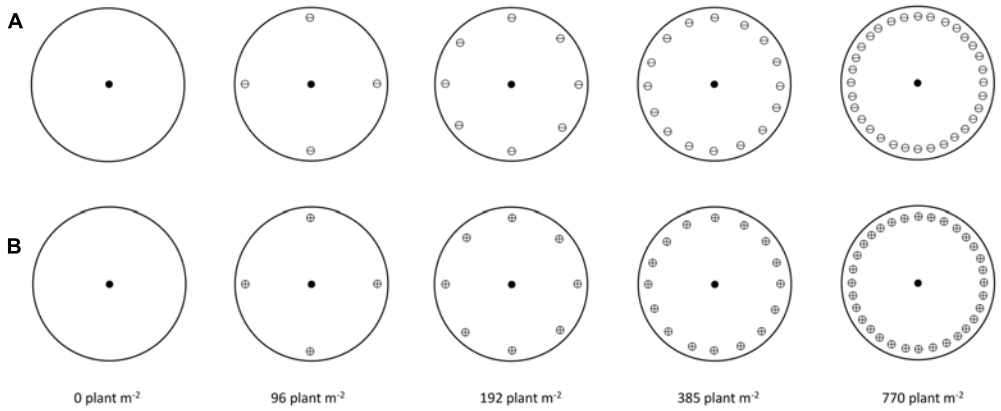

FIGURE 1 | Schematic illustration of target-neighborhood experiment design used to evaluate the competitive responses of S and NTSR sub-populations to increasing density of winter wheat at **(A)** 2-leaf growth stage of winter wheat (⊖) and **(B)** 3-4 leaf growth stage of winter wheat (⊕). Symbols in the middle of circles represent black-grass plants (target plants) at 2-leaf growth stage (●). Symbols close to edge of circle represent winter wheat plants (neighbor plants).

between September and December pots were sub-irrigated to maintain field capacity and a liquid fertilizer was applied as required. The experiment was initiated in the late December 2012 and the pots were kept in a cold greenhouse until spring to vernalize plants and it was terminated in June 2013. The experiment was repeated in the growing season 2013/14.

Above-ground biomass of target and neighbor plants was determined at the tillering stage of black-grass (first harvest time) and the stem elongation stage (second harvest time) by cutting plants at the soil surface. The plants were oven dried for 24 h at 80°C and dry weight was recorded. In addition, the number of tillers of black-grass was measured. Three replicate pots per treatment were harvested at each harvest time.

Three pots per treatment were kept in the greenhouse for evaluation of reproductive ability. Seeds of black-grass mature and shed over a long period, hence it was not possible to measure seed production. Instead, the potential seed production of plants was estimated indirectly, as suggested by Melander (1995). The heads from each pot were cut immediately after emergence (before flowering), kept in small mesh plastic bags at greenhouse temperature to be dried and finally, the cumulative weight of heads was measured. Around 80 heads were used to calculate linear relationship between seeds per head and head weight. The estimated R^2 values for the 2012/13 and 2013/14 experiments were 0.81 and 0.73, respectively. Above-ground biomass was measured at the end of the experiment (third harvest time). Aphids and powdery mildew were controlled twice with the insecticide (imidacloprid (Confidor WG 70; Bayer)) and the fungicide (metrafenon (Flexity; BASF)).

Field Experiment

A target-neighborhood experiment was also conducted in a field in 2013/14 almost similar to the greenhouse experiments. A field (50.5 × 17.5 m) was sown with winter wheat (cv. Herford) on 6 September 2013. Eighty small plots (50 cm × 50 cm) were left bare within the field. The distance between plots was at least 2.5 m. Similar to the greenhouse experiments, five winter wheat densities (0, 96, 192, 385, and 770 plants m^{-1}) were established by sowing 0, 4, 8, 16, and 32 winter wheat in the center of each plot on 11 September 2013 using the same templates as for the greenhouse trial. The S and NTSR black-grass seedlings were grown outdoors using the same method as for the glasshouse experiments. At the 3–4-leaf stage of winter wheat, uniform 3–4-leaf stage seedlings of the two sub-populations of black-grass were transplanted into the center of each plot.

Additional plants of both black-grass sub-populations and winter wheat were grown in a nursery in the same field. Poorly established plants were replaced by new seedlings supplied from the nursery either end of October 2013 and or end of March 2014. The number of replaced seedlings were the same for NTSR and S sub-populations. All plots were sprayed with imidacloprid (Confidor WG 70; Bayer) in late October 2013 to prevent damage from wireworm (*Agriotes* spp.). To avoid pollen dispersal from the NTSR plants to the natural stand of black-grass in the surrounding fields the experiment was terminated at the stem-elongation stage of black-grass (before heading stage) by cutting the plants at the soil surface on 16 May 2014. Dry matter and number of tillers were recorded at the mentioned growth stage.

Statistical Analysis

The greenhouse trials were designed as split-split factorial experiments with four factors (i.e., two black-grass sub-populations × five winter wheat densities × three harvesting times × two growth stages of winter wheat), where winter wheat growth stage was considered as main plot and winter wheat density as subplot. A Completely Randomized Design (CRD) was used with three replicates per treatment, resulting in 180 pots per experiment.

The field trial was a factorial experiment with two factors (two black-grass sub-populations × five winter wheat densities) arranged as a Randomized Complete Block Design (RCBD). There were four replicates per treatment giving 40 plots in total.

Regression analysis was carried out to evaluate the competitive responses of the S and NTSR sub-populations to increasing

density of winter wheat. Specifically, a non-linear hyperbola model was fitted to data, the model equation used was:

$$Y = \frac{a}{1 + \left[\frac{x}{ED_{50}}\right]}$$

where Y denotes the black-grass end-point (biomass, tiller number, and potential seed production), x represents density of winter wheat (plant m^{-2}), a is the upper limit or mean response when winter wheat density is zero (no crop competition), the parameter ED_{50} is the effective density of wheat reducing black-grass end-point by 50%. The sub-populations were compared in terms of the parameters a, ED_{50}, and ED_{90} (the effective density of wheat reducing black-grass end-point by 90%) by means of *post hoc t*-tests. For each harvest time, the model was fitted simultaneously to the data of both sub-populations. Wherever the assumption of homogeneity of variance was not met a transform-both-sides approach (Box–Cox data transformation) was applied (Carroll and Ruppert, 1988; Ritz et al., 2016). The R statistical software with the add on package *drc* was used for the statistical analysis and making graphs (Ritz and Streibig, 2005; R Core Team, 2013).

RESULTS

Greenhouse Experiments

Data were analyzed separately for each year as significant differences were found in the estimated parameters between years. The results showed that the biomass, tiller number, and potential seed production of both the R and S sub-populations significantly decreased with increasing density of winter wheat (**Tables 1**, **2** and Supplementary Figures S1–S3). This response was observed at both growth stages of winter wheat in both years. As expected, suppression of black-grass by increasing densities of winter wheat was more pronounced at the 3–4 leaf stage of winter wheat, i.e., when the winter wheat plants were more developed and, thus, more competitive than the black-grass plants.

More importantly, at the maturity stage (third harvest time) no statistically significant differences were found in fitness traits (potential seed production and final biomass) between the R and S sub-populations either grown alone (no competition) or in competition with winter wheat in either year. A similar result was observed at other growth stages including tillering and stem-elongation stages, i.e., there were no statistically significant differences between the R and S sub-populations. A similar trend to the ED_{50} values was observed for number of tillers, biomass and potential seed production and no significant differences were found between the corresponding ED_{90} values at none of the harvest times, winter wheat growth stages and either year (**Tables 1**, **2**). Overall winter wheat density had more effect on biomass of black-grass at the last harvest time compared to the first harvest time reflecting an increased crop competition in the late growing season. As no significant differences were observed between the R and S sub-populations it can be concluded that no fitness cost in vegetative traits and reproductive ability was found in the R sub-population.

Field Experiment

Similar to the greenhouse experiments, the fitness traits (biomass and tiller number) decreased significantly for both sub-populations with increasing densities of winter wheat. Also, no statistically significant differences were found between the R and S sub-populations in the measured fitness traits either in the absence or presence of winter wheat (**Table 3** and Supplementary Figure S4) confirming the results of the glasshouse experiments.

The potential seed production of plants was not evaluated in the field experiment as we did not want to contribute to the spread of herbicide resistance genes. However, according to the strong relationship usually found between vegetative and reproductive outputs of plants (Weiner, 2004; Weiner et al., 2009), the absence of significant differences in biomass and number of tillers suggest that the R and S sub-populations would also produce similar number of seeds, as it was also observed in the glasshouse experiments.

DISCUSSION

Many studies have investigated the fitness cost of herbicide resistance, however, in 75% of the published studies the genetic background of plants was not controlled possibly leading to flawed results (Vila-Aiub et al., 2009b), while it was revealed that greater control of genetic background increases the probability of identifying resistance costs (Bergelson and Purrington, 1996). Most of the studies that used appropriate protocols to control the genetic background have focused on fitness cost of TSR plants (Vila-Aiub et al., 2005b; Menchari et al., 2008; Ashigh and Tardif, 2009; Wang et al., 2010; Yu et al., 2010; Délye et al., 2013; Giacomini et al., 2014; Vila-Aiub et al., 2014). For instance, no vegetative and fecundity fitness cost were observed in ACCase-TSR black-grass phenotypes carrying the Leu-1781 and Asn-2041 ACCase mutations, while homozygous Gly-2078 ACCase black-grass showed a substantial fecundity and vegetative reduction (Menchari et al., 2008). Four populations of ALS-TSR black nightshade (*Solanum ptychanthum* Dunal) with mutation at position Ala-205 showed negative fecundity fitness, while they did not show any vegetative and seed germinability fitness penalty (Ashigh and Tardif, 2009; Ashigh and Tardif, 2011). High amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene as a TSR mechanism endowing resistance to glyphosate in palmer amaranth and perennial ryegrass (*Lolium perenne* L.) was not associated with any significant reproductive fitness penalty (Giacomini et al., 2014; Vila-Aiub et al., 2014; Yanniccari et al., 2016). Surprisingly, seed production of an ACCase-TSR green foxtail phenotype carrying the Leu-1781 mutation was higher than the wild phenotype (Wang et al., 2010). Possible effects of TSR mechanism on plant fitness were investigated in all above mentioned studies showing that different resistance alleles and genes might have different finesses, i.e., negative, neutral or positive fitness.

Despite importance of NTSR mechanism, generally resistance costs of NTSR biotypes have rarely been studied in a correct way. So far, rigid ryegrass (*Lolium rigidum*) and loose silky bentgrass (*Apera spica-venti*) are the only NTSR weed species where

TABLE 1 | Competitive response of S and NTSR sub-populations selected within a black-grass population (ID914), to increasing density of winter wheat at two different growth stages of winter wheat (GS-I; 2-leaf stage, GS-II; 3–4 leaf stage) in the 2012/13 greenhouse experiment.

Plant traits	Harvest time	ED_{50} (winter wheat plants m^{-2})[a]						ED_{90} (winter wheat plants m^{-2})[b]						D[c]					
		GS-I			GS-II			GS-I			GS-II			GS-I			GS-II		
		S	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*						
Biomass	H 1	270 (70.7)	*ns*	222 (51.8)	44 (9.8)	*ns*	56 (11.9)	2427 (635.9)	*ns*	1998 (466.6)	393 (88.1)	*ns*	505 (107.5)	1.0 (0.11)	*ns*	1.1 (0.12)	0.8 (0.11)	*ns*	0.9 (0.12)
	H 2	74 (22.2)	*ns*	79 (23.3)	15 (4.0)	*ns*	16 (4.1)	667 (200.0)	*ns*	713 (209.3)	136 (36.0)	*ns*	144 (37.2)	9.0 (1.71)	*ns*	10.4 (1.91)	8.0 (1.58)	*ns*	7.8 (1.51)
	H 3	46 (12.1)	*ns*	76 (22.4)	11 (2.9)	*ns*	11 (2.8)	417 (109.1)	*ns*	682 (201.6)	101 (26.3)	*ns*	100.9 (25.3)	146.2 (24.60)	*ns*	140.2 (24.41)	131.2 (22.80)	*ns*	149.9 (25.19)
Tiller No.	H 1	281 (65.0)	*ns*	347 (85.9)	67 (14.3)	*ns*	97 (20.9)	2529 (585)	*ns*	3128 (773)	603 (129)	*ns*	873 (189)	19.5 (1.90)	*ns*	18.9 (1.82)	14.7 (1.83)	*ns*	13.7 (1.66)
	H 2	71 (13.8)	*ns*	92 (17.2)	28 (4.9)	*ns*	26 (4.4)	637 (123.80	*ns*	826 (154.70	251 (44.3)	*ns*	235 (39.7)	56.6 (6.94)	*ns*	67.0 (7.63)	40.8 (5.17)	*ns*	44.1 (5.42)
Potential seed production	H 3	42 (8.6)	*ns*	58 (14.1)	11 (2.6)	*ns*	14 (3.4)	376 (77.4)	*ns*	519 (126.8)	102 (23.3)	*ns*	129 (30.6)	70061 (8429.9)	*ns*	56343 (7608.4)	67984 (8366.2)	*ns*	53136 (7071.2)

The parameters obtained by fitting a nonlinear hyperbola model to the data of the 2012/13 greenhouse experiment. Values in parentheses represent standard error of the estimated parameters. [a]*Effective density of wheat reducing response of target plants by 50%.* [b]*Effective density of wheat reducing response of target plants by 90%.* [c]*Mean response of target plants in the absence of winter wheat.* [ns]*Non-significant (no significant differences between the R and S sub-populations in each growth stages of winter wheat).*

TABLE 2 | Competitive responses of S and NTSR sub-populations selected within a black-grass population (ID914), to increasing density of winter wheat at two different growth stages of winter wheat (GS-I; 2-leaf stage, GS-II; 3-4 leaf stage) in the 2013/14 greenhouse experiment.

Plant traits	Harvest time	ED_{50} (winter wheat plants m^{-2})[a]						ED_{90} (winter wheat plants m^{-2})[b]						D^{c}					
		GS-I			GS-II			GS-I			GS-II			GS-I			GS-II		
		S	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*						
Biomass	H 1	118 (26.7)	*ns*	145 (35.6)	33 (6.5)	*ns*	25 (4.6)	1061 (240.4)	*ns*	1305 (320.2)	299 (58.7)	*ns*	226 (41.4)	0.75 (0.087)	*ns*	0.61 (0.0740	0.75 (0.088)	*ns*	0.96 (0.104)
	H 2	42 (25.5)	*ns*	52 (35.5)	5 (2.4)	*ns*	4 (1.9)	375 (229.1)	*ns*	469 (319.3)	41 (22.1)	*ns*	33 (17.7)	11 (5.3)	*ns*	9 (4.7)	18 (8.6)	*ns*	14 (6.9)
	H 3	42 (20.0)	*ns*	82 (40.5)	4 (2.1)	*ns*	5 (2.6)	375 (180.4)	*ns*	737 (364.8)	37 (18.5)	*ns*	49 (23.0)	67 (20.1)	*ns*	64 (19.0)	94 (27.6)	*ns*	103 (29.3)
Tiller No.	H 1	197 (67.8)	*ns*	230 (78.8)	37 (10.3)	*ns*	36.7 (10.2)	1769 (610.0)	*ns*	2068 (709.4)	332 (93.0)	*ns*	331 (92.3)	10 (1.9)	*ns*	10 (1.8)	18 (4.0)	*ns*	17 (3.8)
	H 2	49 (16.9)	*ns*	46 (17.0)	10 (3.0)	*ns*	14 (4.2)	442 (152.7)	*ns*	410 (153.6)	89.8 (27.3)	*ns*	125 (37.9)	63 (15.6)	*ns*	66 (17.5)	80 (19.7)	*ns*	70 (16.7)
Potential seed production	H 3	41 (30.1)	*ns*	68 (50.3)	3 (1.9)	*ns*	3 (1.8)	368 (271.2)	*ns*	608 (453.1)	26 (17.4)	*ns*	25 (16.2)	49123 (26776.5)	*ns*	47477 (24509.1)	59762 (33022.0)	*ns*	75589 (40936.7)

The parameters obtained by fitting a nonlinear hyperbola model to the data of the 2013/14 greenhouse experiment. Values in parentheses represent standard error of the estimated parameters. [a]*Effective density of wheat reducing response of target plants by 50%.* [b]*Effective density of wheat reducing response of target plants by 90%.* [c]*Mean response of target plants in the absence of winter wheat.* [ns]*Non-significant (no significant differences between the R and S sub-populations in each growth stages of winter wheat).*

TABLE 3 | Competitive responses of S and NTSR sub-populations selected within a black-grass population (ID914) to increasing density of winter wheat (neighbor plants) in field experiment.

	ED_{50} (winter wheat plants m^{-2})[a]			ED_{90} (winter wheat plants m^{-2})[b]			D[c]		
	S		*R*	*S*		*R*	*S*		*R*
Biomass	77 (27.6)	*ns*	148 (55.7)	697 (249)	*ns*	1334 (501)	31 (7.3)	*ns*	24 (5.1)
Tiller No.	120 (37.6)	*ns*	161 (50.0)	1080 (339)	*ns*	1452 (450)	101 (16.4)	*ns*	114 (17.2)

[a]*Effective density of wheat reducing response of target plants by 50%.* [b]*Effective density of wheat reducing response of target plants by 90%.* [c]*Mean response of target plants in the absence of winter wheat.* [ns]*non-significant (no significant differences between the R and S sub-populations). The parameters were obtained by fitting a nonlinear hyperbola model to data. Values in parentheses represent standard error of the estimated parameters.*

fitness cost has been evaluated using appropriate plant material, where genetic background of plants were controlled via the Single Population and the Segregating Population approaches, respectively (Vila-Aiub et al., 2005a,b, 2009a; Pedersen et al., 2007; Babineau et al., 2017). This and our previous study (Keshtkar et al., 2017) are the first ones comparing S and NTSR sub-populations isolated from a resistant black-grass population using the Single Population Protocol providing random homogenous genetic background, as proposed by Vila-Aiub et al. (2011). In the present study, results from two greenhouse experiments and one field trial showed that NTSR mechanism conferring a high level of resistance to fenoxaprop-*P*-ethyl and a moderate level of resistance to pendimethalin and flupyrsulfuron was not associated with any vegetative and potential fecundity fitness cost. In contrast to our results, studies on an enhanced metabolic NTSR rigid ryegrass population resistant to ACCase inhibitors found fitness costs in both vegetative and reproductive outputs in competition with wheat (Vila-Aiub et al., 2005a, 2009a). In agreement with our results, no significant differences were reported in vegetative growth and seed production of S and glyphosate NTSR rigid ryegrass exposed to competition with wheat (Pedersen et al., 2007). In contrast to present study and all other studies evaluated fitness cost of NTSR weed species with appropriate methods of controlling genetic background (Vila-Aiub et al., 2005a,b, 2009a; Pedersen et al., 2007), recently Babineau et al. (2017) did not observe vegetative and fecundity fitness cost in an enhanced metabolic NTSR loose silky bentgrass population and surprisingly some fitness benefits (i.e., earlier germination and earlier flowering time) were observed. These results suggest that also for NTSR it is not possible to make any generalization and each case must be evaluated individually as it was suggested by Lehnhoff et al. (2013). It is worth to note that the exact mechanism endowing NTSR to black-grass and rigid ryegrass were not yet detected hitherto in our study and the study by Pedersen et al. (2007) and remained to be tested. However, we assumed that the enhanced metabolism might be the mechanism endowing NTSR in this population as it was reported in many black-grass populations and importantly the other NTSR mechanisms such as reduced uptake and translocation of herbicide were not detected in black-grass (Kemp et al., 1990; Hall et al., 1995; Menendez and De Prado, 1996; Hall et al., 1997; Letouzé and Gasquez, 2001). It was also suggested that increased herbicide metabolism is the main mechanism in 75% of French black-grass populations evolved resistance to ACCase inhibitors (Délye et al., 2007).

The results are significant because potential seed production, as a major component of plant fitness complex (Menchari et al., 2008; Vila-Aiub et al., 2009b) was evaluated over 2 years under competitive and non-competitive conditions and no differences were found between the S and NTSR sub-populations. Similar seed production potential does, however, not necessarily lead to equal fitness. Fitness of off-springs can be affected by seed germination pattern, seed dispersal, seed longevity, response to pathogens and diseases (Vila-Aiub et al., 2009b). Many pests and diseases attack black-grass that might influence seed production and viability (Moss, 1983). Also, Moss (1983) found a significant relationship between black-grass seed weight and seed viability as the seeds with higher weight had higher seed viability. Black-grass seed dormancy, germinability and rate were affected by cropping system, so that seeds originated from spring crop had lower dormancy and higher germinability (Colbach and Dürr, 2003). Seeds of black-grass produced under dry and warm conditions, also, had lower dormancy (Swain et al., 2006). According to these information, it is suggested that seed weight, seed viability, primary seed dormancy of R and S phenotypes must be measured under contrast conditions in future studies.

It was also suggested that many fitness traits should be considered to avoid misleading conclusions (Giacomini et al., 2014), especially under different conditions. Our previous study provided clear evidence for this, as seedling emergence of the NTSR plants was lower at sub-optimal conditions (low temperature and deep sowing depth) suggesting that the NTSR loci conferring herbicide resistance in the black-grass population were associated with negative fitness (Keshtkar et al., 2017). It should be noted that fitness penalties were only observed under stressful condition. Thus, we can only conclude that NTSR loci conferring resistance to black-grass were not associated with fitness penalty with regard to biomass production, number of tillers and potential seed production under either non-competitive or competitive conditions. Even it was not possible to measure effective see production, however, it is expected that the potential seed production estimated indirectly was a powerful index to measure fecundity fitness of plants. Also, it is said that black-grass seed production linked to the number of tillers (Maréchal et al., 2012). In addition, usually there is a strong relationship between vegetative and reproductive outputs of plants (Weiner, 2004; Weiner et al., 2009).

Environmental condition can affect fitness cost (Menchari et al., 2008), i.e., our results cannot be generalized to other stress conditions such as pathogen and pest infestations and

maybe more importantly to abiotic stress conditions including drought, salinity, and cold. It has been speculated that NTSR biotypes, where resistance is attributed to an increased activity of detoxifying enzymes like cytochrome P-450s and glutathione transferases (GSTs), may possess a higher fitness than the wild type (Cummins et al., 2013; Taylor et al., 2013). For instance, wheat seedlings with induced GST had higher fitness)i.e., seedling length, dry weight and germination rate) than untreated plants under optimum as well as stressful conditions such as a soil contaminated with oil residues and heavy metals (Taylor et al., 2013). So, further studies are needed to confirm the absence of fitness cost under the stressful conditions.

Our study clearly highlighted the effect of crop density on black-grass suppression. Obviously, quantification of these effects can be applied in weed management strategies. On average increasing winter wheat density from 192 to 385 and 192 to 770 plant^{-2} reduced black-grass potential seed production around 50 and 70%, respectively. Other studies have also confirmed the effect of crop density on black-grass, for instance Lutman et al. (2013) reported a decrease in the number of black-grass head m^{-2} up to 37.5% when winter wheat crop density increased from 100 to 350 m^{-2}, while the number of black-grass seedlings m^{-2} was not affected. It must be taken into account that yield and growth of crops can also be affected by the crop density. Optimal wheat density (i.e., the maximum number of plant m^{-2} without yield reduction) depends on many factors such as verity, climate, planting date, special planting pattern. For instance, in Australia the current recommended wheat density is 100–150 plant m^{-2} (Lemerle et al., 2005), while in a Mediterranean climate (Spain) the highest winter wheat yield was obtained at 400–500 seed m^{-2} and it was expected that higher densities might be suggestible (Lloveras et al., 2004). In Denmark, the recommended wheat density is around 250 and 400 plant m^{-2} for early and delayed sowing dates, respectively. Comparing the recommended crop densities with the ED_{50}s presented in the **Table 3**, which is less than 200 plant m^{-2} for both biomass and tiller number, shows the importance of crop density as a herbicide resistance management tool. Another interesting result relates to the growth stage of winter wheat with plants at the 3–4 leaf stage being much more competitive than those at the 2-leaf stage. Effect of crop growth stage was, however, less pronounced than crop density. Overall, in the absence of competition black-grass could potentially produce 59,612 (±6,862) and 63,591 (±3,906) seeds per plant (when the planted winter wheat had 2- and 3–4 leaves, respectively), while the potential seed production of plants exposed to the highest density of winter wheat was 4,293 (±547) and 233 (±69) seeds per plant at 2-leaf and 3–4 leaf stage of winter wheat, respectively (the presented data are raw values, i.e., they were not calculated by the hyperbola model). It should be noted, that potential seed production in this study comprised both viable and non-viable seeds and the reduction in the percentage of viable seeds could be less or higher than the corresponding reduction in potential seed production. Our results suggest that agronomical practices leading to faster establishment of crop could be used to prevent a build-up of the black-grass seed bank. Also increasing the seeding density of winter wheat is an agronomic practice that can be used in an integrated weed management (IWM) strategy.

CONCLUSION

In this study, no significant vegetative and reproductive fitness cost was found with the NTSR black-grass sub-population. Thus, the results of this study do not support the theory of resource-based allocation even under a stressful condition which is expected to magnify fitness cost (Vila-Aiub et al., 2009b). Hence it can be concluded that if effective herbicide resistant management strategies are not implemented, NTSR loci conferring resistance to a range of herbicides in black-grass will persist in the gene pool even after cessation of herbicide use. Consequently, herbicide as an effective tool for control of black-grass will gradually be lost in fields infested by NTSR black-grass. The results of this study revealed that NTSR black-grass sub-population can be expected to persist in the field within population and attention should, therefore, be devoted to IWM programs incorporating herbicide resistant management strategies. The results of this and a previous study (Keshtkar et al., 2017) have highlighted that all aspects of fitness traits should be measured in resistant plants.

AUTHOR CONTRIBUTIONS

EK, SM, and PK conceived and designed the experiments. EK conducted the experiments. EK analyzed the data. EK wrote the manuscript. EK, SM, and PK read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02011/full#supplementary-material

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Overlapping Residual Herbicides for Control of Photosystem (PS) II- and 4-Hydroxyphenylpyruvate Dioxygenase (HPPD)-Inhibitor-Resistant Palmer amaranth (*Amaranthus palmeri* S. Watson) in Glyphosate-Resistant Maize

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A Palmer amaranth (*Amaranthus palmeri* S. Watson) biotype has evolved resistance to photosystem (PS) II- (atrazine) and 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicides (mesotrione, tembotrione, and topramezone) in maize seed production field in Nebraska, USA. The objectives of this study were to determine the effect of soil residual pre-emergence (PRE) herbicides followed by (fb) tank-mixture of residual and foliar active post-emergence (POST) herbicides on PS-II- and HPPD-inhibitor-resistant Palmer amaranth control, maize yield, and net economic returns. Field experiments were conducted in a grower's field infested with PS II- and HPPD-inhibitor-resistant Palmer amaranth near Shickley in Fillmore County, Nebraska, USA in 2015 and 2016. The contrast analysis suggested that saflufenacil plus dimethenamid-P or pyroxasulfone plus saflufenacil applied PRE provided 80–82% Palmer amaranth control compared to 65 and 39% control with saflufenacil and pyroxasulfone applied alone at 3 weeks after PRE (WAPRE), respectively. Among the PRE fb POST herbicide programs, 95–98% Palmer amaranth control was achieved with pyroxasulfone plus safluefenacil, or saflufenacil plus dimethenamid-P applied PRE, fb glyphosate plus topramezone plus dimethenamid-P plus atrazine, glyphosate plus diflufenzopyr plus dicamba plus pyroxasulfone, glyphosate plus diflufenzopyr plus pendimethalin, or glyphosate plus diflufenzopyr plus dicamba plus atrazine applied POST at 3 weeks after POST (WAPOST) through maize harvest. Based on contrast analysis, PRE fb POST programs provided 77–83% Palmer amaranth control at 3 WAPOST through maize harvest compared to 12–15% control with PRE-only and 66–84% control with POST-only programs. Similarly, PRE fb POST programs provided 99% biomass reduction at 6 WAPOST compared to PRE-only (28%) and POST-only (87%) programs. PRE fb POST programs provided higher maize yield (13,617 kg ha^{-1}) and net return (US \$1,724 ha^{-1}) compared to the PRE-only

(2,656 kg ha^{-1}; US $285 ha^{-1}) and POST-only (11,429 kg ha^{-1}; US $1,539 ha^{-1}) programs. The results indicated that effective control of multiple herbicide-resistant Palmer amaranth can be achieved with PRE fb POST programs that include herbicides with overlapping residual activity to maintain season-long control.

Keywords: net return, PRE followed by POST, residual herbicides, resistance management, weed management

INTRODUCTION

Palmer amaranth is a summer annual broadleaf weed species belonging to the family Amaranthaceae that has separate male and female plants (Sauer, 1957). Palmer amaranth is a prolific seed producer and if left uncontrolled, a single female plant can produce as many as 600,000 seeds (Keeley et al., 1987). Palmer amaranth has the highest specific leaf area (149–261 cm^2 g^{-1}), photosynthetic rate (80 μmol CO_2 m^{-2} s^{-1}), and growth rate (0.10–0.21 cm per growing degree day) out of all of the *Amaranthus* species (Horak and Loughin, 2000). Palmer amaranth can tolerate medium to mild water stress conditions using osmotic adjustment as a drought tolerance mechanism (Ehleringer, 1983). Furthermore, Palmer amaranth populations have been reported resistant to microtubule-, acetolactate synthase (ALS)-, photosystem (PS) II-, 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS)-, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-, and protoporphyrinogen oxidase (PPO)-inhibiting herbicides in different states throughout the USA (Heap, 2017). Palmer amaranth biotypes with multiple resistance to two or more herbicide sites of action have also been confirmed (Sosnoskie et al., 2011; Nandula et al., 2012; Heap, 2017). Palmer amaranth's aggressive growth habits and prolific seed production along with its evolution of resistance to different herbicide sites of action has made it the most problematic crop weed in the USA (Horak and Loughin, 2000; Berger et al., 2015; Chahal et al., 2015, 2017; Kohrt and Sprague, 2017).

A PS II- (atrazine) and HPPD-inhibitor-resistant Palmer amaranth biotype has been reported in a continuous maize seed production field in south-central Nebraska, USA (Jhala et al., 2014). While rapid detoxification and increased HPPD gene expression was reported as the mechanism conferring resistance to HPPD-inhibitor in the Palmer amaranth biotype from Nebraska (Nakka et al., 2017), the mechanism of atrazine resistance in this biotype is unknown. PS II- (atrazine) and HPPD-inhibitor (mesotrione, tembotrione, or topramezone) are the most commonly used herbicides for weed control in maize due to their pre-emergence (PRE) and post-emergence (POST) activity, broad-spectrum weed control, and crop safety, particularly in sweet maize, seed maize, and maize for popcorn (Fleming et al., 1988; Swanton et al., 2007; Bollman et al., 2008). The evolution of Palmer amaranth resistant to PS II- and HPPD-inhibitor has reduced the number of herbicide options for Palmer amaranth control in maize in Nebraska, USA.

The management of herbicide-resistant (HR) Palmer amaranth requires PRE followed by (fb) POST herbicide programs with distinct sites of action, herbicide rotation, and rotation of HR crop traits (Jhala et al., 2014; Crow et al., 2016; Chahal et al., 2017). The majority of the maize fields in Nebraska are under glyphosate-resistant (GR) maize production systems using either single or sequential glyphosate applications for POST weed control (Jhala et al., 2014; Chahal et al., 2017). Studies conducted in Nebraska have reported that the PS II- and HPPD inhibitor-resistant Palmer amaranth biotype is sensitive to glyphosate applied at the labeled rate because glyphosate had not been used over the past 8 years while the field was kept under continuous maize seed production (unpublished data). Therefore, glyphosate can be considered as one of the herbicide options for management of PS II- and HPPD inhibitor-resistant Palmer amaranth in GR maize. In Nebraska, GR weed species, including common ragweed (*Ambrosia artemisiifolia L.*), common waterhemp (*Amaranthus rudis Sauer*), horseweed [*Conyza canadensis (L.) Cronq.*], giant ragweed (*Ambrosia trifida L.*), and kochia [*Kochia scoparia (L.) Schrad.*] have been reported (Sarangi et al., 2015; Chahal et al., 2017; Ganie and Jhala, 2017a; Heap, 2017). More recently, GR Palmer amaranth has also been confirmed in Nebraska (Chahal et al., 2017). In view of the widespread occurrence of six GR broadleaf weeds in Nebraska, tank-mixing glyphosate with other site of action herbicides and rotation of GR maize with other HR crop traits has become important to diversify the number of herbicide options for management of HR weeds such as Palmer amaranth (Ganie et al., 2017; Ganie and Jhala, 2017b).

Palmer amaranth has an extended period of emergence (March–October) in the midwestern and southern USA, making it difficult to control, specifically later in the crop season (Keeley et al., 1987). PRE herbicides, also referred to as soil residual herbicides, are applied to the soil after crop planting but before emergence for controlling germinating or emerging weed seedlings. Soil-residual PRE herbicides generally lose their residual activity in the soil in 30–50 days; however, most POST herbicides commonly applied in maize have minimal to no soil residual activity (Jhala et al., 2015; Wiggins et al., 2015). Moreover, late-emerging Palmer amaranth plants often escape POST herbicide applications and produce seeds, leading to the replenishment of the soil seedbank and ensuring weed infestations for the next several seasons (Keeley et al., 1987). Therefore, herbicide programs should be focused on season-long Palmer amaranth control to reduce seed production and infestation during subsequent crop seasons. Though over-the-top (broadcast) application of most foliar active POST herbicides is restricted up to certain maize growth stages (Anonymous, 2017a,b,c), some herbicides such as glyphosate and glufosinate can be applied with drop nozzles in the later maize stages extending up to V8–V12 or the 8- to 12-leaf stage and V8–V10

Abbreviations: fb, followed by; PRE, pre-emergence; POST, post-emergence; WAPRE, weeks after pre-emergence; WAPOST, weeks after post-emergence.

or the 8- to 10-leaf stage in glyphosate- and glufosinate-resistant maize, respectively (Anonymous, 2017b,d). However, the repeated application of herbicides with a single site of action promote the rapid evolution of HR weeds (Délye et al., 2013).

Several soil-residual PRE herbicides have been registered for Palmer amaranth control in maize. For instance, acetochlor, dimethenamid-P, pendimethalin, pyroxasulfone, saflufenacil, or *S*-metolachlor applied PRE provided >80% Palmer amaranth control up to 50 days after application (Johnson et al., 2012; Cahoon et al., 2015; Janak and Grichar, 2016; Meyer et al., 2016). In addition, some soil residual herbicides such as acetochlor, pyroxasulfone, or dimethenamid-P can be applied POST in maize up to certain growth stages (Anonymous, 2017e,f,g). The application of overlapping residual herbicides could be used as an approach for season-long Palmer amaranth control. However, most soil-applied residual herbicides lack foliar activity and are unable to control emerged weeds at the time of application. Therefore, for achieving season-long Palmer amaranth control and to reduce the evolution of HR weeds, different site of action soil-residual herbicides can be applied within 2–3 days of crop planting and in tank-mixture with foliar active herbicides in a POST application.

The cost of herbicide-resistant weed management programs that include different site of action PRE and POST herbicides is usually higher than that of commonly followed weed management practices that involve the use of a single site of action POST herbicide such as glyphosate; therefore, most growers do not consider residual herbicides until they notice the presence of HR weeds in their fields (Peterson, 1999; Norsworthy et al., 2012; Edwards et al., 2014). Additionally, several growers have been avoiding PRE herbicides and relying on POST herbicides to reduce production costs due to low maize and soybean [*Glycine max* (L.) Merr.] commodity prices over the last few years in the USA; however, avoiding PRE herbicides allows early-season crop-weed competition, which could result in a yield penalty (Hall et al., 1992; Schuster and Smeda, 2007). Therefore, it has become crucial to evaluate the economic benefits of implementing herbicide resistant weed management programs to encourage their adoption among growers.

The objectives of this study were to determine the efficacy of soil-residual PRE herbicides fb residual herbicides in tank-mixture with foliar active POST herbicides for PS-II- and HPPD-inhibitor-resistant Palmer amaranth control, crop yield, and net economic return in GR maize. We hypothesized that season-long Palmer amaranth control will be achieved with soil-residual PRE herbicides fb their application in tank-mixture with POST herbicides.

MATERIALS AND METHODS

Experimental Setup

Field experiments were conducted in 2015 and 2016 in a grower's field confirmed with the presence of PS II- and HPPD-inhibitor-resistant Palmer amaranth near Shickley in Fillmore County, Nebraska (40.46°N, 97.80°E). The level of atrazine resistance was 9- to 14-fold, while the level of resistance to mesotrione, tembotrione, and topramezone was 4-, 4- to 6-, and 14- to 23-fold, respectively, compared to two susceptible Palmer amaranth populations (Jhala et al., 2014). Soil texture at the research site was a Crete silt loam (fine, smectitic, mesic Pachic Udertic Argiustolls) with a pH of 6.5, 26% sand, 57% silt, 17% clay, and 3.5% organic matter. A GR maize hybrid (Mycogen 2D351) was seeded at 87,500 seeds ha^{-1} in rows spaced 76 cm apart on May 30, 2015 and June 1, 2016. The experiment was arranged in a randomized complete block design with four replications and the experimental plots were 3 m wide and 9 m long, consisting of four maize rows. Monthly mean air temperature and total precipitation during the 2015 and 2016 growing seasons and the 30 year average in Shickley, Nebraska is provided in **Table 1**.

Herbicide programs in the GR maize included PRE-only, POST-only, and PRE fb their sequential application in tank-mixture with POST herbicides, with a total of 15 treatment combinations including a nontreated control (**Table 2**). The herbicide application timings and rates were based on the label recommendations in maize in Nebraska. Herbicide programs were applied using a CO_2-pressurized backpack sprayer consisting of a four-nozzle boom fitted with AIXR 110015 flat-fan nozzles (TeeJet Spraying Systems Co., P.O. Box 7900, Wheaton, IL 60189) calibrated to deliver 140 L ha^{-1} at 276 kPa. PRE applications were made within 3 days after planting maize and POST herbicides were applied when Palmer amaranth was 12–15 cm tall.

Palmer amaranth control was visually estimated at 3 weeks after PRE (WAPRE), before POST herbicide programs were applied, 3 and 6 weeks after POST (WAPOST) herbicide application, and before maize harvest based on a scale of 0–100%, with 0% corresponding to no control and 100% corresponding to plant death. A similar scale was used to assess maize injury at 1 and 2 weeks after PRE and POST herbicide applications, with 0% corresponding to no injury and 100% corresponding to no seed emergence or plant death. Palmer amaranth density

TABLE 1 | Monthly mean air temperature and total precipitation during the 2015 and 2016 growing seasons and the 30 year (year) average at Shickley, Nebraska, USA[a].

Month	Mean temperature			Total precipitation		
	2015	2016	30 year average	2015	2016	30 year average
	C			mm		
March	7	9	5	12	14	48
April	12	12	11	42	99	68
May	17	16	17	108	200	124
June	23	25	22	264	7	117
July	24	25	25	124	55	86
August	22	23	24	69	147	88
September	22	20	19	104	52	86
October	14	15	12	22	64	59
Annual	12	13	11	908	726	763

[a]*Mean air temperature and total precipitation data were obtained from the National Weather Service and Cooperative Observer Network (2017).*

TABLE 2 | Herbicide products, rates, and application timing for control of Photosystem (PS) II- and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor-resistant Palmer amaranth in glyphosate-resistant maize in field experiments conducted in 2015 and 2016 in Nebraska, USA[a].

Herbicide[b]	Timing	Rate (g ae or ai ha^{-1})	Trade name	Manufacturer
Pyroxasulfone	PRE	110	Zidua	BASF Corporation, Research Triangle Park, NC, USA
Topramezone + dimethenamid-P	POST	750	Armezon PRO	BASF Corporation
Pyroxasulfone fb topramezone + dimethenamid-P	PRE fb POST	110 750	Zidua Armezon PRO	BASF Corporation
Saflufenacil + dimethenamid-P	PRE	586	Verdict	BASF Corporation
Saflufenacil + dimethenamid-P fb topramezone + dimethenamid-P	PRE fb POST	586 750	Verdict Armezon PRO	BASF Corporation
Saflufenacil	PRE	75	Sharpen	BASF Corporation
Saflufenacil fb topramezone + dimethenamid-P	PRE fb POST	75 750	Sharpen Armezon PRO	BASF Corporation
Glyphosate	POST	870	Roundup PowerMax	Monsanto Company, 800 North Lindberg Ave., St. Louis, MO, USA
Dicamba + diflufenzopyr	POST	157	Status	BASF Corporation
Pyroxasulfone + saflufenacil fb glyphosate + topramezone + dimethenamid-P + atrazine	PRE fb POST	110 + 75 870 + 750 + 560	Zidua + Sharpen Roundup PowerMax + Armezon PRO + Aatrex	BASF Corporation Monsanto Company + BASF + Syngenta Crop Protection, Inc., Greensboro, NC, USA
Saflufenacil + dimethenamid-P fb glyphosate + topramezone + dimethenamid-P + atrazine	PRE fb POST	586 870 + 750 + 560	Verdict Roundup PowerMax + Armezon PRO + Aatrex	BASF Corporation Monsanto Company + BASF + Syngenta Crop Protection, Inc.
Saflufenacil + dimethenamid-P fb glyphosate + diflufenzopyr + dicamba + pyroxasulfone	PRE fb POST	780 870 + 157 + 91	Verdict Roundup PowerMax + Status + Zidua	BASF Corporation Monsanto Company + BASF + BASF

[a]*ae, acid equivalent; ai, active ingredient; fb, followed by; PRE, pre-emergence; POST, post-emergence.*
[b]*All POST herbicide programs were mixed with AMS, ammonium sulfate (DSM Chemicals North America Inc., Augusta, GA) at 2.5% wt/v and NIS, nonionic surfactant (Induce, Helena Chemical Co., Collierville, TN) at 0.25% v/v. No AMS or NIS were added to PRE herbicides. PRE applications were made within 3 d after planting and POST herbicides were applied when Palmer amaranth was 12–15 cm tall.*

was assessed from two randomly selected 0.25 m^2 quadrats per plot at 3 WAPRE herbicide programs. The Palmer amaranth's aboveground biomass was harvested from two randomly selected 0.25 m^2 quadrats per plot at 6 WAPOST, oven dried at 65 C for 3 days, and weighed. Palmer amaranth density and biomass data were converted into percent density or biomass reduction compared with the nontreated control (Ganie et al., 2017; Sarangi et al., 2017):

$$\text{Biomass/Density reduction (\%)} = \frac{(C - B)}{C} \times 100 \quad (1)$$

where C is the biomass or density of the nontreated control plot and B is the biomass or density collected from the experimental plot. At maturity, maize was harvested from the middle two rows of each plot using a plot combine, weighed, and the moisture content were recorded. Maize yields were adjusted to 15.5% moisture content (Ganie et al., 2017).

Economic analysis was performed to evaluate the profit and risk associated with each herbicide program. Net return from herbicide programs was calculated using the maize yield from each replication and herbicide program cost (Bradley et al., 2000; Edwards et al., 2014):

$$\text{Net return} = \text{Gross revenue} - \text{Herbicide program cost} \quad (2)$$

Gross revenue was calculated by multiplying the maize yield from each replication for each program by the average grain price ($0.137 kg^{-1}) received in Nebraska at harvest time during the experimental years (USDA-NASS, 2016). Each herbicide program cost included the average herbicide cost per hectare obtained from three agricultural chemical dealers in Nebraska and a custom application cost of $18.11 ha^{-1} $application^{-1}$.

Statistical Analysis

Data of Palmer amaranth control estimates, density and aboveground biomass reduction, maize yield, gross return, and net return were subjected to ANOVA using the PROC GLIMMIX procedure in SAS version 9.3 (SAS Institute Inc., Cary, NC 27513). Herbicide programs and experimental years were considered fixed effects, whereas replications were considered a random effect in the model. Data were combined over years when there was no year-by- program interaction. The nontreated control was not included in the data analysis for control estimates and percent density and biomass reduction. Before analysis, data were tested for normality and homogeneity of variance using Shapiro-Wilks goodness-of-fit and Levene's test in SAS. To meet the normality and homogeneity of variance assumptions of ANOVA, all data, except maize yield, were arc-sine square root transformed before analysis; however, back-transformed data are presented with mean separation based on the transformed

data. Where the ANOVA indicated herbicide program effects were significant, means were separated at $P \leq 0.05$ with Tukey-Kramer's pairwise comparison test to reduce type I error for the series of comparisons. Pre-planned single degree-of-freedom contrast analysis was accomplished to compare the relative efficacy of PRE-only, POST-only, and PRE fb POST herbicide programs for Palmer amaranth control, biomass reduction, maize yield, and net return.

RESULTS

Year-by-herbicide programs interaction was not significant for Palmer amaranth control, density and biomass reduction, maize yield, gross return, and net return; therefore, data were combined over two experimental years.

Palmer Amaranth Control

Saflufenacil applied PRE provided 60–69% Palmer amaranth control compared to 36–42% control with pyroxasulfone at 3 WAPRE; however, saflufenacil plus dimethenamid-P premix or pyroxasulfone tank-mixed with saflufenacil provided 76–85% Palmer amaranth control at 3 WAPRE (**Table 3**). Palmer amaranth control with PRE herbicides applied alone declined to ≤28% later in the season. The contrast analysis suggested that saflufenacil plus dimethenamid-P as well as pyroxasulfone plus saflufenacil provided 80–82% control compared to 65 and

TABLE 3 | Control of photosystem (PS) II- and 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor-resistant Palmer amaranth with PRE and/or POST residual herbicides in glyphosate-resistant maize in field experiments conducted in Nebraska, USA in 2015 and 2016[a].

Herbicide program[b]	Application timing	Rate (g ae or ai ha^{-1})	Control (%)[c,d,e]			
			3 WAPRE	3 WAPOST	6 WAPOST	At harvest
Nontreated Control	–	–	0	0	0	0
Pyroxasulfone	PRE	110	42 de	10 h	5 f	0 f
Topramezone + dimethenamid-P	POST	750	0	57 de	66 b	23 de
Pyroxasulfone fb topramezone + dimethenamid-P	PRE fb POST	110 750	36 e	28 f	39 cd	18 def
Saflufenacil + dimethenamid-P	PRE	586	78 abc	19 gf	26 de	28 de
Saflufenacil + dimethenamid-P fb topramezone + dimethenamid-P	PRE fb POST	586 750	80 abc	64 d	73 b	65 bc
Saflufenacil	PRE	75	60 cd	8 gh	15 ef	12 ef
Saflufenacil fb topramezone + dimethenamid-P	PRE fb POST	75 750	69 bc	36 ef	58 bc	40 cd
Glyphosate	POST	870	0	86 bc	95 a	88 ab
Dicamba + diflufenzopyr	POST	157	0	69 cd	91 a	89 a
Pyroxasulfone + saflufenacil fb glyphosate + topramezone + dimethenamid-P + atrazine	PRE fb POST	110 + 75 870 + 750 + 560	85 a	98 a	99 a	99 a
Saflufenacil + dimethenamid-P fb glyphosate + topramezone + dimethenamid-P + atrazine	PRE fb POST	586 870 + 750 + 560	76 abc	95 a	99 a	99 a
Saflufenacil + dimethenamid-P fb glyphosate + diflufenzopyr + dicamba + pyroxasulfone	PRE fb POST	780 870 + 157 + 91	83 ab	98 a	99 a	99 a
Saflufenacil + dimethenamid-P fb glyphosate + diflufenzopyr + pendimethalin	PRE fb POST	780 870 + 157 + 1,060	83 ab	98 a	98 a	98 a
Pyroxasulfone + saflufenacil fb glyphosate + diflufenzopyr + dicamba + atrazine	PRE fb POST	110 + 75 870 + 157 + 1,120	79 ab	98 a	99 a	99 a
S.E			4.6	5.4	5.2	6.0
CONTRASTS[f]						
PRE vs. POST	–	–	–	12 vs. 71*	15 vs. 84*	13 vs. 66*
PRE vs. PRE fb POST	–	–	–	12 vs. 77*	15 vs. 83*	13 vs. 77*
POST vs. PRE fb POST	–	–	–	71 vs. 77*	84 vs. 83**	66 vs. 77*

[a] *ae, acid equivalent; ai, active ingredient; fb, followed by; PRE, pre-emergence; POST, post-emergence; S.E, standard error.*

[b] *All POST herbicide program were mixed with AMS, ammonium sulfate (DSM Chemicals North America Inc., Augusta, GA) at 2.5% wt/v and NIS, nonionic surfactant (Induce, Helena Chemical Co., Collierville, TN) at 0.25% v/v. No AMS or NIS were added to PRE herbicides. PRE applications were made within 3 d after planting and POST herbicides were applied when Palmer amaranth was 12–15 cm tall.*

[c] *Year-by- program interaction for Palmer amaranth control was not significant; therefore, data were combined over 2 years. Data were arc-sine square-root transformed before analysis; however, data presented are the means of actual values for comparison based on interpretation from the transformed values.*

[d] *The nontreated control data was not included in the statistical analysis.*

[e] *Means within columns with no common letter(s) are significantly different according to Tukey–Kramer's pairwise comparison test at $P \leq 0.05$.*

[f] *Single degree-of-freedom contrast analysis; *significant ($p < 0.05$); **non-significant ($p > 0.05$).*

TABLE 4 | Contrast means for control and density reduction of photosystem (PS) II- and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor-resistant Palmer amaranth at 3 weeks after pre-emergence herbicide application in glyphosate-resistant maize in field experiments conducted in Nebraska, USA in 2015 and 2016[a].

Herbicide program	Control (%)	Density reduction[b] (%)
Pyroxasulfone vs. Saflufenacil	39 vs. 65*	28 vs. 58*
Pyroxasulfone + Saflufenacil vs. Saflufenacil + Dimethenamid-P	82 vs. 80**	78 vs. 66**
Saflufenacil vs. Pyroxasulfone + Saflufenacil	65 vs. 82*	58 vs. 78*

[a] *Single degree-of-freedom contrast analysis; *significant ($p < 0.05$); **non-significant ($p > 0.05$).*

[b] *Palmer amaranth density data were converted into percent density reduction compared with the nontreated control using the formula: Density reduction (%) = $\frac{(C-B)}{C} \times 100$, where C is the density of the nontreated control plot and B is the density collected from the experimental plot.*

39% control with saflufenacil and pyroxasulfone applied alone, respectively, at 3 WAPRE (**Table 4**).

At 3 and 6 WAPOST, Palmer amaranth control ranged from 86 to 95% with glyphosate compared to 57–66% and 69–95% control with topramezone plus dimethenamid-P and diflufenzopyr plus dicamba, respectively. Palmer amaranth control was reduced to 23% at harvest with a POST-only application of topramezone plus dimethenamid-P compared to glyphosate (88%) and dicamba plus diflufenzopyr (89%). However, ≥95% Palmer amaranth control was achieved with pyroxasulfone plus safluefenacil, or saflufenacil plus dimethenamid-P applied PRE fb glyphosate plus topramezone plus dimethenamid-P plus atrazine, glyphosate plus diflufenzopyr plus dicamba plus pyroxasulfone, glyphosate plus diflufenzopyr plus pendimethalin, or glyphosate plus diflufenzopyr plus dicamba plus atrazine at 3 and 6 WAPOST, and at harvest (**Figure 1**; **Table 3**). Most PRE fb POST herbicide programs resulted in ≥95% Palmer amaranth control throughout the season, except pyroxasulfone fb topramezone plus dimethenamid-P (18–39%), saflufenacil fb topramezone plus dimethenamid-P (36–58%), or saflufenacil plus dimethenamid-P fb topramezone plus dimethenamid-P (64–73%) (**Table 3**). The contrast analysis indicated that PRE fb POST programs provided greatest Palmer amaranth control (77%) compared to POST-only programs (66–71%) at 3 WAPOST and at harvest; however, similar control (83–84%) was achieved at 6 WAPOST (**Table 3**). Similarly, POST-only programs provided 66–71% control compared to <15% control with PRE-only programs at 3 and 6 WAPOST and at harvest.

Palmer Amaranth Density and Biomass Reduction

Palmer amaranth density was reduced by 55–82% with saflufenacil, pyroxasulfone plus saflufenacil, or saflufenacil plus dimethenamid-P compared to 27–29% density reduction with pyroxasulfone at 3 WAPRE. Based on the contrast analysis, pyroxasulfone plus saflufenacil or saflufenacil plus dimethenamid-P provided the greatest density reduction (66–78%) compared to saflufenacil (58%) and pyroxasulfone (39%) (**Table 4**).

Saflufenacil or saflufenacil plus dimethenamid-P applied PRE alone provided 29–56% biomass reduction compared to no biomass reduction with pyroxasulfone at 6 WAPOST (**Table 5**). Glyphosate or dicamba plus diflufenzopyr applied POST alone resulted in ≥97% biomass reduction compared to 66% biomass reduction with topramezone plus dimethenamid-P. The PRE fb POST programs provided 76–99% Palmer amaranth biomass reduction (**Figure 1**), except for saflufenacil fb topramezone plus dimethenamid-P (69%), and pyroxasulfone fb topramezone plus dimethenamid-P (44%) at 6 WAPOST (**Table 5**). The contrast analysis indicated that PRE fb POST programs provided 99% Palmer amaranth biomass reduction compared to POST-only (87%) and PRE-only programs (28%) at 6 WAPOST (**Table 5**).

Maize Injury and Yield

Herbicide injury on maize was negligible (0–6%) and transient without impact on maize yield (data not shown). The nontreated control resulted in the lowest maize yield of 1,042 kg ha^{-1} and was comparable with PRE-only programs including pyroxasulfone (1,870 kg ha^{-1}), saflufenacil (1,990 kg ha^{-1}), or saflufenacil plus dimethenamid-P (4,108 kg ha^{-1}). Most of the PRE fb POST programs resulted in greater maize yield varying from 16,031 to 17,161 kg ha^{-1}, except for pyroxasulfone fb topramezone plus dimethenamid-P (5,600 kg ha^{-1}), saflufenacil fb topramezone plus dimethenamid-P (9,194 kg ha^{-1}), and saflufenacil plus dimethenamid-P fb topramezone plus dimethenamid-P (11,450 kg ha^{-1}) (**Table 5**). Maize yield with POST-only programs varied from 8,525 to 14,324 kg ha^{-1} and glyphosate applied alone resulted in a yield comparable with the highest yielding PRE fb POST programs. The contrast analysis indicated that PRE fb POST programs provided higher (13,617 kg ha^{-1}) maize yield compared to POST-only (11,429 kg ha^{-1}) and PRE-only (2,656 kg ha^{-1}) programs (**Table 5**).

Economic Analysis

The cost of PRE-only and POST-only herbicide programs varied from US $61.01 to US $98.11 ha^{-1} and US $29.79 to US $65.90 ha^{-1}, respectively, compared with $133.00 to $215.64 ha^{-1} for PRE fb POST programs (**Table 6**). The gross income and net returns were in consensus with the yield (**Tables 5**, **6**). The PRE fb POST herbicide programs including pyroxasulfone plus safluefenacil, or saflufenacil plus dimethenamid-P applied PRE fb glyphosate plus topramezone plus dimethenamid-P plus atrazine, glyphosate plus diflufenzopyr plus dicamba plus pyroxasulfone, glyphosate plus diflufenzopyr plus pendimethalin, or glyphosate plus diflufenzopyr plus dicamba plus atrazine applied POST provided the highest net returns ranging from $2,023 to $2,246 ha^{-1} (**Table 6**). The net returns with PRE-only programs were <$475 ha^{-1} compared to $1,123 to $1,965 ha^{-1} with POST-only herbicide programs, signifying the importance of POST programs (**Table 6**). The contrast analysis suggested that PRE fb POST programs provided the highest ($1,724 ha^{-1}) net return fb POST-only ($1,539 ha^{-1}), and PRE-only ($285 ha^{-1}) programs.

TABLE 5 | Effect of herbicide programs on photosystem (PS) II- and 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor-resistant Palmer amaranth density reduction at 3 weeks after PRE, biomass reduction at 6 weeks after POST, maize injury at 2 weeks after PRE, and maize yield at harvest in glyphosate-resistant maize in field experiments conducted in Nebraska, USA in 2015 and 2016[a].

Herbicide program[b]	Application timing	Rate (g ae or ai ha^{-1})	Density reduction[c,d,e,f]	Biomass reduction[c,d,e,f]	Maize yield[e,f] (kg ha^{-1})
			3 WAPRE (%)	6 WAPOST (%)	
Nontreated Control	–	–	0	0	1,042 f
Pyroxasulfone	PRE	110	27 bc	0	1,870 ef
Topramezone + dimethenamid-P	POST	750	0	66 bcde	8,525 cd
Pyroxasulfone fb topramezone + dimethenamid-P	PRE fb POST	110 750	29 bc	44 def	5,600 de
Saflufenacil + dimethenamid-P	PRE	586	67 ab	56 bcde	4,108 ef
Saflufenacil + dimethenamid-P fb topramezone + dimethenamid-P	PRE fb POST	586 750	88 a	76 abcd	11,450 bc
Saflufenacil	PRE	75	55 ab	29 ef	1,990 ef
Saflufenacil fb topramezone + dimethenamid-P	PRE fb POST	75 750	60 ab	69 cde	9,194 cd
Glyphosate	POST	870	0	98 a	14,324 ab
Dicamba + diflufenzopyr	POST	157	0	97 a	11,440 bc
Pyroxasulfone + saflufenacil fb glyphosate + topramezone + dimethenamid-P + atrazine	PRE fb POST	110 + 75 870 + 750 + 560	74 a	99 a	16,044 a
Saflufenacil + dimethenamid-P fb glyphosate + topramezone + dimethenamid-P + atrazine	PRE fb POST	586 870 + 750 + 560	49 ab	99 a	17,161 a
Saflufenacil + dimethenamid-P fb glyphosate + diflufenzopyr + dicamba + pyroxasulfone	PRE fb POST	780 870 + 157 + 91	59 ab	99 a	17,114 a
Saflufenacil + dimethenamid-P fb glyphosate + diflufenzopyr + pendimethalin	PRE fb POST	780 870 + 157 + 1,060	64 ab	99 a	16,031 a
Pyroxasulfone + saflufenacil fb glyphosate + diflufenzopyr + dicamba + atrazine	PRE fb POST	110 + 75 870 + 157 + 1,120	82 a	99 a	16,346 a
S.E			19	11	1,082
CONTRASTS[g]					
PRE vs. POST	–	–	–	28 vs. 87*	2,656 vs. 11,429*
PRE vs. PRE fb POST	–	–	–	28 vs. 99*	2,656 vs. 13,617*
POST vs. PRE fb POST	–	–	–	87 vs. 99*	11,429 vs. 13,617*

[a] *ae, acid equivalent; ai, active ingredient; fb, followed by; PRE, pre-emergence; POST, post-emergence; S.E, standard error.*

[b] *All POST herbicide programs were mixed with AMS, ammonium sulfate (DSM Chemicals North America Inc., Augusta, GA) at 2.5% wt/v and NIS, nonionic surfactant (Induce, Helena Chemical Co., Collierville, TN) at 0.25% v/v. No AMS or NIS were added to PRE herbicides. PRE applications were made within 3 d after planting and POST herbicides were applied when Palmer amaranth was 12–15 cm tall.*

[c] *Data were arc-sine square-root transformed before analysis; however, data presented are the means of actual values for comparison based on interpretation from the transformed values.*

[d] *Percent density and biomass reduction data of non-treated control were not included in analysis. Palmer amaranth density and biomass data were converted into percent density or biomass reduction compared with the nontreated control plots using the formula: Biomass/Density reduction (%) = $\frac{(C-B)}{C} \times 100$, where C is the biomass or density of the nontreated control plot and B is the biomass or density collected from the experimental plot.*

[e] *Year-by-program interaction was not significant; therefore, data were combined over 2 experimental years.*

[f] *Means within columns with no common letter(s) are significantly different according to Tukey–Kramer's pairwise comparison test at $P \leq 0.05$.*

[g] *Single degree-of-freedom contrast analysis; *significant ($p < 0.05$).*

DISCUSSION

The results indicated that PRE programs with multiple sites of action, including saflufenacil plus dimethenamid-P premix or pyroxasulfone tank-mixed with saflufenacil provided higher control (80–82%) compared to saflufenacil or pyroxasulfone applied alone (39–65%) at 3 WAPRE. Similarly, Kohrt and Sprague (2017) reported 75% Palmer amaranth control with saflufenacil applied alone and 80–97% control when saflufenacil was tank-mixed with pyroxasulfone at 45 DAPRE in a 3-year field study in Michigan. Janak and Grichar (2016) also reported >95% Palmer amaranth control with saflufenacil plus dimethenamid-P at 95 DAPRE in maize production fields in Texas. Similarly, Aulakh and Jhala (2015) reported 96% common waterhemp control with saflufenacil plus dimethenamid-P at 15 DAPRE in soybean in Nebraska.

FIGURE 1 | Control of Photosystem (PS) II- and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor-resistant Palmer amaranth with **(A)** pyroxasulfone + saflufenacil followed by (fb) glyphosate + topramezone + dimethenamid-P + atrazine, and **(B)** saflufenacil + dimethenamid-P fb glyphosate + diflufenzopyr + pendimethalin compared to **(C)** nontreated control at 3 weeks after post-emergence.

TABLE 6 | Cost of herbicide programs for controlling photosystem (PS) II- and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor-resistant Palmer amaranth and net income from maize yield in glyphosate-resistant maize in field experiments conducted in Nebraska, USA in 2015 and 2016[a].

Herbicide program[b]	Application timing	Rate (g ae or ai ha^{-1})	Program cost[c] ($ ha^{-1})	Gross income[d] ($ ha^{-1})	Net return[e,f,g] ($ ha^{-1})
Nontreated Control	–	–	0	145 f	145 f
Pyroxasulfone	PRE	110	91.76	260.76 ef	169 f
Topramezone + dimethenamid-P	POST	750	65	1,188 cd	1,123 de
Pyroxasulfone fb topramezone + dimethenamid-P	PRE fb POST	110 750	156.76	779.76 de	623 ef
Saflufenacil + dimethenamid-P	PRE	586	98.11	572.11 ef	474 f
Saflufenacil + dimethenamid-P fb topramezone + dimethenamid-P	PRE fb POST	586 750	133.11	1,595 bc	1,462 cd
Saflufenacil	PRE	75	61.01	274 ef	213 f
Saflufenacil fb topramezone + dimethenamid-P	PRE fb POST	75 750	136.01	1,281 cd	1,145 de
Glyphosate	POST	870	29.79	1,995 ab	1,965 abc
Dicamba + diflufenzopyr	POST	157	65.9	1,594 bc	1,528 bcd
Pyroxasulfone + saflufenacil fb glyphosate + topramezone + dimethenamid-P + atrazine	PRE fb POST	110 + 75 870 + 750 + 560	211.18	2,234 a	2,023 ab
Saflufenacil + dimethenamid-P fb glyphosate + topramezone + dimethenamid-P + atrazine	PRE fb POST	586 870 + 750 + 560	144.23	2,390 a	2,246 a
Saflufenacil + dimethenamid-P fb glyphosate + diflufenzopyr + dicamba + pyroxasulfone	PRE fb POST	780 870 + 157 + 91	215.64	2,384 a	2,168 a
Saflufenacil + dimethenamid-P fb glyphosate + diflufenzopyr + pendimethalin	PRE fb POST	780 870 + 157 + 1,060	172.51	2,233 a	2,060 a
Pyroxasulfone + saflufenacil fb glyphosate + diflufenzopyr + dicamba + atrazine	PRE fb POST	110 + 75 870 + 157 + 1,120	212.04	2,276 a	2,064 a
S.E.			–	150	150
CONTRASTS[h]					
PRE vs. POST	–	–	–	–	285 vs. 1,539*
PRE vs. PRE fb POST	–	–	–	–	285 vs. 1,724*
POST vs. PRE fb POST	–	–	–	–	1,539 vs. 1,724*

[a] *ae, acid equivalent; ai, active ingredient; fb, followed by; PRE, pre-emergence; POST, post-emergence; S.E, standard error.*

[b] *All POST herbicide programs were mixed with AMS, ammonium sulfate (DSM Chemicals North America Inc., Augusta, GA) at 2.5% wt/v and NIS, nonionic surfactant (Induce, Helena Chemical Co., Collierville, TN) at 0.25% v/v. No AMS or NIS were added to PRE herbicides. PRE applications were made within 3 d after maize planting and POST herbicides were applied when Palmer amaranth was 12–15 cm tall.*

[c] *Program cost includes the average cost of herbicide, AMS, and NIS; and the cost of application (US $18.11 ha^{-1} application^{-1}) from two independent sources in Nebraska.*

[d] *Gross revenue was calculated by multiplying maize yield for each program by the average grain price received in Nebraska at harvest time during the experimental years ($0.137 kg^{-1}, USDA-NASS, 2016).*

[e] *Net return was calculated as gross income from glyphosate-resistant maize yield minus herbicide program cost.*

[f] *Data were arc-sine square-root transformed before analysis; however, data presented are the means of actual values for comparison based on interpretation from the transformed values. Year-by- program interaction was not significant; therefore, data were combined over two experimental years.*

[g] *Means within columns with no common letter(s) are significantly different according to Tukey-Kramer's pairwise comparison test $P \leq 0.05$.*

[h] *Single degree-of-freedom contrast analysis; *significant ($p < 0.05$); **non-significant ($p > 0.05$).*

The POST herbicide programs including tank-mixture of active ingredients with residual activity and multiple sites of action provided 95–99% Palmer amaranth control compared to topramezone plus dimethenamid-P (57–70%) (**Figure 1**; **Table 3**). Similarly, Wiggins et al. (2015) reported 95–99% control of GR Palmer amaranth with glyphosate plus *S*-metolachlor plus mesotrione plus atrazine, thiencarbazone-methyl plus tembotrione plus atrazine, or glyphosate plus atrazine at 28 DAPOST. However, the unacceptable control with topramezone plus dimethenamid-P may be attributed to a high-level resistance (14- to 23-fold) of Palmer amaranth to topramezone (Jhala et al., 2014). In the same study, Jhala et al. (2014) reported only 58% control of PS II- and HPPD-inhibitor-resistant Palmer amaranth with topramezone compared to 99% control of the susceptible biotypes or 87–99% control of resistant Palmer amaranth when topramezone was tank-mixed with atrazine at 21 DAPOST. Although Palmer amaranth was resistant to PRE and POST applied atrazine or topramezone, the POST application of glyphosate plus topramezone plus dimethenamid-P plus atrazine controlled Palmer amaranth ≥95% throughout the season due to the synergistic interactions of atrazine and topramezone as well as the presence of glyphosate (**Table 3**). Previous studies have reported synergistic interaction when a PS-II inhibitor such as atrazine is applied in tank-mixture with an HPPD inhibitor for POST weed control in maize (Abendroth et al., 2006; Hugie et al., 2008), including control of atrazine- and HPPD-inhibitor-resistant Palmer amaranth (Jhala et al., 2014). Similarly, synergistic interaction between HPPD- and PS II-inhibiting herbicides has been reported for control of giant ragweed (*Ambrosia trifida* L.), common lambsquarters (*Chenopodium album* L.), velvetleaf (*Abutilon theophrasti* Medik.), common waterhemp, and redroot pigweed (*Amaranthus retroflexus* L.) (Abendroth et al., 2006; Hugie et al., 2008; Woodyard et al., 2009a,b). At the physiological level, atrazine binds at the Q_B binding site of DI protein of PS II and inhibits the electron transport during photosynthesis (Fuerst and Normanm, 1991). On the other hand, mesotrione inhibits HPPD enzyme synthesis which leads to depletion of plastoquinone resulting in decreasing electron transport during photosynthesis and also inhibit carotenoids and tocopherols synthesis (Hess, 2000; Mitchell et al., 2001; McCurdy et al., 2008). Both PS II- and HPPD-inhibitors block the electron transport in PS II due to their complementary mode of action and lead to the accumulation of reactive oxygen species and free radicals that damage the foliar tissue membranes (Hess, 2000).

The POST-only programs including glyphosate or dicamba plus diflufenzopyr resulted in 88–95% Palmer amaranth control and were comparable with the PRE fb POST herbicide programs, except at 3 WAPOST (**Table 3**). Jhala et al. (2014) reported 90–99% control of the same Palmer amaranth biotype with glyphosate, glufosinate, or dicamba at 21 DAPOST in a greenhouse study. Similarly, Norsworthy (2004) reported 100% Palmer amaranth control with a single or sequential application of glyphosate at 5 WAPOST. Likewise, Crow et al. (2016) reported >87% control of >20 cm tall Palmer amaranth with dicamba plus diflufenzopyr applied alone or in tank-mixture with glyphosate, mesotrione, tembotrione, mesotrione plus rimsulfuron, or tembotrione plus thiencarbazone at 4 WAPOST. Nonetheless, the dependence on POST herbicides with a single site of action must be avoided to prevent the evolution of HR weeds (Chahal and Jhala, 2015; Chahal et al., 2017; Ganie and Jhala, 2017b). Furthermore, the confirmation of GR Palmer amaranth in a GR soybean/maize production field in south-central Nebraska signifies that dependence on a single POST herbicide program is not a reliable option (Chahal et al., 2017).

Weed density at the time of POST herbicide application plays an important role in determining herbicide efficacy and the number of weeds surviving (Dieleman et al., 1999). Bell et al. (2015) reported that flumioxazin plus pyroxasulfone applied PRE in soybean reduced Palmer amaranth emergence and demonstrated a potential to enhance the efficacy of POST herbicides and reduce selection pressure by exposing a lower number of Palmer amaranth plants to POST herbicides. Similarly, Meyer et al. (2016) reported ≥97% control of Palmer amaranth and common waterhemp for more than 3 weeks of applying isoxaflutole plus *S*-metolachlor plus metribuzin, *S*-metolachlor plus mesotrione, or flumioxazin plus pyroxasulfone. In addition, the application of residual herbicides in a tank-mixture with a foliar active POST herbicide is obligatory for the season-long control of Palmer amaranth because of its extended emergence period that typically begins from early May to late September (Jha and Norsworthy, 2009; Ward et al., 2013).

Palmer amaranth density and biomass reduction were in consensus with the visual estimates of Palmer amaranth control at 3 WAPRE and 6 WAPOST, respectively. Jhala et al. (2014) and Kohrt and Sprague (2017) reported an agreement between Palmer amaranth control estimates and biomass reduction with herbicide programs tested. The PRE fb POST programs resulted in greater yield compared to a PRE- or POST-only herbicide program, excluding a POST-only application of glyphosate. Although higher rainfall was received at the experimental site in 2015 during the critical period of maize growth from the V2 to V8 development stages during June and July compared to 2016 and the 30-year average (**Table 1**), no difference in Palmer amaranth control, density and biomass reduction, and maize yield was observed between the two experimental years. However, previous studies have reported greater weed control and maize yield in years receiving higher rainfall compared to dry years (Whaley et al., 2009; Petcu et al., 2015). A higher level of Palmer amaranth control in this study with glyphosate was due to the fact that glyphosate had not been applied at the research site for the last 8 years because the field was under continuous maize seed production (Jhala et al., 2014). Similarly, the economic analysis indicated higher net returns with PRE fb POST herbicide programs with multiple herbicide sites of action even though the diversified herbicide mixtures were more expensive. Bradley et al. (2000) also reported that PRE fb POST programs including acetochlor or *S*-metolachlor applied PRE fb dicamba or glufosinate plus atrazine applied POST were among the high net income-producing programs with excellent weed control in maize. Norsworthy (2004) also reported greater gross profit with chlorimuron plus metribuzin or sulfentrazone applied PRE

fb glyphosate compared to glyphosate without PRE herbicide applications.

CONCLUSION

The evolution of PS II- and HPPD-inhibitor-resistant Palmer amaranth has become a concern for field maize, maize grown for popcorn and seed production in Nebraska, USA. The results of this study suggested that season-long Palmer amaranth management is possible by including overlapping residual herbicides in synergistic tank-mixtures of PS II- and HPPD-inhibiting herbicides. In addition, application of PS II- and HPPD-inhibiting herbicides in a tank-mixture with glyphosate, dicamba plus dimethenamid-P, or pyroxasulfone provided an effective strategy for Palmer amaranth control due to the synergistic action of atrazine and topramezone; along with the residual activity of atrazine, dimethenamid-P, or pyroxasulfone; and glyphosate or dicamba as additional effective sites of action to reduce selection pressure. However, Culpepper (2006) emphasized that no single herbicide program will provide a consistently satisfactory control of Palmer amaranth for more than a 4- to 5-year period. Therefore, it has become crucial to incorporate feasible non-chemical weed control tools including tillage, rotation of different HR cultivars with conventional crop cultivars, row spacing, and harvest weed seed control etc., for an integrated HR Palmer amaranth management.

AUTHOR CONTRIBUTIONS

PC conducted the experiments, analyzed the data, and edited the manuscript; ZG assisted in the experiments and writing of the manuscript; and AJ conceptualized and designed the research and edited the manuscript.

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Population Genetic Structure in Glyphosate-Resistant and -Susceptible Palmer Amaranth (*Amaranthus palmeri*) Populations Using Genotyping-by-sequencing (GBS)

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Palmer amaranth (*Amaranthus palmeri*) is a major weed in United States cotton and soybean production systems. Originally native to the Southwest, the species has spread throughout the country. In 2004 a population of *A. palmeri* was identified with resistance to glyphosate, a herbicide heavily relied on in modern no-tillage and transgenic glyphosate-resistant (GR) crop systems. This project aims to determine the degree of genetic relatedness among eight different populations of GR and glyphosate-susceptible (GS) *A. palmeri* from various geographic regions in the United States by analyzing patterns of phylogeography and diversity to ascertain whether resistance evolved independently or spread from outside to an Arizona locality (AZ-R). Shikimic acid accumulation and *EPSPS* genomic copy assays confirmed resistance or susceptibility. With a set of 1,351 single nucleotide polymorphisms (SNPs), discovered by genotyping-by-sequencing (GBS), UPGMA phylogenetic analysis, principal component analysis, Bayesian model-based clustering, and pairwise comparisons of genetic distances were conducted. A GR population from Tennessee and two GS populations from Georgia and Arizona were identified as genetically distinct while the remaining GS populations from Kansas, Arizona, and Nebraska clustered together with two GR populations from Arizona and Georgia. Within the latter group, AZ-R was most closely related to the GS populations from Kansas and Arizona followed by the GR population from Georgia. GR populations from Georgia and Tennessee were genetically distinct from each other. No isolation by distance was detected and *A. palmeri* was revealed to be a species with high genetic diversity. The data suggest the following two possible scenarios: either glyphosate resistance was introduced to the Arizona locality from the east, or resistance

evolved independently in Arizona. Glyphosate resistance in the Georgia and Tennessee localities most likely evolved separately. Thus, modern farmers need to continue to diversify weed management practices and prevent seed dispersal to mitigate herbicide resistance evolution in *A. palmeri*.

Keywords: Palmer amaranth, population genetics, glyphosate, herbicide resistance, genetic relatedness, SNP molecular markers, phylogeography

INTRODUCTION

Since the introduction of transgenic soybean, corn, and cotton in the mid-1990s, herbicide-resistant varieties of these crops have largely replaced conventional varieties in the United States (Coupe and Capel, 2016). In 1996, glyphosate-resistant (GR) (Roundup Ready) crops were commercialized and as a result global glyphosate usage rose by about 15-fold (Benbrook, 2016), dominating the current herbicide market (Duke, 2017). The widespread reliance on glyphosate to the exclusion of all other weed control methods has resulted in high selection pressure and the evolution of GR weeds, including Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Culpepper et al., 2006), which is now a major threat to many U.S. food production systems (Beckie, 2011).

Amaranthus palmeri is a dioecious, annual species with prolific seed production, pollen-mediated gene flow due to obligate outcrossing, and high genetic variability (Franssen et al., 2001; Sellers et al., 2003; Ward et al., 2013). As a member of the *Amaranthaceae* family, *A. palmeri* is native to the southwestern United States and northwestern Mexico, having first been documented in Sonora, California, Arizona, New Mexico, and Texas in the late 19th century. During the early 20th century, the species started to spread east and northeast, probably because of human mediated seed dispersal (Sauer, 1957; Ward et al., 2013). In recent years, *A. palmeri* has expanded its distribution as far north as Ontario, Canada and as far east as Massachusetts, United States (Kartescz, 2014). The species made its first occurrence on the annual listing of most troublesome weeds in South Carolina in 1989 (Webster and Coble, 1997). By 2009 the weed was ranked the most troublesome weed in cotton in the Southern United States (Webster and Nichols, 2012; Ward et al., 2013).

Resistance to glyphosate in *A. palmeri* was first reported from a GR cotton field in Georgia in 2004. Shortly after, another case was reported from North Carolina in 2005 (Culpepper et al., 2006, 2008). As of 2017, GR *A. palmeri* was found in 27 U.S. states, Argentina, and Brazil (Scott et al., 2007; Norsworthy et al., 2008; Steckel et al., 2008; Berger et al., 2016; Heap, 2017; Küpper et al., 2017). The primary mechanism of glyphosate resistance in *A. palmeri* has been identified as the amplification of the gene encoding the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which produces increased *EPSPS* transcription and protein activity (Gaines et al., 2010). The same glyphosate resistance mechanism has independently evolved in six other species (Salas et al., 2012; Jugulam et al., 2014; Lorentz et al., 2014; Chatham et al., 2015; Chen et al., 2015; Wiersma et al., 2015; Malone et al., 2016; Ngo et al., 2017). *EPSPS* gene amplification has also transferred via pollen-mediated inter-specific hybridization from *A. palmeri* to *A. spinosus* (Nandula et al., 2014).

Evolutionary models have identified that herbicide resistance dynamics are largely influenced by gene flow, seed immigration, and fitness cost (Maxwell et al., 1990). Further factors include mutation rate, the mode of inheritance, dominance of the resistance trait, seed bank turnover rate, herbicide chemistry and persistence, as well as herbicide usage patterns (Georghiou and Taylor, 1986; Jasieniuk et al., 1996; Neve, 2008). For instance, glyphosate used prior to crop emergence is predicted to have a low risk of resistance evolution while post-emergence use increases the risk, and reliance on glyphosate exclusively increases the risk even further (Neve, 2008). A simulation model for *A. palmeri* predicted that five applications of glyphosate each year with no other herbicides would result in resistance evolving in 74% of the simulated populations (Neve et al., 2011).

Amaranthus palmeri management is complicated by the fact that this species evolved resistance to five different modes of action (Chahal et al., 2015; Heap, 2017; Nakka et al., 2017; Schwartz-Lazaro et al., 2017), the lack of discovery of new modes of action for the past three decades, and the high cost of bringing new herbicides to the market (Duke, 2012). The overuse of and sole reliance on glyphosate and the resulting evolution of resistant weeds exhausted the lifespan of a once-in-a-century herbicide (Duke and Powles, 2008) and threatens current crop production practices by diminishing available weed management options further. Therefore, knowledge about the origin and geographical pathways of glyphosate resistance in *A. palmeri*, one of the most problematic GR weeds in the United States, is crucial to avoid repeating the same mistakes made with glyphosate with other modes of action that are still successful at controlling weeds in the field.

This study focuses on a GR population identified in a no-till cotton-wheat double crop system near Phoenix, Arizona (AZ), United States. Glyphosate was used as the sole weed management technique for the cotton portion of the production cycle for more than 10 year before glyphosate resistance was first suspected in 2012, 8 years after the first report in the species. The objective was to determine whether GR *A. palmeri* immigrated to the AZ locality from an outside location via seed or pollen-mediated gene flow, or if resistance evolved at or nearby the location in AZ independently via parallel evolution. To answer this question, single nucleotide polymorphisms (SNPs) generated by genotyping-by-sequencing (GBS) to identify numerous sequence differences at presumably random parts of the genome (Brumfield et al., 2003), were used. The GR population from AZ and seven other populations from different locations in

the United States were investigated for their degree of genetic relatedness to identify patterns of phylogeography and variation on an intraspecific level.

MATERIALS AND METHODS

Plant Material and DNA Isolation

Twelve *A. palmeri* individuals (six males and six females) from each of eight different locations in the United States were used for the analyses (**Table 1**), except for AZ-S2 for which only eleven individuals were used to leave a blank on the plate. Locations AZ-S1, AZ-S2, KS-S, GA-S and NE-S were verified as glyphosate-susceptible (GS) and locations AZ-R (Molin et al., 2017b), GA-R (Culpepper et al., 2006), and TN-R (Steckel et al., 2008) were verified as GR. The populations were collected between 2004 and 2012, except for AZ-S2 which was maintained by the USDA-ARS Germplasm Resource Information Network (accession number: Ames 5370) since its collection in 1981 and serves as an outgroup to prevent ascertainment bias (Wakeley et al., 2001; Akey et al., 2003). AZ-S1 was collected about 240 km southeast of Buckeye, AZ (AZ-R) where no agronomic crop production has occurred since the 1960s to provide a recently collected Arizona-native GS population that is fairly sympatric with AZ-R.

For DNA extraction, young leaf tissue was collected, immediately frozen in liquid nitrogen, and stored at −80°C. For GR samples only individuals that survived 800 g a.e. ha^{-1} glyphosate (Roundup WeatherMAX, Monsanto) were used. DNA extraction was performed following a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Doyle, 1991; Küpper et al., 2017) and quantified on a NanoDrop spectrophotometer (Thermo Scientific) followed by normalization. Gel electrophoresis and enzyme digestion with *Hind*III (Thermo Scientific) were performed on all or 10% of the samples, respectively, to confirm DNA quality and normalization.

Herbicide Resistance Characterization

To confirm glyphosate resistance and susceptibility for the individuals used for GBS, an *in vivo* shikimate accumulation assay with excised leaf tissue (Shaner et al., 2005) was conducted. Additionally, *EPSPS* gene copy number was determined for all samples. Four-mm leaf disks from each individual were exposed to glyphosate at 0, 100, 500, and 1000 μm glyphosate for 16 h. Shikimate accumulation was measured on a spectrophotometer (Synergy 2 Multi-Mode Reader, BioTek). A shikimate standard curve was used to calculate the ng shikimate μl^{-1} accumulation above the background level. Each biological sample was run in three technical replicates for each dose.

For *EPSPS* gene copy number determination, DNA concentrations were adjusted to 5 ng μl^{-1} and primer sets (ALSF2 and ALSR2, EPSF1 and EPSR8) and qPCR conditions were used as previously described (Gaines et al., 2010). Quantitative PCR was performed using SYBR green master-mix (BioRad) on a CFX Connect™ Real-Time PCR Detection System (BioRad). *EPSPS* gene copy number relative to *ALS* was determined using the $2\Delta C_T$ method where $\Delta C_T = C_{T(ALS)} - C_{T(EPSPS)}$. Each biological sample was run in three technical replicates.

Greenhouse dose response studies were conducted to confirm pyrithiobac-sodium [acetolactate synthase (ALS inhibitor)] resistance in AZ-R with AZ-S1 as a susceptible control. The experiments took place at the University of Arizona Campus Agricultural Center in Tucson, AZ, United States. Seeds were planted in artificial soil mix in 10 cm pots and after emergence seedlings were thinned, fertilized, and irrigated as needed. ALS-inhibitor treatments included 0, 0.0001, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 kg a.i. ha^{-1} pyrithiobac-sodium (Staple LX, DuPont) with 0.25% v/v non-ionic surfactant (Activator 90, Loveland Products). Plants were sprayed at the six-leaf stage using a CO_2 pressurized backpack sprayer equipped with a three nozzle (TeeJet XR8001VS) boom delivering a carrier volume of 112 L ha^{-1} at 172 kPa at 4 km h^{-1}. The experimental design was random with five replications per dose. Above-ground biomass was harvested 27 days after treatment (DAT), dried at 60°C and dry weight was measured.

The *ALS* gene was sequenced from three individuals each of the AZ-R, GA-R, and TN-R populations using the same DNA used for the *EPSPS* copy number test and SNP calling. *ALS* gene sequencing was conducted as previously described (Küpper et al., 2017).

TABLE 1 | *Amaranthus palmeri* populations used in the study, their origin and time of collection.

Abbreviation	Origin	Collection year
AZ-R	Buckeye, Arizona	2012
AZ-S1	Sahuarita, Arizona	2012
AZ-S2	Tucson, Arizona	1981
GA-R	Macon, Georgia	2006
GA-S	Worth County, Georgia	2004
KS-S	Ottawa, Kansas	2005
NE-S	Shickley, Nebraska	2011
TN-R	Jackson, Tennessee	2007

Genotyping and SNP Filtering

After DNA extraction, GBS and bi-allelic SNP calling was conducted by the Biotechnology Resource Center at Cornell University, Ithaca, NY, United States (Elshire et al., 2011). A total of 95 samples (eleven samples for AZ-S2 and twelve samples for the remaining populations) were digested with *Ape*KI, individually barcoded, run on an Illumina HiSeq2500 single-end 100 bp sequencing lane, and later trimmed to 64 bp for analysis. The GBS UNEAK pipeline in TASSEL v. 3.0.173 (Bradbury et al., 2007; Lu et al., 2013; Glaubitz et al., 2014) was used for *de novo* clustering of the sequences. The resulting SNP calls were then filtered for depth and missing values at any given locus with VCFtools v. 0.1.11 (Danecek et al., 2011) after which 4,566 filtered SNPs remained. Through further pruning, 70.4% of the filtered SNPs were excluded due to percentage of missing data points (>5%), minor allele frequency (MAF) values lower than 0.05, or more than 80% loci with more than one allele, leaving 1,351 SNPs which were informative (Supplementary Information Figure 2). Except

where indicated, all analyses were performed on the panel of 1,351 SNPs.

EPSPS gene copies in GR *A. palmeri* individuals are randomly dispersed throughout the whole genome (Gaines et al., 2010). They can be found embedded in a complex array of repetitive elements and putative helitron sequences referred to as the '*EPSPS* cassette' (Molin et al., 2017a). Because SNPs are called genome-wide, an overrepresentation of called SNPs within these sequences could potentially lead to clustering of GR individuals regardless of their actual genetic relatedness. To avoid such bias, the sequences flanking the 1,351 SNPs were aligned to the *A. palmeri* 1,044 bp *EPSPS* sequence (Gaines et al., 2010) and the 297,445 bp *A. palmeri EPSPS* cassette (Molin et al., 2017a). The 1,351 SNP sequences were also aligned to the chloroplast genome of spinach (*Spinacia oleracea*) and the mitochondrial genome of sugar beet (*Beta vulgaris*) to identify SNPs specific to the cytoplasmic regions.

Analysis of Genetic Structure

The putative population genetic structure was explored using the model-based Bayesian analysis implemented in STRUCTURE v2.3.4 (Pritchard et al., 2000). The number of sub-populations K in the dataset was determined by the averaged likelihood at each K $\left[\ln \Pr(X \mid K) \text{ or } \ln(K_n)\right]$ and the variance between replicates was determined by running a continuous series of K = 1–15 to determine the optimal number of populations present within the 95 individuals. The analysis was carried out using a burn-in of 30,000 iterations and a run length of 100,000 Markov Chain Monte Carlo (MCMC) replications in ten independent runs. Prior knowledge about the number of populations was not included. The optimum number of clusters was predicted following the *ad hoc* statistic ΔK (Evanno et al., 2005) using Structure Harvester v0.6.94 (Earl, 2012). For the final K analysis a burn-in of 30,000 with a run length of 500,000 MCMC replications and 20 independent runs were used. To be conservative, the analyses were run assuming admixture and correlated allele frequencies (Porras-Hurtado et al., 2013). The Greedy algorithm by CLUMPP v1.1.2 (Jakobsson and Rosenberg, 2007) was used to obtain the individual and cluster membership coefficient matrices over the 20 runs which were then plotted using *distruct* 1.1 (Rosenberg, 2004).

The following information and tests were calculated in R v3.4.1. The number of alleles (N_a) and allelic richness (A_R) per population were calculated using the package 'PopGenReport.' Observed (H_O) and expected heterozygosity (H_E) were calculated with 'adegenet' (Jombart and Ahmed, 2011; Adamack and Gruber, 2014). The inbreeding coefficient (F_{IS}) was calculated following the formula $1 - \left(H_o / H_E\right)$. Principal component analysis (PCA) was conducted using 'SNPRelate' and 'gdsfmt' (Zheng et al., 2012). Calculations for Nei's distance (D_{ST}) (Nei, 1972) and pairwise fixation index (F_{ST}) among populations were performed with 1,000 bootstrap replications using 'StAMPP' (Pembleton et al., 2013). The analysis of molecular variance (AMOVA) (10,000 permutations) and the Mantel test (10,000 permutations) for isolation by distance analysis were performed using 'poppr' (Kamvar et al., 2014) and 'adegenet' (Dray and Dufour, 2007), respectively. The phylogenetic analysis was based on the UPGMA clustering method using the Hasegawa-Kishino-Yano (HKY) genetic distance model in the software Geneious v10.0.6.

RESULTS

Herbicide Resistance Characterization

Glyphosate-susceptible *A. palmeri* populations showed higher shikimate accumulation (11.8–146.3 ng μl^{-1} at 500 μm glyphosate) than GR populations (0–3.8 ng μl^{-1}) (**Figure 1A**) while GR populations showed higher genomic *EPSPS* copy number (individuals measured from 25- to 250-fold) than GS populations (individuals measured from onefold to twofold) (mean *EPSPS* copy number shown in **Figure 1B**). Thus, the mechanism of glyphosate resistance was determined to be *EPSPS* gene duplication in all the sampled GR populations (Gaines et al., 2010). The average copy numbers for the GR populations were within a similar range (**Figure 1B**). The 500 μm glyphosate concentration was a clear discriminating dose between GR and GS individuals.

Resistance to the ALS-inhibitors, commonly used in cotton, was suspected in AZ-R as well, thus a dose response with pyrithiobac-sodium and sequencing of the *ALS* gene was conducted. The ED_{50} values for dry weight (pyrithiobac-sodium dose causing 50% reduction in dry weight) were 6.9 and 1.3 g a.i. ha^{-1} for AZ-R and AZ-S1, respectively (P = 0.027) (Supplementary Information Figure 1).

Sequencing the *ALS* gene in three GR individuals each from AZ-R, GA-R and TN-R, showed that one TN and one AZ plant were heterozygous for a mutation from TGG → TTG resulting in an amino acid change from tryptophan to leucine at position 574 ($W_{574}L$). A different AZ plant was heterozygous for a AGC → AAC mutation resulting in a change from serine to asparagine at position 653 ($S_{653}N$). No individual carried both mutations within the same allele. The remaining individuals tested showed no mutations at these positions (Supplementary Information Table 1). Both mutations have been reported before in *A. palmeri* from Mississippi, United States, and Brazil (Molin et al., 2016; Küpper et al., 2017) while only $S_{653}N$ was reported from GA (Berger et al., 2016). The mutation at $W_{574}L$ is known to confer resistance to triazolopyrimidines, sulfonylureas, imidazolinones, and pyrimidinylthio-benzoates (including pyrithiobac-sodium), whereas the $S_{653}N$ mutation confers resistance to imidazolinones and the pyrimidinylthio-benzoates only (McNaughton et al., 2005; Whaley et al., 2006; Patzoldt and Tranel, 2007; Laplante et al., 2009; Yu et al., 2012). Both mutations are known to be inherited as a dominant trait (Tranel and Wright, 2002; Powles and Yu, 2010). It is suspected that a non-target site mechanism conferring resistance to ALS inhibitors exists (Küpper et al., 2017) and such a mechanism may also be present in AZ-R ALS-resistant individuals that lack target-site ALS mutations.

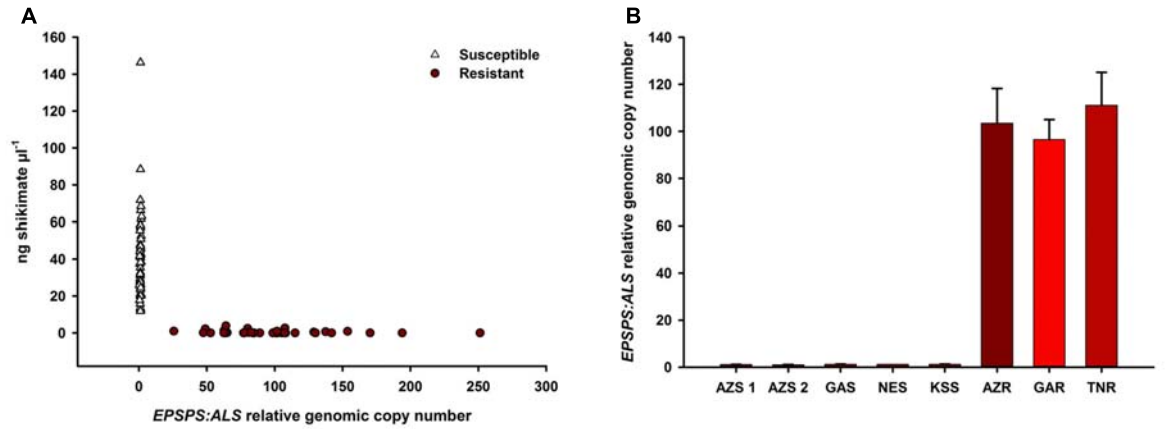

FIGURE 1 | Correlation of shikimate accumulation and *EPSPS* genomic copy number in all individuals of each of the glyphosate-resistant and -susceptible *A. palmeri* populations. Shikimate accumulation was measured after incubation in 500 µm glyphosate in an *in vivo* leaf disk assay Increase in genomic copy number of *EPSPS* is relative to *ALS* as measured using qPCR on genomic DNA **(A)**. *EPSPS* genomic copy number by population **(B)**.

Influence of Glyphosate Resistance Mechanism on GBS Analysis

The *EPSPS* gene was found to have five potential cutting sites for the enzyme *Ape*KI used in this GBS study, while the entire *EPSPS* cassette has 289 potential cutting sites. No SNPs were called within the *EPSPS* gene and only one SNP was called from within the *EPSPS* cassette which was removed from further analysis. Thus, the mechanism of glyphosate resistance (repetitive *EPSPS* gene copies) is not expected to influence the analysis of genetic relatedness in this case.

Within Population Genetic Diversity

The 1,351 loci used for this study had an average percentage of missing data of 1.07% and an average MAF of 0.159. A high degree of polymorphism (MAF $\geq$ 0.30) was found in 14.41% of the dataset. The proportion of MAF $<$ 0.1 was 45.37%. One AZ-S2 individual was removed from all future analysis because it was an extreme outlier. The observed number of alleles within a population ranged from 2,017 (AZ-S2) to 2,395 (KS-S), with an average of 2,217. Levels of heterogeneity were compared among populations to examine genetic variability within populations. Allelic richness (A_R) ranged from 1.445 (AZ-S2) to 1.654 (KS-S) with an average of 1.560. The observed (H_O) and expected heterozygosity (H_E) values ranged from 0.161 (AZ-S1) to 0.219 (TN-R) and from 0.163 (AZ-S2) to 0.211 (KS-S/GA-R), respectively, with an average of 0.193. Low values for H_O indicate small effective population sizes or population bottlenecks. The H_O values in most populations were less than the H_E values (Supplementary Information Figure 3), with the exception of GA-S, TN-R and AZ-S2. The inbreeding coefficient (F_{IS}) for these three populations was negative. AZ-R was the population with the highest F_{IS} value (0.121) (**Table 2**).

Consensus Tree

The consensus tree separated GA-S, TN-R, NE-S, and AZ-S2 with over 86% certainty with GAS, TN-R, and AZ-S2 being the most divergent populations. AZ-S1, AZ-R, and KS-S clustered together. Except for KS-S and AZ-R, all individuals clustered within their sampling location (**Figure 2**), The long branch lengths for the individuals indicate high within-individual genetic variability.

Principal Component Analysis

To confirm this clustering, a similar pattern of differentiation among populations was constructed using PCA which is used to bring out strong patterns in the dataset based on their variance. The first two principal component (PC) axes cumulatively accounted for 16.69% of the total variation. PCA showed that all individuals clustered according to their collection site. Three distinct outgroups (GA-S, TN-R, and AZ-S2) emerged while the remaining individuals from the other five populations clustered into one group. The first dimension (PC 1) accounted for 8.91% of the variation and roughly separated GR from GS individuals (**Figure 3A**). After removing GA-S, TN-R, and AZ-S2, AZ-R did not separate from the cluster with KS-S and AZ-S1, while GA-R and NE-S clustered distinctively according to PC 2,

TABLE 2 | Population information and genetic variability estimates based on 1,351 SNP loci in eight populations of *A. palmeri*.

Population	*n*	N_a	A_R	H_O	H_E	F_{IS}
AZ-R	12	2,381	1.650	0.182	0.207	0.121
AZ-S1	12	2,299	1.581	0.161	0.181	0.110
GA-R	12	2,307	1.617	0.193	0.211	0.085
GA-S	12	2,031	1.474	0.191	0.183	−0.044
NE-S	12	2,245	1.579	0.184	0.199	0.075
KS-S	12	2,395	1.654	0.194	0.211	0.081
TN-R	12	2,059	1.477	0.219	0.183	−0.197
AZ-S2	10	2,017	1.445	0.180	0.163	−0.104

n, number of individuals per population; N_a, observed number of alleles; A_R, allelic richness; observed (H_O) and expected (H_E) heterozygosity; F_{IS}, inbreeding coefficient.

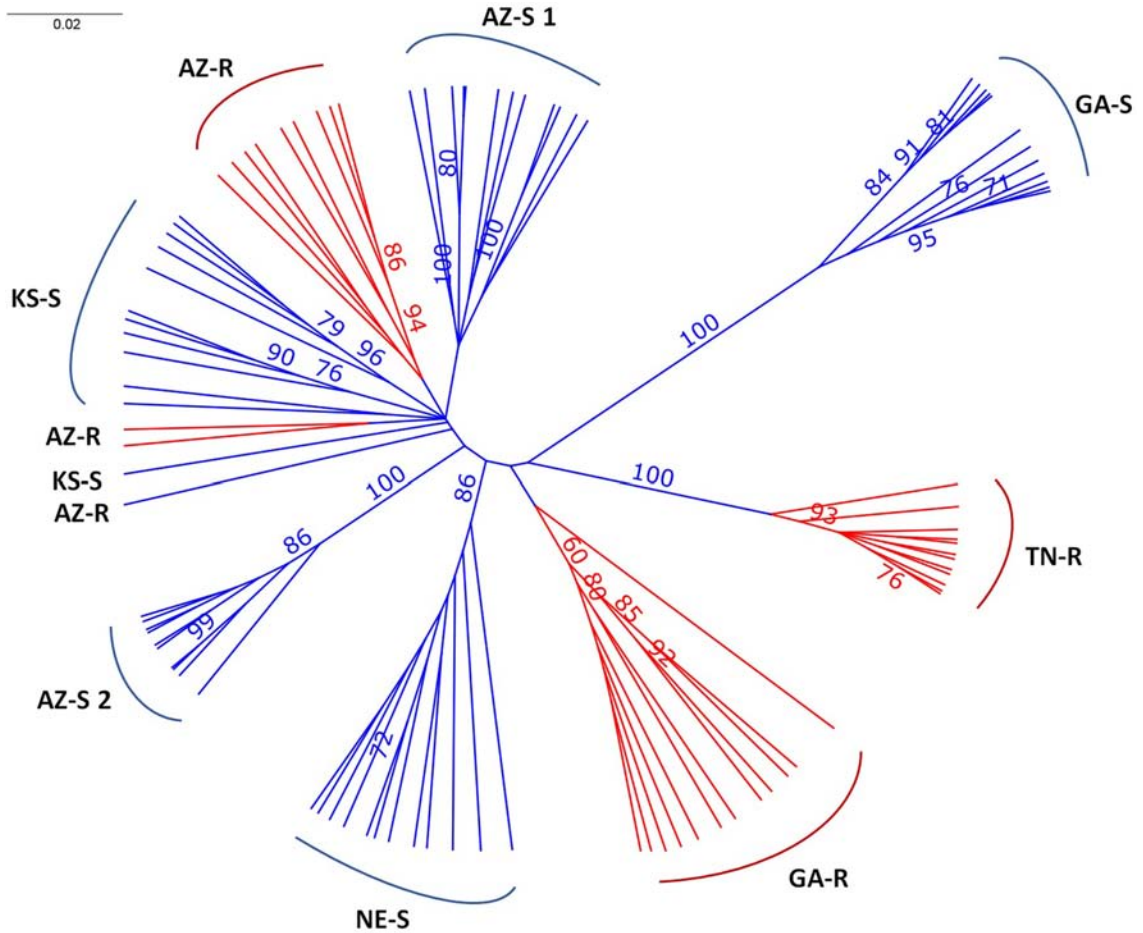

FIGURE 2 | Unrooted UPGMA consensus tree after 1,000 bootstrap replications depicting the relationships of *A. palmeri* individuals from eight populations. Bootstrap values > 70% at nodes are indicated.

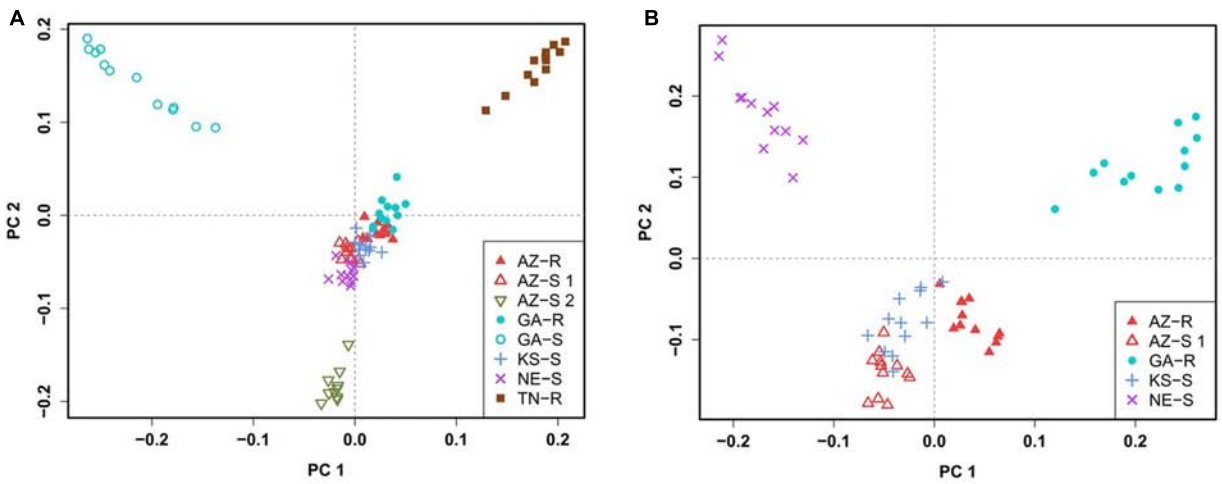

FIGURE 3 | Clustering of *A. palmeri* populations based on principal component analysis (PCA) using the filtered and pruned whole dataset of 1,351 SNPs. Analysis was done on all eight populations **(A)** and on a subset of populations removing the three outlier groups AZ-S2, GA-S, and TN-R **(B)**. Each point represents an individual colored according to the collection site. Glyphosate-resistant individuals are marked by filled symbols and susceptible individuals are marked by empty symbols. Individuals from the same U.S. state have the same symbols.

supporting the UPGMA consensus tree. PC 1 in the second PCA on the subset of populations accounted for 6.87% of the variation in the dataset and again roughly separated GR from GS individuals. Individuals from the same population occupied different areas of the cluster, which indicates a population substructure (**Figure 3B**).

Bayesian Analysis

Model based clustering was used to assign individuals to sub-populations based on allele frequency differences. Initially, the putative number of populations (K) in the dataset required to explain the total sum of genetic variation observed was determined. Evanno's test (Evanno et al., 2005) on the whole dataset of 1,351 SNPs indicated that the K distribution was bimodal and that the most informative numbers of subpopulations were four and six with $K = 6$ being most probable (Supplementary Information Figure 4). At $K = 4$, consistent with the previous findings, sub-population structure analysis revealed that individuals from GA-S, TN-R, and AZ-S2 appeared distinct from the other populations. The same analysis also showed that individuals from AZ-R and GA-R shared a proportion of their alleles with TN-R while AZ-S1 shared a small proportion with AZ-S2. At $K = 6$, AZ-R, KS-S, and AZ-S1 showed the highest membership coefficient for a shared cluster (beige) while the remaining populations contained unique alleles. This is supported by a high number of shared alleles among these three populations at $K = 8$ where KS-S displayed a high degree of admixture with AZ-R and AZ-S1. Although less than with KS-S and AZ-S1, AZ-R still shared alleles with GA-R while AZ-S1 and GA-R shared none (**Figure 4**). Investigating the dataset without the three outgroups GA-S, TN-R, and AZ-S2 at $K = 5$ supports that AZ-R shares alleles with KS-S, AZ-S1, and GA-R and very few with NE-S (Supplementary Information Figure 5).

Pairwise Comparison of Genetic Distances

As expected, very high genetic distances (D_{ST}) (Nei, 1972) and F_{ST} values were found for the three outgroups (TN-R, AZ-S2, and GA-S) while the genetic distance was lower among AZ-R, AZ-S1, and KS-S. Thus, AZ-R was most closely related to AZ-S1 ($F_{ST} = 0.052$, $D_{ST} = 0.026$) and KS-S ($F_{ST} = 0.049$, $D_{ST} = 0.028$) and most distantly related to the three outgroups GA-S ($F_{ST} = 0.201$, $D_{ST} = 0.079$), TN-R ($F_{ST} = 0.176$, $D_{ST} = 0.067$), and AZ-S2 ($F_{ST} = 0.179$, $D_{ST} = 0.067$). This was further visualized by a heatmap in Supplementary Information Figure 6. The bootstrap analysis of F_{ST} values indicated that all populations were significantly different from each other, except for AZ-R and KS-S, where only 5% of the genetic differences between populations were attributable to their geographic origin (**Table 3**).

Analysis of Molecular Variance and Isolation by Distance

An AMOVA revealed that 17.78% ($P < 0.001$) of the total genetic variation was among populations, 4.87% was among individuals within a population ($P < 0.01$) and the remaining 77.35% ($P < 0.001$) of the genetic variation was within individuals (Supplementary Information Figure 7). Population differentiation exists at all levels but the variation within individuals was the largest. The high genetic variation within individuals suggests a lack of population structure, even though F_{ST} values up to 0.324 (**Table 3**) indicate that genetic differentiation between populations was high.

The geographical distance between any two populations ranged from about 60 to 1,930 km. The Mantel test revealed that no pattern of isolation by distance was evident between genetic and geographic distance ($R^2 = 0.006$, $P = 0.259$). The observed correlation of 0.076 further suggests that the two distances are not associated (Supplementary Information Figure 8).

Genetic Relatedness Based on SNPs within the Chloroplast and Mitochondrial Genome

Forty-two SNPs specific to the chloroplast genome and fifty-four SNPs specific to the mitochondrial genome were identified. PCA with chloroplast SNPs identified GA-S and TN-R as distinct groups (**Figure 5A**). Structure analysis with the identified sub-populations of $K = 4$ and $K = 5$ (Supplementary Information Figure 9A) supported this observation. AZ-S2, however, shared considerably more alleles with NE-S, AZ-S1, KS-S, and GA-R than previously observed when including the loci from the nuclear and mitochondrial genomes (**Figure 6A**). At $K = 8$ AZ-R was closest related to AZ-S1 ($F_{ST} = 0.058$, $D_{ST} = 0.279$) (Supplementary Information Table 2).

Consistent with the analysis with all 1,351 SNPs, mitochondrial SNPs identified GA-S, TN-R, NE-S, and also AZ-S2 as distinct groups (**Figure 5B**), while the remaining populations AZ-R, AZ-S1, GA-R, and KS-S clustered together (**Figure 6B**) leaving $K = 5$ identifiable clusters among the eight populations (Supplementary Information Figure 9B). At $K = 8$, AZ-R was closest related to KS-S ($F_{ST} = 0.053$, $D_{ST} = 0.23$) (Supplementary Information Table 3).

DISCUSSION

Previous population genetics studies investigating the phylogeographic structure of pesticide resistant organisms reveal either a single origin (Raymond and Callaghan, 1991; Linda and Alan, 1997; Daborn et al., 2002) or, more frequently, redundant independent, parallel evolution events shaped by variations in selection pressure (Cavan et al., 1998; Anstead et al., 2005; Menchari et al., 2006; Chen et al., 2007; Pinto et al., 2007; Délye et al., 2010). As an example, it was found that glyphosate resistance in horseweed (*Conyza canadensis*) from California had multiple independent origins within the Central Valley and evolved many years before its first detection. From there it spread, possibly due to increased selection by the herbicide (Okada et al., 2013). The resistance mechanism(s) for the *C. canadensis* populations used in this study were unknown but most likely involved reduced translocation (Wang et al., 2014) and vacuolar sequestration (Ge et al., 2010). Similarly, investigations into the frequency of target site mutations in

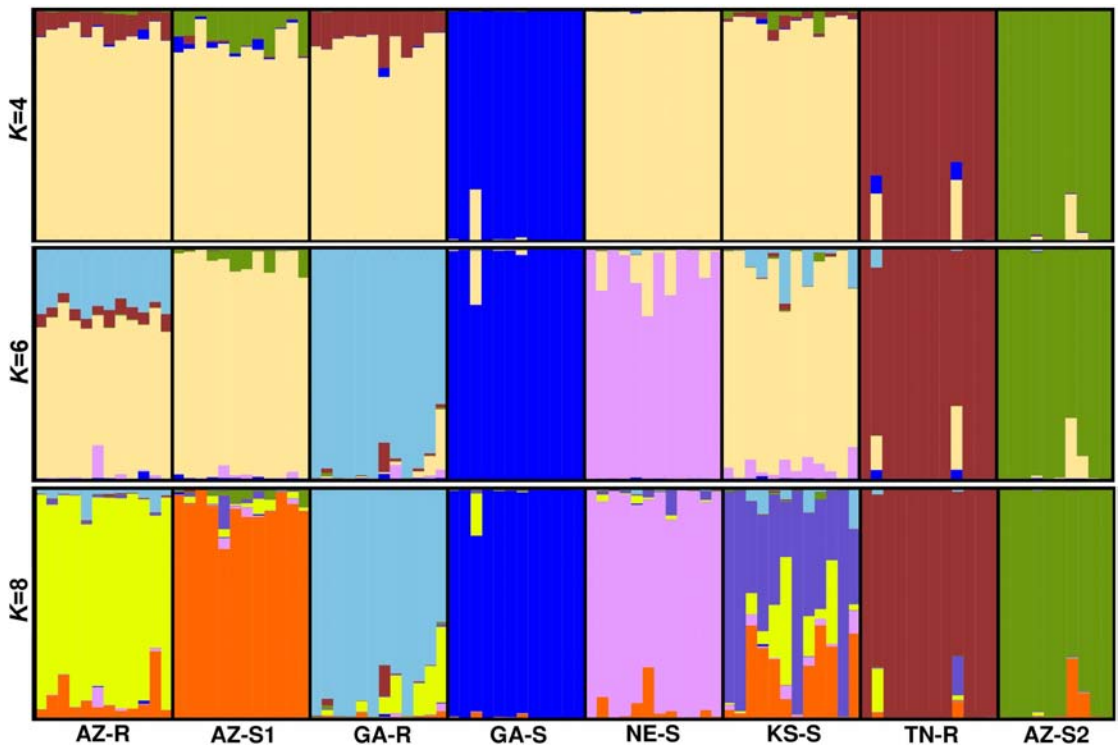

FIGURE 4 | Population structure analysis with K = 4, K = 6, and K = 8 based on 1,351 SNPs of eight *A. palmeri* populations. Each individual is represented by a vertical bar that is divided by K colored segments representing the likelihood of a membership to each cluster.

the *EPSPS* gene of GR Italian ryegrass (*Lolium perenne* L. ssp. *multiflorum*) populations (Karn and Jasieniuk, 2017) as well as simple sequence repeats (SSR) genotyping of GR common morning glory (*Ipomoea purpurea*) (Kuester et al., 2015), and GR Johnsongrass (*Sorghum halepense* L. Pers) (Fernández et al., 2013) found multiple evolutionary origins for glyphosate resistance.

Two previous studies have examined population genetics in *A. palmeri* with glyphosate resistance due to *EPSPS* gene amplification. An investigation using four genomic loci as markers of GR *A. palmeri* from several sampling sites within North Carolina suggested that adaptation to glyphosate application took place in parallel. The authors based this conclusion on the fact that four out of five identified population clusters were statistically associated with increased glyphosate resistance (Beard et al., 2014). In contrast, sequencing of selected regions of the 287 kb *EPSPS* cassette in GR populations from geographically distant locations within the United States showed strong homology between sequences and the authors concluded that the conserved nature of the cassette indicated that glyphosate resistance via amplification evolved once from a point source and then rapidly spread across the United States (Molin et al., 2017b).

Information on the factors that influence the evolutionary origin, demographic history, and geographical pathways of glyphosate resistance in *A. palmeri* is crucial for the formulation of successful strategies to delay and manage herbicide resistance.

TABLE 3 | Pairwise estimates of F_{ST} and Nei's standard genetic distance (D_{ST}) between eight *A. palmeri* populations.

	AZ-R	AZ-S1	GA-R	GA-S	NE-S	KS-S	TN-R	AZ-S2
AZ-R		0.052	0.080	0.201	0.106	0.049†	0.176	0.179
AZ-S1	0.026		0.126	0.202	0.108	0.051	0.215	0.170
GA-R	0.038	0.050		0.225	0.154	0.100	0.209	0.228
GA-S	0.079	0.072	0.090		0.233	0.196	0.316	0.308
NE-S	0.046	0.042	0.064	0.090		0.085	0.257	0.228
KS-S	0.028	0.026	0.046	0.077	0.039		0.191	0.171
TN-R	0.067	0.077	0.082	0.125	0.101	0.074		0.324
AZ-S2	0.067	0.058	0.088	0.115	0.084	0.064	0.122	

Pairwise estimates of F_{ST} and D_{ST} are shown above and below the diagonal, respectively. †*Non-significant value ($P > 0.05$).*

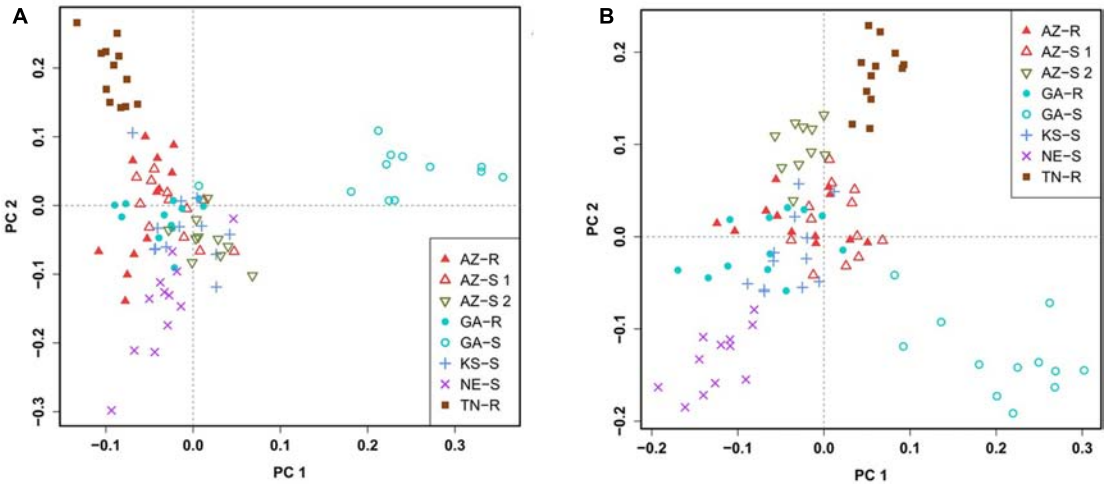

FIGURE 5 | Clustering of *A. palmeri* populations based on principal component analysis (PCA) for SNPs found in all eight populations in the chloroplast genome **(A)** and the mitochondrial genome **(B)** using the filtered and pruned whole dataset. Each point represents an individual colored according to the collection site. Glyphosate-resistant individuals are marked by filled symbols and susceptible individuals are marked by empty symbols. Individuals from the same U.S. state have the same symbols.

The aim of this study was to investigate population structure and genetic differentiation among eight geographically distant *A. palmeri* populations to assess if glyphosate resistance evolved in the southeastern United States and migrated to the southwestern United States, or if it evolved independently in AZ as a result of local management practices. Glyphosate resistance and susceptibility were determined by *EPSPS* copy number and shikimate assay test in all sampled individuals confirming that the resistance mechanism was *EPSPS* gene amplification. *EPSPS* genomic copy number was similar among the resistant populations; thus, spread of glyphosate resistance from a single origin is possible. GBS was used to identify numerous genome-wide sequence differences used as putative neutral markers due to its fast and simple application, cost-effectiveness and high resolution (Brumfield et al., 2003; Morin et al., 2004; Deschamps et al., 2012; Narum et al., 2013). The technique is widely applicable for studying non-model organisms, such as weeds, because the consensus of read clusters around the sequence site becomes the reference sequence and therefore a complete reference genome sequence is not required (Baxter et al., 2011; Elshire et al., 2011; Reitzel et al., 2013). For this study, 1,351 SNPs were used that remained after filtering.

Genetic diversity for each of the *A. palmeri* populations was estimated by the number of alleles, allelic richness, observed and expected heterozygosity, as well as inbreeding coefficient. The varying levels of heterozygosity found can most likely be attributed to differing collection dates and subsequent seed increase events which may have caused inbreeding depression. In particular AZ-S2, collected in 1981, is expected to have undergone severe inbreeding.

UPGMA phylogenetic tree analysis, PCA, Bayesian model-based clustering, and pairwise comparisons of genetic distances were used to determine the genetic relationship among the eight different *A. palmeri* populations and yielded congruent results. GA-S, TN-R, and AZ-S2 were genetically distinct while the remaining populations AZ-R, KS-S, AZ-S1, GA-R, and NE-S clustered together more closely. AZ-R was most closely related to KS-S, followed by AZ-S1, with GA-R being the next most similar population to AZ-R.

Cytoplasmic genomes are maternally inherited and do not undergo recombination. Thus, they permit a more conserved examination of intraspecific phylogeography in plants. They further have the potential to allow for higher differentiation (Petit et al., 2005). Chloroplast and mitochondrial SNPs were evaluated separately because they might support different phylogenies (Washburn et al., 2015; Zhu et al., 2016), since mitochondrial genomes have lower nucleotide sequence variation than chloroplast genomes (Wolfe et al., 1987). Analyses with SNPs in cytoplasmic genomes supported GA-S and TN-R to be genetically distinct. Chloroplast SNPs, however, placed AZ-S2 closer to the remaining populations than NE-S, consistent with the geographical distribution. AZ-R was closest to AZ-S1 based on chloroplast SNPs while mitochondrial SNPs placed the population closest to KS-S. Sequencing of the *ALS* gene revealed that two out of three AZ-R individuals carried a $W_{574}L$ and $S_{653}N$ mutation each, showing high diversity of the *ALS* sequence within the population. Since only the $W_{574}L$ mutation was found in one out of three individuals from TN-R, while GA-R and GA-S individuals had none (Küpper et al., 2017), mutations in the *ALS* gene do not support clustering of AZ-R and GA-R.

According to the observed population genetic structure, two scenarios are possible for AZ-R: Either glyphosate resistance evolved independently in AZ or GR *A. palmeri* from GA migrated west via KS to AZ, against the species expansion direction (Supplementary Information Figure 10). The small amount of shared sequences with GA-R and the moderate amount of shared sequences with KS-S individuals support such an introduction route, as does the chronological order

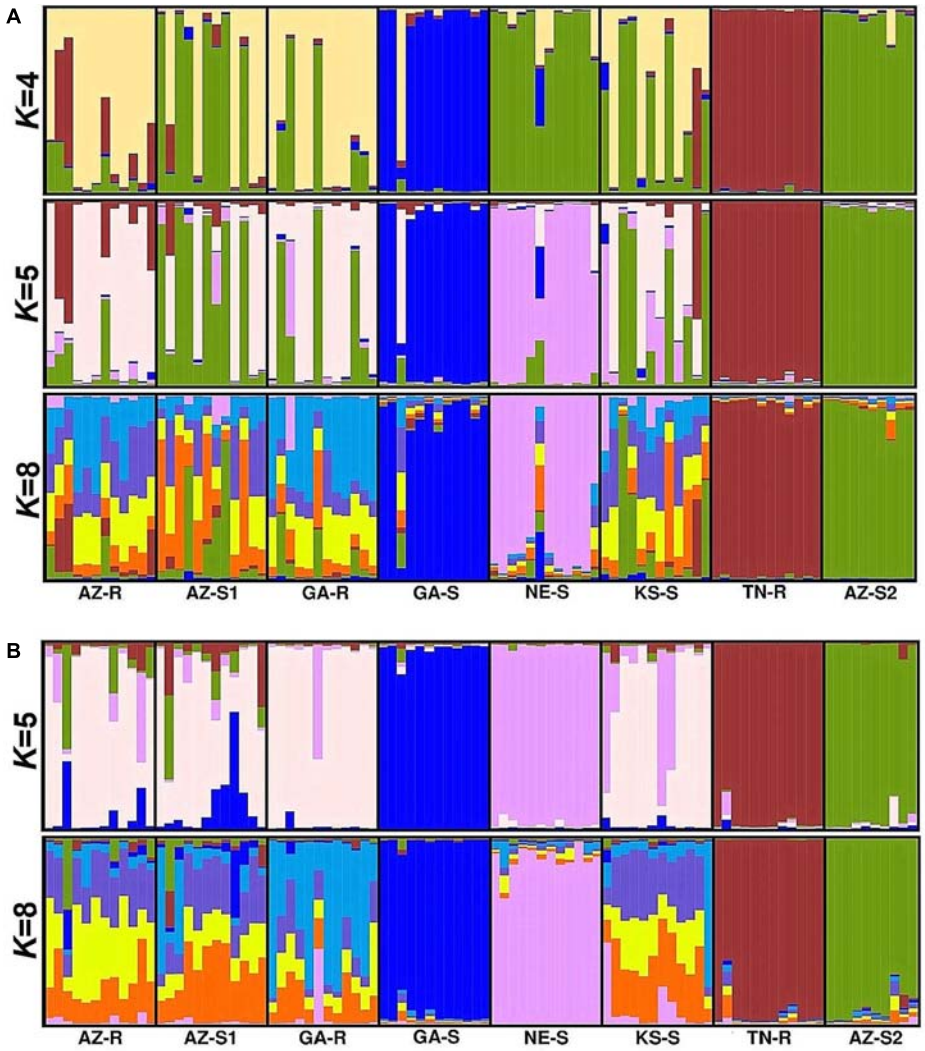

FIGURE 6 | Population structure analysis with $K = 4$, $K = 5$, and $K = 8$ based on SNPs from the chloroplast genome for eight *A. palmeri* populations **(A)** and $K = 5$, and $K = 8$ based on SNPs from the mitochondrial genome for eight *A. palmeri* populations **(B)**. Each individual is represented by a vertical bar that is divided by K colored segments representing the likelihood of a membership to each cluster.

of reports of glyphosate resistance (Georgia: 2004, Kansas: 2011, Arizona: 2012). AZ-R individuals shared alleles with AZ-S1 which could be attributed to crossing events with the native, GS population since resistance is likely to have been reported some time after the introduction event (Cavan et al., 1998). The high degree of unique sequence in AZ-R suggests that the exact origin of the population could not be identified. It can, however, be predicted that AZ-R individuals were not introduced from around the sampling location in TN.

Interestingly, TN-R and GA-R did not share any alleles and seemed unrelated in all analyses. Such strong population differentiation and monophyly can stem from a past divergence event and subsequent adaptation to environmental conditions through intraspecies convergent evolution (Ralph and Coop, 2015) or isolation due to limited dispersal and low connectivity (Reitzel et al., 2013). Further, agricultural practices might have strongly modified weed communities and disturbed genetic equilibrium (Menchari et al., 2007). TN and GA/NC coastal regions are geographically separated by the Appalachian mountain range and have dissimilar cropping systems with one primarily focusing on soybean and corn production and the other on cotton. As resistance to glyphosate was reported within a time frame of 2 years in these states, it is very possible that the populations represent independent glyphosate resistance origins. GA-R and GA-S, however, were genetically distinct from each other in all analyses even though collected from about 115 km apart and without any major geographical obstacles in the way. If glyphosate resistance evolved at the GA-R location, a more panmictic structure would have been expected (Chauvel and Gasquez, 1994). Such differences could be attributed to locally differing conditions, a high degree of natural spatial genetic

diversity within the species, or the possibility that glyphosate resistance did not originate in GA. It is also possible that continuous selection with glyphosate created a genetic bottleneck and subsequent inbreeding of resistant individuals.

Amaranthus palmeri is a species with high genetic variability which makes it a challenge to draw a definite conclusion about an introduction event without very specific sampling. This study has shown that genetic relatedness does not decrease with distance. Hence, if GS individuals collected from within a 50 km radius can have the high level of genetic differentiation observed in this study (e.g., AZ-S1 and AZ-S2), it may be difficult to identify a parallel adaptation event. The nativity of *A. palmeri* to the southwestern United States and adaptation to local and heterogenous environments (Clements et al., 2004) as well as the species' obligate outcrossing nature are drivers for heterozygosity. Genetic diversity, in turn, increases the likelihood of resistance to evolve, as does high selection pressure due to frequent usage of glyphosate which has been the case in all areas of GR *A. palmeri* collection sites. Future research should incorporate a more extensive collection of GR *A. palmeri* populations, always coupled with at least one geographically close GS population. Furthermore, all seed should be collected by the exact same sampling technique to increase the precision and accuracy with which questions of genetic relatedness and geographic migration patterns can be answered.

CONCLUSION

A major management question for growers is how much of the resistance issue results from previous selection intensity from management practices in their own fields, and how much results from gene flow from neighboring fields. Although this study was not able to definitively determine whether AZ-R evolved independently or if glyphosate resistance migrated to AZ, the recent geographical expansion of *A. palmeri* to the upper United States Midwest (Karteszc, 2014), Argentina (Berger et al., 2016), and Brazil (Küpper et al., 2017) shows that migration via seed movement is an important factor for *A. palmeri*. Long-distance seed dispersal is possible through irrigation and rainfall events (Norsworthy et al., 2014), buying and selling of used harvest equipment, custom harvesting crews moving around the country (Schwartz et al., 2016), contaminated crop seed and feed, as well as transportation through migrating wildlife such as ducks and geese (Farmer et al., 2017). Aside from harvest equipment hygiene requirements, it is difficult to prevent such seed dispersal. Early detection and rapid response approaches already used in invasive species management (Westbrooks, 2004) and disease outbreaks (Fasina et al., 2014) could be useful to adopt for herbicide resistance management. Delaying resistance evolution and prolonging the utility of remaining effective modes of actions for which resistance is not yet widespread, such as synthetic auxins, glutamine synthetase-, and phytoene desaturase (PDS)-inhibitors, is critical for future *A. palmeri* management.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AK, HM, WM, and TG. Performed the experiments: AK, WM, and DG. Analyzed the data: AK and EP. Contributed materials: WM and TG. Drafted the manuscript and figures: AK. All authors contributed to the revision of the final manuscript. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the National Institute of Food and Agriculture (NIFA) or the United States Department of Agriculture (USDA).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00029/full#supplementary-material

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Inheritance of Mesotrione Resistance in an *Amaranthus tuberculatus* (var. *rudis*) Population from Nebraska, USA

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A population of *Amaranthus tuberculatus* (var. *rudis*) evolved resistance to 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor herbicides (mesotrione, tembotrione, and topramezone) in Nebraska. The level of resistance was the highest to mesotrione, and the mechanism of resistance in this population is metabolism-based likely via cytochrome P450 enzymes. The increasing number of weeds resistant to herbicides warrants studies on the ecology and evolutionary factors contributing for resistance evolution, including inheritance of resistance traits. In this study, we investigated the genetic control of mesotrione resistance in an *A. tuberculatus* population from Nebraska, USA. Results showed that reciprocal crosses in the F1 families exhibited nuclear inheritance, which allows pollen movement carrying herbicide resistance alleles. The mode of inheritance varied from incomplete recessive to incomplete dominance depending upon the F1 family. Observed segregation patterns for the majority of the F2 and back-cross susceptible (BC/S) families did not fit to a single major gene model. Therefore, multiple genes are likely to confer metabolism-based mesotrione resistance in this *A. tuberculatus* population from Nebraska. The results of this study aid to understand the genetics and inheritance of a non-target-site based mesotrione resistant *A. tuberculatus* population from Nebraska, USA.

Keywords: waterhemp, herbicide resistance evolution, polygenic, 4-hydroxyphenylpyruvate dioxygenase, metabolism-based resistance

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INTRODUCTION

Waterhemp [*Amaranthus tuberculatus* (var. *rudis*)] is a classic example of herbicide resistance evolution. *A. tuberculatus* is a native species of the Midwestern United States, and it has become a predominant weed in corn-soybean cropping systems (Owen, 2008; Waselkov and Olsen, 2014). The biology of *A. tuberculatus* is an important factor contributing to its adaptation in row-crop production systems. The dioecious nature of *A. tuberculatus* enforces cross-pollination and the potential for gene flow of herbicide resistance genes (Trucco et al., 2005, 2006; Sarangi et al., 2017). Moreover, a single *A. tuberculatus* female plant can produce over a million seeds depending on density (Hartzler et al., 2004); therefore, herbicide resistance may evolve and spread faster in *A. tuberculatus* than other monoecious weedy *Amaranthus*. *A. tuberculatus* is ranked among the

worst herbicide resistant weeds (Tranel et al., 2011; Heap, 2014). As of 2017, populations of *A. tuberculatus* have evolved resistance to six herbicide sites-of-action (SOA) in the United States (Heap, 2017).

Herbicides inhibiting 4-hydroxyphenylpyruvate dioxygenase (HPPD) represent the latest introduced SOA for weed control in corn, commercialized in the late 1980's (Mitchell et al., 2001; Duke, 2012). Mesotrione, an HPPD-inhibitor herbicide, was introduced as an effective preemergence (PRE) and postemergence (POST) herbicide for controlling various broadleaf weeds, including *A. tuberculatus* (Mitchell et al., 2001; Sutton et al., 2002). However, resistance to mesotrione evolved recently (Hausman et al., 2011; McMullan and Green, 2011), and it is increasing across the north-central United States (Schultz et al., 2015). The persistence and adaptation of HPPD-inhibitor herbicide-resistant *A. tuberculatus* populations to cropping-systems is a concern. There will be a potential increased use of these herbicides by the use of transgenic HPPD-inhibitor-resistant crops in the United States. Therefore, tactics are needed to minimize the evolution of resistance to this herbicide group.

The mechanism of herbicide resistance discovered in *A. tuberculatus* varies according to the herbicide SOA, which can be either target site resistance (TSR) or non-target site resistance (NTSR). Target-site amino acid substitutions, codon deletion, and gene amplification are the major mechanisms of TSR (Patzoldt and Tranel, 2007; Thinglum et al., 2011; Lorentz et al., 2014; Chatham et al., 2015), which are often caused by a dominant gene in a single locus (Délye et al., 2013). In contrast, NTSR includes mechanisms that are not TSR, frequently resulting from multiple genes conferring reduced herbicide penetration, herbicide differential translocation, and enhanced herbicide metabolism (Powles and Yu, 2010; Délye, 2013; Délye et al., 2013). Enhanced metabolism (NTSR) is the only reported mechanism of mesotrione resistance in *A. tuberculatus* (Ma et al., 2013; Kaundun et al., 2017).

A population of *A. tuberculatus* (hereafter referred as R) has evolved resistance to POST-applied HPPD-inhibitor herbicides (mesotrione, tembotrione, and topramezone) in a corn/soybean production system in northeast Nebraska (Oliveira et al., 2017a). Mesotrione detoxification to 4-hydroxymesotrione has been confirmed as the mechanism of resistance in this population (Kaundun et al., 2017). Further research characterized the role of cytochrome P450 enzymes in this R population, as malathion (a cytochrome P450 inhibitor) did not synergize mesotrione (Oliveira et al., 2017b). This result is different from what was previously reported in a different mesotrione resistant *A. tuberculatus* population from Illinois, in which malathion synergized mesotrione (Ma et al., 2013). It is likely that different P450 enzymes are endowing mesotrione resistance in different *A. tuberculatus* populations. Therefore, empirical studies are needed to understand the eco-evolutionary dynamics causing weed evolution (Neve et al., 2009, 2014). For example, inheritance studies can improve our knowledge of the genetic structure of weed populations under herbicide selection and aid to create appropriate herbicide resistance simulation models (Neve et al., 2014; Renton et al., 2014; Menalled et al., 2016).

Inheritance of herbicide alleles contributing to pesticide (e.g., herbicide) resistance can vary with different genetic backgrounds and dose environment (Ffrench-Constant et al., 2004; Neve et al., 2014). Thus, the objective of this study was to determine the mode of inheritance and number of alleles controlling mesotrione resistance in the R population from Nebraska with two experiments: (1) dose-response studies with parental [mesotrione R and susceptible (S)] and F1 families generated from the S × R cross; and (2) segregation studies in pseudo-F2 and back cross (BC) families with low, recommended, and high mesotrione dose.

METHODS

Plant Material and Growth Condition

Two *A. tuberculatus* were the originating populations in this study. This species is an obligate outcrosser so inbred lines could not be developed to establish parents for the inheritance studies. The seeds of the R population were collected in 2014 from a field in Platte County, NE, where mesotrione resistance was confirmed (Oliveira et al., 2017a). The S population seeds were collected from Dixon County, NE; this population was known to have high sensitivity to mesotrione. For both populations, seeds were collected from at least 20 plants. The seeds were cleaned and stored separately (R and S populations) at 5 C until used in the greenhouse study in 2015 and 2016 at the University of Nebraska-Lincoln. Seeds were planted in 1200 cm^3 plastic pots for pairwise crosses containing peat:soil:sand:vermiculite (4:2:2:2) potting mix. In addition, seeds were planted in 164 cm^3 cone-tainers (Ray Leach "Cone-tainer" SC10®, Stuewe and Sons Inc, Tangent, OR, USA) for dose-response and segregation analysis with the same potting mix described. Plants were supplied with adequate water and kept under greenhouse conditions at 28/20 C day/night temperature with 80% relative humidity. In addition, twice per week, plants were fertilized with 3 mg of NPK (20-10-20 Peters® Professional, JR Peters Inc., Allentown, PA, USA) for each 100 cm^3 of the potting mix until plants were 8–10 cm tall. Artificial lighting was provided using metal halide lamps (600-μmol photons m^{-2} s^{-1}) to ensure a 15-h photoperiod.

Generations of F1, Pseudo-F2, and Back-Cross Families

The R population was grown from seeds in a greenhouse and selected with a POST application of the recommended rate of 105 g ai ha^{-1} of mesotrione (Callisto®, Syngenta Crop Protection, Research Triangle Park, NC, USA) when R seedlings were 10–15 cm tall. The recommended mesotrione rate caused low injury (<30%) on R plants. Therefore, the R plants were kept for individual pairwise crosses with the S plants. Single S and R plants growing in pots were paired according to floral synchrony and enclosed with pollination bags (100 by 185-cm) to exclude external pollen. Male plant inflorescences were gently shaken in the morning during pollination period (∼3 weeks) to ensure cross-fertilization between S and R individuals. At maturity, seeds were collected from each female plant,

cleaned, and bagged separately, then designated as F1 families (**Figure 1**).

The F1 families were derived from 20 parental crosses. Four F1 families were advanced to the next generation of crosses (Table S1), including three S × R and one R × S (female × male). The F1 plants were termed RS/F1-5, SR/F1-8, SR/F1-9, and SR/F1-13 (Table S1). The R parent of the SR/F1-13 family was derived from an R × R cross of the R population seeds, and the R × R cross was performed under greenhouse conditions, similarly as described for the S × R cross. The remaining F1 families were derived from field collected R and S seeds. The F1 plants were treated with 105 g ai ha^{-1} of mesotrione when plants were 10 to 15 cm tall. The F1 plants survived with variable mesotrione injury (data not shown) confirming that they were crosses between R and S. *A. tuberculatus* is dioecious, preventing F1 self-pollination to produce true F2 plants (**Figure 1**). Therefore, the F1 family SR/F1-9 and SR/F1-13 individuals were separately cross-pollinated using the procedures described above to make pseudo-F2 plants (hereinafter referred as F2). The F2 plants were designed F2-9 (from SR/F1-9) and F2-13 (from SR/F1-13). In addition, F1 male plants were also allowed to pollinate the parental susceptible female plants (S) to produce backcross susceptible (BC/S) families (**Figure 1**). Three BC/S families were made and designed BC-8/S (from SR/F1-8), BC-9/S (from SR/F1-9), and BC-13/S (from SR/F1-13; Table S1).

Dose-Response of Mesotrione in F1 Families and Parental Population

The greenhouse experiments were set in completely randomized design with four replications and repeated twice in 2016 at the University of Nebraska-Lincoln. Mesotrione dose-response studies were conducted separately with R and S (parent); and F1 families, including RS/F1-5, SR/F1-9, and SR/F1-13.

The mesotrione dose was 0, 0.125×, 0.25×, 0.5×, 1×, 2×, 4×, 8×, 16×, where 1× represents 105 g ai ha^{-1} (labeled use rate of mesotrione). Mesotrione was mixed with 1% (v/v) crop oil concentrate (Agri-Dex®, Helena Chemical Co, Collierville, TN, USA) and 20.5 g L^{-1} of ammonium sulfate (DSM Chemicals North America Inc., Augusta, GA, USA). Herbicide treatments were applied with a single tip chamber sprayer (DeVries Manufacturing Corp, Hollandale, MN, USA) fitted with an 8001 E nozzle (Spraying Systems Co., North Avenue, Wheaton, IL 60139), calibrated to deliver 140 L ha^{-1} spray volume at 210 kPa at a speed of 3.7 km h^{-1}. Control was assessed visually 21 d after treatment (DAT) using a scale of 0 to 100% (where 0 indicates no injury and 100 indicates plant death). Control ratings were based on symptoms such as bleaching, necrosis, and stunting of plants compared to non-treated control plants. Aboveground biomass was harvested at 21 DAT from each experimental unit and oven-dried at 65 C until reaching constant weight before the weight of biomass (g $plant^{-1}$) was recorded.

The effective mesotrione doses needed to control and reduce biomass by 50% (ED_{50}) and 90% (ED_{90}) for the parental and F1 families were determined using the three-parameter log-logistic curve of the drc package of the R statistical software (Knezevic et al., 2007).

$$Y = d/1 + \exp\{b[\log(x) - \log(e)]\} \quad (1)$$

In this model, Y is the control (%) or biomass reduction (g $plant^{-1}$), d is the upper limit, and e represents the ED_{50} value relative to d. The parameter b is the relative slope around the parameter e, and x is the mesotrione dose in g ai ha^{-1}.

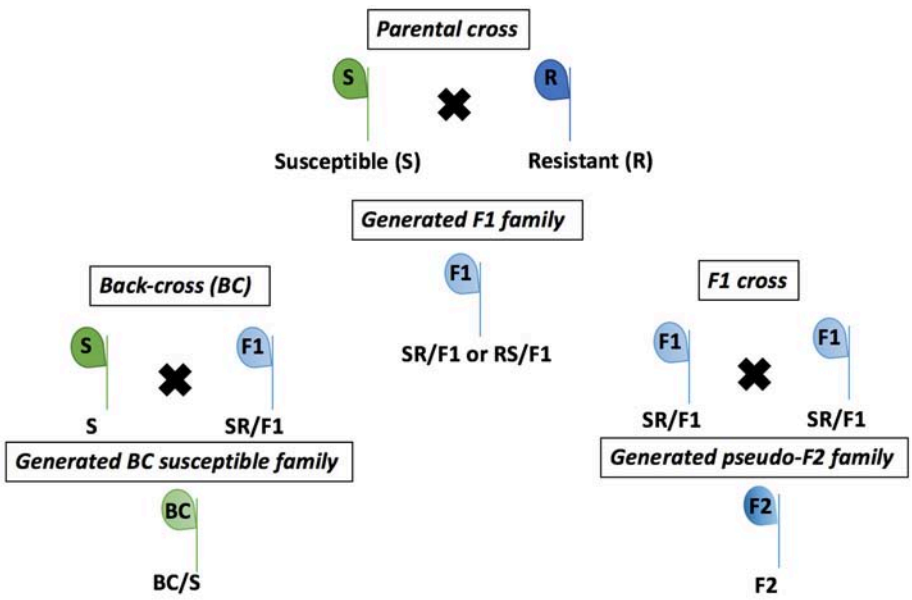

FIGURE 1 | Crosses of *Amaranthus tuberculatus* populations (R and S) to generate F1, F2, and BC/S families conducted in greenhouse at the University of Nebraska-Lincoln.

The resistance level was calculated by dividing the effective dose for providing 50% control or 50% biomass reduction (ED_{50}) of the R or F1 populations by the ED_{50} of the S population. The resistance level indices for the respective ED_{50} between the R or F1, and the S were compared using the *EDcomp* function of package drc in R software (Ritz and Streibig, 2005). The *EDcomp* function compares the ratio of ED_{50} using t-statistics, where *P*-value < 0.05 indicates that herbicide ED_{50} values are different between the R or F1 and the S.

The degree of dominance (D) was calculated following the formula

$$D = [(2X_2 - X_1 - X_3)/(X_1 - X_3)] \quad (2)$$

Where D is the degree of dominance and X_1, X_2, and X_3 are the log(ED_{50}) of R, F1, and S population, respectively. When $D = 0$, the F1 has a resistance level that is the mean of the R and S (additive). When $D = 1$ there is an indication of complete dominance, $0 < D < 1$ supports the model of incomplete dominance, $-1 < D < 0$ of incomplete recessive, and $D = -1$ of complete recessive (Stone, 1968).

Mesotrione Segregation in *Amaranthus tuberculatus* F2 and BC/S Families

The R and S parent, SR/F1 (SR/F1-8, SR/F1-9, and SR/F1-13), F2 (F2-9 and F2-13) and BC/S (BC-8/S, BC-9/S, and BC-13/S) families were treated with three mesotrione doses attempting to discriminate S from R plants. These doses were 26, 105, and 420 g ai ha^{-1}, which represent 0.25×, 1×, and 4× the recommended rate, respectively. The 0.25× dose was chosen to assess segregation at a below-label dose. The 1× dose was chosen to evaluate segregation at recommended label dose at which

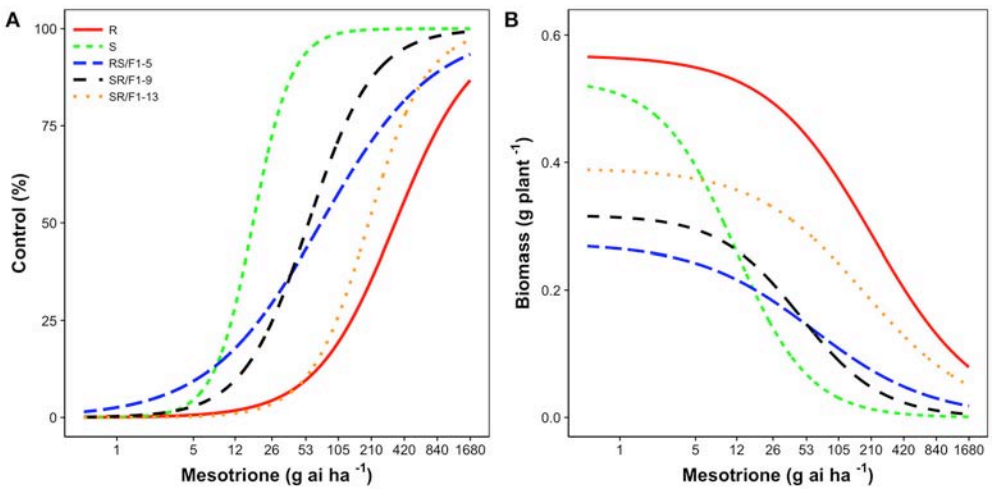

FIGURE 2 | Mesotrione dose-response on **(A)** Control (%) and **(B)** Biomass (g $plant^{-1}$) of *Amaranthus tuberculatus* families (R and S parent populations; and F1 families, RS/F1-5, SR/F1-9, and SR/F1-13) conducted in greenhouse at the University of Nebraska-Lincoln.

TABLE 1 | Estimated parameters (*b*, *d*, and *e*) and effective dose to control 90% (ED_{90}) of *Amaranthus tuberculatus* (R and S parent populations; and F1 families, RS/F1-5, SR/F1-9, and SR/F1-13) conducted in greenhouse at the University of Nebraska-Lincoln.

Population[a]	Parameter[b]			ED_{90} (±SE)	*P*-value[c]	Resistance level[d]
	b (±SE)	*d* (±SE)	*e* (ED_{50}) (±SE)			
			g ai ha^{-1}			
S	−2.4 (0.2)	100	18 (1)	43 (4)	–	–
R	−1.2 (0.1)	100	348 (19)	2199 (257)	0.00	19
RS/F1-5	−0.8 (0.0)	100	73 (4)	981 (143)	0.00	4
SR/F1-9	−1.5 (0.1)	100	56 (2)	255 (27)	0.00	3
SR/F1-13	−1.6 (0.1)	100	198 (9)	759 (80)	0.00	11

[a] *S, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-susceptible A. tuberculatus collected from a field in Dixon County, NE in 2014; HPPD-R, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicide-resistant A. tuberculatus collected from a field in Platte County, NE in 2014. RS/F1-5, SR/F1-9, and SR/F1-13 are crosses from SxR parents made under greenhouses conditions.*

[b] *b, the slope; d, the upper limit (locked at 100); and e (ED_{50}), the inflection point relative to the upper limit. The ED_{50} is an effective dose of mesotrione needed to reach 50% HPPD-R control. SE, standard error.*

[c] *R and F1 families vs. S population t-statistics comparison of e (ED_{50}), P-value > 0.05 means non-significant difference between treatments.*

[d] *Resistance level was calculated dividing e (ED_{50}) value of R and F1 families by S population.*

plants are selected under field conditions. The higher dose (4×) was selected to likely allow mesotrione resistance segregation to occur with minimal interference of minor resistance loci (Busi et al., 2014). There were two runs (repetitions in time) of this experiment for each mesotrione dose, and each run included BC/S and F2 populations represented by 29 to 98, and 56 to 98 plants, respectively. In addition, parent (R and S) populations were represented by 8 to 17 plants, and F1 populations were represented by 8 to 24 plants. A total of 2,908 *A. tuberculatus* plants were screened with mesotrione in this study. Herbicide application was the same as described above in dose-response studies. At 21 DAT, all populations were visually evaluated and assessed as dead or alive. Alive plants were separated into different injury level groups, including low (<40%), medium (41 to 79%), high (80 to 98%), and dead (>98%; Figure S1). Plant aboveground biomass was harvested, oven-dried until constant weight, and biomass (g plant^{-1}) was recorded. The plant biomass and injury level for parental, SR/F1, F2, and BC/S families were represented in violin plots with a rotated kernel density plot using package *ggplot2* in R statistical software (Wickham, 2009).

The experimental null hypothesis was that mesotrione resistance segregates as controlled by a single major gene (one locus). The segregation analysis in F2 and BC/S families was based on the observed survival ratio (alive/total treated plants) compared to expected survival assuming one gene locus segregation (Table S2). For the F2 and BC/S families, the expected number of surviving plants was described by the following equations (Tabashnik, 1991; Busi et al., 2013; Han et al., 2014):

F2: One gene locus model (1R:2F1:1S),

$$\text{Exp. F2} = 0.25 \times \text{Obs } R + 0.5 \times \text{Obs F1} + 0.25 \times \text{Obs S} \quad (3)$$

BC/S: One gene locus model (1F1:1S),

$$\text{Exp. BC/S} = 0.5 \times (\text{F1} + \text{S}) \quad (4)$$

where R, F1, and S are the number of observed surviving plants of the R, F1, and S families at each mesotrione dose (26, 105, and 420 g ai ha^{-1}). Thus, for each dose, the expected number of survivors for F2 and BC/S populations was calculated with the total number of treated plants multiplied by the theoretical one locus segregation ratio for F2 and BC/S families.

A chi-square goodness-of-fit test (χ^2) was used to compare the observed and expected plant survival based on a one locus segregation model. The *P*-values were obtained to indicate the probability in rejecting the null hypothesis for F2 and BC/S families at one locus segragation. For example, for one locus segregation, the null hypothesis (H_0) is that the BC segregates as

TABLE 3 | Degree of dominance based on logarithm of parameter *e* (ED_{50}) control and biomass (g plant^{-1}) of *Amaranthus tuberculatus* (R and S parent populations; and F1 families, RS/F1-5, SR/F1-9, and SR/F1-13) conducted in greenhouse at the University of Nebraska-Lincoln.

Population[a]	Control	Degree of dominance[b]	Biomass	Degree of dominance[b]
	log(ED_{50})		log(ED_{50})	
R	5.85	–	5.37	–
S	2.86	–	2.45	–
RS/F1-5	4.29	−0.04	4.18	0.14
SR/F1-9	4.03	−0.22	3.84	−0.05
SR/F1-13	5.29	0.62	5.21	0.88
F1-pooled	4.53	0.11	4.39	0.32

[a] *S, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-susceptible A. tuberculatus collected from a field in Dixon County, NE in 2014; HPPD-R, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicide-resistant A. tuberculatus collected from a field in Platte County, NE in 2014. RS/F1-5, SR/F1-9, and SR/F1-13 are crosses from SxR parents made under greenhouses conditions.*

[b] *Degree of dominance was calculated using the formula $D = [(2X_2\text{-}X_1\text{-}X_3)/(X_1\text{-}X_3)]$, where X1, X2, and X3 represent the e (ED_{50}) values of R, S, and respective F1 family.*

TABLE 2 | Estimated parameters (*b*, *d*, and *e*) and effective dose to reduce biomass (g plant^{-1}) 90% (ED_{90}) of *Amaranthus tuberculatus* (R and S parent populations; and F1 families, RS/F1-5, SR/F1-9, and SR/F1-13) conducted in greenhouse at the University of Nebraska-Lincoln.

Population[a]	Parameter[b]			ED_{90} (±SE)	*P*-value[c]	Resistance level[d]
	b (±SE)	*d* (±SE)	*e* (ED_{50}) (±SE)			
	Biomass (g plant^{-1})			g ai ha^{-1}		
S	1.3 (0.2)	0.5 (0.0)	12 (1)	66 (12)	–	–
R	0.9 (0.1)	0.6 (0.0)	216 (23)	2665 (408)	0.0	18
RS/F1-5	0.8 (0.1)	0.3 (0.0)	62 (15)	956 (340)	0.0	5
SR/F1-9	1.2 (0.2)	0.3 (0.0)	47 (7)	314 (86)	0.0	4
SR/F1-13	1.0 (0.1)	0.4 (0.0)	183 (31)	2354 (686)	0.0	15

[a] *S, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-susceptible A. tuberculatus collected from a field in Dixon County, NE in 2014; HPPD-R, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicide-resistant A. tuberculatus collected from a field in Platte County, NE in 2014. RS/F1-5, SR/F1-9, and SR/F1-13 are crosses from SxR parents made under greenhouses conditions.*

[b] *b, the slope; d, the upper limit (locked at 100); and e (ED_{50}), the inflection point relative to the upper limit. The ED_{50} is an effective dose of mesotrione needed to reach 50% HPPD-R control. SE, standard error.*

[c] *R and F1 families vs. S population t-statistics comparison of e (ED_{50}), P-value > 0.05 means non-significant difference between treatments.*

[d] *Resistance level was calculated dividing e (ED_{50}) value of R and F1 families by S population.*

0.5F1:0.5S. The significant level is $\alpha = 0.05$ and if P-value < 0.05, the null hypothesis is rejected.

RESULTS

Mode of Inheritance of Mesotrione Resistance

The R population displayed a high level of resistance whereas S was susceptible (**Figures 2A,B**). The level of resistance of R compared to S was 19-fold based on ED_{50} values (**Table 1**). The generated F1 families provided an intermediate and relatively lower level of resistance (3-4-fold) based on ED_{50} values, except the SR/F1-13 family (11-fold). The biomass (g plant^{-1}) of parental and F1 families corroborated with visual control, resulting in similar ED_{50}, ED_{90}, and resistance levels (**Table 2**).

The F1 families expressed a variable degree of dominance (D). For example, based on control, the degree of dominance of SR/F1-13 family was 0.62 (**Table 3**). In contrast, the SR/F1-9 family had a negative degree of dominance ($D = -0.22$). The RS/F1-5 family had a degree of dominance close to zero ($D = -0.04$). The pooled-F1 family resulted in $D = 0.11$. Similar degree of dominance trend was also observed with plant response to mesotrione based on biomass (g plant^{-1}) of the F1 families (**Table 3**).

Mesotrione Segregation in *Amaranthus tuberculatus* F2 and BC/S Families

The segregation at below-label mesotrione dose (26 g ai ha^{-1}) resulted in 77% survival of S plants when averaging both runs, and survived S plants showed high injury (>80%; **Figure 2A**). In addition, the 26 g ai ha^{-1} resulted in high survival rate (≥87%) of the SR/F1, F2, and BC/S families across runs. Therefore, this dose was not the best to describe a segregation analysis for this R population. Nonetheless, a single major gene model would not fit (P-value < 0.05) the majority of F2 and BC/S families (**Table 4**).

The recommended (105 g ai ha^{-1}; **Table 5**) and a high mesotrione rate (420 g ai ha^{-1}; **Table 6**) effectively distinguished R and S plant survival. At these two rates, R plants survived with low injury (< 40%), and S plants died (**Figures 3C,E**). However, F1 family survival and level of injury at 105 and 420 g ai ha^{-1} varied within families (**Figures 3D,F**). At mesotrione rate of 105 g ai ha^{-1}, segregation in each family deviated from

TABLE 4 | Phenotypic resistance segregation observed in two pseudo-F2 (F2) and three back-cross susceptible (BC/S) families at below-label mesotrione dose (26 g ai ha^{-1}).

Run	Dose (g ai ha^{-1})	Family[a]	Plants treated	Survivors (observed)	Survival ratio	Survivors (expected)	χ^2	*P*-value
1	26	R	10	10	1.00			
		S	8	6	0.75			
		SR/F1-8	13	13	1.00			
		SR/F1-9	9	9	1.00			
		SR/F1-13	8	8	1.00			
		F2 segregation				1R:2F1:1S		
		F2-9	75	65	0.87	70.3	6.4	0.01
		F2-13	98	98	1.00	91.8	6.6	0.01
		BC/S segregation				1F1:1S		
		BC-8	93	93	1.00	81.3	13.4	0.00
		BC-9	94	94	1.00	82.2	13.4	0.00
		BC-13	60	57	0.95	52.5	3.1	0.07
2	26	R	16	16	1.00			
		S	14	11	0.78			
		SR/F1-8	24	24	1.00			
		SR/F1-9	14	14	1.00			
		SR/F1-13	24	24	1.00			
		F2 segregation				1R:2F1:1S		
		F2-9	97	97	1.00	91.8	5.5	0.02
		F2-13	98	98	1.00	92.7	1.8	0.02
		BC/S segregation				1F1:1S		
		BC-8/S	29	27	0.97	25.9	0.8	0.38
		BC-9/S	95	95	1.00	84.8	11.4	0.00
		BC-13/S	49	48	0.98	43.7	5.9	0.04

Chi-square (χ^2) analysis for expected plant survival assuming control for mesotrione resistance by a single major gene.

[a]*S, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-susceptible A. tuberculatus collected from a field in Dixon County, NE in 2014; HPPD-R, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicide-resistant A. tuberculatus collected from a field in Platte County, NE in 2014. SR/F1-8 (generated BC-8/S), SR/F1-9 (generated F2-9 and BC-9/S), and SR/F1-13 (generated F2-13 and BC-13/S) are crosses originated from SxR parents made under greenhouses conditions.*

TABLE 5 | Phenotypic resistance segregation observed in two pseudo-F2 and three back-cross susceptible (BC/S) families at recommended label mesotrione dose (105 g ai ha^{-1}).

Run	Dose (g ai ha^{-1})	Family[a]	Plants treated	Survivors (observed)	Survival ratio	Survivors (expected)	χ^2	*P*-value
1	105	R	8	8	1			
		S	8	0	0			
		SR/F1-8	24	19	0.79			
		SR/F1-9	14	14	1			
		SR/F1-13	13	13	1			
		F2 segregation				1R:2F1:1S		
		F2-9	96	80	0.83	72.0	10.6	0.00
		F2-13	98	87	0.89	73.5	9.9	0.00
		BC/S segregation				1F1:1S		
		BC-8/S	96	85	0.89	37.9	96.7	0.00
		BC-9/S	96	90	0.94	48.0	73.5	0.00
		BC-13/S	49	40	0.82	24.5	19.6	0.00
2	105	R	17	17	1			
		S	13	0	0			
		SR/F1-8	24	13	0.55			
		SR/F1-9	10	10	1			
		SR/F1-13	24	23	0.96			
		F2 segregation				1R:2F1:1S		
		F2-9	88	85	0.97	66.0	21.9	0.00
		F2-13	95	91	0.96	69.3	25.1	0.00
		BC/S segregation				1F1:1S		
		BC-8/S	66	59	0.89	17.8	130.6	0.00
		BC-9/S	95	94	0.99	47.5	91.0	0.00
		BC-13/S	49	33	0.67	23.5	7.4	0.00

Chi-square (χ^2) analysis for expected plant survival assuming control for mesotrione resistance by a single major gene.
[a]S, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-susceptible A. tuberculatus collected from a field in Dixon County, NE in 2014; HPPD-R, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicide-resistant A. tuberculatus collected from a field in Platte County, NE in 2014. SR/F1-8 (generated BC-8/S), SR/F1-9 (generated F2-9 and BC-9/S), and SR/F1-13 (generated F2-13 and BC-13/S) are crosses originated from SxR parents made under greenhouses conditions.

the corrected 1R:2F1:S (F_2) and 1F1:1S (BC) ratios expected for one major gene in the two experimental runs (**Table 5**). Two and three loci segregation model tested fitted for most of F2 and BC/S, families (data not shown). For 420 g ai ha^{-1}, only F2-13 (first run) and BC-8/S (second run) families did not deviate from the expected one major locus (**Table 6**). However, BC-8/S (first and second run), F2-13 (second run), and BC-13/S (first run) also did not deviate from two and three loci segregation (data not shown). In general, at 105 and 420 g ai ha^{-1} doses, the plant mortality was variable but higher than expected.

The plant biomass (g) and injury levels (%) of the segregation analysis at different mesotrione doses on *A. tuberculatus* families was illustrated with combined (**Figures 3A,C,E**) or separated (**Figures 3B,D,F**) violin plots. The plant biomass of the generated families overlapped the R and S parent phenotypic ranges. The violin plots showed either continuous or bell-shaped distribution of biomass (g) across families and mesotrione rates (**Figures 3A–F**). For example, at 105 g ai ha^{-1} of mesotrione, the F2-9 family showed a majority of plants with low injury and highly variable biomass (**Figure 3D**). The F2-13 family had a medium to high injury with relatively low uniform biomass. Nonetheless, the F2 families resulted in high plant survival (**Table 5**). In contrast, the BC/S families resulted in a uniform relatively low biomass across families and mesotrione doses (**Figures 3B,D,F**). In addition, the shape of the phenotype was more continuous and thinner as the mesotrione dose increased (**Figures 3A–F**).

DISCUSSION

The individual plants representing the R population have been under continual selection pressure for mesotrione resistance, but they have not been through inbreeding that would increase homozygosity. The F1 families showed a lack of dominance or recessivity (**Table 3**), and the degree of dominance in F1 families varied from additive (SR/F1-5), incomplete recessive (SR/F1-9), and incomplete dominance (SR/F1-13; **Table 3**). The closest to complete dominance was SR/F1-13, which was the only F1 family for which the R parent was previously crossed under greenhouse conditions (R × R). Therefore, further inbreeding would be needed to establish families that could be used in future studies to test for allelism by crossing with other resistant populations

TABLE 6 | Phenotypic resistance segregation observed in two pseudo-F_2 and three back-cross susceptible (BC/S) families at high mesotrione dose (420 g ai ha^{-1}).

Run	Dose (g ai ha^{-1})	Family[a]	Plants treated	Survivors (observed)	Survival ratio	Survivors (expected)	χ^2	*P*-value
1	420	R	8	8	1.00			
		S	8	0	0.00			
		SR/F1-8	13	1	0.08			
		SR/F1-9	9	3	0.33			
		SR/F1-13	12	9	0.75			
		F2 segregation				1R:2F1:1S		
		F2-9	91	86	0.95	37.9	104.6	0.00
		F2-13	96	56	0.58	60.0	0.7	0.40
		BC/S segregation				1F1:1S		
		BC-8/S	98	10	0.10	3.8	11.1	0.00
		BC-9/S	96	84	0.88	16.0	346.8	0.00
		BC-13/S	86	54	0.63	32.3	23.3	0.00
2	420	R	16	16	1.00			
		S	15	0	0.00			
		SR/F1-8	24	1	0.04			
		SR/F1-9	9	4	0.44			
		SR/F1-13	24	22	0.92			
		F2 segregation				1R:2F1:1S		
		F2-9	56	56	1.00	26.4	62.8	0.00
		F2-13	94	82	0.87	66.6	12.2	0.00
		BC/S segregation				1F1:1S		
		BC-8/S	91	0	0.00	1.9	1.9	0.16
		BC-9/S	94	68	0.72	20.9	136.5	0.00
		BC-13/S	85	20	0.24	38.9	16.9	0.00

Chi-square (χ^2) analysis for expected plant survival assuming control for mesotrione resistance by a single major gene.

[a]*S, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-susceptible A. tuberculatus collected from a field in Dixon County, NE in 2014; HPPD-R, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicide-resistant A. tuberculatus collected from a field in Platte County, NE in 2014. SR/F1-8 (generated BC-8/S), SR/F1-9 (generated F2-9 and BC-9/S), and SR/F1-13 (generated F2-13 and BC-13/S) are crosses originated from SxR parents made under greenhouses conditions.*

and to further characterize the genotype × herbicide treatment environment.

The majority of weed species show either semi-dominance or dominance of inherited herbicide resistant alleles (Mallory-Smith et al., 1990; Lorraine-Colwill et al., 2001; Busi and Powles, 2017). For example, lack of dominance and high degree of genetic complexity within population has been documented in cross-pollinated (dioecious) weed species, including *Lolium rigidum*, *A. tuberculatus*, and *Alopecurus myosuroides* (Petit et al., 2010; Busi et al., 2013; Huffman et al., 2015). In contrast, the majority of insecticide resistance is recessive (Sandrock and Vorburger, 2011; Shen et al., 2017; Amusa et al., 2018). In theory, insect mating (S × R) would result in heterozygote susceptible (F1 families) phenotypes. Recessive inheritance was a key factor for the success of refugee strategy in Bt crops for delaying insecticide resistance evolution (Tabashnik et al., 2013; Jin et al., 2014). Therefore, tactics for combating herbicide resistance may be more complex than for insecticide resistance evolution. In our study, semi-dominance of mesotrione resistance was evident (**Figures 2A,B**). In such cases, theoretically, the heterozygote progeny (F1) would survive the field recommended herbicide dose (105 g ai ha^{-1}).

Similar results from the reciprocal crosses (S × R and R × S) in F1 families indicated nuclear inherited resistance alleles (**Figures 2A,B**). As a result, nuclear inheritance allows seed and pollen movement carrying herbicide resistance alleles (Busi et al., 2011). Pollen-mediated gene flow plays an important role in dispersing herbicide resistance traits, which was previously documented by intra- and inter-specific hybridization studies in *Amaranthus* species (Gaines et al., 2012; Liu et al., 2012; Sarangi et al., 2017). This result highlighted the potential gene flow for spreading the metabolism-based mesotrione resistance from this *A. tuberculatus* population from Nebraska, and the likelihood to increase the frequency of resistance genes within the population (Jasieniuk et al., 1996).

Herbicide resistance traits in weeds are usually nuclear inherited (Jasieniuk et al., 1996; Gaines et al., 2012). In addition, our results support the conclusion that a single major gene did not control metabolism-based resistance in this *A. tuberculatus* population from Nebraska (**Tables 4–6**). Regardless of the apparent heterogeneity, there was no mortality in the R population at the three mesotrione doses applied. In fact, the R population is highly resistant as R plants survived 420 g ai ha^{-1} of mesotrione with low injury (**Figure 3E**). Even with the

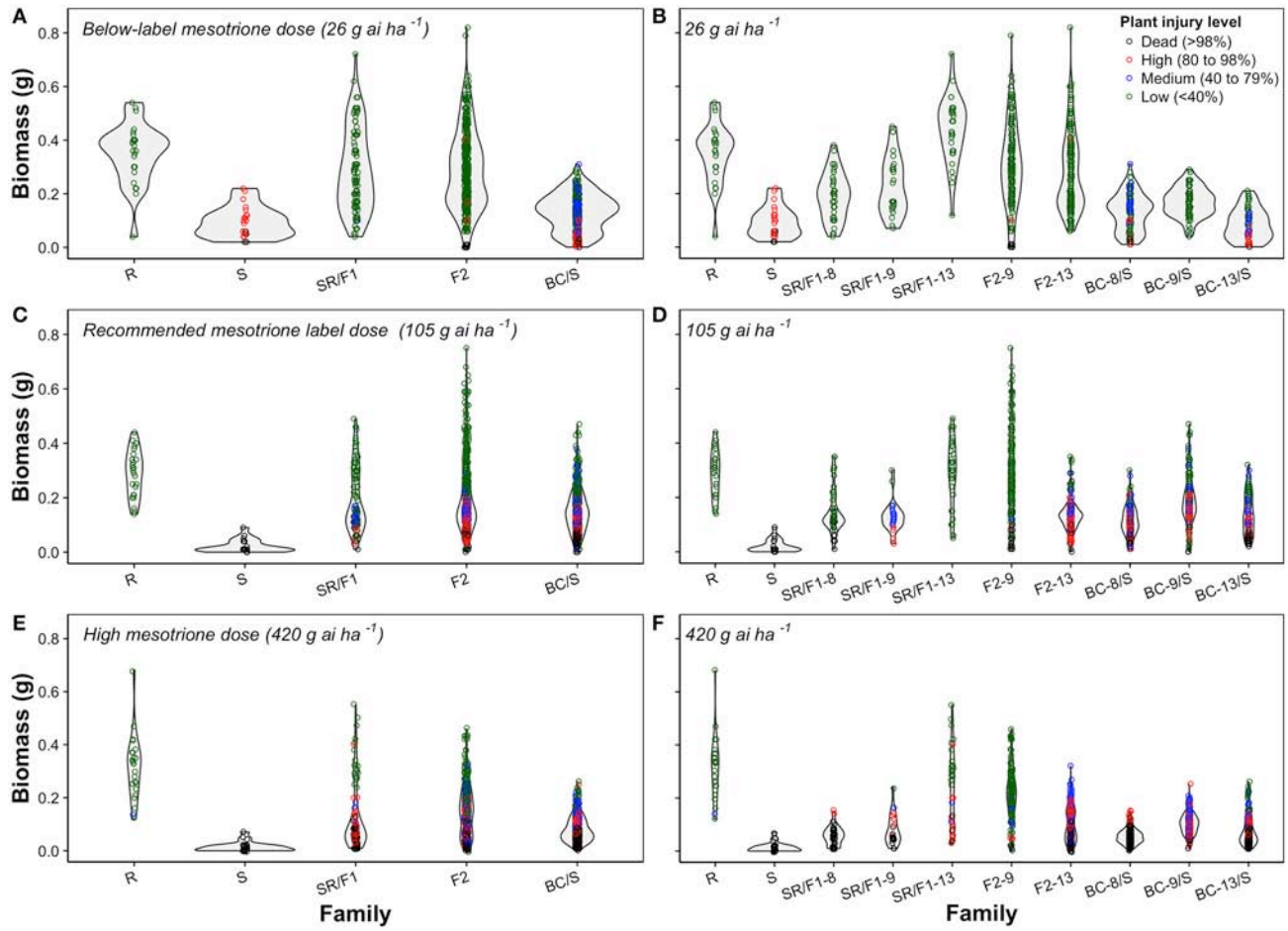

FIGURE 3 | Violin plots combining a rotated kernel density plot on each side of mesotrione inheritance of *Amaranthus tuberculatus* families at below-label mesotrione dose (26 g ai ha^{-1}) of combined **(A)** or separated families **(B)**; recommended mesotrione label dose (105 g ai ha^{-1}) of combined **(C)** or separated families **(D)**; and high mesotrione dose (420 g ai ha^{-1}) of combined **(E)** or separated families **(F)**. The dots represent biomass (g) for an individual plant within a population and colors denote the mesotrione injury level in experiment conducted at the University of Nebraska-Lincoln.

apparent lack of homogeneity in the R parent population, the variability of the phenotype in each generated family would not be explained by a single major gene (e.g., segregation pattern of BC and F2 families; **Figures 3A–F**). Also, mesotrione segregation analysis in the parent (R and S) populations and in the F1, F2, and BC/S families showed either continuous or bell-shaped distributions for *A. tuberculatus* biomass (**Figures 3A–F**), which is a typical response of quantitative (polygenic) traits (Morton and MacLean, 1974; Huffman et al., 2015). Furthermore, in our study, plant mortality was lower than expected at lower doses and higher than expected at higher doses (**Tables 4–6**), suggesting that inheritance of mesotrione resistance alleles is additive (Tabashnik, 1991).

In weeds, herbicide metabolism-based resistance is not well-understood and usually conferred by multiple alleles (Délye et al., 2013). For example, polygenic resistance was reported in NTSR mechanisms of ACCase- and ALS-inhibitor herbicides in *L. rigidum* populations (Busi et al., 2011, 2013; Han et al., 2014) and to ACCase-inhibitors in *Avena fatua* (Burns et al., 2018). However, monogenic resistance was found to endow NTSR mechanisms as well. A single major gene explained auxinic, glyphosate, and pyroxasulfone resistance in *L. rigidum* populations (Lorraine-Colwill et al., 2001; Busi et al., 2014; Busi and Powles, 2017) and atrazine resistance in *A. tuberculatus* (Huffman et al., 2015). Therefore, a single gene or multiple genes can endow NTSR mechanisms in weeds, suggesting that the number of alleles controlling NTSR varies according to the ecology and evolutionary factors contributing for weed resistance evolution. Also, these results demonstrated the complexity of NTSR mechanisms in weed species, which might be influenced by epigenetic effects (Yu and Powles, 2014; Markus et al., 2018).

Previous research in this R population suggested that HPPD-resistance is likely due to multiple cytochrome P450 enzymes, which is also indicative of polygenic resistance (Oliveira et al., 2017b). The cytochrome P450s comprise a large plant gene family and have repeatedly been associated with metabolism-based resistance in grass weed species (Powles and Yu, 2010). Examples of metabolism-based resistance via cytochrome P450s in grass

weed species include *L. rigidum* (Christopher et al., 1991; Busi et al., 2011, 2013, 2014; Han et al., 2014); *A. myosuroides* (Letouzé and Gasquez, 2003); and *Echinochloa phyllopogon* (Yun et al., 2005; Iwakami et al., 2014). In dicotyledon species, metabolism-based resistance is still under-studied (Powles and Yu, 2010). In such cases, often the presence of TSR mechanisms might mask the metabolism-based resistance (Yu and Powles, 2014). Nonetheless, metabolism-based resistance via cytochrome P450s gained attention with the evolution of 2,4 D (Figueiredo et al., 2017) and HPPD resistance (Ma et al., 2013; Kaundun et al., 2017; Nakka et al., 2017) in *Amaranthus* species. Multiple genes were found to confer resistance in a metabolism-based mesotrione resistant *A. tuberculatus* population from Illinois (Huffman et al., 2015). Despite different genetic background, farming system, and potentially different cytochrome P450 enzymes causing resistance, the genetics of mesotrione resistance is consistent with polygenic inheritance in the Nebraska (Oliveira et al., 2017a) and Illinois (Huffman et al., 2015) *A. tuberculatus* populations. Therefore, there are likely multiple genes involved in mesotrione resistant-*A. tuberculatus* populations across the north-central United States.

In summary, we confirmed that mesotrione resistance in an *A. tuberculatus* from Nebraska is nuclear inherited and likely mediated by multiple genes. It remains unknown if another mechanism of herbicide resistance has arisen in the R population from Nebraska. Other proteins play an important role in herbicide compartmentalization (transporter proteins), degradation (glutathione-*S*-transferases, glycosyl-transferases, esterases, hydrolases), and compensation (oxidases, peroxidases; Délye, 2013; Ghanizadeh and Harrington, 2017). For example, other steps in herbicide metabolism in addition to oxidation by P450s, such as initial oxidation followed by conjugation (e.g., to a sugar) may be part of a metabolic resistance mechanism. The multiple metabolic steps would be consistent with additive inheritance, due to the requirement of upregulation of more than one gene to confer high level resistance and partial resistance conferred from upregulation of only one.

AUTHOR CONTRIBUTIONS

MO and TG: designed the experiments; MO: conducted the experiments, analyzed the data, and wrote the manuscript; TG, SK, and AJ: conceptualized the research. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00060/full#supplementary-material

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Multiple Resistance Evolution in Bipyridylium-Resistant *Epilobium ciliatum* After Recurrent Selection

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The use of herbicides with different modes of action is the primary strategy used to control weeds possessing resistance to a single mechanism of action (MOA). However, this practice can lead to selection for generalist resistance mechanisms and may cause resistance to all MOAs. In this research, we characterized the resistance to diquat/paraquat (bipyridiliums) in an *Epilobium ciliatum* biotype (R1) collected in an olive orchard from Chile, where alternatives herbicides (2,4-D, glyphosate, glufosinate, flazasulfuron and pyraflufen-ethyl) with different MOAs were used, but they have also showed failure in controlling this species. Because the resistance/susceptibility patterns of the R1 biotype to glufosinate, 2,4-D and pyraflufen-ethyl were not clear, a recurrent resistance selection was carried out in field and greenhouse using these herbicides on R1 plants for three generations (R2 biotype). One biotype that was never treated with herbicides (S) was included as control. Results indicated that the S biotype was controlled at the field dose of all herbicides tested. The biotype R1 exhibited resistance to diquat, paraquat and flazasulfuron and natural tolerance to glyphosate. The R2 biotype displayed resistance to glufosinate, 2,4-D and pyraflufen-ethyl with LD_{50} (herbicide dose to kill 50% of plants) values higher than field doses in all assays. Physiological and biochemical studies determined the resistance to diquat of the R1 biotype, which was due to impaired translocation. The resistance to flazasulfuron in the R1 and R2 biotypes was confirmed by the low sensitivity of the acetolactate synthase (ALS) activity compared to the S biotype. The similar accumulation of shikimate in treated S, R1, and R2 plants with glyphosate supported the existence of innate tolerance to this herbicide in *E. ciliatum*. Resistance to glufosinate, 2,4-D and pyraflufen-ethyl in the R2 biotype, acquired after recurrent selection, was determined by low sensitivity of the glutamine synthetase, low accumulation of ethylene and protoporphyrinogen IX oxidase, respectively, in comparison

to the S biotype. *Epilobium ciliatum* from Chilean olive orchards had resistance to only two MAOs (photosystem I and ALS inhibitors), but resistance to five MOAs could occur in the next cropping seasons, if alternatives to weed management, other than herbicides, are not included.

Keywords: 5-enolpyruvylshikimate-3-phosphate synthase, acetolactate synthase, glutamine synthetase, fringed willowherb, photosystem I, protoporphyrinogen oxidase, synthetic auxins

INTRODUCTION

Epilobium ciliatum Raf. (fringed willowherb or American willowherb) is a troublesome annual weed belonging to Onagraceae, known as the willowherb or evening primrose family that contains approximately 170 species. This family is exceptional for its morphological, ecological and cytological variety (Myerscough and Whitehead, 1966). *Epilobium ciliatum* was first recorded in Britain in 1891 and was then spread globally. This species has a wide ecological niche and can produce thousands of seeds in a season. Its growth can be found in areas at sea level or 3,000 m above sea level in dry soils, open or disturbed woodlands, grasslands, and along roadsides (Myerscough and Whitehead, 1967).

Epilobium ciliatum is normally controlled with post-emergence application of paraquat and/or diquat (PS I electron diverter, also known as bipyridyliums) in perennial crops such as nurseries, orchards and hops in Northern Europe (Bulcke et al., 1987). Although paraquat is one of the most toxic herbicides used in the last 60 years, it is used extensively in approximately 100 countries on more than 100 crops without restrictions. Paraquat is a non-selective post-emergence herbicide that causes peroxidative stress in plants (Vartak and Bhargava, 1999).

The repeated application of bipyridylium herbicides can easily cause selection of resistant and tolerant weed biotypes (Hawkes, 2014). Both herbicide resistance and tolerance implies that there was no selection or genetic manipulations that allowed survival of weeds. To date, 32 species have evolved bipyridylium resistance globally, and *E. ciliatum* was detected as being resistant to paraquat in Belgium and Unite Kingdom in 1982 and 1989, respectively (Heap, 2018). Usually resistance to these herbicides is associated with reduced translocation of bipyridylium out of treated tissues (Moretti and Hanson, 2017). Additional reports indicated that protective enzymes also confer paraquat resistance (Hawkes, 2014).

The occurrence of bipyridylium resistance has led to an increase in the use of the herbicides glyphosate [5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor], glufosinate [glutamine synthetase (GS) inhibitor], 2,4-D (synthetic auxin), flazasulfuron [acetolactate synthase (ALS) inhibitor], and pyraflufen-ethyl [protoporphyrinogen oxidase (PPO) inhibitor] among others. Using numerous herbicides allows a broad herbicidal spectrum that includes activity against monocotyledonous and dicotyledonous weeds, and rapid onset of action and long persistence of some herbicides (Ganie and Jhala, 2017).

The use of herbicides with different mechanism of action (MOA), alone or in mixture, is the main tool to combat resistance to a specific group of herbicides (Tornisielo et al., 2013). The proper implementation of this practice can lead to selection of generalist target or non-target site resistance mechanisms, inducing the evolution of weed biotypes resistant to multiple MOAs (Neve and Powles, 2005). In cases of resistance multiple to herbicides, weeds evolved from monogenetic to polygenetic resistance (Heap, 2014). Many plants, particularly weeds, can reach this condition, because they contain a tremendous amount of genetic variation that allows them to survive under different biotic and abiotic conditions (Neve et al., 2009).

Since the early 1990s, herbicides have been the main tool for weed control in Chile (Valverde, 2007). In some cases, herbicides with different MOAs were applied widely to control bipyridylium-resistant *E. ciliatum* in olive trees in Chile; however, some of them have also shown failure in controlling this species. Here, we investigated the resistance to bipyridylium and the evolution of multiple resistance in *E. ciliatum* biotypes harvested in the Lolol province in Chile.

MATERIALS AND METHODS

Chemicals

Commercially formulated diquat, paraquat, glyphosate, glufosinate, flazasulfuron, 2,4-D and pyraflufen-ethyl (**Table 1**), were used for spraying *E. ciliatum* plants. Analytical grade (>99.5%) was used to determine the herbicide effects on physiological and biochemical studies in plants.

Plant Material

Epilobium ciliatum mature seeds of a biotype that is resistant (R1) to diquat/paraquat were collected from an olive orchard in the Lolol province, Chile (34°44′07″S71°42′16″W) in 2014. This orchard field had been treated for more than 20 years using PSI inhibiting herbicides, but in recent years, other herbicides (from the ALS, EPSPS, GS, PPO inhibitors and synthetic auxins chemical groups) have been used as alternatives to the first one. Seeds of a susceptible (S) biotype were also collected in 2014 from a closed area in which herbicides had never been applied.

The seeds were germinated in Petri dishes containing filter paper that was moistened with distilled water. Petri dishes were placed in a growth chamber at 28/18°C (day/night) with a photoperiod of 16 h, 850 μmol m^{-2} s^{-1} photosynthetic photon flux, and 80% relative humidity. All seedlings were transplanted into pots (one plant per pot) containing sand/peat in a 1:1 (v/v) ratio, and placed in a greenhouse with a 16 h photoperiod.

TABLE 1 | Herbicides, formulation type (FT), percentage of concentration (PC), WSSA/HRAC group (Group), mechanism of action (MOA), field doses in g ai ha^{-1} (Dose), doses used in the curve dose-response in g ai ha^{-1} (Dose-response) and application time (Time) evaluated on the bipyridylium-resistant *Epilobium ciliatum* biotypes from Chile.

Herbicide	Trade name (FT-PC)[a], Manufacturer	Group[b]	MOA[c]	Dose	Dose-response	Application time[d]
Diquat	Reglone® (SL, 17% w/w), Syngenta	22/D	PSI	400	0, 12.5, 25, 50, 100, 250, 500, 1,000, and 2,000	POST
Paraquat	Paratex® (SL, 20% w/v), Aragonesas Agro (ADAMA)	22/D	PSI	500	0, 25, 50, 100, 500, 1,000, 2,000, 4,000, and 6,000	POST
Glyphosate[e]	Roundup® Energy (SC, 50.9% w/w), Monsanto	9/G	EPSPS	720	0, 100, 200, 400, 600, 800, 1,000, 2,000, and 4,000	POST
Glufosinate	Finale® (SL, 20% w/v), Bayer CropScience	10/H	GS	750	0, 12.5, 25, 50, 100, 250, 500, 1,000, and 2,000	POST
Flazasulfuron	Terafit® (WG, 25% w/w), Syngenta	2/B	ALS	50	0, 1.25, 2.5, 5, 10, 20, 40, 80, and 160	PRE and early POST
2,4-D	U46® D Complet (SL, 60% w/v), Nufarm	4/O	SA	800	0, 12.5, 25, 50, 100, 250, 500, 1,000, and 2,000	POST
Pyraflufen-ethyl	Gozal® (CE, 2.65% w/v), Belchim Crop Protection	14/E	PPO	5	0, 0.15, 0.3, 0.6, 1.2, 2.4, 6.0, 12, and 24	Early POST

[a] *FT: SL, soluble (liquid) concentrate; SC, suspension concentrate; WG, water dispersible granules, and EC, emulsifiable concentrate. PC: w/w = weight/weight or w/g = weight/volume. Mention of trade names in this publication is solely for providing specific information and does not imply their recommendation.*

[b] *WSSA, Weed Science Society of America and HRAC, Herbicide Resistance Action Committee.*

[c] *MOA: PSI, photosystem I inhibitor (electron diverter); EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase inhibitor; GS, glutamine synthetase inhibitor; ALS, acetolactate synthase inhibitor; SA, synthetic auxins, and PPO, protoporphyrinogen oxidase inhibitor.*

[d] *POS, post-emergence and PRE, pre-emergence.*

[e] *Doses expressed as g acid equivalent (ae) ha^{-1} (50.9% potassium salt of glyphosate equals 450 g ae L^{-1}).*

Dose Response to Herbicides

Experiments were conducted using eight replicates (individual plants) for each *E. ciliatum* biotype (R1 and S) at eight doses of paraquat and diquat, and including one set of non-treated plants as controls (**Table 1**). The herbicide doses were applied at two different growth stages: rosette (BBCH14-16) and 10 cm height (tillering, BBCH55-60) plants. Glyphosate, glufosinate, flazasulfuron, 2,4-D and pyraflufen-ethyl were only applied at the rosette stage with eight doses of each herbicide (**Table 1**). The herbicides were applied in a laboratory chamber (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with 8002 flat fan nozzles delivering 200 L ha^{-1} at the height of 50 cm from plant level. Experiments with alternative herbicides were repeated including the biotype R2, obtained from recurrent selection for resistance, as will be described later.

Plant mortality (LD) and fresh weight reduction (GR) were measured 21 days after treatment (DAT). After estimating LD_{50} (herbicide dose required to kill by 50% a weed population) and GR_{50} (dose required to reduce shoot weight by 50% relative to non-treated plants) values using log-logistic models ($Y= c+\{(d-c)/[1+(x/g)^b]\}$ or $Y =(d)/1+(x/g)^b$), the resistance factors (RF = R/S) were computed as R-to-S GR_{50} or LD_{50} ratios.

Recurrent Selection

Because the resistance/susceptibility patterns of the R1 biotype to 2,4-D, glufosinate and pyraflufen-ethyl were not clear in the first set of dose-response assays, a recurrent selection for resistance to these three herbicides was conducted.

For the first generation, two thousand seedlings of R1 biotype were transplanted into plots (2 × 5 m) in the experimental field at the University of Cordoba (Spain). When plants reached the rosette stage, they were treated with pyraflufen-ethyl (PPO inhibitor) at 5 g ai ha^{-1}. Two weeks later the surviving plants were treated with 2,4-D (synthetic auxin) at 400 g ai ha^{-1}. Finally, surviving plants that were 10 cm in height (tillering stage) were treated with glufosinate (GS inhibitor) at 750 g ai ha^{-1}. The herbicides were applied using a Pulverex backpack sprayer with a T coupling for the wand equipped with four flat fan nozzles, calibrated to deliver 200 L ha^{-1} at a spraying pressure of 200 kpa.

The effect of pyraflufen-ethyl was very fast. Growth stopped and leaves exhibited burns 2 DAT. Resistant plants (5%) began to regrow producing leaves. During the first days after 2,4-D treatment the plants stopped growing and classical uncontrolled tissue growth and epinasty appeared, followed by growth inhibition and death. However, more than 20% of the total plants recovered their normal growth 14 DAT. Finally, treatment with glufosinate caused a rapid effect after the first DAT, with appearance of plant chlorosis and necrosis. Between 40 and 50% of plants finished their reproductive cycle and mature seeds with multiple resistance (to PS I + GS + PPO + synthetic auxins) were harvested and used for the next generations.

For the second generation, approximately 500 seedlings were transplanted into trays (40 × 100 × 15 cm) containing the same soil mixture and growth conditions described in Plan Material section. Approximately 80% of the treated plants finished their reproductive cycle. Six months later the third generation was initiated and plant survival was 95% in this generation. The F_3 progeny, hereinafter are referred as R2 biotype, was included together with biotypes R1 and S to repeat the dose-response experiments, as described above, and to conduct the physiological and biochemical studies to characterize multiple resistance.

Physiological and Biochemical Studies

Seedlings of *E. ciliatum* R1, R2, and S biotypes were transplanted into pots and grown in growth chambers under the same set of conditions described in Plant Material section.

Diquat

^{14}C-diquat absorption and translocation were evaluated at 3, 6, 12, and 24 h after treatment (HAT). R1 and S plants were treated when reached the rosette stage with a solution of ^{14}C-diquat (specific activity 6.2789 MBq/mg, American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) plus commercial diquat formulation. The solution applied contained ^{14}C-diquat providing 0.834 kBq μL^{-1} at the final concentration of 100 g ia ha^{-1} of diquat in 300 L. One drop of 1 μL $plant^{-1}$ of solution was applied on the adaxial surface of the second youngest fully expanded leaf. After treatment, the plants were maintained in the growth chamber during 12 h in the dark before light was initiated (Moretti and Hanson, 2017).

To determine the absorption, the ^{14}C-diquat treated plants were harvested (at the previous times) and the treated leaves were washed three times separately with 1 mL of water to recover the non-absorbed ^{14}C-diquat. The washing solution was mixed with 2 mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard). Samples were reserved to analysis the radioactivity. To determine the translocation, the whole plants were carefully removed from the pot (at the previous times) and washed, mainly the roots. The plants were individually divided into treated leaf, remainder of the plant and root system. The samples were stored in flexible combustion cones (Perkin-Elmer, BV BioScience Packard, Groningen, Netherlands), dried in an oven at 60°C for 48 h. Later, the samples were combusted in a Packard Tri Carb 307 biological oxidizer (Packard Instrument Co., Downers Grove, IL, USA). The CO_2 released from the combustion was captured in 18 mL of a mix of Carbo-Sorb E and Permafluor (1:1 v/v) (Perkin-Elmer, BV BioScience Packard, Groningen, Netherlands).

The radioactivity in washing solution and combustion samples was quantified by liquid scintillation spectrometry in a LS 6500 scintillation counter (Beckman Coulter Inc., Fullerton, CA, USA) during 5 min per sample, and the measurement was repeated again 24 h later. The radioactive values were used to calculate recovery percentage as: % ^{14}C-diquat recovered= (kBq in treated leaf + kBq reminder plant + kBq in root system + kBq from washes/ kBq total applied) × 100. Experiments were arranged in a completely random design with five replicates per biotype at each time evaluated.

Glyphosate

The shikimic acid accumulation produced by glyphosate was studied in the R1, R2, and S biotypes of *E. ciliatum* according to Shaner et al. (2005). Leaf disks (5 mm diameter) were harvested from the youngest fully expanded leaves from a batch of 15 plants of each biotype at the rosette stage. The glyphosate concentrations used were: 0.1, 10, 50, 500, and 1,000 μM. Absorbance was measured using a DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, USA) at 380 nm. The experiments had a completely random design using three tissue samples of 50 mg from each *E. ciliatum* biotype per glyphosate concentration. Experiments were repeated twice and results were expressed in mg of shikimic acid per gram of fresh tissue. The amount of shikimic acid was determined by comparison to a set of standard samples of known shikimic concentrations used for plotting calibration curve.

Flazasulfuron

ALS activity was measured using the product acetolactate estimation after conversion by decarboxylation in the presence of acid to acetoin (Hatami et al., 2016). Two grams of young leaves of *E. ciliatum* R1, R2, and S biotypes were ground using extraction buffer (3 mL g^{-1}). The de-salted protein extract was used in ALS enzyme assays, with flazasulfuron as representative of the ALS inhibitor herbicides. The herbicide concentrations used were: 0, 1, 5, 10, 50, 100, 500, 1,000, 2,000, and 3,000 μM. ALS activity was assayed colorimetrically (at 520 nm) by measuring acetoin production and expressing this as a percentage in respect to the control. The experiment was repeated at least three times from independent protein extractions and the I_{50} values (inhibition of the ALS enzyme by 50%) were estimated. The protein concentration of the crude extract was measured using the Bradford method (Bradford, 1976).

Glufosinate

The glutamine synthetase (GS) response to glufosinate was determined using crude protein extracts isolated from *E. ciliatum* R1, R2, and S leaves (rosette stage) as described by Rojano-Delgado et al. (2013). The glufosinate concentrations used were: 0, 1, 10, 50, 100, 500, 1,000, and 5,000 μM. The glufosinate concentration that reduced the GS activity by 50% (I_{50}) was used to calculate the resistance values (RF) values. The total protein (nmol of glutamine mg^{-1} of protein h^{-1}) was measured following the Bradford's method (Bradford, 1976). This experiment was repeated twice using three replications per herbicide concentration.

2,4-D

Plants at the rosette stage were sprayed with 2,4-D solutions (0, 200, 400, 600, 800, and 1,000 g ai ha^{-1}) as in dose response curves. Twenty-four HAT, seedlings were excised and 400 g shoot fresh weight were placed into a 10 mL syringe with 1 mL distilled water and sealed (De Prado et al., 2000). The syringes were placed in a dark incubator at 27°C for 4 h and 1 mL of the headspace gas was analyzed for ethylene (C_2H_4) by gas chromatography (Shimabukuro and Hoffer, 1996). The C_2H_4 was expressed as nanoliter per gram of fresh weight by hour. There were five replicates per treatment and the experiment was repeated twice.

Pyraflufen-Ethyl

Protoporphyrin oxidase IX (Protox IX) levels were determined following the method proposed by Dayan et al. (2015). Approximately 0.1 g leaf-disks (4 mm-diameter) of R1, R2, and S biotypes were incubated in a Petri dishes containing 6 mL of 2% (w/v) sucrose, 1 mM 2-(N-morpholine) ethanesulfonic acid and 100 μM technical pyraflufen-ethyl for 20 h at 25°C in darkness. After incubation, leaf disks were homogenized and centrifuged (Fernandez-Moreno et al., 2017). The supernatants

were concentrated and reconstituted in 1 mL of methanol and filtered through a 0.2 μm nylon syringe filter to clean the samples. The extracts were stored in opaque glass vials at 20°C. An aliquot of 50 μL was injected into the HPLC system (Beckman Coulter 126 Gold System, Fullerton, California, USA). Protox IX concentrations in the extracts were quantified using a calibration curve obtained from a Protox IX standard (Sigma Aldrich, St. Louis, Missouri, USA). The results were expressed as nanomoles per gram of fresh weight. Treatments were carried out three times, with three repetitions per treatment.

RESULTS

Dose Response to PS I Inhibiting Herbicides

Dose-response studies with diquat and paraquat on plants were concluded with 100% mortality in the *E. ciliatum* S biotype at doses well below the field doses. In contrast, the R1 biotype was markedly less affected by PS I inhibiting herbicides and required doses higher than those normally used by Chilean farmers in the Lolol area. The LD_{50} and GR_{50} values for R1 were higher than for S biotype (**Figure 1**, **Table 2**).

When both herbicides were applied in R1 and S plants before flowering (BBCH55-60 stage), herbicide efficacy decreased and resulted in a LD_{50} that was notably higher than younger plants (BBCH14-16 stage). The GR_{50} values also established high resistant factors (RF) to PS I inhibiting herbicides (**Figure 1**, **Table 2**).

Response to Alternative Herbicides Before and After Recurrent Resistance Selection

For 20 years, *E. ciliatum* has been exposed to the PS I inhibiting herbicides (diquat/paraquat), and eventually herbicides with different MOAs (glyphosate, flazasulfuron, glufosinate, 2,4-D and pyraflufen-ethyl) were used as alternative. However, Chilean farmers have also reported failures in controlling *E. ciliatum* with these herbicides. Our results of the first set of dose-response assays were ultimately inconclusive to determine the status of resistance or susceptibility to glufosinate, 2,4-D and piraflufen-ethyl, because the GR_{50} and LD_{50} values of the R1 biotype were similar to the S biotype. R1 plants showed low resistance to glufosinate (FR = 2.9) and intermediate resistance to 2,4-D (FR = 3.9) and pyraflufen-ethyl (FR = 1.7) (**Table 3**). However, some individuals in the R1 biotype survived at doses equal to or

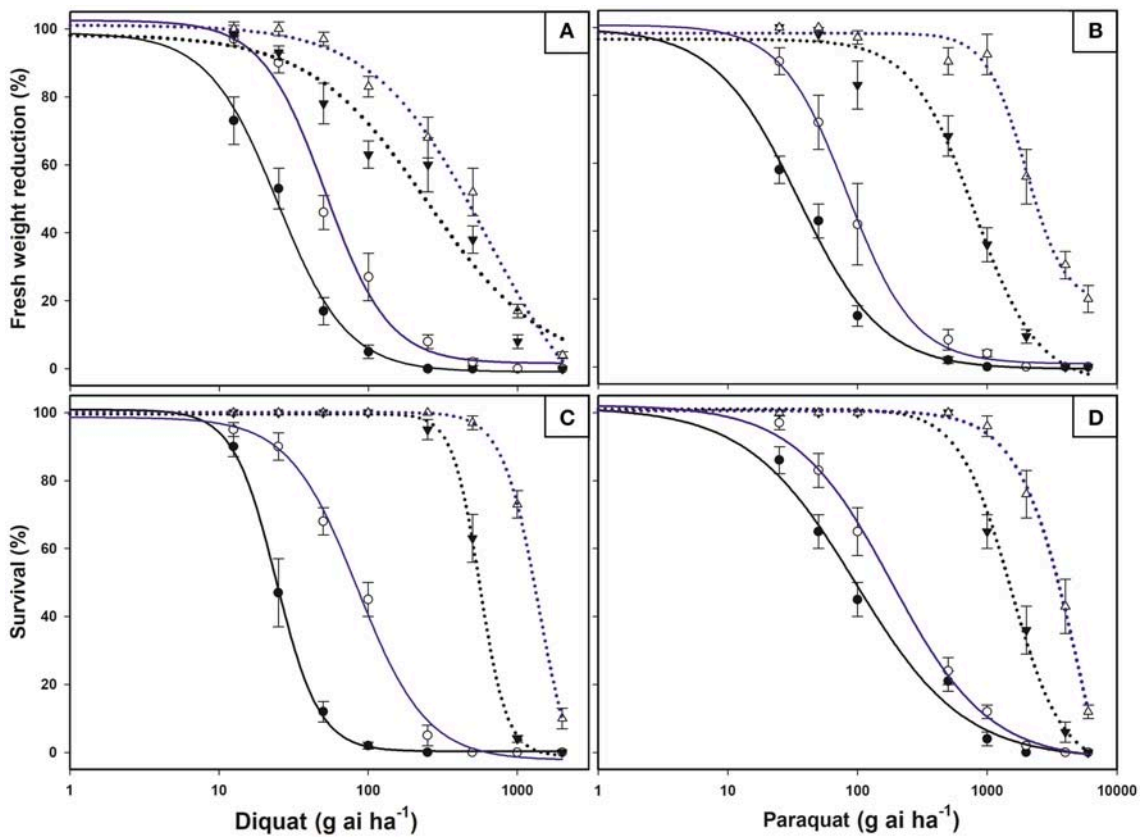

FIGURE 1 | Log-logistic curves of bipyridylium-resistant *Epilobium ciliatum* S (solid lines) and R1 (dotted lines) biotypes, treated at the BBCH14-16 (black lines) and BBCH55-60 (blue lines) stages. Diquat **(A)** and Paraquat **(B)** dose-response curves based on the fresh weight reduction percentage with respect to untreated controls. Diquat **(C)** and Paraquat **(D)** dose-response curves of survival percentage with respect to untreated controls. Evaluations were conducted 21 days after treatments. Vertical bars are ± standard errors of the mean ($n = 8$).

TABLE 2 | LD_{50} and GR_{50} values of the R1 and S *Epilobium ciliatum* biotypes using diquat and paraquat at different growth stages (BBCH 14–16 and 55–60).

Herbicide	BBCH	Biotype	GR_{50} ± SE	RF	*c*	*d*	*b*	R^2	LD_{50} ± SE	RF	*c*	*d*	*b*	R^2
Diquat	14–16	S	24.6 ± 1.7	10.1	−0.86	98.78	1.86	0.99	37.2 ± 2.4	15.1	0.27	100.79	3.00	0.99
		R1	247.6 ± 31.6		0.0	98.27	1.11	0.93	560.2 ± 22.5		−1.03	99.56	4.76	0.99
	55–60	S	51.4 ± 5.2	12.1	1.57	102.42	2.04	0.98	84.9 ± 6.3	16.5	−0.28	98.60	1.87	0.99
		R1	619.6 ± 42.4		−3.31	101.11	1.15	0.98	1401.7 ± 37.6		−0.77	100.03	3.51	1.00
Paraquat	14–16	S	35.0 ± 3.0	22.7	−0.68	99.63	1.34	0.99	96.9 ± 17.4	15.7	−1.91	101.49	1.03	0.98
		R1	796.0 ± 48.4		−1.82	96.49	1.78	0.98	1521.2 ± 158.6		−0.65	101.02	2.18	0.98
	55–60	S	83.8 ± 7.8	23.7	0.91	100.66	1.69	0.99	184.1 ± 19.6	18.7	0.30	102.15	1.16	0.99
		R1	1986.6 ± 191.8		8.1	98.24	2.84	0.98	3447.4 ± 216.2		−1.71	100.54	1.85	0.99

Data were pooled and fitted using the non-linear regression model: $Y = c+[(d–c)/[1+(x/g)^b]]$, where Y is the is the fresh weight reduction or survival percentage; x (independent variable) the herbicide dose; c and d are the lower and upper limits, respectively; b is the slope of the curve; GR_{50} and LD_{50} expressed in g ai ha^{-1}. [b]RF = Resistance factor = GR_{50} or LD_{50} of the R biotype/GR_{50} or LD_{50} of the S biotype. ± Standard error of the mean (n = 8).

higher than the field doses of these herbicides. For this reason, we conducted the recurrent selection for resistance in field and greenhouse during three generations, which gives origin to the R2 biotype. These experiments were aimed at testing if multiple resistance condition can occur in Chilean olive orchards in the next cropping seasons.

Glyphosate showed low levels of efficiency on S, R1 and R2 biotypes. The three *E. ciliatum* biotypes had LD_{50} values close to the glyphosate field dose (1,080 g ea ha^{-1}) used by farmers. The RF values of the R1 and R2 biotypes in respect to the S biotype were 1.1 and 1.0, respectively (**Table 3**).

The S biotype was very sensitive to flazasulfuron; and the LD_{50} and GR_{50} values the R1 and R2 biotypes, which presented similar profiles of resistance, were close to the field dose of this herbicide (**Table 3**). The resistance to flazasulfuron in *E. ciliatum* had already been selected in field by showing poor control.

The R1 biotype had low resistance to glufosinate based on both the LD_{50} and GR_{50} parameters, compared to the S biotype. The R2 biotype had LD_{50}, GR_{50}, and RF values that were higher than the R1 biotype. The LD_{50} of R2 biotype was higher than the field dose confirming its resistance to glufosinate after the recurrent selection (**Table 3**).

Data associated with survival and growth reduction show that the S biotype was more susceptible to 2,4-D than the R1 and R2 biotypes. The R1 and R2 *E. ciliatum* biotypes were 3.3 and 9.2 more resistant then the S biotype (**Table 3**), showing that the resistance to 2,4-D increased markedly after the recurrent selection.

The S and R1 biotypes 48 HAT presented visible symptoms with pyraflufen-ethyl, meanwhile, the R2 plants had no presented damage. At 96 HAT, chlorosis and necrosis corona damages became evident in the S biotype and less so in some plants in the R1 biotype. The R2 biotype remained green with little damage in adult leaves. After 1 week, all S plants and only a small number of R1 plants died and all R2 plants survived. Based on the LD_{50} and GR_{50} values, the biotypes R1 and R2 were 1.7 and 13.8 or 5.9 and 41.4 more resistant, respectively, than the S biotype (**Table 3**).

Physiological and Biochemical Studies

Diquat

The average total recovery of ^{14}C-diquat applied was >93% in both R1 and S biotype. ^{14}C-diquat absorption was similar in S and R1 *E. ciliatum* biotypes throughout the measurement period, increasing over time up to 80% at 24 HAT. However, the translocation of ^{14}C-diquat from the treated leaf to the rest of the shoot and roots was greater and faster in the S biotype than in the R1 biotype. Thus, we can observe a translocation of 34.9% in the rest of the plant and 18.3% in the root system in the *E. ciliatum* S biotype after 24 HAT. In the biotype R1, translocation was reduced with values of 3.1% in the rest of plant and a negligible amount of ^{14}C-diquat in the root system (**Table 4**).

Glyphosate

Epilobium ciliatum plants treated with increasing glyphosate concentrations accumulated shikimic acid. However, no differences in shikimate accumulation were observed between the three biotypes (**Figure 2**). These results are consistent with those obtained previously from the dose-response assays (**Table 3**).

Flazasulfuron

The inhibition of the ALS enzyme in the R1 and R2 biotypes was similar, and they were 16.4 and 14.9 less susceptible, respectively, than the S biotype (I_{50} = 30.9 μM flazasulfuron) (**Figure 3**). The specific activity of the ALS enzyme was similar across the three biotypes (318.2, 322.1, and 316.9 nmol acetoin per mg protein per hour for S, R1, and R2, respectively).

Glufosinate

The glufosinate doses required to reduce GS activity by 50% (I_{50}) were 18.5, 25.8, and 1252.3 μM for the S, R1, and R2 biotypes, respectively. The RF values indicated that R2 was 67.7-fold more resistant than the S biotype; whereas GS activity was inhibited by glufosinate in a similar way in the S and R1 biotypes (**Figure 4**).

2,4-D

The ethylene accumulation in *E. ciliatum* S, R1, and R2 plants responded positively to the dose of herbicide applied (**Figure 5**).

TABLE 3 | LD_{50} and GR_{50} values of the R1, R2 and S *Epilobium ciliatum* biotypes using different herbicides, applied at the BBCH 14–16 growth stage.

Herbicide	Biotype	GR_{50} ± SE	RF	*d*	*b*	R^2	LD_{50} ± SE	RF	*d*	*b*	R^2
Glyphosate	R1	310.1 ± 23.1	1.1	102.06	1.42	0.98	931.8 ± 37.1	1.0	99.56	3.85	0.98
	R2	281.0 ± 28.8	1.0	104.11	1.40	0.99	989.1 ± 41.6	1.1	99.19	4.32	0.99
	S	270.9 ± 19.5	–	103.78	1.39	0.99	904.0 ± 58.0	–	94.63	3.22	0.97
Glufosinate	R1	60.4 ± 19.7	1.6	102.05	1.14	0.98	167.1 ± 22.8	2.9	100.18	3.91	0.99
	R2	269.8 ± 41.4	7.3	99.72	1.85	0.97	1096.1 ± 38.9	19.3	100.12	2.94	0.99
	S	36.9 ± 4.7	–	101.95	1.52	0.98	56.9 ± 3.4	–	101.66	1.73	0.98
Flazasulfuron	R1	37.4 ± 3.9	7.8	93.35	2.12	0.99	63.4 ± 6.6	3.4	100.31	4.73	0.99
	R2	41.7 ± 4.1	8.7	95.88	2.33	0.98	66.5 ± 4.5	3.5	101.23	4.76	0.97
	S	4.8 ± 0.4	–	102.09	1.58	0.99	18.9 ± 2.0	–	99.16	3.21	0.99
2,4-D	R1	164.8 ± 24.8	3.3	101.31	1.77	0.97	469.5 ± 28.5	3.9	101.07	4.69	0.97
	R2	461.3 ± 38.1	9.2	99.16	1.79	0.99	1076.6 ± 57.3	9.0	100.01	6.01	0.98
	S	50.1 ± 3.1	–	101.12	1.17	0.98	119.1 ± 9.0	–	103.05	3.51	0.99
Pyraflufe-ethyl	R1	0.41 ± 0.08	5.9	100.04	2.13	0.97	1.05 ± 0.31	1.7	99.33	1.53	0.99
	R2	2.9 ± 0.16	41.4	99.23	1.38	0.99	8.42 ± 1.05	13.8	100.42	2.37	0.98
	S	0.07 ± 0.01	–	100.01	0.68	0.97	0.61 ± 0.06	–	99.66	1.34	0.99

Data were pooled and fitted using the non-linear regression model: $Y = (d)/1+(x/g)^b$, where Y is the is the fresh weight reduction or survival percentage; x (independent variable) the herbicide dose; d is the upper limit; b is the slope of the curve; LD_{50} and GR_{50} expressed as g ai ha^{-1} or g ae ha^{-1}, in the case of glyphosate. [b]RF = Resistance Factor = GR_{50} or LD_{50} of an R biotype/GR_{50} or LD_{50} of the S biotype. ± Standard error of the mean (n = 8).

TABLE 4 | ^{14}C-diquat absorption and translocation from three to 24 h after treatment (HAT) in R1 and S *Epilobium ciliatum* plants.

Biotype	HAT	Absorption(%)[a]	Translocation (% absorbed)[b]		
			TL	RP	RS
S	3	10.3 ± 1.4 E	97.1 ± 2.3 A	2.3 ± 0.4 D	0.6 ± 0.2 C
	6	21.4 ± 3.1 D	81.4 ± 5.9 B	10.2 ± 2.5 C	8.4 ± 1.9 B
	12	49.7 ± 6.8 C	68.3 ± 6.4 C	17.1 ± 3.4 B	14.6 ± 2.1 A
	24	81.2 ± 5.9 A	46.8 ± 3.9 D	34.9 ± 6.8 A	18.3 ± 3.4 A
R1	3	8.4 ± 2.2 E	98.3 ± 0.1 A	1.5 ± 0.3 D	ND
	6	23.2 ± 5.7 D	97.9 ± 1.8 A	1.8 ± 0.9 D	ND
	12	61.3 ± 4.8 B	98.1 ± 1.5 A	1.4 ± 0.4 D	ND
	24	79.2 ± 3.5 A	96.4 ± 3.3 A	3.1 ± 1.1 D	0.5 ± 0.09 C

[a]Percentage of ^{14}C-diquat absorbed from total applied.
[b]TL, treated leaf; RP, remainder of the plant; RS, root system. Means followed by the same letter per column do not differ by the Tukey test ($P < 0.05$). ± Standard error (n = 5). ND, no detected.

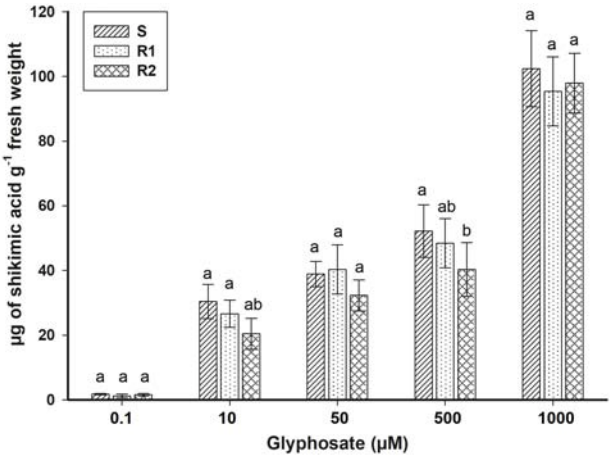

FIGURE 2 | Shikimic acid accumulation in bipyridylium-resistant *Epilobium ciliatum* biotypes at different glyphosate concentrations. Means followed by the same letter do not differ by the Tukey test ($P < 0.05$). Vertical bars are ± standard errors of the mean ($n = 3$).

Non-treated plants from the three biotypes had similar ethylene accumulation (0.14 nL g^{-1} fresh weight h^{-1}). The results of accumulation of ethylene at 800 g ai ha^{-1} of 2,4-D were 2.0 and 5.0 times smaller in the R1 and R2 biotypes, respectively, than accumulation in the biotype S.

Pyraflufen-Ethyl

After 100 µM piraflufen-ethyl application, the R2 biotype accumulated significantly less Protox IX than the S and R1 biotypes of *E. ciliatum*. The Protox IX accumulation values were 0.258, 1.420, and 3.062 nmol g^{-1} fresh weight in R2, R1, and S *E. ciliatum* biotypes, respectively.

DISCUSSION

The olive (*Olea europaea* L.) is one of the oldest fruit trees used by man. At present, this is an innovative and expanding crop in Chile (20,000 ha in 2017), which has been integrated into the new olive growing locations around the globe (Barranco et al., 2017). The use of herbicides is the most widely used tool for Chilean weed control (Valverde, 2007), in some cases, rotating between herbicides with different MOA. However, this practice has lead

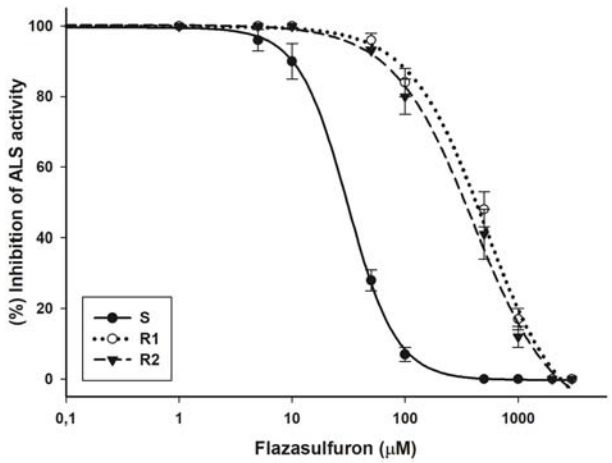

FIGURE 3 | Acetolactate synthase (ALS) enzyme activity in bipyridylium-resistant *Epilobium ciliatum* biotypes determined using flazasulfuron. The equations of log–logistic curves to estimates the I_{50} values are: S: $Y= 0.31+\{(99.53–0.31)/[1+ (dose/I_{50})^{2.00}]\}$, ($R^2 = 0.99$); R1: $Y = -3.40+\{(99.94+3.40)/[1+(dose/I_{50})^{1.30}]\}$, ($R^2 = 0.99$): R2: $Y = -1.17+\{(100.26+1.17)/[1+(dose/I_{50})^{1.22}]\}$, ($R^2 = 0.99$). Vertical bars are ± the standard errors of the mean ($n = 3$).

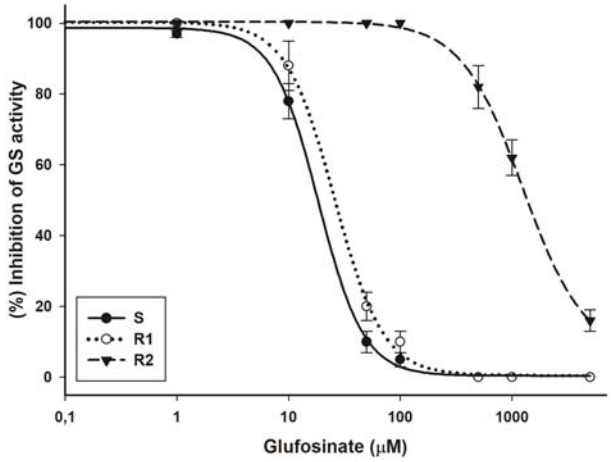

FIGURE 4 | Glutamine synthetase (GS) inhibition by glufosinate in bipyridylium-resistant *Epilobium ciliatum* biotypes. The equations of log–logistic curves to estimates the I_{50} values are: S: $Y= 0.39+\{(98.64–0.39)/[1+ (dose/I_{50})^{2.14}]\}$, ($R^2 = 0.99$); R1: $Y = 0.50+\{(100.30–0.50)/[1+(dose/I_{50})^{1.99}]\}$, ($R^2 = 0.99$); R2: $Y=6.55+\{(100.39–6.55)/[1+(dose/I_{50})^{1.57}]\}$, ($R^2 = 0.99$). Vertical bars are ± the standard errors of the mean ($n = 3$).

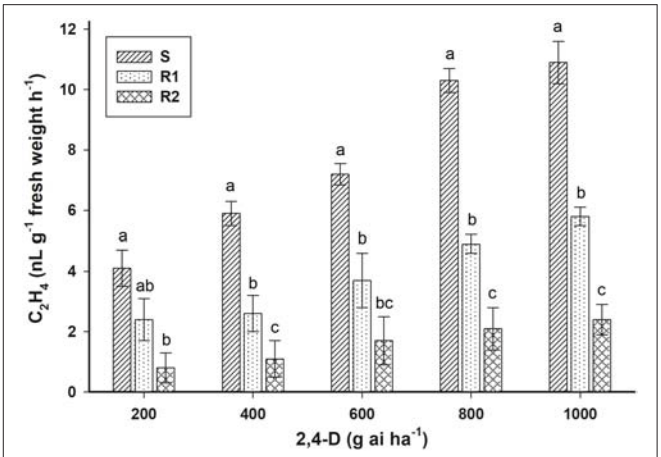

FIGURE 5 | Ethylene accumulation induced by 2,4-D in S, R1 and R2 plants of the bipyridylium-resistant *Epilobium ciliatum* biotypes. Means followed by the same letter do not differ by the Tukey test ($P < 0.05$). Vertical bars are ± the standard errors of the mean ($n = 5$).

to the evolution of resistance to multiple herbicides that utilize different resistance mechanisms.

The R1 *E. ciliatum* biotype, which had been treated for consecutive years by bipyridium herbicides (diquat/paraquat), survived at doses higher than those used by the farmer. However, the S biotype, harvested in an area that was never treated by diquat/paraquat, was efficiently controlled at these doses. These results evidence the evolution of resistance of the R1 *E. ciliatum* biotype to bipyridium herbicides. Presently, there have been 67 reports of resistance to paraquat and only 10 to diquat (Heap, 2018). *Epilobium ciliatum* was detected as being resistant to paraquat in Belgium in 1986, but not to diquat (Bulcke et al., 1987). Our results demonstrate cross-resistance to both herbicides. Applications at the tillering stage were much less efficient than those applied at the rosette stage, which indicates that these herbicides need to be applied at the early stages for greater efficiency.

The plant resistance to bipyridyliums may be due to protective enzymes, which minimizes reactive oxygen species, although, resistance to these herbicides is usually due to reduced movement of the herbicide into plants (Preston, 1994; Hawkes, 2014; Moretti and Hanson, 2017). The results with ^{14}C-diquat demonstrated that both biotypes, S and R1, have similar capacities for herbicide absorption during the first 24 HAT. However, the reduced translocation of ^{14}C-diquat observed within the leaf in the R1 biotype in the basipetal direction, compared to the highest and most rapid translocation found in the S biotype, indicates that this physiological alteration contributes to resistance. The lack of translocation was characterized as being the mechanism responsible for resistance to paraquat in species of the genera *Conyza* (Kato and Okuda, 1983; Fuerst et al., 1985) and *Hordeum* (Powles, 1986; Turcker and Powles, 1991). In *Lolium perenne*, translocation of paraquat from the treated leaf was lower in a resistant biotype than in a susceptible one (Brunharo and Hanson, 2017). This reduced translocation was the result of a higher transport of the herbicide into the vacuole, which could also be considered a resistance mechanism.

Chilean farmers have always complained about the poor control of *E. ciliatum* with glyphosate, even if used at high doses. The similar susceptibility patterns found in the S, R1, and R2 biotypes, according to the dose-response and shikimate accumulation results, allow us to conclude that *E. ciliatum* has natural tolerance to glyphosate, which can be explained

by mechanisms of the non-target site type (Bracamonte et al., 2018). The lack of ^{14}C-glyphosate absorption and translocation, and in other cases, the glyphosate metabolism into non-toxic compound, has been characterized as the mechanisms responsible for the tolerance to this herbicide (Cruz-Hipolito et al., 2011; Ribeiro et al., 2015).

The R1 and R2 *E. ciliatum* biotypes presented LD_{50} values of flazasulfuron higher than the field dose, which supports that the resistance to this herbicide was already selected for in the field. The similar inhibition of the ALS enzyme in R1 and R2 biotypes suggests that a mutation in the target site may be responsible for the resistance found in both R biotypes. There are 159 weeds species that are resistant to the ALS inhibiting herbicides (Heap, 2018), but only *Senecio vulgaris* and *Cyperus brevifolius* were detected being resistant to flazasulfuron (Okuno et al., 2015; Délye et al., 2016). In most cases, the resistance to these herbicides is due to mutation(s) in different position(s) of the ALS gene, but due to the action of specific enzymes, herbicide metabolic processes may also be involved (Yu and Powles, 2014).

Resistance to glufosinate, 2,4-D and pyraflufen-ethyl in the bipyridylium-resistant *E. ciliatum* (biotype R1), as mentioned above, was not clear in our first set of dose-response assays, nor has been confirmed by the farmers. However, the F_3 progeny (R2 biotype) of *E. ciliatum* displayed great resistance after the recurrent selections, with LD_{50} values that were above field doses. Obviously, our experimental conditions were extreme by selecting only the most resistant R1 individuals that gave origin to the R2 biotype, and by suppressing the genetic variability existing in natural conditions (Neve and Powles, 2005). Therefore, this condition of multiple resistance will take in appear more than three cropping season or selections, but due to the signs of control failure of *E. ciliatum* in field, this situation could be a reality in Chilean olive orchards, if alternatives to weed management, other than herbicides, are not included. Physiological and biochemical tests suggested the primary mechanisms that are likely involved in the multiple resistance to these herbicides in the R *E. ciliatum* biotypes.

The similar susceptibility to glufosinate in the R1 and S biotypes observed in the dose-response and GS activity assays, suggests that the R1 biotype was sensitive to this herbicide, and resistance in R2 was due to recurrent selection. These results suggest there may be a mutation in the GS gene of the progeny R2 biotype. To date, there are only four glufosinate-resistant weed species confirmed (Avila-Garcia and Mallory-Smith, 2011; Ghanizadeh et al., 2015; Fernandez et al., 2017; Jalaludin et al., 2017), but only in *Lolium perenne* ssp. *multiflorum* from Oregon (USA), a mutation was found in the GS gene endowing resistance to this herbicide (Avila-Garcia et al., 2012).

Plants treated with auxin herbicides, such as 2,4-D, show a fast accumulation of ethylene that is a function of the greater or lesser toxicity of these herbicides in treated plants (Mithila et al., 2011; Busi et al., 2017). The results obtained in our experiments indicated that 2,4-D was not reaching its target, a nuclear auxin receptor (F-box family), as determined by the ethylene synthesis pathway (ACC synthase) in the R2 biotype. There are 31 weeds with resistance to fenoxy-carboxylic acids (16 to 2,4-D) worldwide (Heap, 2018), and in the majority of these, non-target site resistance acts as the mechanisms (Busi et al., 2017; Torra et al., 2017). Among those, impaired translocation in resistant plants, compared to susceptible plants, is related to reduced ethylene production because 2,4-D is not reaching its nuclear target, such as *Papaver rhoeas* (Rey-Caballero et al., 2016).

Pyraflufen-ethyl, a novel inhibitor of Protox IX, is an effective herbicide in early post-emergence acting quickly on broad-leaved weeds at very low rates. The accumulation of the Protox IX can be used to determinate the efficacy of PPO inhibiting herbicides, where the greater accumulation of this enzyme corresponding to susceptible plants (Dayan et al., 2015). The accumulation of the Protox IX in plants treated with piraflufen-ethyl was lower in R2 plants than in R1 and S plants, confirming the higher resistance of the R2 biotype. These results support that the S and R1 biotypes were susceptible to pyraflufen-ethyl and that the R2 biotype evolved resistance after three recurrent selections. *Ambrosia artemisiifolia* was documented as the first resistance case to PPO inhibiting herbicides in 2004 (Rousonelos et al., 2012). To date, 13 cases of PPO-resistant weeds have been documented, 10 belonging to dicotyledonous weeds and three to monocotyledonous (Heap, 2018). The levels of resistance are quite variable between the different species and the herbicides tested (Dayan et al., 2014). The genus *Amaranthus* (*A. hybridus, A. palmeri, A. retroflexus,* and *A. tuberculatus*) detected in several cropping areas in the USA that has multiple resistance to glyphosate, ALS and PPO has been studied in depth (Legleiter and Bradley, 2008; Salas et al., 2016). Molecular studies have shown that a mutation in the PPO gene at position 98 is the most likely resistance mechanism involved in *A. artemisiifolia* (Rousonelos et al., 2012) and *A. palmeri* (Giacomini et al., 2017).

CONCLUSION

Based on our results, we can conclude that *E. ciliatum* harvested directly in field was resistant to bipyridyliums and flazasulfuron. Glyphosate is not an alternative in controlling *E. ciliatum* due to its innate tolerance to this herbicide. The condition of multiple resistance to five MOAs (ALS, GS, PPO, PSI inhibitors, and synthetic auxin) could occur in next cropping seasons, as demonstrated by the recurrent selection, if alternatives to weed management, other than herbicides, are not included.

AUTHOR CONTRIBUTIONS

RD: idea and designed of the research. BT, RA-dlC, and AR-D performed the experiments. BT, RA-dlC, EA, JT, JD-V, HC-H and AR-D interpreted and analyzed the raw data. BT, RA-dlC, EA, JT, JD-V, HC-H, AR-D and RD wrote and approved the manuscript.

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