ARTIFICIAL POLYPLOIDY IN PLANTS

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PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88966-407-8
DOI 10.3389/978-2-88966-407-8

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ARTIFICIAL POLYPLOIDY IN PLANTS

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Citation: Chen, J.-T., Coate, J., Meru, G., eds. (2021). Artificial Polyploidy in Plants. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-407-8

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frontiers in Plant Science

EDITORIAL
published: 07 December 2020
doi: 10.3389/fpls.2020.621849

Editorial: Artificial Polyploidy in Plants

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Keywords: antimitotic agents, artificial polyploidy, flow cytometry, plant biotechnology, whole genome duplication

Editorial on the Research Topic

Artificial Polyploidy in Plants

In higher plants, polyploidy plays a significant role in diversification and speciation to drive evolution. Using biotechnology approaches, polyploidy can be induced artificially, chiefly by antimitotic agents, and is a powerful strategy for plant breeding. Duplicating whole sets of chromosomes not only increase copies of existing genes but produces additional alterations of the genome such as epigenetic changes and modulated gene expression that influences a wide range of phenotypes. Consequently, agricultural and horticultural crops with increased ploidy often exhibit anatomical and morphological changes and enhanced biomass, yield, vigor, and stress tolerance, which are potentially valuable for the commercial success of their products. This Research Topic explores current advances in artificial polyploidy and consists of 12 publications that are further divided into three subtopics, as summarized below.

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *27 October 2020*
Accepted: *12 November 2020*
Published: *07 December 2020*

Citation:
Chen J-T, Coate JE and Meru G (2020) Editorial: Artificial Polyploidy in Plants. Front. Plant Sci. 11:621849. doi: 10.3389/fpls.2020.621849

DEVELOPMENT OF NOVEL PROTOCOLS FOR INDUCING POLYPLOIDY

The technology for inducing polyploidy is well-established in some plants, however, there is still a large vacancy for the development of novel protocols in induction, evaluation, and screening.

Triploid breeding is a revolutionizing tool in *Populus* breeding that has resulted in improved growth-rate and wood quality for the timber industry, as well-enhanced stress tolerance. However, limited work has been done to improve efficiency of triploid development in *Populus*. In an effort to optimize triploid breeding in *Populus*, Zhou et al. determined that the rate of 2n pollen production was dependent upon the meiotic stage, injection time, and the interaction between the two factors. In the study, the authors report the most effective method for inducing 2n pollen in *Populus*, and conclude that the rate of triploid production is primarily limited by restricted growth of 2n pollen tubes.

Walnuts are economically important trees and their fruits have been part of the human diet for thousands of years. Luo and Chen used fluorescence *in situ* hybridization, an early-fruiting gene fragment-based analysis, and simple sequence repeats analysis to differentiate walnut cultivars from Sichuan Province in China. The authors found Sichuan walnut cultivars have great variation but not sufficient to recognize the cultivars as a new taxon. They proposed the resulting knowledge could be applied in the identification and breeding of Sichuan walnut cultivars. Additionally, such

techniques might be applied in the identification of novel genetic variations resulting from induced polyploidy, particularly in tree species.

Currently, there are limited sexual polyploids in the field of floriculture. Zeng et al. evaluated nine cultivars of an orchid, *Cymbidium* Swartz, and found that 2n male gametes occurred in frequencies ranging from 0.15 to 4.03%, and was genotype dependent. They performed seven pairs of crosses between cultivars and obtained five triploids and two tetraploids. The triploid hybrids showed enhanced *in vitro* regeneration capacity, growth, and flowering. The authors concluded that using 2n gametes to produce triploids is reliable for crop improvement in floriculture.

THE IMPLICATIONS OF POLYPLOIDY IN PLANT BREEDING

Synthetic polyploidy is crucial to plant breeding, therefore, it is valuable to explore the implications including disease resistance, stress tolerance, and production enhancement, and so on.

In a study of *Hydrangea macrophylla*, Alexander reported that triploids had improved growth, flower size, and larger stoma than related diploids cultivars. The author further studied reproductive biology on interploidy crosses between diploid and triploid cultivars of *H. macrophylla*. Genome size evaluation of the resulting hybrids revealed that the progeny were genetically unstable, and may have broad potential in breeding programs based on their genetic and phenotypic variability.

Calendula officinalis L. is a multipurpose plant for horticulture, cosmetics, and medicinal use, with a complex karyotype. Esmaeili et al. estimated genome sizes in nine commercial cultivars and concluded that they were of allotetraploid background. They induced artificial chromosome doubling in three allotetraploid cultivars by treatment with antimitotic agents. The resulting polyploids had enhanced cell volume and leaf size when compared to the original diploids.

Coffee beans are a popular product globally from plants that belong to the *Coffea* genus. Venial et al. induced polyploids from *C. canephora* and *C. arabica* through indirect somatic embryogenesis, and optimized the conditions, including the timing and concentration of the antimitotic agent, colchicine. Using the adopted procedure, they successfully obtained stable polyploids in both *Coffea* species. The authors proposed the protocol might be applied to other shrubbery and woody species for generating novel polyploids.

Lippia alba has economic and medicinal value, but currently, there are no reports of polyploidy induction. Julião et al. established a protocol to induce synthetic polyploids using colchicine, and successfully obtained triploid and tetraploids with altered composition of essential oils when compared with the unimproved individuals.

Triploid citrus produces seedless fruits of great economic value, and has been reported to improve tolerance to abiotic stresses, such as cold stress, which is the major impediment to citrus production. Lourkisti et al. reported that triploid citrus was more tolerant to natural chilling temperatures when compared to diploids, parental cultivars, and one diploid clementine tree. They found triploids to have better photosynthetic properties under natural low temperatures and higher contents of E-β-ocimene and linalool that may contribute to enhanced adaptation to cold stress.

Alien chromosome introgression is a valuable chromosome engineering tool for increasing genetic variation in plant breeding programs. Kwiatek et al. found that lines of *Aegilops kotchyi* Boiss obtained through monosomic addition or substitution had significantly improved resistance to leaf rust. The authors propose a similar approach for developing novel triticale varieties with resistance to leaf rust.

The use of synthetic polyploids in commercial production is currently limited, and the underlying mechanisms are poorly understood. A review of synthetic polyploidy by Ruiz et al. provides a brief history of artificial polyploidy in grafted crops, and refines the methodology for inducing polyploidy. The authors point to the potential application of artificial polyploidy in combating climate change in the future.

MECHANISTIC INSIGHTS INTO POLYPLOIDY BIOLOGY

Based on our knowledge in the field of polyploidy biology, the deep mechanistic insights are still lacking. Hence, the use of advanced technology toward deciphering mechanisms of alterations of growth and development, morphology, yield, metabolism, sterility, and genome structure and function is crucial.

Allopolyploids can be occasionally obtained by crossing distantly related species. The resulting hybrids often exhibit genetic instability and infertility. Park et al. analyzed meiotic chromosome behaviors in pollen mother cells in a synthesized intergeneric allotetraploid, *xBrassicoraphanus*, which is derived from a cross between *Brassica rapa* ($2n = 20$) and *Raphanus sativus* ($2n = 18$). According to the findings, the authors suggest that the prevention of non-homologous interactions between the parental chromosomes in *xBrassicoraphanus* is mainly due to structural dissimilarity of chromosomes and the suppression of crossovers between non-homologous chromosomes which is required for correct meiotic progression and gamete formation.

The hypothesis of "hybridization followed by chromosome doubling" suggests that it is possible to develop a viable hybrid zygote by providing each chromosome with a pairing homolog. Tel-Zur et al. attempted to provide evidence supporting this hypothesis by evaluating a series of behaviors in a group of putative hybrids obtained from embryo rescue following controlled crosses of *H. megalanthus* × *H. undatus*. They found that allohexaploids were formed via chromosome doubling events at a very early development stage, consistent with the "hybridization followed by chromosome doubling" hypothesis.

CONCLUSIONS AND PERSPECTIVES

This Research Topic collects recent advances in the field of Artificial Polyploidy in Plants, and broadly covers critical

topics including novel methodologies, plant breeding strategies, and mechanistic insights. We hope that the current era, characterized by rapid development of molecular tools and approaches, such as multi-omics, high-throughput biology, and emerging biotechnology such as CRISPR genome editing will facilitate further unraveling of artificial polyploidy biology, and in turn result in novel mechanistic insights and cultivars for growers.

AUTHOR CONTRIBUTIONS

J-TC, JC, and GM drafted the manuscript. All authors revised and approved the final version.

ACKNOWLEDGMENTS

We greatly appreciate the invaluable contribution of all authors and reviewers as well as the efforts of Specialty Chief Editor Diego Rubiales of Frontiers in Plant Science: Plant Breeding.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 19 February 2020
doi: 10.3389/fpls.2020.00100

Ploidy Level Influences Pollen Tube Growth and Seed Viability in Interploidy Crosses of *Hydrangea macrophylla*

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All *Hydrangea macrophylla* cultivars tested to date are diploid or triploid and triploid *H. macrophylla* have thicker stems, larger flowers, and larger stoma compared to related diploids. It is unknown whether interploidy crosses between diploid and triploid hydrangeas can be used to develop triploid varieties. The objective of this study was to compare pollen tube development, fruit formation, and seed viability among intra- and interploidy pollinations of *H. macrophylla* and evaluate the genome size and pollen viability of resultant progeny. By 24 h post-pollination, pollen tubes had reached the ovaries of diploid flowers in 48.7% of samples while pollen tubes reached the ovaries in only 8.7% of triploid flowers (χ^2 = 30.6, $p < 0.001$). By 48 h post-pollination pollen tubes reached the ovaries of diploid and triploid flowers in 72.5% and 53.8% of samples, respectively (χ^2 = 26.5, p = 0.001). There was no difference in percentage of flowers with pollen tubes reaching the ovaries in diploid and triploid flowers at 72 h after pollination (χ^2 = 7.5, p = 0.60). Analysis of covariance showed that pollen tube length at 24 and 48 h post-pollination was significantly influenced by ploidy and flower length of the female parent. Progeny of interploidy crosses was diploid and aneuploid; no triploid progeny were recovered from crosses using triploid parents. Mean genome sizes of offspring from each cross type ranged from 4.56 pg for 2x × 2x offspring to 5.17 pg for 3x × 3x offspring. Estimated ploidy of offspring ranged from 2x for 2x × 2x crosses to 2.4x for 3x × 3x crosses. Pollen stainability rates of flowering offspring using a modified Alexander's stain ranged from 69.6% to 76.4%.

Keywords: pollen tube, polyploid, mating barrier, triploid, hydrangea, ornamental plant breeding

OPEN ACCESS

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *27 September 2019*
Accepted: *22 January 2020*
Published: *19 February 2020*

Citation:
Alexander L (2020) Ploidy Level Influences Pollen Tube Growth and Seed Viability in Interploidy Crosses of Hydrangea macrophylla. Front. Plant Sci. 11:100. doi: 10.3389/fpls.2020.00100

INTRODUCTION

Polyploidy—also known as whole genome duplication—represents a major mechanism of adaptation and speciation in plants (Grant, 1981; Ramsey and Schemske, 1998). It is widespread among plant families and an estimated 47 to 70% of angiosperm species are polyploid (Grant, 1981). Polyploidy has also been associated with changes to ornamental traits in plants. Increasing chromosome copy number leads to an increase in the number of each gene, a phenomenon

known as "genetic redundancy". The genetic redundancy present in polyploids enables them to undergo extensive genetic rearrangements that lead to the stable expression of novel traits (Kumari and George, 2008). Polyploids can serve as a bridge that allows interspecific hybridization between two species previously separated by ploidy differences and can be used to restore fertility to sterile hybrids (Lim et al., 2000). Polyploidization has also been used to develop sterile cultivars, enhance stress tolerance to drought, cold, herbicides, and poor soils, and enhance flower size, color, and other ornamental attributes (Takamura and Miyajima, 1996; Griesbach, 2000; Van Tuyl and Lim, 2003; Kumari and George, 2008).

Hydrangea macrophylla is one of the most economically important nursery crops worldwide, with U.S. sales of *Hydrangea* species topping $120,000,000 in 2014 (USDA-NASS, 2014). Hydrangeas, prized for their large showy flowers and lush green foliage, are sold for landscape use, patio and container use, and as cut flowers in the floral industry. Chromosome number within the genus ranges from 2n = 2x = 30 in *H. involucrata* Sieb. to 2n = 4x = 72 or 2n = 6x = 108 in *H. paniculata* Sieb. (Funamoto and Tanaka, 1988). All *H. macrophylla* cultivars tested to date are diploid (2*n*=2*x*=36) or triploid (2*n*=3*x*=54; Demilly et al., 2000; Cerbah et al., 2001; Zonneveld, 2004; Jones et al., 2007). Alexander (2017) showed that triploid hydrangeas had thicker stems, larger flowers, and larger stoma compared to full-sibling diploids. These triploids were meiotic polyploids produced using unreduced gamete breeding, where a source of n and 2n male gametes combined with meiotically normal female gametes to form both diploid and triploid full-sibling offspring (Bretagnolle and Thompson, 1995; Alexander, 2017). As observed in other species, triploids may also be obtained through somatic fusion or sexual hybridization between a diploid and tetraploid parent. Sexual hybridization between triploids and diploids has been used in a few, diverse ornamental species with fertile triploids like tulip (*Tulipa*) and pear (*Pyrus*) to generate genetic variability or new ploidy levels for interspecific hybridization (Cao et al., 2002; Marasek-Ciolakowska et al., 2014). It is unknown whether interploidy crosses between diploid and triploid hydrangeas can be used to develop triploid varieties, although a recent reconstruction of an *H. macrophylla* pedigree identified at least four putative interploidy crosses that produced high-quality diploid and/or triploid progenies (Hempel et al., 2018).

Fertility of triploids varies widely among plant species and is dependent on frequency and type of trivalents formed at metaphase I and the tolerance of gametes to aneuploidy (Singh, 1993). Stainable pollen for the diploid and triploid *H. macrophylla* ssp. macrophylla cultivars averaged 70% and 63%, respectively, and results of controlled pollinations indicate that triploid cultivars can produce viable seed (Jones et al., 2007). Breeding improvement using mixed ploidy levels requires the adoption of cytological methods to assess fertility of potential parents and suitability of potential cross combinations. Pollen staining is widely used to assess male fertility and pollen viability; however, pollen staining only assesses fertilization potential because not all stainable grains may actually be viable. Alexander (2019) showed that aniline-blue staining overestimated pollen viability by an average of 2.3x for *H. macrophylla* when compared to *in vitro* germination. Results of pollen germination using growth medium are considered more accurate, but still fail to account for pollen-pistil interactions. Using fluorescent microscopy to monitor pollen germination and growth of the pollen tube most closely simulates *in vivo* conditions (Kho and Baër, 1968; Atlagić et al., 2012). Further, visualization of pollen germination and pollen tube growth provides a measure of "cross" incompatibility among species or cultivars. The fruit of a hydrangea is a capsule containing dozens to hundreds of ovules that are capable of maturation into viable seeds (Hufford, 2001). Hybrid seed production is often low in *H. macrophylla*, possibly due to inbreeding in the cultivated gene pool (Wu and Alexander, 2019). Jones et al. (2007) reported from 0 to 20 seeds/capsule while other researchers have reported as little as 0–5 seeds/capsule resulting from crosses among *H. macrophylla* cultivars (Venturieri et al., 2017). Visualization of pollen germination and pollen tube growth allows for the differentiation between pre and post zygotic reproductive barriers which in turn influences the choice of plant breeding techniques (somatic hybridization or embryo culture) that may be used to mitigate incompatibility (Atlagić et al., 2012).

Interploidy hybridization which results in fertile progenies may generate new, desirable traits in established hydrangea cultivars or allow for the transfer of traits between hydrangea species. The objective of this study was to 1) compare pollen tube development, fruit formation, and seed viability among intra- and interploidy pollinations of *H. macrophylla*, and 2) evaluate the genome size and pollen viability of resultant progeny. Results will be used to identify reproductive barriers between ploidy levels and determine the efficacy of using triploids in *H. macrophylla* hybrid breeding. Progeny from interploidy hybridizations would be beneficial to expand the relatively narrow gene pool of cultivated hydrangea.

MATERIALS AND METHODS

Plant Material

Three triploid *H. macrophylla* cultivars (Blaumeise, Kardinal, and Taube) and three diploid cultivars (Decatur Blue, Oakhill, and Zaunkoenig) were used as parents in this experiment. Ten 1 year-old clones per cultivar were used in this experiment. Clones were produced by rooting softwood cuttings in pine bark using intermittent mist and 1,500 ppm indole 3-butyric acid. Plants were grown indoors in 3-gallon containers under 56% shade and micro-irrigated using spray stakes. Growing media consisted of pine bark amended with 6.6 kg·m^{-3} 19N-2.1P-7.4K Osmocote Pro fertilizer (Scotts-Sierra Horticultural Products Co., Maryville, Ohio), 0.6 kg·m^{-3} Micromax (Scotts-Sierra Horticultural Products Co.), 0.6 kg·m^{-3} iron sulfate, and 0.2 kg·m^{-3} Epsom salts. Greenhouse temperatures were maintained at 24°C during the d and 20°C at night.

Pollinations

Controlled pollinations were made following the method of Reed (2004). Briefly, pollen receptor flowers were emasculated 1–2 days

before pollination and covered with breathable bags (DelStar, Inc., Middleton, DE). Pollen from donor flowers was collected in glass vials the day of pollination, pollen was applied to exposed receptor stigmas using a camel hair brush, and the bag was replaced. A new brush was used for each pollination. Five inflorescences on each plant were used for pollination. To reduce pollen contamination, only one male parent was used on each inflorescence. Six flowers were pollinated on each inflorescence. Three pollinated flowers were harvested for pollen tube measurements—one after 24 h, one after 48 h, and one after 72 h. The other three pollinated flowers were left to develop into capsules. Bags were removed after 1–2 weeks. For estimations of seed set and seed viability, approximately 30 flowers were pollinated for each cross.

Pollen Staining and Pollen Tube Visualization

To estimate parental pollen viability, fresh pollen from a single flower was placed on a microscope slide using a camel-hair brush (n = 3 flowers per plant). A 30 μL drop of modified Alexander's stain (Alexander, 1969; Peterson et al., 2000; Atlagić et al., 2012) was pipetted onto the slide and a coverslip was applied. Slides were observed after 30 min at 10× magnification using an Olympus BX-60 compound microscope with an Olympus Q Color 5 digital camera for image capture. Percent stained pollen was calculated as: (number of stained pollen grains/total number of pollen grains) x 100%.

To analyze pollen tube growth, approximately 30 flowers were pollinated for each cross as above. Flowers were collected 24, 48, or 72 h after pollination and prepared for visualization. Briefly, pistils were removed from flowers and fixed in Carnoy I (3:1; ethanol:glacial acetic acid) for at least 24 h at room temperature. The fixative was removed by pipetting and pistils were rinsed twice for 10 min in 70% ethanol with gentle agitation. Fresh 70% ethanol was added to cover pistils and pistils remained in ethanol until visualization (between 24 h and 6 d). On the day of visualization, ethanol was removed and pistils were rinsed in deionized water, hydrolyzed in 8 N NaOH for 1 – 3 h, and rinsed again with deionized water. Fully hydrolyzed pistils appeared translucent. Pistils were placed in a petri dish containing decolorized aniline blue (0.1% aniline blue dissolved in 0.1 N K_3PO_4) for 1 h, removed to a microscope slide with one 30 uL drop of stain (2:1; decolorized aniline blue:glycerin), and a cover-slip was applied to spread the tissues. Slides containing stained pistils were visualized using a fluorescent microscope (BX-60; Olympus America, Inc.) equipped with a 100 W high-pressure Hg lamp and a U-MNV near ultraviolet (400–410 nm) filter. Magnification depended on size of the flower. After slide preparation, between 6 and 10 intact flowers were available for each cross at each time point. Data recorded for three to six flowers per cross included length of longest three pollen tubes (mm) and whether pollen tubes reached the ovary (Y/N). Density of pollen tubes in the ovaries was scored as follows: 0 = none, 1 = Few (1–10 tubes), 2 = Some (11–20 tubes), 3 = Many (≥20 tubes; i.e., too many to count). Total length of each style was recorded. A total of 279 pollinated flowers were visualized, measured, and used for further analysis.

Seed Collection and Germination

Capsules were harvested November 2017 and stored at 4°C for 35–50 days. One capsule from each clone was harvested to determine average number of seeds per capsule. The seed from all 10 capsules of a cross (one capsule from each clone) was pooled to determine germination percentages. Up to 100 seeds of each full-sib family were sown in trays (1,020 flats) containing #1 starter mix and placed into a germinator on 4 January 2018. Germination conditions were 16 h light/8 h dark and 22°C. Flats were placed on trays and water was added to each tray when media in control flats appeared dry. Germination began ~7 d after sowing and was recorded for 28 days. Up to 30 seedlings per family were transferred to 2" pots. Seedlings were moved to 4" pots in April 2018, to 1.5 gal pots in July 2018, and were maintained in 1.5 gal pots in a greenhouse throughout the remainder of the experiment under the same conditions as the parent cultivars.

Seedling Pollen Viability and Genome Size Determination

Flowering, pollen viability, and genome size of seedlings was measured April–June 2019. For each flowering seedling, pollen was collected and stained in the same manner as the parental plants. Percent stained pollen was calculated as: (number of stained pollen grains/total number of pollen grains) x 100%.

Up to 30 offspring per cross and each parent cultivar were sampled for genome size determination. Approximately 0.5 cm^2 of growing leaf tissue of sample and standard were chopped for 30 to 60 s in a plastic petri dish containing 0.4 mL extraction buffer (Partec CyStain ultraviolet precise P Nuclei Extraction Buffer; Partec GMBH Muenster, Germany). The resulting extract was passed through a 30 μM filter into a 3.5-mL plastic tube to which was added 1.6 mL Partec CyStain ultraviolet precise P Staining Buffer containing the fluorochrome 4′, 6-diamidino-2-phenylindole (DAPI). The relative fluorescence of the total DNA was measured for each nucleus using a Partec PA-1 ploidy analyzer (Partec GMBH, Muenster, Germany). Results were displayed as histograms showing the number of nuclei grouped in peaks of relative fluorescence intensity. For each sample, at least 3000 nuclei were analyzed revealing a single peak with a coefficient of variation (CV) less than 4.9%. Genome sizes were calculated as nuclear DNA content for unreduced tissue (2C) as: 2C DNA content of tissue = (mean fluorescence value of sample ÷ mean fluorescence value of standard) × 2C DNA content of standard. *Pisum sativum* L. "Ctirad" with a 2C content of 9.09 pg was used as the internal standard (Doležel and Bartoš, 2005). Ploidy and genome sizes are the averages of two subsamples per plant.

Statistics

Pearson's chi-square test of association was used to determine the relationship between cross type and number of flowers with pollen tubes reaching the ovaries. Initial investigation of the data showed a strong correlation between style length and pollen tube length such that analysis of covariance (ANCOVA) with style length as a covariate was chosen over analysis of variance (ANOVA) to more accurately represent the influence of ploidy level. ANCOVA was used to partition variance in pollen tube

length into sources attributable to female ploidy level, male ploidy level, and environment (error) with female style length as a covariate. Means for each ploidy level or cross were compared using Tukey's studentized range test with an α = 0.05 significance level. Data for all analyses were checked for normality and non-constancy of variance using Shapiro-Wilk's and Levene's tests, respectively. All data analysis was performed using SAS® software, Version 9.4 of the SAS system for Microsoft (Copyright © 2013, SAS Institute Inc., Cary, NC, USA).

RESULTS

Pollen Viability of Parents

Percent stained pollen averaged 65 and 61% for diploid and triploid parent cultivars, respectively. One diploid parent, "Decatur Blue", only had 44.1 ± 0.041% stainable pollen while the other diploid parents, "Oakhill" and "Zaunkoenig" produced 86.8 ± 0.064% and 65.2 ± 0.054% stainable pollen. Percent stained pollen for triploid parents was 75.3 ± 0.001%, 59.2 ± 0.026%, and 48.4 ± 0.12% for "Blaumeise", "Nachtigall", and "Taube", respectively (**Figure 1**).

Pollen Tube Growth

Pollen germinated on the stigma of all flowers in this study, and all pairs of reciprocal crosses had some pollinations where pollen tubes reached the ovaries (**Figure 2**). There were significant associations between cross type and percentage of flowers with pollen tubes reaching the ovaries at 24 h (χ^2 = 30.6, $p < 0.001$) and 48 h (χ^2 = 26.5, p = 0.001) post-pollination. After 24 h, pollen tubes had reached the ovaries of diploid flowers in 44.4% of 2x × 2x crosses and 52.9% of 2x × 3x crosses. In contrast, pollen tubes reached the ovaries of triploid flowers in only 3.6% of 3x × 2x crosses and 13.8% of 3x × 3x crosses after 24 h (**Figure 3**). The trend continued at 48 h after pollination where pollen tubes reached the ovaries of diploid flowers in 65.0% of 2x × 2x crosses and 80.0% of 2x × 3x crosses. Pollen tubes reached the ovaries of triploid flowers in 60.6% of 3x × 2x crosses and 46.9% of 3x × 3x crosses after 48 h. There was no difference in percentage of flowers with pollen tubes reaching the ovaries in diploid and triploid flowers at 72 h after pollination (χ^2 = 7.5, p = 0.60).

Analysis of covariance showed that pollen tube length was significantly influenced by ploidy of the female parent and the style length of the female parent (**Table 1**). The 3x × 2x crosses had the longest pollen tubes at 24 and 48 h post-pollination (**Table 2**). The 3x × 3x pollinations had the shortest pollen tubes at 24 h and 2x × 2x and 3x × 3x crosses had the shortest pollen tubes at 48 h. There was no significant difference in pollen tube length among cross types by 72 h post-pollination. These comparisons were made adjusting for flower length, which accounted for most of the variation in pollen-tube length. Longer flowers tended to have longer pollen tubes; that is, pollen tubes grew until they reached the ovary regardless of the total size of the flower. Based on ANCOVA results, the influence of female ploidy disappeared after 48 h; flower length was the only significant source of variation in pollen tube length by 72 h post-pollination (**Table 1**).

Seed Set, Germination, and Seedling Growth

Number of flowers pollinated for each cross ranged from 23 to 37. All cross types except 3x × 2x had at least one cross fail as defined by the absence of rounded capsules (**Table 3**). Many crosses had fully-formed capsules that contained no seed. For example, only 1 seed was obtained from the cross 3x × 3x cross "Kardinal" × "Taube" even though 11 capsules appeared mature. Average

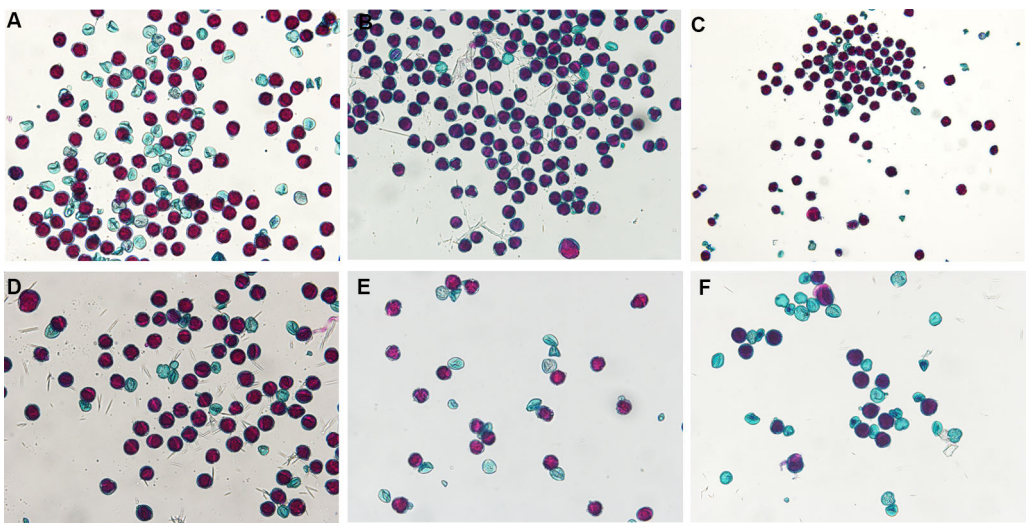

FIGURE 1 | Stained pollen of *Hydrangea macrophylla* diploid cultivars Decatur Blue **(A)**, Oakhill **(B)**, and Zaunkoenig **(C)**, and triploid cultivars Blaumeise **(D)**, Nachtigall **(E)**, and Taube **(F)**. Fresh pollen was stained with modified Alexander's stain and observed after 30 min at 10× magnification using an Olympus BX-60 compound microscope with an Olympus Q Color 5 digital camera for image capture.

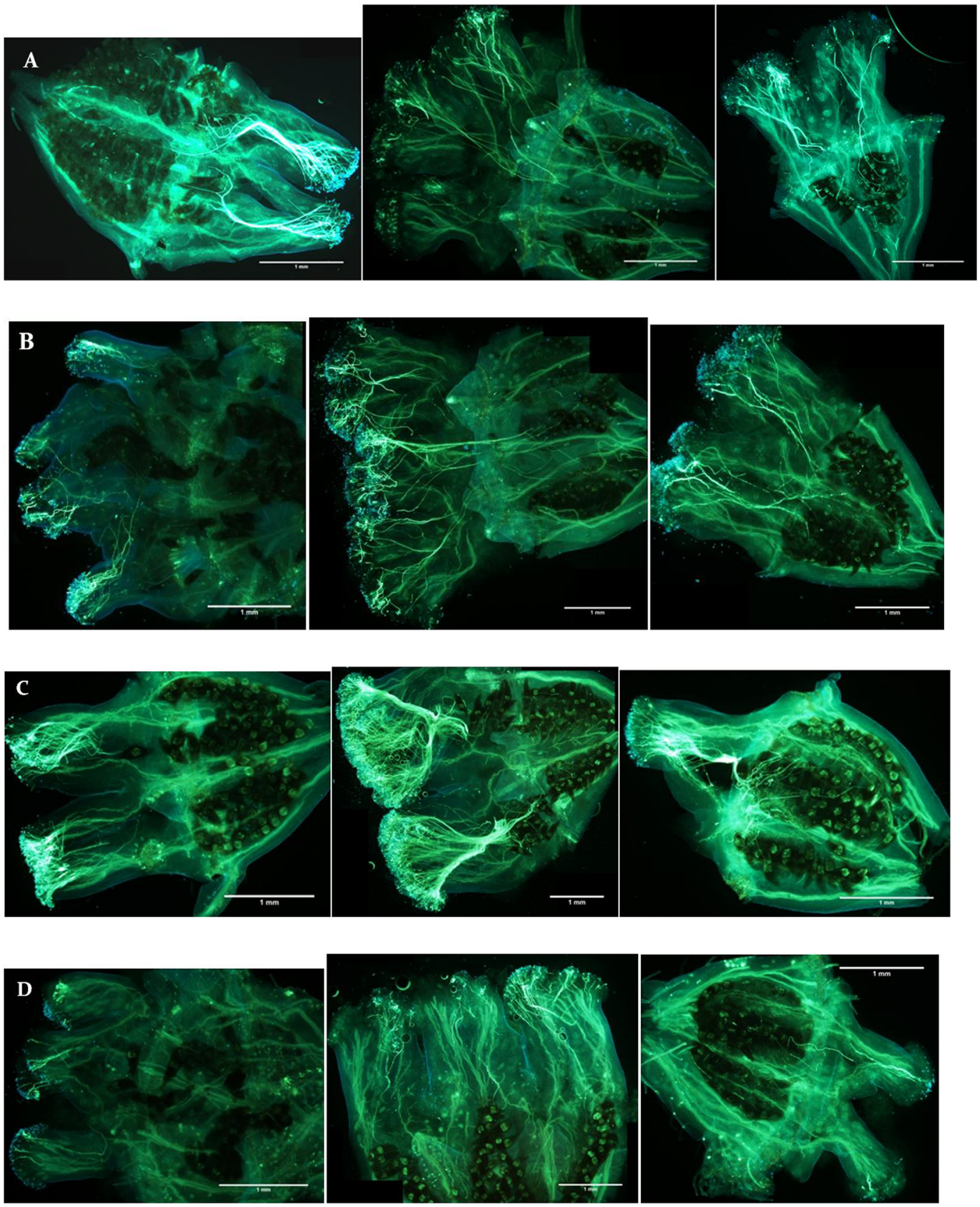

FIGURE 2 | Stained pistils showing pollen tube growth from **(A)** 2x × 2x, **(B)** 2x × 3x, **(C)** 3x × 2x, and **(D)** 3x × 3x controlled crosses of *Hydrangea macrophylla* collected 24 h (left), 48 h (center), and 72 h (right) post-pollination. Crosses shown from top to bottom are: "Decatur Blue" x "Oakhill", "Decatur Blue" x "Kardinal", "Kardinal" x "Decatur Blue", and "Taube" x "Kardinal". Pistils were fixed, rinsed, hydrolyzed, stained with decolorized aniline blue, and placed on microscope slides. Slides were observed after 1 h using an Olympus BX-60 compound microscope with an Olympus Q Color 5 digital camera for image capture.

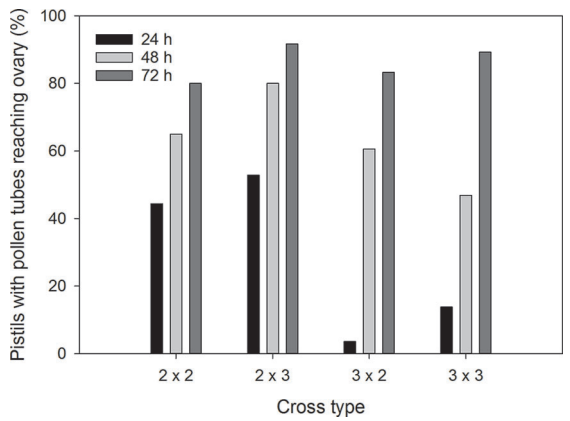

FIGURE 3 | Mean percentage of *Hydrangea macrophylla* flowers with pollen tube reaching the ovaries at 24, 48, and 72 h post-pollination. 2 = 2*x* = diploid; 3 = 3*x* = triploid. Means are based on six crosses per cross type per time period. Error bars represent standard error of the mean. Pearson's chi-square values for association between cross type and percentage of flowers with pollen tubes reaching the ovaries were $\chi^2 = 30.6$, $p < 0.001$ at 24 h, $\chi^2 = 26.5$, $p = 0.001$ at 48 h, and $\chi^2 = 7.5$, $p = 0.60$ at 72 h post-pollination.

TABLE 1 | Analysis of covariance for sources of variation in pollen tube length for interploidy and intraploidy crosses of *Hydrangea macrophylla*.

Time	Source	DF	Mean Square	F Value	Pr > F
24 h	Female ploidy	1	1.9	3.7	0.0578
	Male ploidy	1	1.1	2.2	0.1433
	Flower length	1	12.6	24.0	<0.0001
	Error	95	0.5		
48 h	Female ploidy	1	4.6	4.1	0.0461
	Male ploidy	1	0.0	0.0	0.9597
	Flower length	1	34.0	30.0	<0.0001
	Error	96	1.1		
72 h	Female ploidy	1	1.9	2.3	0.1332
	Male ploidy	1	0.1	0.2	0.7012
	Flower length	1	69.5	84.9	<0.0001
	Error	79	0.8		

number of seeds per capsule ranged from 0.1 for the 3x × 3x cross "Kardinal" × "Taube" to 63.8 for its reciprocal, "Taube" × "Kardinal" (**Table 3**). Germination rate ranged from 74% for the 2x × 2x cross "Zaunkoenig" × "Decatur Blue" to 0% for two 2x × 3x, three 3x × 2x, and one 3x × 3x cross (**Table 3**).

Seedlings grew slowly after germination, especially those resulting from 2x × 3x and 3x × 3x crosses (**Figure 4**). Percent mortality for each cross type was calculated by adding the survival rates for each cross, dividing by the number of crosses, and subtracting from 100. Only crosses that produced seed were considered. By six months post-germination, mortality for 2x × 2x, 2x × 3x, 3x × 2x, and 3x × 3x seedlings was 1.8%, 45.5%, 10.5%, and 88.8%, respectively (**Table 3**).

Genome Size and Pollen Viability of Seedlings

All six parental cultivars and from 2 to 30 seedlings from each cross type were analyzed for genome size (**Table 4**). Genome

TABLE 2 | Average pollen tube length (mm) and density (in italics) of pollen tubes from flowers of interploidy and intraploidy crosses of *Hydrangea macrophylla* collected 24, 48, and 72 h post-pollination.

Parents	Ploidy level of parents	Hours after pollination					
		24		48		72	
D × O[z]	2 × 2[y]	2.06 abc[x]	*0.6*[w]	2.97 bc	*2.2*	3.13 cd	*2.5*
O × D	2 × 2	1.31 bc	*0.0*	1.68 d	*0.5*	1.71 f	*1.0*
D × Z	2 × 2	2.52 a	*1.0*	3.01 bc	*1.5*	3.19 cd	*2.0*
Z × D	2 × 2	1.61 bc	*0.0*	1.72 de	*0.4*	1.89 ef	*1.5*
O × Z	2 × 2	1.15 cd	*1.4*	1.47 e	*1.9*	1.56 f	*2.5*
Z × O	2 × 2	2.24 ab	*0.2*	2.55 c	*0.5*	2.92 de	*1.0*
D × K	2 × 3	1.51 bc	*0.8*	2.37 cd	*1.0*	2.98 de	*1.7*
O × T	2 × 3	1.22 bc	*0.3*	1.88 d	*1.6*	2.16 def	*2.1*
Z × T	2 × 3	2.12 abc	*0.8*	2.90 bc	*1.0*	3.12 cd	*1.9*
D × T	2 × 3	2.32 ab	*1.0*	3.44 b	*2.0*	3.99 bc	*2.3*
O × K	2 × 3	1.76 abc	*0.5*	3.02 bc	*0.7*	3.17 cd	*1.1*
Z × B	2 × 3	1.04 cde	*0.8*	2.19 cd	*1.4*	2.68 def	*2.0*
K × Z	3 × 2	2.57 a	*0.0*	3.72 b	*2.0*	5.04 abc	*2.4*
K × O	3 × 2	1.69 abc	*0.1*	1.74 d	*0.4*	2.47 ef	*1.0*
K × D	3 × 2	2.27 ab	*0.0*	4.57 a	*2.1*	4.78 b	*2.5*
B × Z	3 × 2	2.42 a	*0.0*	3.51 b	*0.6*	4.26 bc	*1.4*
T × D	3 × 2	2.48 a	*0.0*	3.83 b	*1.7*	5.81 a	*2.3*
T × Z	3 × 2	1.95 bc	*0.0*	3.21 bc	*0.4*	5.12 ab	*1.0*
T × B	3 × 3	1.05 d	*0.0*	2.40 cd	*0.8*	3.91 bc	*2.2*
B × T	3 × 3	0.74 e	*0.0*	1.60 de	*1.1*	3.02 cd	*1.9*
B × K	3 × 3	1.20 cd	*0.0*	2.30 cd	*0.4*	4.13 bc	*1.5*
K × B	3 × 3	0.98 de	*0.0*	1.93 d	*0.5*	2.33 ef	*1.0*
K × T	3 × 3	2.25 ab	*0.0*	3.45 b	*0.6*	4.23 b	*2.0*
T × K	3 × 3	1.90 abc	*0.9*	2.27 cd	*1.8*	3.75 bc	*2.5*

[z]D, Decatur Blue; O, Oakhill; Z, Zaunkoenig; B, Blaumeise; K, Kardinal; T, Taube.
[y]2, 2x, diploid; 3, 3x = triploid.
[x]Pollen tube lengths were analyzed via ANCOVA with female flower lengths as a covariate. Cross means were separated using Tukey's studentized range test. Means followed by the same letter were not significantly different at the α = 0.05 significance level. The longest three pollen tubes of three flowers were measured for each cross.
[w]Density of pollen tubes in the ovaries was scored as follows: 0 = none, 1 = Few, 2 = Some, 3 = Many.

sizes of diploid parents measured approximately 4.5 pg while triploid cultivars ranged between 6.2 and 6.4 pg. Overall mean 1C genome size was 2.17 pg. Offspring ploidy levels were calibrated with genome sizes based on these representative cytotypes using the equation: mean estimated ploidy level (x) = mean 2C genome size ÷ mean 1C genome size (Beck and Ranney, 2014). Mean genome sizes of offspring from each cross type ranged from 4.56 for 2x × 2x offspring and 5.17 for 3x × 3x offspring. Estimated ploidy of offspring ranged from 2x for 2x × 2x crosses to 2.4x for 3x × 3x crosses (**Table 4**). DAPI stain, which we used, has a higher affinity for AT bases than propidium iodide (PI) stain which shows no base preference. Thus, the

TABLE 3 | Percent fruit set, average number of seeds/fruit, seeds used for germination, germination rate, and six-month seedling survival of progenies resulting from interploidy and intraploidy crosses of *Hydrangea macrophylla*.

Parents	Ploidy level of parents	Fruit set (%)	Average number of seeds/ fruit	Seeds used for germination	Germination (%)	Number (%) alive after six months
D × O[z]	2 × 2[y]	0.0	0.0	0	0.0	0 (0.0)
O × D	2 × 2	44.0	6.7	74	46.1	34 (100.0)
D × Z	2 × 2	46.7	4.6	65	50.8	32 (97.0)
Z × D	2 × 2	51.6	6.9	100	74.0	72 (97.3)
O × Z	2 × 2	41.2	52.4	100	61.0	61 (100.0)
Z × O	2 × 2	50.0	9.1	91	35.2	31 (96.9)
D × K	2 × 3	0.0	0.0	0	0.0	0 (0.0)
O × T	2 × 3	16.2	9.7	58	37.9	12 (54.5)
Z × T	2 × 3	18.4	10.3	62	8.0	0 (0.0)
D × T	2 × 3	0.0	0.0	0	0.0	0 (0.0)
O × K	2 × 3	35.0	0.9	6	0.0	0 (0.0)
Z × B	2 × 3	35.0	0.7	5	0.0	0 (0.0)
K × Z	3 × 2	78.0	1.5	17	0.0	0 (0.0)
K × O	3 × 2	100.0	15.4	100	21.0	18 (85.7)
K × D	3 × 2	71.4	5.0	100	15.0	14 (93.3)
B × Z	3 × 2	91.7	0.3	3	0.0	0 (0.0)
T × D	3 × 2	59.0	3.1	62	17.7	0 (0.0)
T × Z	3 × 2	38.5	0.8	4	0.0	0 (0.0)
T × B	3 × 3	100.0	9.0	100	6.0	0 (0.0)
B × T	3 × 3	0.0	0.0	0	0.0	0 (0.0)
B × K	3 × 3	0.0	0.0	0	0.0	0 (0.0)
K × B	3 × 3	85.7	8.9	100	8.0	1 (12.5)
K × T	3 × 3	64.7	0.1	1	0.0	0 (0.0)
T × K	3 × 3	23.5	63.8	100	10.0	1 (10.0)

[z]*D, Decatur Blue; O, Oakhill; Z, Zaunkoenig; B, Blaumeise; K, Kardinal; T, Taube.*
[y]*2, 2x, diploid; 3 = 3x, triploid.*

genome size estimates presented herein likely slightly overestimate the total nuclear DNA content of the *H. macrophylla* we tested (Zonneveld, 2004; Jones et al., 2007).

The number of offspring that produced flowers ranged from 0% for 3x × 3x crosses to 73% for 2x × 2x crosses (**Table 5**). Each of the three cross types with flowering offspring had some offspring that produced viable pollen. Sterility rates of flowering offspring ranged from 0% for 2x × 2x crosses to 50% for 2x × 3x crosses. Pollen stainability rates of flowering offspring ranged from 69.6% to 76.4% (**Table 5**). There were no significant differences in mean stainable pollen among cross types or between offspring and parent cultivars. However, comparisons of pollen viability between parents and offspring should be considered as rough estimates only as growth stage may influence pollen viability. The parent plants were one year-old cuttings taken from mature plants, and the offspring were grown from seed and measured the first time they flowered.

DISCUSSION

Pollen Tube Growth

Sympatric occurrences of two or more ploidy levels within a single morphologically defined species have been documented in many groups of vascular plants (Herben et al., 2016). Sympatry of different ploidy levels primarily arises through repeated formation of unreduced gametes by diploids (primary origin) or from secondary contact between previously allopatric diploid and polyploid populations (secondary origin; Petit et al., 1999). *H. macrophylla* shows both high rates of 2n gamete formation (Tränkner et al., 2019) and weak internal pre-zygotic reproductive barriers between ploidy levels as both diploids and triploids are nearly identical with respect to morphology, phenology, and likely habitat preferences (McClintock, 1957). The current study provides insight into a potential pre-zygotic barrier relating to pollen tube growth, where tubes of pollen from triploid parents grew more slowly than tubes of pollen from diploid parents, regardless of the ploidy level of the female parent. This may be because tubes from aneuploid pollen grains grow more slowly than tubes from haploid (n) gametes or diploid (2n) gametes, and all of the pollen produced by triploids in this study appeared to be aneuploid based on progeny genome sizes.

Seed Set, Germination, and Seedling Growth

Triploid block is a well-studied post-zygotic reproductive barrier that operates in the endosperm, a terminal tissue surrounding the embryo (Brink and Cooper, 1947; Ramsey and Schemske, 1998; Köhler et al., 2010). In crosses between diploid individuals, a haploid pollen grain combines with a haploid egg cell to form a diploid embryo, while the surrounding endosperm tissue is

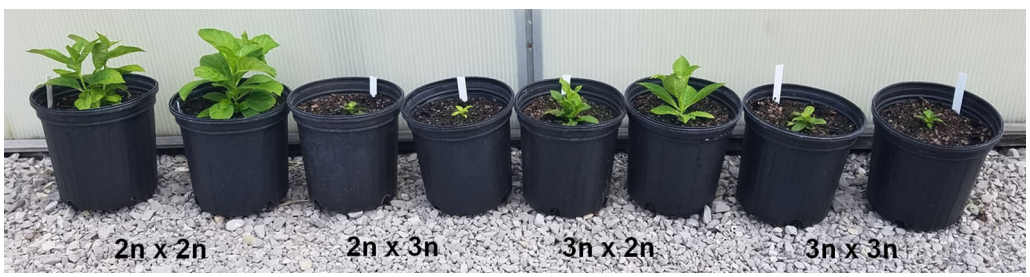

FIGURE 4 | *Hydrangea macrophylla* seedlings from interploidy and interploidy crosses after five months of growth in a greenhouse under 56% shade at 24°C/20°C day/night and natural light. Photo taken 11 July 2018 in McMinnville, TN, USA.

TABLE 4 | Genome size and estimated ploidy for parent cultivars and offspring of *Hydrangea macrophylla* interploidy and intraploidy crosses.

Parent cultivar or cross type	N[z]	2C genome size (pg)	SD[y]	Min.[x]	Max.[w]	Estimated ploidy level (*x*)[t]
Parent cultivar						
Decatur Blue	1	4.54	0.05	–	–	2
Oakhill	1	4.59	0.01	–	–	2
Zaunkoenig	1	4.56	0.05	–	–	2
Blaumeise	1	6.24	0.03	–	–	3
Kardinal	1	6.39	0.01	–	–	3
Taube	1	6.22	0.12	–	–	3
Cross type[v]						
2 x 2	30	4.56	0.07	4.37	4.65	2
2 x 3	12	4.79	0.02	4.52	5.06	2.2
3 x 2	30	4.98	0.29	4.48	5.74	2.3
3 x 3	2	5.17	0.02	5.14	5.20	2.4

[z]*Number of individuals sampled. Individual plant values are the mean of two subsamples.*
[y]*Standard deviation of genome size.*
[x]*Minimum individual plant genome size.*
[w]*Maximum individual plant genome size.*
[v]*2 = 2x = diploid; 3 = 3x = triploid.*
[t]*Mean estimated ploidy level* (extend) *= mean 2C genome size ÷ mean 1C genome size.*

TABLE 5 | Pollen stainability of offspring from intraploidy and interploidy crosses of *Hydrangea macrophylla*.

Cross type[y]	No. plants	Fertility		Pollen stainability[z] (%)	
		No. (%) plants flowered	No. (%) sterile plants	Mean ± SD[x]	Range
2n × 2n	30	22 (73.3%)	0 (00.0%)	76.4 ± 0.09	(62.1–96.4)
2n × 3n	12	4 (33.3%)	2 (50.0%)	69.6 ± 0.06	(65.1–73.4)
3n × 2n	30	16 (53.3%)	2 (12.5%)	73.8 ± 0.13	(50.9–95.2)
3n × 3n	2	0 (00.0%)	–	–	–

[z]*Fresh pollen was stained with modified Alexander's stain and observed after 30 min at 10× magnification using an Olympus BX-60 compound microscope.*
[y]*2, 2x = diploid; 3, 3x = triploid.*
[x]*Standard deviation.*

triploid. In interploidy hybridizations, the parental genome contributions are altered, which affects endosperm development and the formation of viable seeds (Schatlowski and Köhler, 2012). Jones et al. (2007) reported that mean number of seed per cross and percent seed germination were higher when triploid cultivars were used as the pistillate (female) rather than the staminate (male) parent in controlled crosses. Results herein indicated that not only does parental ploidy influence seed set and seed germination, but that parental influence continues throughout plant growth and flowering. Interploidy crosses with triploids as opposed to diploids as the female parent showed higher seed set, higher germination rates, higher survival rates, and higher rates of flowering. After double-fertilization, the endosperm of a 3x × 2x cross will have a multiple of a complete set of chromosomes (4x) while the endosperm of a 2x × 3x cross will contain an imbalanced chromosome complement (3.5x). These data support the idea that endosperm imbalance is a major post-zygotic barrier in *H. macrophylla*. Another major post-zygotic reproductive barrier is fitness of offspring (Herben et al., 2016). The current study showed slow growth and low survival of offspring resulting from interploidy crosses, supporting the primary origin hypothesis of triploid *H. macrophylla*. However, as proportion of unreduced gametes (determined by pollen visualization and hybridization experiments) is genotype-specific (Herben et al., 2016; Alexander, 2017) more triploid cultivars should be screened for unreduced gamete production.

Sexual hybridization among ploidy levels has played a key role in breeding improvement for a large number of woody ornamental species where crosses between tetraploid and diploid parents are used to produce sterile triploid hybrids with unique traits (Kumari and George, 2008). Breeders often face strong or absolute sterility of triploid hybrids which prevents further breeding using triploid individuals. In the case of *H. macrophylla*, triploids have nearly identical fertility to diploids and that fertility is cultivar specific (Jones et al., 2007; Alexander, 2017). All three triploid cultivars used as parents in this study had a higher fertility (as measured by percent stainable pollen) than the least fertile diploid parent. Fertility of triploids determined by staining is corroborated by the presence of offspring in this and other studies, and by the results of pollen tube growth presented herein. The offspring were also fertile such that progeny with unique traits could be used for future breeding. However, the high number of aneuploid progeny indicates that aneuploid gametes appear viable using the staining method described herein.

Possible Origin of Triploid Cultivars

Progeny of interploidy crosses was diploid and aneuploid; no triploid progeny was recovered from crosses using triploid parents. Based on the genome size of surviving progeny, it can be inferred that few 1x and many aneuploid gametes were produced by triploids, with no evidence of successful pollination by 2n gametes that would be necessary to produce triploid offspring. Success of using triploid parents to produce triploid progeny varies across species. The results of Cao et al. (2002) indicate that triploids were not useful in polyploid breeding of pear, while high numbers of triploids were obtained in 2x × 3x crosses of hybrid tulips (Marasek-Ciolakowska et al., 2014). Crosses between pentaploid and tetraploid *H. paniculata* tended to produce progeny with genome sizes within or near the tetraploid range, indicating a bias towards euploidy/isoploidy (Beck and Ranney, 2014). While no triploid *H. paniculata* were available for comparison, it can be inferred that the pentaploids produced aneuploid and 1x gametes, similar to the triploid *H. macrophylla* in the current study. In both cases, the polyploid hydrangea of uneven ploidy (i.e., 3x and 5x) failed to produce offspring reflective of the unreduced number of the closest lower ploidy (i.e., 2n gametes for triploids and 4n gametes for pentaploids).

Several hundred *H. macrophylla* cultivars were bred in the twentieth century based on seven or eight different plants that were brought to Europe from plant explorations to Japan and China in the 18th and 19th centuries (Bertrand, 2000). The origin of triploid *H. macrophylla* cultivars is unclear. Several *H. macrophylla* triploids were produced by a few breeders. Two German breeders, H. Schadendorff and F. Matthes, were responsible for six triploid cultivars, whereas another six were released by the Federal Research Institute for Horticulture in Switzerland (Mallet, 1994; Van Gelderen and Van Gelderen,

2004). It is not known whether these and other European breeders had access to tetraploid forms of *H. macrophylla* that were not included in previous ploidy analyses (Demilly et al., 2000; Cerbah et al., 2001; Zonneveld, 2004) or if they were using parental stocks that produced unreduced gametes. An additional possibility, considering the fertility of the triploids, is that a triploid parent may have been used in the breeding of additional triploids. Hempel et al. (2018) suggested that sexual hybridization between diploids and triploids was present in the pedigree of many well-known diploid and triploid cultivars. In the current study, controlled crosses between triploid and diploid *H. macrophylla* plants resulted in aneuploid offspring with differing chromosome numbers and poor growth compared to their parent cultivars. Therefore, the hypothesis of interploidy crosses as the source of triploid cultivars was not supported by this research, though it cannot be ruled out as a possibility. Additionally, high rates of unreduced gamete formation have been documented in *H. macrophylla*. An F1 mapping population from two diploid parents contained 103 diploids and 317 triploids (Tränkner et al., 2019). Alexander (2017) showed that controlled crosses using a cultivar known to produce unreduced male gametes resulted in 97% triploid offspring in the progeny. Considering the difficulty of obtaining interploidy crosses combined with their low survival rates, it appears that unreduced gamete breeding is the most suitable method for producing marketable triploids and is the most likely source of triploid *H. macrophylla* cultivars.

In conclusion, interploidy crosses among *H. macrophylla* diploid and triploid cultivars are associated with genetically unstable progeny. Viable seed from controlled interploidy crosses is difficult to obtain, survival is low, and progeny show poor growth and ornamental quality compared to their parent cultivars. Using triploid plants as the maternal parent improves success. *H. macrophylla* seems highly tolerant to aneuploidy and several aneuploid plants showed unique phenotypes and flowers with stainable pollen. The progeny from interploidy hybridizations have broad potential in breeding programs to provide genetic and phenotypic variability for the production of novel varieties.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

LA conceived, designed, and supervised the experiment, analyzed the data, and wrote the manuscript.

ACKNOWLEDGMENTS

Research presented in this article was supported in part by funds from the Floral and Nursery Research Initiative administered through the United States Department of Agriculture, Agricultural Research Service (USDA-ARS). The author would like to thank Carrie Witcher for assistance with pollen collection and microscopy. Benjamin Moore and Joseph Davis maintained the plants used in this experiment. Mention of trade names of commercial products in the publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 February 2020
doi: 10.3389/fpls.2020.00027

Distinguishing Sichuan Walnut Cultivars and Examining Their Relationships with *Juglans regia* and *J. sigillata* by FISH, Early-Fruiting Gene Analysis, and SSR Analysis

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *18 October 2019*
Accepted: *13 January 2020*
Published: *25 February 2020*

Citation:
Luo X and Chen J (2020) Distinguishing Sichuan Walnut Cultivars and Examining Their Relationships with Juglans regia and J. sigillata by FISH, Early-Fruiting Gene Analysis, and SSR Analysis. Front. Plant Sci. 11:27. doi: 10.3389/fpls.2020.00027

Walnuts are economically important tree species in Sichuan Province (China) that provide heathy nuts. Fluorescence *in situ* hybridization (FISH) and analyses of an early-fruiting gene fragment and simple sequence repeats (SSRs) were used to distinguish Sichuan walnut cultivars and examine their relationships with *Juglans regia* L. and *Juglans sigillata* Dode. Thirty-four small chromosomes were counted in four Sichuan walnut cultivars. In the four cultivars, 5S rDNA was located in the proximal regions of two chromosomes (5 and 6), while $(AG_3T_3)_3$ was located at both ends of each chromosome. The existence of the signal at both chromosome ends ensured accurate chromosome counts. 5S rDNA and $(AG_3T_3)_3$ were not effective in identifying Sichuan walnut cultivars. Evolutionary analysis involving 32 early-fruiting nucleotide sequences from Sichuan walnut materials were performed with the maximum likelihood method. There were a total of 602 positions. All positions with gaps and missing data were eliminated, resulting in a final dataset of 562 positions. The ML tree with the highest log likelihood (–1607.82) revealed two obvious groups: one including materials of *J. regia*, which fruits 1 year after grafting, and another including materials of *J. sigillata*, which fruits >3 years after grafting. The early-fruiting gene fragment divided 22 walnut materials (10 walnut cultivars and 12 walnut accessions) into two groups, indicating that it was somewhat effective for distinguishing Sichuan walnut cultivars. Furthermore, 22 SSR loci were revealed to identify nine walnut cultivars. Eight cultivars were exclusively discerned by one SSR locus each: Chuanzao 1 [CUJRB307 (116) or CUJRA206a (182)], Chuanzao 2 [JSI-73 (154)], Shuangzao [CUJRB103a (123), CUJRB218 (144), JSI-71 (146), or CUJRA206a (176)], Shimianju [ZMZ11 (138)], Meigupao [CUJRB218 (149), CUJRB103a (151), or CUJRA206a (190)], Muzhilinhe [CUJRB220 (136), ZMZ11 (147), CUJRC310 (156), or JSI-73 (166)], Maerkang [CUJRA124 (154), CUJRB218 (159), or CUJRA123 (182)], Yanyuanzao [CUJRA124 (150) or CUJRA206a (192)]. The Shuling cultivar was identified by the combination of

ZMZ11 (148) and other SSR loci, which distinguished and excluded the Chuanzao 1 and Yanyuanzao cultivars. Our results will guide the identification and breeding of Sichuan walnut cultivars.

Keywords: microsatellite marker, karyotype analysis, natural hybrids, artificial hybrids, walnuts

INTRODUCTION

Fruits are genetically very diverse groups grown in temperate, subtropical, and tropical regions and have been recognized for their human health benefits. Most of the fruits have high content of non-nutritive, nutritive, and bioactive compounds such as flavonoids, phenolics, anthocyanins, phenolic acids, and as well as nutritive compounds such as sugars, essential oils, carotenoids, vitamins, and minerals (Halasz et al., 2010; Alibabic et al., 2018; Butiuc et al., 2019). The species *Juglans regia* L. and *Juglans sigillata* Dode belong to *Juglans* in Juglandaceae, which consists of perennial woody plants that produce healthy nuts with a unique flavor and nutrient-rich composition (Kim et al., 2014). Sichuan Province is located at the boundary which is adjacent to the natural distributions of northern and southern walnuts in China. Northern walnuts (north of the Nibashan-Qinling Mountains), which grow under cold, dry, and high-sunshine conditions, and southern walnuts (south of the Erlangshan-Nibashan-Huangmaogeng-Wumengshan Mountains), which grows under warm, dry, and low-sunshine conditions (Xi and Zhang, 1996), have been introduced to Sichuan. In this area, the introduced cultivars are exposed to high-temperature, high-humidity, and low-sunshine stresses, resulting in weak trees, severe fruit drop or fruitlessness. Northern walnut is classified as *J. regia*, while southern walnut is classified as *J. sigillata*. Sichuan walnut is highly likely to be a hybrid of these two varieties. The shapes of the nuts and compound leaves of these types of walnuts are shown in **Figure 1**. *J. sigillata* has more leaflets (~7 pairs) than *J. regia* (~2 pairs), and the hybrids usually have ~4 pairs of leaflets. The compound leaf of *J. sigillata* contains a small, double parietal lobe (**Figure 1A**), while that of *J. regia* and the hybrids commonly has a large, single parietal lobe (**Figures 1B, C**). The walnut shell of *J. sigillata* has a deep texture (**Figure 1D**), while that of *J. regia* has little texture or may even be smooth (**Figure 1E**), and the shell of their hybrids has an intermediate texture (**Figure 1F**). Walnut cultivars vary greatly in quality (Pei and Lu, 2011), and tools for distinguishing among them are lacking. The engraftment scions (Chuanzao 1, Shuling, Chuanzao 2, Shuangzao, and Zaofeng) and the stock plants lack distinct characters (**Figure 2**). Therefore, a method to easily and accurately identify Sichuan walnut cultivars is greatly needed; such a method would also benefit breeders and planters. Walnut cultivars are usually distinguished based on morphology (Zenelli et al., 2005), supplemented by cytology (Tulecke et al., 1988), isozyme analysis (Fornari et al., 2001), restriction fragment-length polymorphism (RFLP) analysis (Fjellstrom and Parfitt, 1994), randomly amplified polymorphic DNA (RAPD) analysis (Ross-Davis et al., 2008), inter-simple sequence repeat (ISSR) analysis (Christopoulos et al., 2010), amplified fragment-length polymorphism (AFLP) analysis (Mozaffarian et al., 2008), simple sequence repeat (SSR) analysis (Zhou et al., 2018a; Zhou et al., 2018b; Ebrahimi et al., 2019; Wu and Gmitter, 2019; Yan et al., 2019), single-nucleotide polymorphism (SNP) analysis (You et al., 2012), and high-throughput sequencing (Wu et al., 2019; Zhang et al., 2019; Zhu et al., 2019). However, morphological studies are preliminary breeding approaches, and morphological traits are usually quantitative, meaning that they are easily controlled by the environment and thus exhibit high variability (Andersen and Lübberstedt, 2003). In addition, similar characters cannot be distinguished by morphology (Zhao, 2007), and minor differences in tree cell structure cannot be detected by cytology (Luo et al., 2018). Furthermore, cytology is dependent on the experimental conditions and procedures used as well as on the methods used to prepare slides, with large differences among individual methods (Han et al., 2018). Elucidation of effective molecular markers for the identification of walnut cultivars requires sufficient genomic data. In addition, sample collection of late-fruiting walnut (which fruits after >3 years) is time consuming. Currently available methods are insufficient for the identification of Sichuan walnut cultivars; thus, there is a great need to establish an accurate and easy way to distinguish Sichuan walnut cultivars and to determine their relationships with *J. regia* and *J. sigillata*.

Chromosome number is a basic attribute used to establish the taxonomic statuses of walnut cultivars. Species in the genus *Juglans* have 32 chromosomes (Woodworth, 1930; Hans, 1970; Mu and Xi, 1988; Tulecke et al., 1988; Mu et al., 1990). At least five molecular genetic linkage maps for walnut have been established (Fjellstrom and Parfitt, 1994; Woeste et al., 1996; Malvolti et al., 2001; Luo et al., 2015; Zhu et al., 2015). Fluorescence *in situ* hybridization (FISH) with probe-labeled chromosome ends enables accurate counting of small walnut chromosomes (Deng et al., 2019; Liu and Luo, 2019; Luo and Liu, 2019a). Karyotype analysis aids in chromosome physical map construction and chromosome assembly (Hizume et al., 2002; Luo et al., 2017; Luo et al., 2018; Pereira et al., 2018), thereby providing a guide for distinguishing Sichuan walnut cultivars to a certain degree. However, no walnut cultivars have been subjected to FISH analysis. Furthermore, DNA sequencing is much more sensitive than FISH to species differences. Early fruiting, which is a dominant character controlled by at least two alleles, is an important attribute of walnut (Xi, 1987). Early fruiting walnut cultivars are those that fruit within three years after grafting. Sequence variation in early fruiting genes occurs in response to environmental stress. The harsh northern environment favours accelerated reproduction (Charrier et al., 2011); hence, early-fruiting genes are commonly found in northern walnut (*J. regia*)

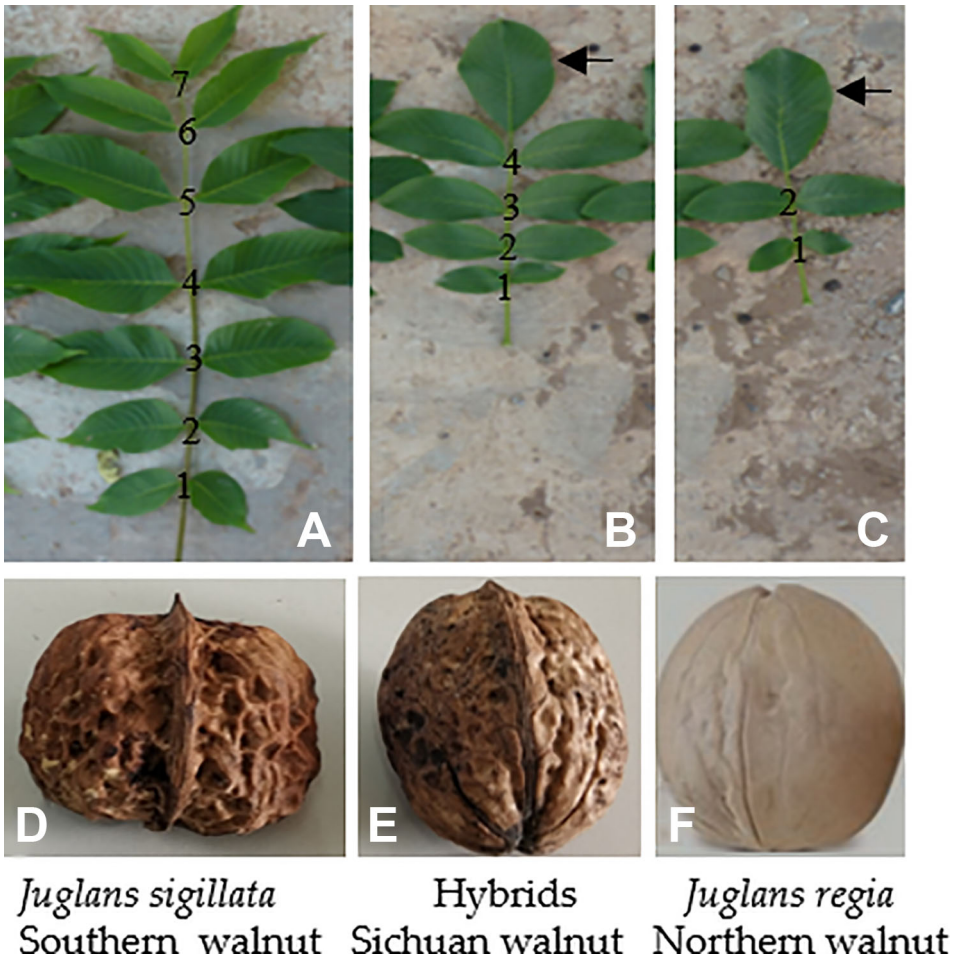

FIGURE 1 | Morphological comparison of the leaves and nuts of *J. sigillata*, *J. regia* and their hybrids. *J. sigillata* has more leaflets (**A**, ~7 pairs) than *J. regia* (**C**, ~2 pairs), and their hybrids usually have ~4 pairs of leaflets **(B)**. The compound leaf of *J. sigillata* **(A)** has a small, double parietal lobe, while that of *J. regia* and the hybrids **(B, C)** commonly has a large, single parietal lobe (arrowed). The walnut shell of *J. sigillata* has a deep texture **(D)**, while that of *J. regia* has little texture or may even be flat **(F)**; the shells of the hybrids have intermediate textures **(E)**.

but seldom found in southern walnut (*J. sigillata*). Sichuan walnuts, as hybrids, have both early-fruiting (<3 years) and late-fruiting (>3 years) characteristics. Artificial hybrids, such as Chuanzao 1 (Pu et al., 2011) and Shuangzao (Xiao et al., 2013), and natural hybrids, such as Shimianju and Yanyuanzao (Sun et al., 2011) fruit 1 year after grafting. Natural hybrids, such as Meigupao and Muzhilinhe (Wang et al., 2017) do not fruit until >3 years after grafting or planting. Early-fruiting genes have high heritability (Ye et al., 2010). The SCAR marker linked to early bearing genes in the walnut was developed by Niu et al. (2008), and its terminal sequence was cloned by Zhu et al. (2011). The early-fruiting genes *AGAMOUS*, *APETALA3*, *Ap1,* and *LFAFY* in *J. regia* were cloned and their expression analyzed by Breton et al. (2004), Ye and Niu (2011; 2012). However, no early-fruiting gene DNA sequences have been identified to distinguish Sichuan walnut cultivars. Analysis of SSRs, which are repeated units of 1 to 6 nucleotides, is a rapid, accurate, and effective technique that has been widely and successfully used to distinguish numerous walnut cultivars. Molecular studies on SSRs began to increase in the late 2000s. In 2016, the number of SSR loci identified in *Juglans* reached 15,000 (Bernard et al., 2018a), and this number has continued to increase (Shi et al., 2016; Ebrahimi et al., 2017; Bernard et al., 2018b; Chen et al., 2018; Zhou et al., 2018a; Zhou et al., 2018b; Ebrahimi et al., 2019; Chen et al., 2019; Wu et al., 2019). Although SSRs have been widely used for walnut identification, Sichuan walnut cultivars have not been well distinguished.

A total of 22 Sichuan walnut materials were used in this study. The karyotypes of four cultivars were analyzed in detail by FISH. The early-fruiting gene nucleotide sequences of 22 walnut materials (10 walnut cultivars and 12 walnut accessions) were subjected to evolutionary analysis, and nine walnut cultivars were subjected to SSR analysis. This work aimed to distinguish Sichuan walnut cultivars and examine their relationships with *J. regia* and *J. sigillata* with the goal of providing a guide for the identification and breeding of Sichuan walnut cultivars.

FIGURE 2 | Morphological comparison of engraftment scions of Chuanzao 1, Shuling, Chuanzao 2, Shuangzao, and Zaofeng and their stocks. No distinct characters were observed for any of the engraftment scions.

MATERIALS AND METHODS

In total, 10 walnut cultivars and 12 walnut accessions (belonging to *J. regia* or *J. sigillata*) were collected in Sichuan Province, China, for this study (**Table 1**). These materials included eight certified elite cultivars (Chuanzao 1, Chuanzao 2, Shuangzao, Shuling, Zaofeng, Shimianju, Muzhilinhe, and Yanyuanzao). Among these, there are five artificial hybrid cultivars, including ① *J. regia* L. 'Chuanzao 1' = (*J. regia* L. × *J. sigillata* Dode 'Yunxin7926') × (*J. regia* L. 'Xiazao') 'Chuanzao 1', ② *J. regia* L. 'Chuanzao 2' = (*J. regia* L. × *J. sigillata* Dode 'Yunxin7926') × (*J. regia* L. 'Shahe') 'Chuanzao 2', ③ *J. regia* L. 'Shuangzao' = (*J. regia* L. × *J. sigillata* Dode 'Yunxin7926') × (*J. regia* L. 'Xiazao') 'Shuangzao', ④ *J. regia* L. 'Shuling' = (*J. regia* L. × *J. sigillata* Dode 'Yunxin7926') × (*J. regia* L. 'Xiazao') 'Shuling', and ⑤ *J. regia* L. 'Zaofeng' = (*J. regia* L. × *J. sigillata* Dode 'Yunxin7926') × (*J. regia* L. 'Xiazao') 'Zaofeng'. The other seventeen walnut materials were likely natural hybrids.

FISH Analysis

Four Sichuan walnut cultivars, namely, Chuanzao 1, Muzhilinhe, Maerkang, and Yanyuanzao, were used in this experiment. Nuts from these cultivars were germinated in wet sand at room temperature. When the lateral roots reached a length of ~2 cm, their tips were cut off, treated with nitrous oxide, and stored in 75% alcohol. Chromosome slides were prepared by digesting meristems with cellulase and pectinase (2:1), stirring the meristems, and dropping the meristems in suspension onto slides. An Olympus CX23 microscope (Olympus, Japan) was used to examine the air-dried slides. A 5S rDNA sequence (41 bp) (Luo et al., 2017) and a chromosome-end repeat oligonucleotide $(AG_3T_3)_3$ (21 bp) (Qi et al., 2002) were used for FISH analysis. The two probes were first tested in *Juglans* species. The oligonucleotide probes were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The synthetic oligonucleotides were 5′ end-labeled with 6-carboxyfluorescein (6-FAM) or 6-carboxytetramethylrhodamine (6-TAMRA). The synthesized probes were diluted using a 1× TE solution, maintained at a concentration of 10 μM and then stored at −20°C.

Furthermore, well-spread slides were subjected to chromosome fixation, dehydration, and denaturation before being dehydrated again and then hybridized with the denatured probe mixture at 37°C for 2 h. Subsequently, the chromosomes on the slides were washed, counterstained, and finally examined under an Olympus BX63 fluorescence microscope coupled to a Photometric SenSys Olympus DP70 CCD camera (Olympus, Japan). The raw images were examined in Photoshop version 7.1 (Adobe Systems Incorporated, San Jose, CA, USA). The chromosome nomenclature was based on length from the longest chromosome to the shortest chromosome. The chromosome ratio was calculated (over 3 metaphases) by dividing the length of the longest chromosome by the length of the shortest chromosome.

Early-Fruiting Gene Analysis

We examined 22 Sichuan walnut materials, including 10 cultivars and 12 accessions (**Table 1**). Total genomic DNA extraction was performed as described by Doyle and Doyle (1987), with a slight change. The early-fruiting gene was amplified with the forward primer 5′-TTTGTTGTTAGACTGAATGC-3′ and the reverse

TABLE 1 | The 22 materials used in this study, including 10 cultivars and 12 accessions.

Walnut material	License number	Species	GenBank accession number
Chuanzao 1_1	Chuan S-SV-JSJR-001-2012	*Juglans regia* L. 'Chuanzao 1'	MN548314
Chuanzao 1_2			MN548322
Chuanzao 2	Chuan S-SC-JSJR-001-2016	*Juglans regia* L. 'Chuanzao 2'	MN548311
Shuangzao_1	Chuan R-SC-JSJR-002-2009	*Juglans regia* L. 'Shuangzao'	MN548308
Shuangzao_2			MN548316
Shuling	Chuan R-SV-JSJR-003-2007	*Juglans regia* L. 'Shuling'	MN548335
Zaofeng_1	Chuan R-SC-SJR -008-2013	*Juglans regia* L. 'Zaofeng'	MN548325
Zaofeng_2			MN548326
Shimianju	Chuan R-SC-JS-035-2011	*Juglans regia* L. 'Shimianju'	MN548307
Muzhilinhe	Chuan R-SC-JR-002-2015	*Juglans sigillata* Dode 'Muzhilinhe'	MN548332
Yanyuanzao_1	Chuan S-SC-JR-003-2009	*Juglans regia* L. 'Yanyuanzao'	MN548306
Yanyuanzao_2			MN548309
Maerkang	–	*Juglans sigillata* Dode 'Maerkang'	MN548330
Meigupao	–	*Juglans sigillata* Dode 'Meigupao'	MN548331
4	–	*Juglans sigillata* Dode	MN548327
5	–	*Juglans sigillata* Dode	MN548317
6	–	*Juglans sigillata* Dode	MN548333
9_1	–	*Juglans sigillata* Dode	MN548318
9_2			MN548329
10	–	*Juglans sigillata* Dode	MN548337
16_1	–	*Juglans regia* L.	MN548315
16_2			MN548319
16_3			MN548320
20_1	–	*Juglans regia* L.	MN548312
20_2			MN548313
32	–	*Juglans sigillata* Dode	MN548334
42	–	*Juglans regia* L.	MN548310
45	–	*Juglans sigillata* Dode	MN548321
46_1	–	*Juglans sigillata* Dode	MN548328
46_2			MN548336
50_1	–	*Juglans regia* L.	MN548323
50_2			MN548324

primer 5′-GTGGATTTAAGGAAGGTTTG-3′. The primers were designed based on known early-fruiting sequences described by Ye et al. (2010). Each PCR (50 μL) contained 15 μL of polymerase mix, 1 mM each primer, and ~ 20 ng of template DNA complemented with ddH_2O. The amplification protocols included 1 cycle of 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C; and 1 cycle of 10 min at 72°C. The PCR products were visualized on 0.8% agarose gels, purified with an ENZA™ gel extraction kit (Omega, Georgia, USA), and then sequenced by the Beijing Genomics Institute (Beijing, China).

The sequences outside the primers were removed from the raw sequence data. The bidirectional sequences were matched in DNAMAN 6.0.3.99 (http://www.lynnon.com/). Thirteen materials had a single sequence, eight materials had two sequences, and one material had three sequences. Therefore, the 22 materials included 32 sequences, and these clean sequences were submitted to the National Center of Biotechnology Information (NCBI) and given GenBank accession numbers ranging from MN548306-MN548337 (**Table 1**). Subsequently, evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The evolutionary history of the early-fruiting gene was inferred by using the maximum likelihood method and the Tamura-Nei model (Tamura and Nei, 1993). The initial trees used for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with the superior log likelihood value. The tree was drawn to scale, with the branch lengths indicating the number of substitutions per site. The codon positions included were 1st+2nd+3rd+noncoding.

SSR Analysis

Nine Sichuan walnut cultivars, namely, Chuanzao 1, Chuanzao 2, Shuangzao, Shuling, Shimianju, Meigupao, Muzhilinhe, Maerkang, and Yanyuanzao, were used for SSR analysis. Total genomic DNA extraction was performed as described by Doyle and Doyle (1987), with a slight change. Each PCR (15 μL) contained 5 μL of polymerase mix, 1 mM each primer, and ~20 ng of template DNA complemented with ddH_2O. The amplification protocols included 1 cycle of 4 min at 95°C; 25 cycles (or 30 cycles depending on the primers) of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and 1 cycle of 7 min at 72°C. The forward primers were labeled with 6-FAM, HEX, and ROX.

Based on the genomic data for walnut (*J. regia* and *J. sigillata*) available from the NCBI (Martinez-Garcia et al., 2016; Luo and Liu, 2019b), 21 pairs of primers (**Table 2**) were obtained after two rounds of screening. Then, the fluorescent amplification products were sequenced on an ABI 3730 DNA analyser (Applied Biosystems), and allele sizing was performed using Gene Mapper™ 4.1 software (Applied Biosystems). The obtained allele sizes and relative peak heights were analyzed in Excel 2019.

TABLE 2 | The 21 SSR loci distinguish Sichuan walnut materials used in this study.

SSR loci	Repeat motif	Product size (bp)	Forward (F) and reverse (R) primers (5'-3')
CUJRA123	$(AC)_{12}$	193	F-TTGGTCTCTTCTTTCCTCTATG R-TCGAACGTACAATAACGTACAG
CUJRA124	$(GT)_{13}$	151	F-CGTTGCCTGAACAAGTAAGAT R-GAAGGAGGCTAACTCCCTATG
CUJRA206a	$(AC)_{16}$	197	F-GCCGAGAGAGGAAGAGAGACT R-CGACTACAGGGACCAATCAAC
CUJRB012	$(AG)_{15}$	102	F-ACTCATCAAGATCCCCGACTAC R-CCACATCGTCTGGGTTCAT
CUJRB103a	$(TC)_{17}$	148	F-CATGCTATGGACTACCTCCTCR-AAGAGAGACGAACGAAGAGTG
CUJRB218	$(GA)_{13}$	159	F-CTAGCGTCGAAGAAGAAGATG R-TTGTTTCTCCTCTGTCATGTTT
CUJRB220	$(TC)_{25}$	163	F-AGCATGTATAGGCCAATGATG R-TCGTTCTATCTACAAGCACTCG
CUJRB305	$(GA)_{19}$	132	F-GCTGCTTTATTAGCCATGATC R-GGTTCAATGTGCAACAAGAG
CUJRB307	$(GA)_{23}$	146	F-CTGGGCTGAAGGAGAATC R-TTGGATGTCTGCTTTTTTAGAG
CUJRB317	$(TC)_{17}$	125	F-TGCCCACTAACCCTAACC R-GAGAAAAAGAATGGCTGTATTG
CUJRC310	$(TTG)_{7}$	142	F-GCTGTTAGTGGAATCCCAACT R-TAAACGTGATCGAAGTGAAATG
CUJRD204a	$(CTC)_{4}$	154	F-CAGCCAATCTTCTTCTGCTTC R-GAGACCTACGACCACGATCAC
JH2753	$(GCT)_{6}$	188–212	F-CAGTTTTGGCCAGCTGCAAT R-TGTGCCCATGCTAAGACTGG
JUG-13	$(GGA)_{5}$	240–250	F-GAAGAGACTCCGTTGCCACA R-ACTCCGTCGTTTCCCTGAAC
JSI-15	$(TC)_{6}$	180–200	F-ATGAGAGCCAGCCAACAGAC R-CGAGCGAGCAAGAGAGAGAG
JSI-63	$(GATCG)_{5}$	180–200	F-TCCGGACAACTCCTCATCCT R-CTCTCCGCCGAGTCATGTAC
JSI-71	$(GCAGTA)_{8}$	135–155	F-AGCTAGCTCTCAAACAACAAGC R-ACAAACATGGCAACCTTCGTG
JSI-73	$(TGCTCG)_{5}$	160–175	F-AGCTCAACGGTCAAGGAAGG R-GGAGAGAGAGAGCTCGGCTA
JRE-28	$(AAG)_{5}$	150–170	F-CCGGGAAGCTCAGTTCAAGA R-GGTTCTTCCGCAGTTGGTCT
JRE-46	$(GAA)_{18}$	190–220	F-GCCTCTCCTCGTGCTCATTT R-ACTCGCTACTTTTCAGGCCC
ZMZ11	$(CTG)_{5}$	160	F-CCAGAACCAGGAGCCAGCAA R-GACCATCGGCCCGAAAGTAA

RESULTS

FISH Analysis

The mitotic metaphase chromosomes of four walnut cultivars (Chuanzao 1, Muzhilinhe, Maerkang, and Yanyuanzao) after FISH are shown in **Figure 3**. Furthermore, the chromosomes shown in **Figure 3** are presented individually in **Figure 4**. A total of 34 chromosomes were counted in all four walnut cultivars. 5S rDNA oligonucleotides were located in the proximal regions of two chromosomes (5 and 6) in each cultivar (red), while $(AG_3T_3)_3$ was located at both ends of each chromosome in each cultivar (green). The existence of a signal at both chromosome ends ensured accurate chromosome counts. The fluorescence intensity of 5S rDNA was stronger than that of $(AG_3T_3)_3$. The fluorescence intensity of 5S rDNA varied, but that of $(AG_3T_3)_3$ was consistent among the four cultivars. Among the cultivars, the Yanyuanzao cultivar exhibited the strongest fluorescence intensity of 5S rDNA, the Maerkang cultivar exhibited the weakest fluorescence intensity, and the other two cultivars (Chuanzao 1 and Muzhilinhe) exhibited intermediate intensity. There were no number differences or obvious intensity differences in the fluorescence signals among the four cultivars. Since 5S rDNA and $(AG_3T_3)_3$ were insufficient for identifying Sichuan walnut cultivars, further methods for detection were necessary.

The chromosomes of each cultivar were aligned by length in one column from longest (#1) to shortest (#34). The chromosome lengths of the Chuanzao 1, Muzhilinhe, Maerkang, and Yanyuanzao cultivars were 2.16 to 0.97 μm, 2.54 to 1.00 μm, 2.65 to 0.98 μm, and 2.13 to 0.98 μm, respectively. Since these lengths were less than 3 μm, the chromosomes of these four walnut cultivars are small chromosomes. The chromosomes of the Muzhilinhe and Maerkang cultivars were slightly larger than those of the other cultivars (Chuanzao 1 and Yanyuanzao). The chromosome ratios of the Chuanzao 1, Muzhilinhe, Maerkang, and Yanyuanzao cultivars were 2.23, 2.54, 2.70, and 2.17, respectively, indicating the following order in terms of chromosome asymmetry: Maerkang> Muzhilinhe> Chuanzao 1> Yanyuanzao. Due to the hidden centromere in a large proportion of the chromosomes, the long arm and short arm were not determined, and further karyotype analysis was not carried out. These limited karyotype data contributed little to the identification of the walnut cultivars.

Early-Fruiting Gene Analysis

To further distinguish Sichuan walnut cultivars, evolutionary analysis involving thirty-two early-fruiting nucleotide sequences from 22 walnut materials was performed with the maximum likelihood method; the results are shown in **Figure 5**. The tree with the highest log likelihood (−1607.82) is shown. There were a total of 602 positions. All positions with gaps and missing data were eliminated, resulting in a final dataset of 562 positions. There were two obvious groups in the tree. The first group included nineteen early-fruiting nucleotide sequences covering eleven *J. regia* materials with fruiting one year after grafting. The second group included thirty early-fruiting nucleotide sequences covering the other eleven *J. sigillata* materials with fruiting >3 years after grafting or planting. The seven cultivars Chuanzao 1, Chuanzao 2, Yanyuanzao, Shuling, Zaofeng, Shuangzao, and Shimianju belonged to the first group, while the other three cultivars (Meigupao, Maerkang, and Muzhilinhe) belonged to the second group. Since the early-fruiting gene fragment divided the ten walnut cultivars into two groups, it was able to distinguish the cultivars to some degree, but further methods of detection were needed.

SSR Analysis

To effectively distinguish the walnut cultivars, we further developed 21 SSR loci to detect nine walnut cultivars. A scatter diagram of these 21 SSR loci is provided in **Figure 6**. The allele

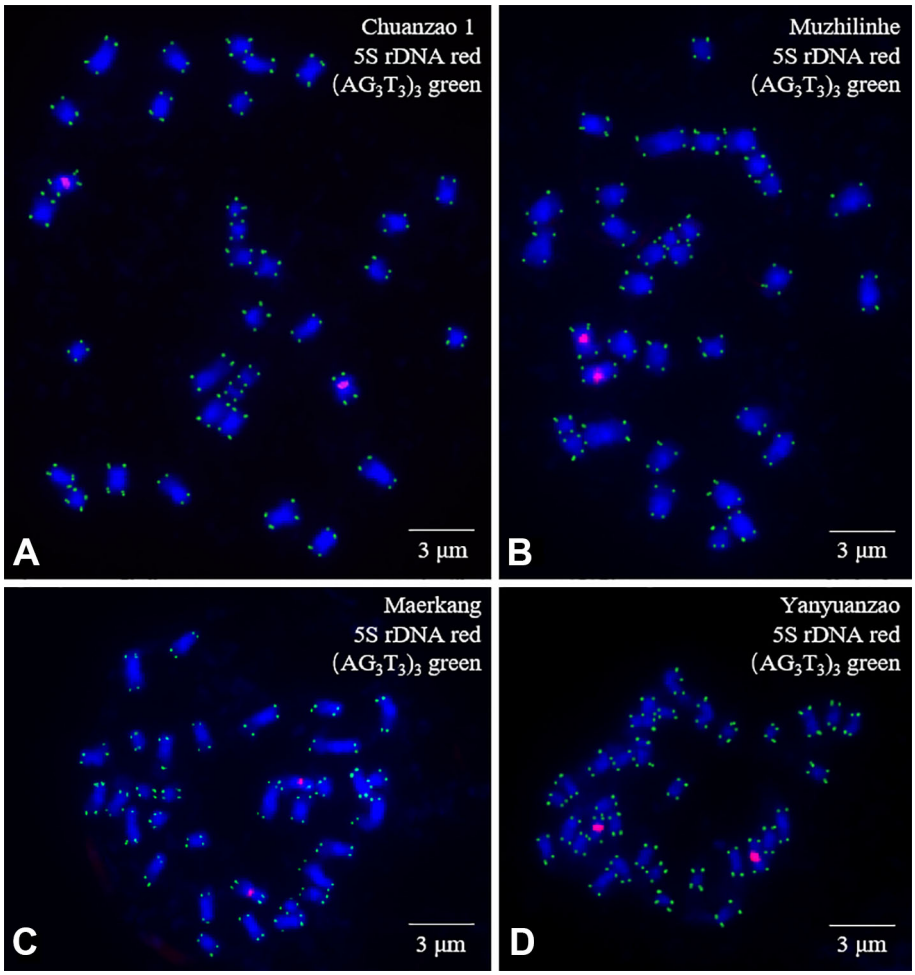

FIGURE 3 | Mitotic metaphase chromosomes of four walnut cultivars: **(A)** Chuanzao 1, **(B)** Muzhilinhe, **(C)** Maerkang, and **(D)** Yanyuanzao. Red represents 5S rDNA, while green represents $(AG_3T_3)_3$. The blue chromosomes were counterstained by DAPI. Scale bar = 3 μm.

sizes ranged from 82 to 237 nucleotides, while the relative peak heights ranged from 201 to 32,722. Most alleles were 135 to 200 nucleotides in length, while no alleles were 210 to 235 in size. Among the alleles, 3% were less than 100 nucleotides, 9% were greater than 200 nucleotides, and 87% were between 100 and 200 nucleotides in length. Half of the relative peak heights ranged from 25,000 to 32,722, while the others ranged from 200 to 25,000. Among these relative peak heights, 21% were less than 10,000, 15% were from 10,000 to 20,000, 17% were from 20,000 to 30,000, and 47% were greater than 30,000.

Furthermore, the allele size of each of the 21 SSR loci is shown in **Figure 7**. In total, 183 allele sizes were obtained, excluding six missing allele sizes (white) for the Shuling cultivar. The SSR loci were aligned based on the allele size of the Chuanzao 1 cultivar from smallest (red) to largest (blue). To precisely and easily discern the four artificial hybrid cultivars and five natural hybrid cultivars, repeated allele sizes and relative peak heights less than 14,000 were filtered out. Subsequently, several effective allele sizes were maintained, as indicated by the purple ovals. Eleven SSR loci helped discern eight of the cultivars (excluding the Shuling cultivar); these SSR loci were CUJRB307, CUJRA124, CUJRA123a, CUJRB220, ZMZ11, CUJRC310, JSI-71, JSI-73, CUJRB218, CUJRA206a, and CUJRA213. Each of the eight cultivars (Chuanzao 1, Chuanzao 2, Shuangzao, Shimianju, Meigupao, Muzhilinhe, Maerkang, and Yanyuanzao) was exclusively discerned by one SSR locus. Due to the lack of allele sizes, the Shuling cultivar was discerned by a repeat allele size of 148 nucleotides, as shown by the dashed oval. Hence, it was possible to discern the Shuling cultivar with a combination of ZMZ11 and other SSR loci, which distinguished and excluded the Chuanzao 1 and Yanyuanzao cultivars.

Figure 8 further shows the nine cultivars discerned by the 21 selected SSR allele sizes from eleven SSR loci summarized in **Figure 7**. The allele sizes ranged from 116 to 192 nucleotides, while the relative peak heights ranged from 14,643 to 32,607. The Chuanzao 1 cultivar was exclusively discerned by CUJRB307 (116) or CUJRA206a (182); the Chuanzao 2 cultivar was exclusively discerned by JSI-73 (154); the Shuangzao cultivar

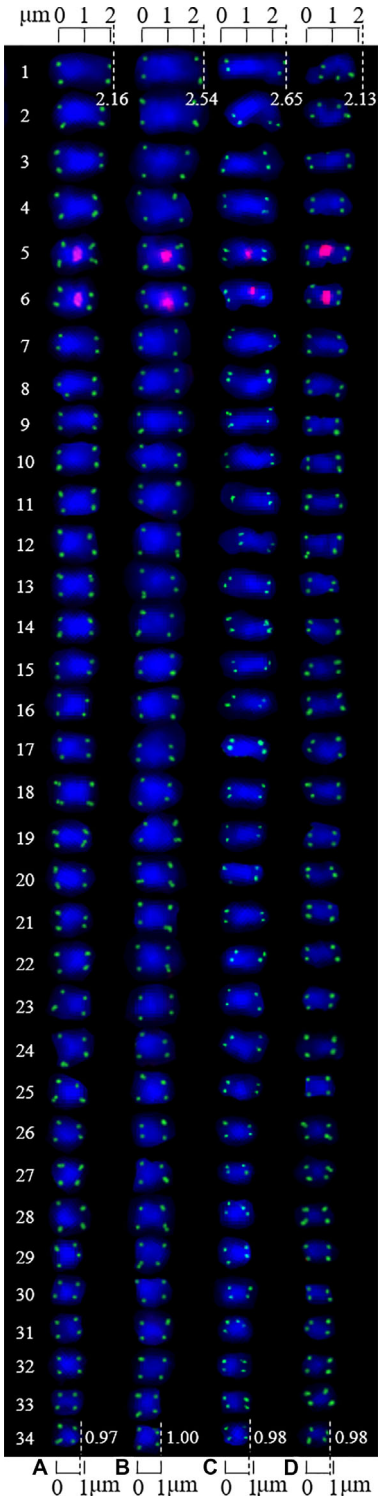

FIGURE 4 | Chromosomes from **Figure 3** presented individually. The chromosomes were aligned by length from longest to shortest. The left number represents the chromosome number, while the top/bottom number represents the chromosome length. **(A)** Chuanzao 1, 2.16–0.97 μm; **(B)** Muzhilinhe, 2.54–1.00 μm; **(C)** Maerkang, 2.65–0.98 μm; **(D)** Yanyuanzao, 2.13–0.98 μm.

was exclusively discerned by CUJRB103a (123), CUJRB218 (144), JSI-71 (146), or CUJRA206a (176); the Shimianju cultivar was exclusively discerned by ZMZ11 (138); the Meigupao cultivar was exclusively discerned by CUJRB218 (149), CUJRB103a (151), or CUJRA206a (190); the Muzhilinhe cultivar was exclusively discerned by CUJRB220 (136), ZMZ11 (147), CUJRC310 (156), or JSI-73 (166); the Maerkang cultivar was exclusively discerned by CUJRA124 (154), CUJRB218 (159), or CUJRA123 (182); the Yanyuanzao cultivar was exclusively discerned by CUJRA124 (150) or CUJRA206a (192); and the Shuling cultivar was discerned by the combination of ZMZ11 (148) and other SSR loci, which distinguished and excluded the Chuanzao 1 [CUJRB307 (116) or CUJRA206a (182)] and Yanyuanzao [CUJRA124 (150) or CUJRA206a (192)] cultivars.

DISCUSSION

Rapid and accurate identification of Sichuan walnut cultivars is particularly important in grafting propagated nut tree species both for practical breeding purposes and for cultivar proprietary rights protection. In this study, we used FISH, early-fruiting gene analysis and SSR analysis to identify Sichuan walnut cultivars. We will discuss the results with respect to the following two aspects: *i*) distinguishing Sichuan walnut cultivars by molecular cellular inheritance and *ii*) determining the relationships of Sichuan walnuts with *J. regia* and *J. sigillata*.

Distinguishing Sichuan Walnut Cultivars by Molecular Cellular Inheritance

In contrast to morphological analysis, FISH, early-fruiting gene analysis and SSR analysis are three methods of molecular cellular inheritance analysis. The Sichuan walnut cultivars showed strong differences in morphological characters, such as leaf and nut shell characters. However, these characters are easily controlled by the environment. Therefore, we first performed FISH to distinguish Sichuan walnut cultivars. In contrast to the findings of previous studies, thirty-four chromosomes were detected by chromosome-end fluorescence signals in all four cultivars in this study. Several previous works have reported that *Juglans* has thirty-two chromosomes (Woodworth, 1930; Hans, 1970; Tulecke et al., 1988; Mu and Xi, 1988; Mu et al., 1990). The original chromosome images obtained by Hans (1970); Tulecke et al. (1988), and Woodworth (1930) have not been checked. However, thirty-four chromosomes were counted in the original image of *J. regia* (**Figure 9A**), but Mu and Xi (1988) reported thirty-two chromosomes. Similarly, thirty-four chromosomes were counted in the original image of *J. sigillata* (**Figure 9B**), whereas Mu et al. (1990) reported thirty-two chromosomes. A similar phenomenon has been observed in the *Carya* species *C. aquatica* (2n = 32), *C. dabieshanensis* (2n = 32), *C. hunanensis* (2n = 32), *C. myristiciformis* (2n = 32), *C. tonkinensis* (2n = 32), *C. illinoinensis* (2n = 34), *C. ovata* (2n = 34), *C. floridana* (2n = 64), and *C. texana* (2n = 64) (Xu, 2017). During adaptive evolution, the chromosome numbers and forms of several plants change *via* polyploidization, aneuploidization (caused by

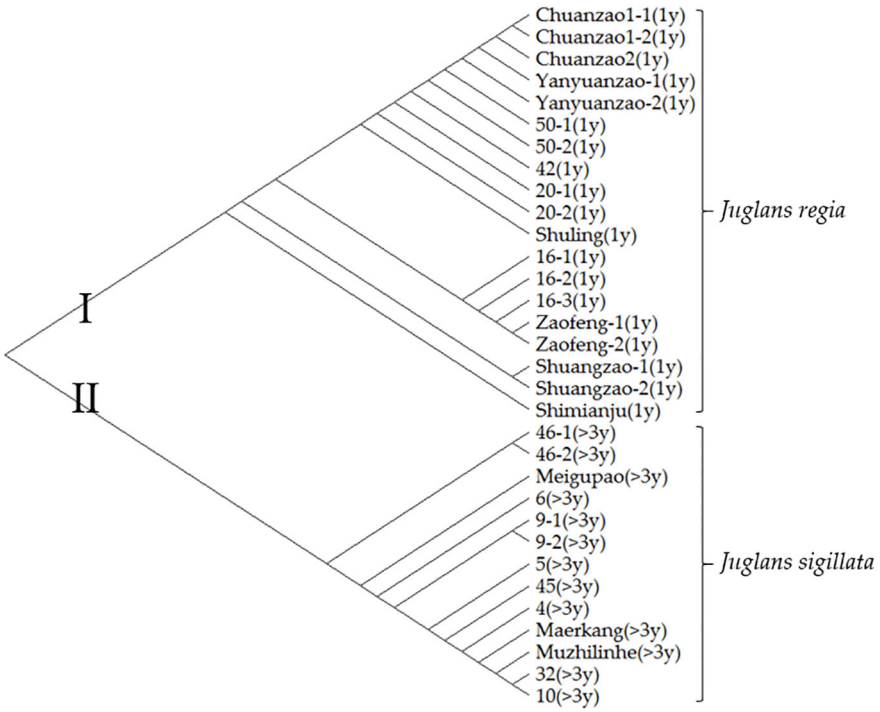

FIGURE 5 | Evolutionary analysis involving thirty-two early-fruiting nucleotide sequences from 22 walnut materials was performed with the maximum likelihood method. Evolutionary history was inferred by using the maximum likelihood method and the Tamura-Nei model. Evolutionary analyses were conducted in MEGA X. The information in parentheses represents the number of years after planting before fruiting occurs.

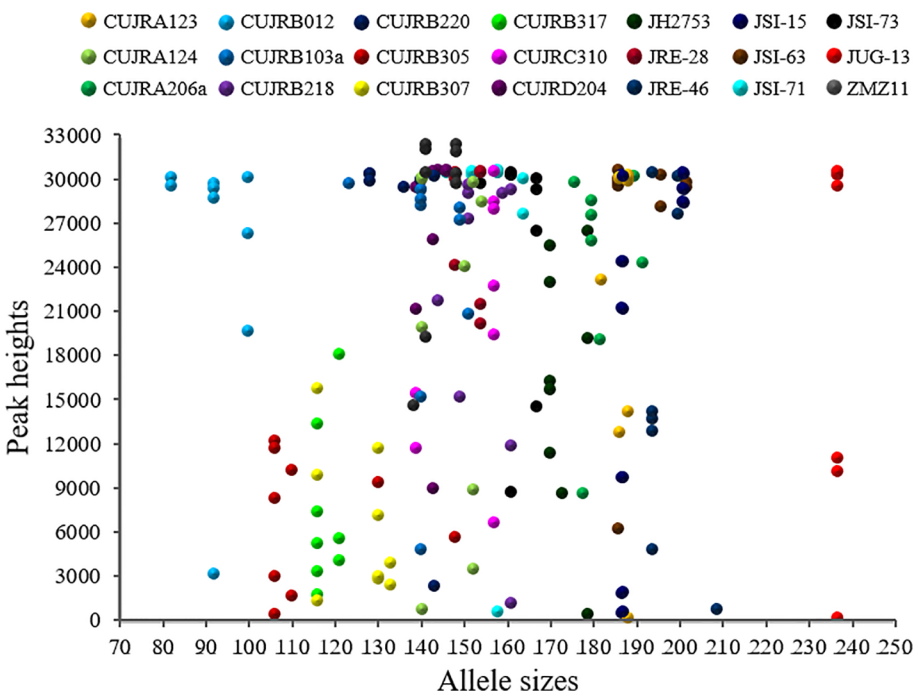

FIGURE 6 | Scatter diagram of 21 SSR loci detected in the nine walnut cultivars. The SSR loci are represented by different colors and symbols. The x-axis indicates allele size, while the y-axis indicates relative peak height. The allele sizes ranged from 82 to 237 nucleotides, while the relative peak heights ranged from 201 to 32,722.

	Artificial hybrids				Natural hybrids				
SSR loci	Chuanzao1	Chuanzao2	Shuangzao	Shuling	Shimianju	Meigupao	Muzhilinhe	Maerkang	Yanyuanzao
CUJRB012	92	92	100	92	82	100	92	82	100
CUJRB305	106	110	106	106	110	106	148	129	106
CUJRB307	116	129	135	117	129	129	117	129	135
CUJRB317	121	116	116	-	117	121	121	116	117
CUJRA124	140	152	140	140	152	152	140	154	150
CUJRB103a	140	140	123	140	140	151	149	140	149
CUJRB220	143	141	128	143	128	141	136	128	141
CUJRD204a	147	143	139	-	143	147	139	143	143
ZMZ11	148	141	141	148	138	141	147	141	148
JRE-28	154	154	154	-	148	154	147	148	147
CUJRC310	157	157	139	-	157	139	156	157	157
JSI-71	158	152	146	158	152	158	163	163	158
JSI-73	161	154	160	161	167	167	166	167	160
CUJRB218	161	151	144	161	161	149	151	159	151
JH2753	179	170	179	179	173	170	170	171	171
CUJRA206a	182	180	176	-	178	190	180	180	192
JSI-15	187	187	201	187	201	187	201	187	187
CUJRA123	186	188	188	189	188	186	186	182	188
JSI-63	186	196	201	186	186	196	186	201	186
JRE-46	200	200	194	-	209	194	194	194	194
JUG-13	237	236	237	237	237	237	236	237	237

FIGURE 7 | Allele sizes of 21 SSR loci of the four artificial hybrid cultivars and five natural hybrid cultivars. The SSR loci were aligned based on the allele size of the Chuanzao 1 cultivar from smallest to largest. Color gradients of red, blue, and green were used to mark all the allele sizes in Excel 2019. Red represents small allele sizes, green represents large allele sizes, and light blue represents intermediate allele sizes. Six SSR loci of the Shuling cultivar were not obtained, as shown by "-" in the column for this cultivar. The allele sizes in purple ovals are those that can be used to discern the cultivars (except for allele size 148 in the dashed oval, because Shuling can be discerned by the combination of ZMZ11 (148) and other SSR loci, which distinguish and exclude Chuanzao 1 and Yanyuanzao.

deletion, duplication, inversion, or translocation), and B chromosome generation (Schubert, 2007; Guerra, 2008; Raskina et al., 2008; Weiss-Schneeweiss et al., 2008). For example, in plants in the genera *Carex* and *Prospero*, chromosomes commonly undergo breakage and fusion (Hipp and Roalson, 2009; Chung et al., 2011; Jang et al., 2013), especially *Carex* species, in which chromosome numbers range from 6 to 46 (Roalson, 2008). Both *Trifolium subterraneum* (2n = 16) and *Trifolium israeliticum* (2n = 12) are produced by aneuploidization of *T. israeliticum* (2n = 14) (Falistocco et al., 2013). In Brassicaceae plants, a chromosome count of n = 8 can be reduced to a count of n = 7 (Mandakova and Lysak, 2008). The numbers of chromosomes vary among individuals within populations of *Phaseolus vulgaris* (Pedrosa-Harand et al., 2006). Hence, the possible reasons for the differences in walnut chromosome numbers between previous works and this study are as follows: *i*) Adaptive evolution. Aneuploidization variation in the four Sichuan walnut cultivars is likely because the chromosome numbers varied from 32 to 34. *ii*) Among-population variation. Similar to those of *P. vulgaris*, *C. illinoinensis,* and *C. ovata*, the chromosome numbers of the four Sichuan walnut cultivars probably vary among populations of individuals, and we determined the walnut cultivars to have 2n = 34. *iii*) Hybridization. Continuous hybridization has probably caused the chromosomes of the four Sichuan walnut cultivars to become mismatched and to misdivide during meiosis and mitosis because the parents have different genetic backgrounds. *iv*) Inaccurate chromosome number counts. Due to a lack of available technology for effectively discerning intact chromosomes, the chromosomes did not spread very well in previous study.

Although walnut chromosome numbers have been reported previously, the FISH technique has not yet been used to analyse walnut chromosomes. The FISH 5S rDNA oligonucleotide with two signals and $(AG_3T_3)_3$ with an end signal on every chromosome showed almost no differences among the four walnut cultivars in this study. Similar distribution patterns have also been observed in *Citrus* species (Deng et al., 2019); *Berberis* species (Liu and Luo, 2019); and *Fraxinus*, *Syringa*, and *Ligustrum* species (Luo and Liu, 2019a). FISH probes $(AG_3T_3)_3$ located at chromosome ends ensure accurate chromosome counts (Pereira et al., 2018), although $(AG_3T_3)_3$ is also occasionally observed in the internal positions of chromosomes (Murray et al., 2002; Majerová et al., 2014; Vasconcelos et al., 2018). Because 5S rDNA and $(AG_3T_3)_3$ were inadequate for identifying the Sichuan walnut cultivars, it was necessary to perform further detection. The hidden centromeres of the small chromosomes hampered further karyotype analysis. A similar phenomenon has also been observed in *Fraxinus pennsylvanica*, *Syringa oblata*, *Ligustrum lucidum*, and *Ligustrum* × *vicaryi* (Luo and Liu, 2019a) as well as in *Zanthoxylum armatum* (Luo et al., 2018). The limited karyotype data contributed little to the identification of the walnut cultivars.

It is difficult for FISH to discern interspecific and intraspecific differences (Liu and Luo, 2019; Luo and Liu, 2019a). In addition, it is rather hard to prepare well-distributed chromosomes and

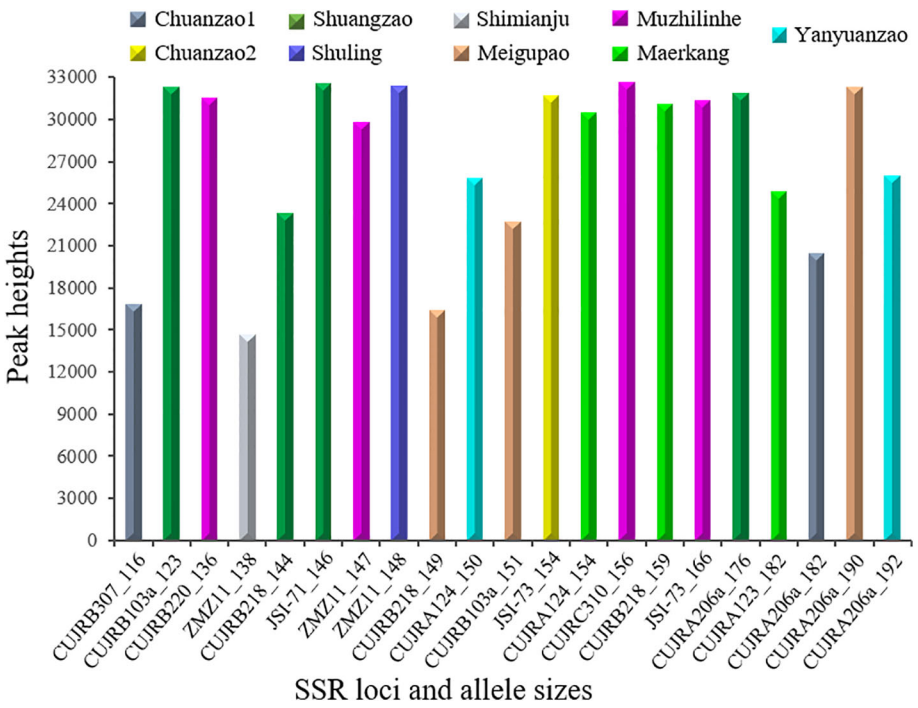

FIGURE 8 | Nine cultivars were discerned by 21 selected SSR allele sizes. The x-axis indicates SSR locus and allele size, while the y-axis indicates relative peak height. The allele size ranged from 116 to 192 nucleotides, while the relative peak height ranged from 14,643 to 32,607. The Chuanzao 1 cultivar was discerned by CUJRB307 (116) or CUJRA206a (182); Chuanzao 2 was discerned by JSI-73 (154); Shuangzao was discerned by CUJRB103a (123), CUJRB218 (144), JSI-71 (146), or CUJRA206a (176); Shimianju was discerned by ZMZ11 (138); Meigupao was discerned by CUJRB218 (149), CUJRB103a (151), or CUJRA206a (190); Muzhilinhe was discerned by CUJRB220 (136), ZMZ11 (147), CUJRC310 (156), or JSI-73 (166); Maerkang was discerned by CUJRA124 (154), CUJRB218 (159), or CUJRA123 (182); Yanyuanzao was discerned by CUJRA124 (150) or CUJRA206a (192); and Shuling was discerned by the combination of ZMZ11 (148) and other SSR loci, which distinguished and excluded Chuanzao 1 [CUJRB307 (116) or CUJRA206a (182)] and Yanyuanzao [CUJRA124 (150) or CUJRA206a (192)].

explore the ability of probes to identify walnut varieties. Root samples for chromosome counts should be collected from cuttings of sample trees. However, walnut plants rarely produce fat young roots; even seedlings usually grow all thin old roots. Hence, the root materials of the four walnut cultivars used in this study originated from germinated seeds. It takes approximately at least two months for walnuts to germinate, and then the lateral roots must branch from the main root. Root tips from lateral roots were used in this study. It was quite difficult to obtain even one well-spread slide. Furthermore, when potential sequences with differences between cultivars are obtained, these sequences must be converted into FISH probes. If these sequences are oligonucleotides (< 60 bp), the FISH probes are inexpensive, easy to prepare, and effective. However, if these sequences are longer, preparation of FISH probes is expensive and more time consuming. When the probes are ready, additional slides are needed to test if the probes produce signals. In addition, for visualization of the fluorescence signal, the probes must be repeatedly used in adjacent chromosomes up to 10 kbp long in metaphase (Pedersen and Linde-Laursen, 1995). Therefore, it is not easy to explore the identification ability of probes. In this study, we prepared well-spread chromosomes and developed probes located at chromosome ends and proximal regions, but the probes were not able to distinguish the walnut cultivars. Hence, it is necessary to explore more probes to identify walnut cultivars.

Furthermore, an evolutionary analysis of early-fruiting nucleotide sequences clearly revealed two groups of *J. regia* and *J. sigillata* in this study. DNA sequences of gene fragments can ordinarily be used to distinguish species in different genera; however, they are less well suited to reveal interspecies differences within the same genus and can rarely be used to discern cultivars in the same species. Previous works on early-fruiting genes have mainly concentrated on *J. regia* and have included *in vitro* cultivation (Breton et al., 2004), molecular marker analysis (Niu et al., 2008; Ye et al., 2010), and cloning and expression analysis (Niu et al., 2008; Zhu et al., 2011). Few studies on early-fruiting genes in *J. sigillata* are available. The two species, *J. regia* and *J. sigillata,* have also been identified by restriction site-associated DNA SNPs (RAD-SNPs) (Yan et al., 2019) and RAPD (Chen et al., 2007). However, the chloroplast genomes of these two species differ little (Hu et al., 2017). In addition, early-maturing walnuts are more diverse than and are distinct from normally maturing walnuts (Ebrahimi et al., 2017). Walnuts feature precocious genotypes and exhibit a tendency towards homozygosity due to self-pollination (Chen et al., 2018). Since the early-fruiting gene fragment divided the ten walnut

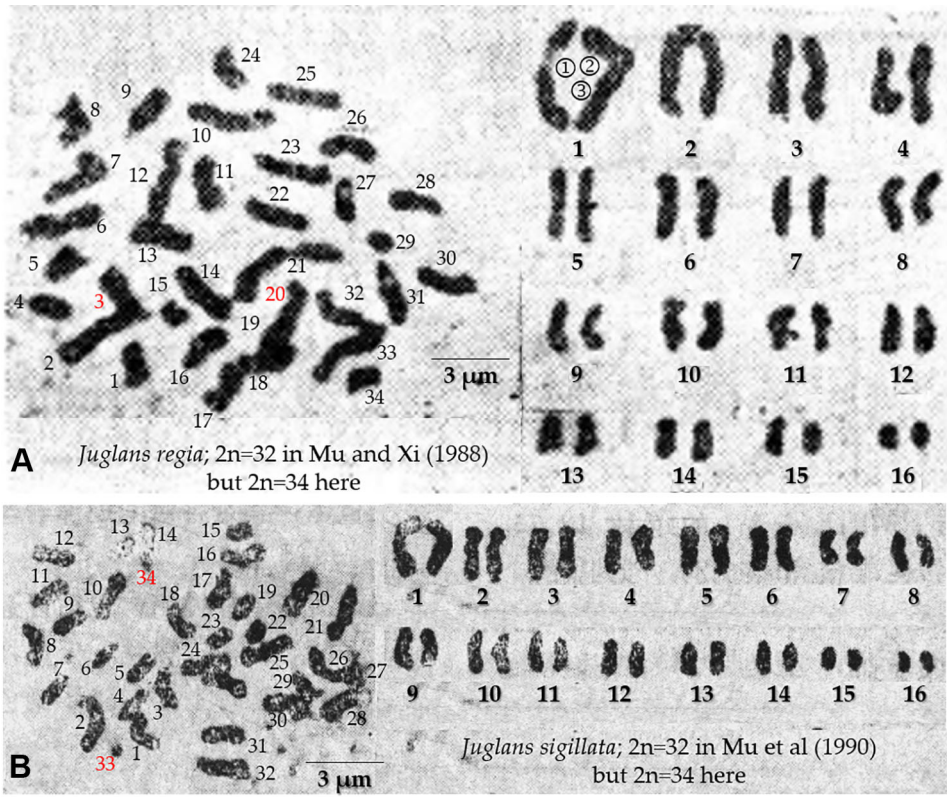

FIGURE 9 | Metaphase chromosomes of *J. regia* **(A)** and *J. sigillata* **(B)**. Mu and Xi (1988) reported 2n = 32 *J. regia* chromosomes, but 2n = 34 were counted here. The first pair of chromosomes in **(A)** probably consists of three chromosomes, and the chromosomes with red numbers are likely disputed extra chromosomes. Mu et al. (1990) reported 2n = 32 *J. sigillata* chromosomes, but 2n = 34 were counted here. The chromosomes with red numbers in **(B)** are likely disputed extra chromosomes.

cultivars into two groups, it was somewhat useful for recognition; however, additional methods of detection are still needed.

We further developed 21 SSR loci to detect nine Sichuan walnut cultivars in this study. Each of eight cultivars (Chuanzao 1, Chuanzao 2, Shuangzao, Shimianju, Meigupao, Muzhilinhe, Maerkang, and Yanyuanzao) was exclusively discerned by one SSR locus, and the Shuling cultivar was discerned by a combination of SSR loci. Shi et al. (2016) identified seven Sichuan walnut cultivars by selecting combinations of SSR loci from previous works (Woeste et al., 2002; Dangl et al., 2005; Ross and Woeste, 2008; Wang et al., 2011). Zhou et al. (2018a; 2018b) constructed SSR fingerprints of elite Sichuan cultivars of *J. regia* and *J. sigillata*. Although several walnut cultivars were examined by Shi et al. (2016) and Zhou et al. (2018a; 2018b), this study provided the following novel contributions: *i*) new SSR loci were developed, *ii*) each of eight cultivars was exclusively discerned by one SSR locus, and *iii*) a few cultivars were tested for the first time. In total, approximately fifty elite walnut cultivars are licensed in Sichuan Province (http://www.sclmzm.com:88/sczmz/linmuliangzhong/index.jhtml). To date, no study has distinguished all of these cultivars. The main barriers to doing so are difficulties in sample collection from all the cultivars and inadequate identification techniques. Hence, more work is needed to explore professional identification techniques for all the walnut cultivars in Sichuan. We are devoting to testing oligonucleotides probes from our SSR repeat motifs, developing more SSR loci that exclusively distinguish all the walnut cultivars in Sichuan, using additional methods such as Hi-C (High-throughput chromosome conformation capture) to construct chromosome conformation of Sichuan walnuts.

Relationships of Sichuan Walnuts with *J. regia* and *J. sigillata*

The geographical location of Sichuan has led to the development of natural hybrids of Sichuan walnuts, while social development and human demands have led to the production of artificial hybrids of Sichuan walnuts (Xiao and Pu, 2015). It is unclear whether each hybrid Sichuan walnut belongs to one of its parent species (*J. regia* or *J. sigillata*) or represents a new taxon of *Juglans*. For example, the Shimianju cultivar was treated as *J. regia* by Sun et al. (2011); Shi et al. (2016), and Wu (2009) but as *J. sigillata* by Yan et al. (2019) and Zhou et al. (2018a; 2018b). Sichuan walnuts are intermediate between *J. regia* and *J. sigillata* in terms of their morphological characters (Xiao and Pu, 2015; Zhou et al., 2018a; 2018b). Sichuan walnuts (2n = 34) have

been reported to possess two more chromosomes than *J. regia* and *J. sigillata* in previous works (Woodworth, 1930; Hans, 1970; Tulecke et al., 1988; Mu and Xi, 1988; Mu and Xi, 1990). A probable reason for this increase in chromosome number is chromosome mismatching or misdivision during meiosis and mitosis caused by multiple hybridizations between *J. regia* and *J. sigillata*, which have genetic differences. Sichuan walnuts are different from *J. regia* and *J. sigillata* in terms of their morphological characters, and their chromosome numbers seem to provide some support for their classification in a new taxon of *Juglans*. However, the evolutionary analysis of the early-fruiting gene of Sichuan walnut cultivars placed some of them within *J. regia* and some of them within *J. sigillata*, which does not support the grouping of Sichuan walnut cultivars in a new *Juglans* taxon. The SSR allele sizes of the Sichuan walnut cultivars were similar and did not support the existence of a new taxon of *Juglans*. In summary, Sichuan walnut cultivars vary greatly: some are similar to *J. regia,* some are similar to *J. sigillata*, and some are intermediate. However, these differences are not sufficient to treat these cultivars as a new *Juglans* taxon.

DATA AVAILABILITY STATEMENT

The DNA sequencing generated in this study was submitted to the National Center of Biotechnology Information (NCBI) and given GenBank accession numbers ranging from MN548306-MN548337.

AUTHOR CONTRIBUTIONS

Conceptualization: XL. Methodology: JC. Software: JC. Validation: XL. Formal analysis: XL. Investigation: JC. Resources: XL. Data curation: XL. Writing—original draft preparation: XL. Writing—review and editing: XL. Visualization: JC. Supervision: XL. Project administration: XL. Funding acquisition: XL. All authors consented to this submission.

FUNDING

This research was funded by the Natural Science Foundation of China (grant number 31500993).

ACKNOWLEDGMENTS

The authors thank Professor Zhou Yonghong for laboratory equipment support.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 March 2020
doi: 10.3389/fpls.2020.00154

Autotetraploid *Coffea canephora* and Auto-Alloctaploid *Coffea arabica* From *In Vitro* Chromosome Set Doubling: New Germplasms for *Coffea*

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Edited by:
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Reviewed by:
Sezai Ercisli, Atatürk University, Turkey
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *05 December 2019*
Accepted: *31 January 2020*
Published: *04 March 2020*

Citation:
Venial LR, Mendonça MAC, Amaral-Silva PM, Canal GB, Passos ABRdJ, Ferreira A, Soares TCB and Clarindo WR (2020) Autotetraploid Coffea canephora and Auto-Alloctaploid Coffea arabica From In Vitro Chromosome Set Doubling: New Germplasms for Coffea. *Front. Plant Sci. 11:154. doi: 10.3389/fpls.2020.00154*

Polyploidy is more than two chromosomal sets per nucleus, as the allotetraploid *Coffea arabica*. Due to allotetraploidy, *C. arabica* shows different phenotypes compare to diploid *Coffea* species, highlighting by beverage quality produced from its grains. Looking for the possibility of new phenotypes coupled with economic feature, considerable progress since 60's was reached for synthetic chromosome set doubling (CSD) *in vitro*, involving especially the antitubulin compounds, biological material, and used tissue culture pathway as the indirect somatic embryogenesis (ISE). Here, we aimed to regenerate autotetraploid and auto-alloctaploid plantlets of *Coffea canephora* and *C. arabica*, respectively, from a novel *in vitro* CSD procedure for *Coffea*. Exploring the ISE pathway, we treated the cellular aggregate suspensions (CAS) with 0.0 (control), 0.5, 1.5, or 2.5 mM of colchicine solution for 48, 72, or 96 h and maintained in liquid medium under constant orbital shaking. After transferring the CAS to semisolid media for somatic embryo regeneration, we considered it as cellular mass. Mature cotyledonary somatic embryos were only regenerated from cellular masses treated with 2.5 mM/48 h and 2.5 mM/72 h for *C. canephora* and with 0.5 mM/48 h for *C. arabica*. Evaluating the DNA ploidy level and the chromosome counting revealed that 36 (34.9%) plantlets of *C. canephora* were autotetraploids (4C = 2.86 pg, 2n = 4x = 44) and 61 (21.1%) of *C. arabica* were auto-alloctaploids (4C = 5.24 pg, 2n = 8x = 88). The CSD procedure, exploring the CAS proliferation and ISE pathway, promoted whole-genome duplication and resulted in a relatively high number of solid polyploids of both *Coffea* species. Due to distinct responses, DNA sequence fidelity (genetic) and global level of 5-methylcytosine (epigenetic) were evaluated. We observed that the increase of 5-methylcytosine levels was associated with somatic embryo regeneration from cells

showing DNA sequence fidelity for the tested SSR primers. In conclusion, the adopted procedure for *in vitro* CSD is reproducible for induction, regeneration and propagation of *Coffea* polyploids and potentially other shrubbery and woody species. In view of the novelty of this procedure to generate new germplasm, we show the key issues and the steps of the CSD procedure.

Keywords: coffee, polyploidy, plant tissue culture, flow cytometry, cytogenetics, whole-genome duplication

HIGHLIGHTS

We established a novel chromosome set doubling procedure for *Coffea* treating cellular aggregate suspensions with colchicine. From this procedure, new *Coffea canephora* autotetraploids and *Coffea arabica* auto-alloctaploids were regenerated.

INTRODUCTION

Polyploidization leads to more than two complete chromosome sets per nucleus in a cell, naturally occurring through autopolyploidy or allopolyploidy (Stebbins, 1947). As a result of "omic" changes (genomic, epigenomic, transcriptomic, and metabolomic), polyploids may exhibit new physiological, morphological and reproductive phenotypes and/or traits (Sattler et al., 2016; Li et al., 2019; Iannicelli et al., 2020). Because of this, polyploidy has been considered an important trigger in plant diversification and evolution (Soltis et al., 2009; Iannicelli et al., 2020), including the saltational speciation (Mallet, 2007; Iannicelli et al., 2020).

The impact of natural polyploidy on plant diversity and evolution, but also in worldwide economy and breeding programs, have inspired several research groups to establish different strategies for synthetic polyploidization through chromosome set doubling (CSD). In an agronomic scenario, the *ex vitro* and *in vitro* procedures to induce synthetic polyploidy lead to new and/or improved germplasms, enhancing the breeding programs of crop, ornamental, medicinal and forest species (Dhooghe et al., 2011; Sattler et al., 2016; Iannicelli et al., 2020). Synthetic polyploids have been obtained mainly from CSD in *in vitro* environments following Murashige and Nakano (1966), under controlled physical and chemical conditions. Biological material showing proliferative cells, mainly shoot tips, is exposed to the antitubulinic agent (e.g., colchicine, oryzalin, trifluralin, amiprophos-methyl) added to the tissue culture medium. These compounds prevent the mitotic or meiotic spindle microtubule (fuse) formation through binding to α- and/or β-tubulin (Planchais et al., 2000). Due to this cytotoxic effect, the sister chromatids (mitotic anaphase, meiotic anaphase II) and homologues chromosomes (meiotic anaphase I) disjunction as well as the cytokinesis do not occur, resulting in cells with duplicated chromosome set. Regarding the *in vitro* strategies, the chromosome set has been successfully duplicated for trees and shrubs, like *Acacia dealbata* Link., *Acacia mangium* Willd. (Blakesley et al., 2002), *Platanus acerifolia* (Ait.) Willd. (Liu et al., 2007), *Jatropha curcas* L. (de Oliveira et al., 2013), *Ziziphus jujuba* Mill. (Shi et al., 2015), *Eriobotrya japonica* (Thunb.) Lindl. (Blasco et al., 2015), allotriploid "Híbrido de Timor" (*Coffea canephora* Pierre ex A. Froehner *x Coffea arabica* L., Sanglard et al., 2017), *Eucalyptus grandis* W. Hill ex Maiden, *Eucalyptus urophylla* S. T. Blake, *Eucalyptus benthamii* Maiden & Cambage, and homoploid *E. urophylla x E. grandis* (Silva et al., 2019).

In order to expand the applicability, improvements have been made to solve the main bottlenecks of the *in vitro* CSD procedure: low rate of solid polyploids and high rate of mixoploids, as well as propagule mortality. Nowadays, the more promising *in vitro* procedure associates the indirect somatic embryogenesis (ISE) pathway with the antitubulin agent treatment. This *in vitro* pathway is based on somatic embryo recovery—the possibility of regenerating a plantlet from a single cell (Stewart et al., 1958)—which maximizes the occurrence of only solid polyploids from CSD exploring the ISE (Wu and Mooney, 2002; Dutt et al., 2010; Acanda et al., 2015; Sanglard et al., 2017).

Pro-embryogenic cells of friable calli in semisolid medium (Wu and Mooney, 2002; Petersen et al., 2003; Zhang et al., 2007; Sanglard et al., 2017) or of cellular aggregate suspensions (CAS) in liquid medium (Dutt et al., 2010; Acanda et al., 2015) have been exposed to different antitubulin agents for different times and concentrations. CSD was performed from semisolid system for *Spathiphyllum wallisii* Regel (Eeckhaut et al., 2004), *Citrus* L. (Wu and Mooney, 2002; Petersen et al., 2003; Zhang et al., 2007), homoploid *Vitis* x *Muscadinia* (Xie et al., 2015), anorthoploid *Coffea* "Híbrido de Timor" (Sanglard et al., 2017), *Lilium distichum* Nakai, and *Lilium cernuum* Komar (Fu et al., 2019). Differently, Dutt et al. [(2010), for *Citrus reticulata* Blanco] and Acanda et al. [(2015), for *Vitis vinifera* L. "Mencía"] conducted the CSD from CAS, which they established from friable calli propagated in liquid medium. CAS show a high cell proliferation rate (van Boxtel and Berthouly, 1996) that is the feedstock for CSD. The gradient of nutrients in the liquid medium is considered another advantage over semisolid system (Dutt et al., 2010), increasing cell contact with the tissue culture compounds and with the antitubulin used for CSD.

Variations in the ISE response have been observed after treatment with antitubulin agents, as the rate of somatic embryos and plantlets (responsive or unresponsive) recovered from the friable calli or CAS (Wu and Mooney, 2002; Zhang et al., 2007; Sanglard et al., 2017). The causes of these variations can be associated with the *in vitro* conditions, the occurrence of somaclonal variation during the ISE pathway and/or the

cytotoxic effect of the antitubulin treatment that involves a pulse using different compounds and concentrations (Dhooghe et al., 2011). Thus, genetic (SSR markers) and epigenetic (global methylated cytosine) features are appointed as possible factors that interfere in the somatic embryo and plantlet regeneration (Dutt et al., 2010). Therefore, these aspects should be investigated in order to understand the distinct ISE responses.

Owing to the new genomic and phenomic features of the polyploids in relation to their ancestors, there is great interest to achieve synthetic polyploids. The aim of this work was to establish a new procedure for CSD from CAS of the agronomic relevant *Coffea* species *C. canephora* and *C. arabica*. Additionally, we evaluate the DNA sequence stability (SSR markers) and epigenetic (5-methylcytosine) level to find possible causes for the varying ISE responses. We choose *C. canephora* and *C. arabica* because of the previously established ISE for these species by our research group (Sanglard et al., 2019). Besides, *C. canephora* and *C. arabica* are relevant for the economy of some countries, like Brazil, Vietnam, Colombia, Mexico, Indonesia, India, Guatemala, Uganda, and Ethiopia. The grains produced by *C. arabica* represent 70–75% of the exported coffee for Brazil, and 25–30% by *C. canephora*. Thus, we also expected to provide new *C. canephora* and *C. arabica* polyploid germplasms for future breeding approaches involving selection of the individuals based on their grain production, coffee beverage quality and tolerance and resistance to abiotic and biotic stresses, as well as for crossing with other *Coffea*.

MATERIALS AND METHODS

Plant Material

One *C. canephora* plantlet, which has been propagated in an *in vitro* germplasm bank (Universidade Federal do Espírito Santo, Espírito Santo, Brazil), and one *C. arabica* plant, which has been maintained in a greenhouse bank (Universidade Federal de Viçosa, Minas Gerais, Brazil), were used as explant donor plants. Chromosome number, ploidy level and DNA content of the explant donor plants were confirmed as 2n = 2x = 22 chromosomes and 2C = 1.43 pg for *C. canephora*, and 2n = 4x = 44 chromosomes and 2C = 2.62 pg for *C. arabica*. These *Coffea* crops were chosen due to agronomic and evolutive relevance. *C. canephora* is a diploid species with 2n = 2x = 22 chromosomes and 2C nuclear DNA content of 1.43 pg, and *C. arabica* is a true allotetraploid that possesses 2n = 4x = 44 chromosomes and 2C = 2.62 pg (Sanglard et al., 2019). *C. arabica* is the only polyploid species of the *Coffea* genus, which was probably originated from crossing between the diploid species *Coffea eugenioides* S. Moore and *C. canephora*. Besides the karyotype, the divergences between these species also include the reproductive mechanism (*C. canephora* is allogamous, like the other diploid *Coffea* species, and *C. arabica* is autogamous, Yu et al., 2011), morphological and physiological aspects (Charrier and Berthaud, 1985), and commercial relevance mainly associated to the beverage quality of coffee (Farah and Donangelo, 2006).

Friable Calli Induction and Cellular Aggregate Suspensions Establishment

We collected and disinfected leaves of *Coffea arabica* according to Sanglard et al. (2019) before inoculation. We excised five leaf fragments of 1cm² from both species and inoculated them in M1 medium (**Table 1**) in 60 × 15 mm Petri dishes. The culture was conducted in the dark at 25 ± 2°C for friable calli induction. After 60 days, 0.5 g of friable calli was transferred to 125 ml Erlenmeyers containing 30 ml of M2 medium (**Table 1**). The Erlenmeyers were maintained in the dark at 25 ± 2°C on a 100 rpm orbital shaker. For establishment of the CAS, the material was subcultured every 15 days into a fresh medium, respecting the 0.5 g of cellular aggregates per Erlenmeyer. All procedures were performed under aseptic conditions.

Chromosome Set Doubling and Plantlet Recovering

After the fourth subculture, the CAS were treated with colchicine, an alkaloid compound isolated from *Colchicum autumnale* L. seeds and bulbs (Planchais et al., 2000). We applied the colchicine treatment 7 days after the fourth subculture, according to growth curves of the *C. canephora* and *C. arabica* CAS (van Boxtel and Berthouly, 1996). For this, we added filter-sterilized colchicine solution to each Erlenmeyer: 0.0 (control), 0.5, 1.5, and 2.5 mM, respectively for each treatment. The CAS were maintained under colchicine treatment in the dark, on a 50 rpm orbital shaker at 25 ± 2°C for 48, 72, and 96 h. One Erlenmeyer referred to each treatment (colchicine/time), adding up to 12 in total. After colchicine

TABLE 1 | Tissue culture media used for friable calli induction (M1), CAS establishment (M2), CSD (M2), somatic embryo (M3 and M4) and plantlet regeneration (M5) of *C. canephora* and *C. arabica*.

Components	Medium				
	M1	M2	M3	M4	M5
MS (Sigma®)	2.15 g L^{-1}	2.15 g L^{-1}	4.30 g L^{-1}	4.30 g L^{-1}	4.30 g L^{-1}
Gamborg's B5 vitamins	10 ml L^{-1}	10 ml L^{-1}	10 ml L^{-1}	10 ml L^{-1}	10 ml L^{-1}
Sucrose (Sigma®)	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}
L-cysteine (Sigma®)	0.08 g L^{-1}	0.04 g L^{-1}	0.04 g L^{-1}	0.04 g L^{-1}	0.04 g L^{-1}
Malt extract (Sigma®)	0.4 g L^{-1}	0.4 g L^{-1}	0.4 g L^{-1}	0.4 g L^{-1}	0.4 g L^{-1}
Casein hydrolysate (Sigma®)	0.1 g L^{-1}	0.1 g L^{-1}	0.1 g L^{-1}	0.1 g L^{-1}	0.1 g L^{-1}
2,4-D (Sigma®)	9.06 μM	9.06 μM	–	–	–
BAP (Sigma®)	4.44 μM	4.44 μM	4.44 μM	4.44 μM	–
GA_3 (Sigma®)	–	–	–	–	2.89 μM
Phytagel (Sigma®)	2.8 g L^{-1}	–	2.8 g L^{-1}	2.8 g L^{-1}	2.8 g L^{-1}
Activated charcoal (Isofar®)	–	–	2.0 g L^{-1}	4.0 g L^{-1}	–
pH	5.6	5.6	5.6	5.6	5.6

MS, Murashige and Skoog (1962); Gamborg's B5 vitamins, Gamborg et al. (1968); 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP: 6-benzylaminopurine; GA_3, gibberellic acid. Culture media were sterilized in an autoclave at 121°C and 1.5 atm for 20 min.

exposition, the CAS of each Erlenmeyer was separately filtered through a 40 µm cell strainer (BD Falcon™), and carefully washed with at least 50 ml of autoclaved dH_2O for residual elimination of colchicine (**Figure 1**). Cellular aggregates of each Erlenmeyer were subdivided in 60 × 15 mm Petri dishes containing M3 or M4 medium (**Table 1**). After transferring the CAS to semisolid medium, the cellular aggregates were denominated as cellular mass. After regeneration of somatic embryos, only the mature cotyledonary somatic embryos (MCSE) were transferred to tubes containing M5 medium for germination (**Table 1**).

Ploidy Level of the Recovered Plantlets

Initially, we determined the DNA ploidy level of the regenerated *C. canephora* and *C. arabica* plantlets from nuclei suspensions extracted from leaf fragments (~1 cm^2) by chopping (Galbraith et al., 1983) and staining according to Otto (1990) and Praça-Fontes et al. (2011). Nuclei suspensions obtained from leaves of the explant donor *C. canephora* and *C. arabica* were used as control for DNA ploidy level determination. The suspensions were analyzed with a Partec PAS® cytometer (Partec® GmbH, Münster, Germany).

In addition, roots were excised and treated according to Sanglard et al. (2017) to determine the 2n chromosome number of the plantlets previously screened by flow cytometry. From these roots, we prepared slides by cell dissociation and air-drying. All slides were stained with 5% Giemsa for 20 min, washed two times in dH_2O and analyzed under a Nikon Eclipse Ci-S microscope (Nikon). Prometaphases and metaphases were captured using 100x objective and a CCD camera (Nikon Evolution™) coupled to a Nikon 80i microscope (Nikon).

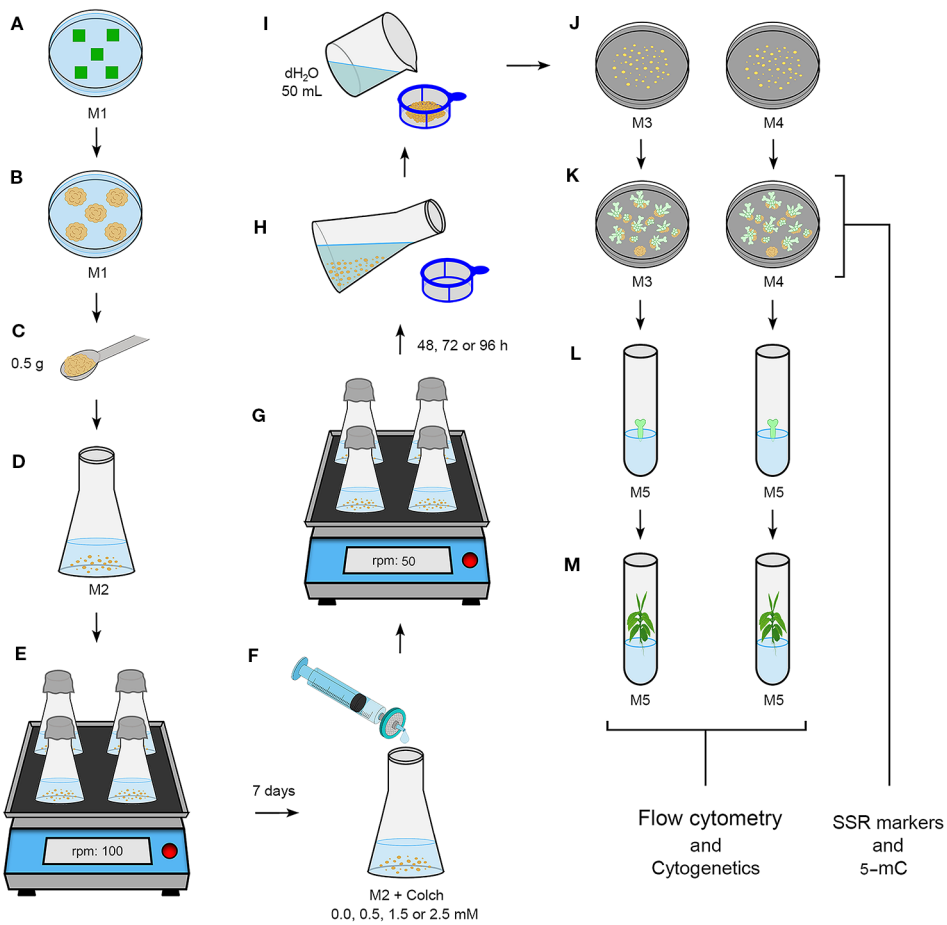

FIGURE 1 | Novel procedure for CSD from CAS treated with colchicine. **(A)** *Coffea* leaf fragment explants (~1 cm^2) in M1 medium (**Table 1**). **(B)** Friable calli after ~30 days in M1. **(C, D)** 0.5 g of friable calli in 125 ml Erlenmeyers containing 30 ml of M2 medium (**Table 1**). **(E)** CAS on orbital shaker at 100 rpm. **(F)** Filter sterilized colchicine solution addition at different concentrations after seven days, according growth curve reported for *Coffea* (van Boxtel and Berthouly, 1996). **(G)** CAS under colchicine treatment for distinct times on orbital shaker at 50 rpm. **(H, I)** Careful and continous washing of the CAS with 50 ml sterile dH_2O. **(J)** Cellular mass (CM) in M3 or M4 medium (**Table 1**). **(K)** CM showing *C. canephora* or *C. arabica* globular, heart, torpedo and cotyledonary embryos in M3 and M4. CM samples of "j" and "l" were collected to evaluate and compare the DNA sequence variability and 5-methylcytosine level. **(L)** MCSE in M5 medium (**Table 1**). For *C. arabica*, these embryos were obtained after 120 days, and for *C. canephora* after 180 days. **(M)** Regenerated plantlets of the two *Coffea* species, of which we excised leaves for DNA ploidy level determination and root meristems for chromosome counting.

DNA Sequence Stability and 5-Methylcytosine Level

Due to different responses obtained during the somatic embryo recovery in M3 and M4, DNA sequence stability (SSR markers) and 5-methylcytosine level (5-mC%) were evaluated in order to identify genetic (SSR markers) and epigenetic (5-mC%) differences related to *in vitro* responses. For this, besides of leaf of the explant donor plants, the following DNA samples of the cellular mass were used: (a) not colchicine-exposed (control – friable calli of the M1), (b) colchicine-exposed and without somatic embryos, and (c) colchicine-exposed and with MCSE, totalling to 32 samples for DNA sequence stability and 21 for 5-mC% analyses (**Table S1**). Because at least 30 μg of genomic DNA are necessary to accomplish the 5-mC% measurement, cellular masses of the same treatment and *in vitro* response (a, b, or c—above) were put together. Genomic DNA was extracted from the explant donor *Coffea* plants and the cellular mass and macerated in MagNALyser (Roche®, Germany) for 70 s at 6,300 rpm (Doyle and Doyle, 1990), with addition of 7.5 M $C_2H_3O_2NH_4$ and excluding the overnight period for DNA precipitation. DNA purity and concentration were estimated using NanoDrop (Thermo Scientific® 2000c).

DNA sequence stability was evaluated employing ten SSR primers (SSR Ca002, SSRCa021, SSRCa045, SSRCa091, SSRCa006, SSRCa084, SSRCa085, SSRCa087, SSRCa088, and SSRCa095) developed and validated for *C. arabica* (Missio et al., 2009). PCR reactions were performed in a final volume of 15 μL composed of: 3 μL of 5×buffer, 1.5 mM of dNTPs, 0.2 μM of primers, 50 ng of DNA, 1.6 mM of $MgCl_2$, 1 U of Taq DNA polymerase, and sufficient quantity of dH_2O to 15 μl. Amplifications were carried out in a Bio-Rad® 96-Well Thermal Cycler C1000™ by touchdown PCR procedure, as performed by Sanglard et al. (2017) for allotriploid and hexaploid "Híbrido de Timor". PCR products were submitted to electrophoresis on 10% polyacrylamide gel in 1X TBE buffer for 4 h at 100 V. The gels were stained with ethidium bromide solution (0.25 mg ml^{-1}) for 20 min, and photo-documented in a Bio-Rad Molecular Imager® Gel Doc ™ using the Image Lab program. The allele forms were tabulated considering the number and position of the bands.

Global 5-mC% was measured through high-performance liquid chromatography (HPLC) using 30 μg of DNA diluted in sterile dH_2O for 100 μl of solution. DNA samples were hydrolyzed with 50 μl of 70% perchloric acid at 100°C for 1 h and the pH 4 (Chen et al., 2013). The solutions were analyzed in Prominence HPLC (Shimadzu®, Japan). The global 5-mC% of each sample was determined by comparison with standards of cytosine (C) and 5-mC for HPLC (Sigma®). The global 5-mC% in the DNA was calculated by %5-mC = [5-mC/(C + 5-mC)] × 100.

Statistical Analysis

The total number of MCSE was compared by *F* test ($P \leq 0.05$) and represented in graphics. Cellular masses [(a) not colchicine-exposed (control–friable calli of the M1), (b) colchicine-exposed and without somatic embryos and (c) colchicine-exposed and with MCSE] were compared in relation to their DNA sequence stability and their global 5-mC%. A contingency table was generated considering all allele forms found for each SSR primer and for each defined cellular mass. After analysis of variance (ANOVA), we performed a correspondence analysis from the contingency table to verify the relation between the allele forms and cellular masses. The mean global 5-mC% values were compared by ANOVA, followed by Dunnett's test ($P \leq 0.05$). Statistical comparisons were performed using the software R (R CORE TEAM, Version 3.1.1, 2014-07-10).

RESULTS

Indirect Somatic Embryogenesis Response

After 90 days in M1 (**Table 1**), the mean number of responsive explants, which were defined by leave fragments with friable calli, was 1.07 for *C. canephora* and 2.37 for *C. arabica*. In M2 (**Table 1**), CAS were established from friable calli of the two *Coffea* species after the third subculture, equivalent to 45 days. So, the CSD procedure was conducted in the seventh day during the fourth subculture. After transferring the cellular aggregates to M3 or M4, they were denominated cellular mass (**Figure 1**).

C. canephora somatic embryos were regenerated from cellular mass treated with 2.5 mM/48 h colchicine and maintained in M3 or M4 and 2.5 mM/72 h colchicine in M3. For *C. arabica,* somatic embryos were recovered only from 0.5 mM/72 h colchicine in M3 or M4 (**Figure 2**). The cellular mass of these *Coffea* species presented globular somatic embryos (**Figure 3A**), which were converted in heart (**Figure 3B**), torpedo (**Figure 3C**) and cotyledonary stages (**Figures 3D–F**). The cotyledonary somatic embryos matured into MCSE (**Figure 3F**). Somatic embryos in different development stages were recorded in the same responsive cellular mass, evidencing an asynchronized ISE response (**Figures 2** and **3**). The responsive cellular masses were statistically identical in relation to somatic embryo number.

In M3 and M4, we recovered 324 MCSE for *C. arabica* after 90 days and 76 for *C. canephora* after 120 days (**Table 2**, **Figure S1**). Thus, the regeneration response occurred in different moments for the two *Coffea* species, the colchicine treatments (time and concentration) and for M3 and M4 media. We counted a total of 878 MSCE in the experiment, of which 621 were from *C. arabica* and 257 from *C. canephora*. The plantlets were recovered gradually from the MCSE in M5 medium (**Table 1**) after 60 days, resulting in 392 (44.6%) plantlets out of which 103 plantlets belonged to *C. canephora* and 289 to *C. arabica* (**Table 1**). This reduced number of plantlets in relation to MCSE is due to failure or inadequate morphological development of the root and shoot. The cellular masses showed an almost continuous production of somatic embryos for both species, over a time span of more than one year, with potential for plantlet recovery over several months. However, we only regarded embryos until 150 days in M3 or M4 in this study.

Plantlet Screening for Chromosome Set Doubling

DNA ploidy level was determined for individual plantlet (392 plantlets, **Table 2**, **Figure S1**) upon comparison with the G_0/G_1

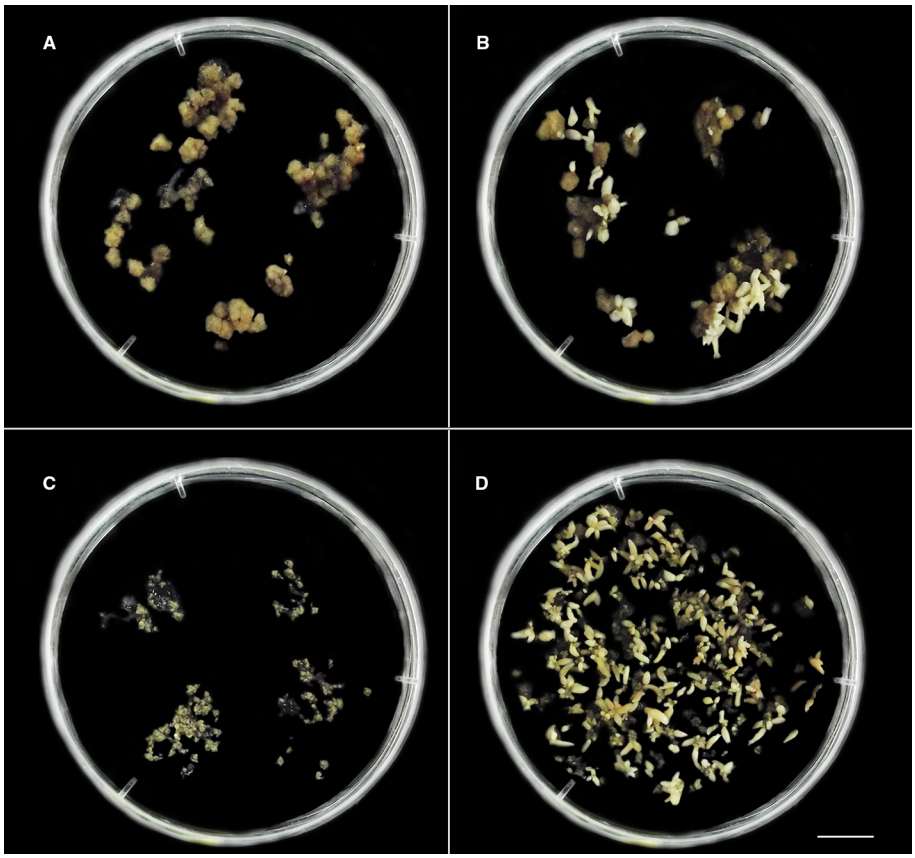

FIGURE 2 | The distinct *in vitro* responses from cellular mass of *C. canephora* **(A, B)** and *C. arabica* **(C**, **D)** observed after colchicine treatment in liquid medium M2 (**Table 1**, **Figure 1**). **(A, B)** *C. canephora* cellular mass after 150 days (**Table 2**) treated with 2.5 mM/48 h colchicine (**Figure 1**) and maintained in M4 medium (**Table 1**). **(A)** Petri dish showing predominance of unresponsive cellular mass and others with few globular somatic embryos (spotlight – right/above). **(B)** Petri dish exhibiting one unresponsive cellular mass and all others with somatic embryos in distinct development stages (globular, heart, torpedo, and cotyledonary). **(C)** Unresponsive cellular mass of *C. arabica* treated with 1.5 mM/72 h colchicine and maintained in M4 during 90 days. **(D)** Several *C. arabica* somatic embryos in different development stages. This result was obtained after 90 days from CAS treated with 0.5 mM/72 h colchicine and the resulting cellular mass maintained in M4. Bar = 1 cm.

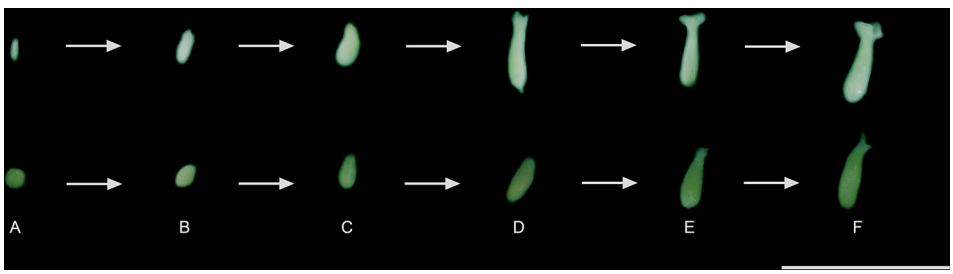

FIGURE 3 | Regeneration, conversion, and maturation of the somatic embryos of *C. canephora* and *C. arabica*: **(A)** globular, **(B)** heart, **(C)** torpedo, **(D)** initial, **(E)** middle, and **(F)** mature cotyledonary. The MCSE **(F)** were transferred to M5 (**Table 1**) and, thus, considered for comparison of the treatments. Bar = 1 cm.

nuclei peak of the explant donor *Coffea* plant (**Figure 4**). For the recovered plantlets screened for CSD, the DNA ploidy level was newly determined after two months and after six months. So, the polyploid condition was verified for all *Coffea* plantlets (**Table 2**) in all flow cytometry analyses, confirming the solid polyploid condition. We found no mixoploid plantlets.

We screened plantlets with a DNA ploidy level equivalent to tetraploidy (4C = 2.86 ± 0.053 pg) for *C. canephora* (explant

TABLE 2 | Number of recovered MCSE, number (%) of plantlets regenerated, and number (%) of autotetraploid *C. canephora* and auto-alloctaploid *C. arabica* regenerated from the CSD procedure (**Figure 1**).

Coffea species	Colchicine treatment	Medium[1]	MCSE in 90 days[2]	MCSE in 120 days[2]	MCSE in 150 days[2]	Total of MCSE	Total of plantlets[3]	Total (%) of plantlets with chromosome set doubling[4]
C. canephora	2.5 mM/48 h	M3	–	25	100	125	45 (36.0%)	12 (26.7%)
		M4	–	29	70	99	37 (37.4%)	16 (43.2%)
	2.5 mM/72 h	M3	–	22	11	33	21 (63.6%)	8 (38.1%)
				76	181	257	103 (40.1%)	36 (34.9%)
C. arabica	0.5 mM/72 h	M3	89	70	78	237	102 (43.0%)	27 (26.5%)
		M4	235	40	109	384	187 (48.7%)	34 (18.2%)
			324	110	187	621	289 (46.5%)	61 (21.1%)

*1—**Table 1**.*

*2—Total number and % of MCSE regenerated in M3 or M4 and transferred to M5 (**Table 1**).*

3—Total number and % of plantlets showing leaves for DNA ploidy level assessment after two months in M5.

4—Number and % of plantlets that showed the DNA ploidy level equivalent to the double in relation to the respective explant donor plant.

donor plant with 2C = 1.43 pg, 2n = 2x = 22 chromosomes), being 12 plantlets (26.7%) from 2.5 mM/48 h/M3, 16 (43.2%) from 2.5 mM/48 h/M4, and 8 (38.1%) from 2.5 mM/72 h/M3. So, we found a total of 36 (34.9%) tetraploid plantlets for *C. canephora* (**Table 2**). The tetraploidy of these plantlets was confirmed by chromosome counting, showing 2n = 4x = 44 chromosomes. Therefore, these new germplasms represent *C. canephora* autotetraploids (**Figure 4**).

The CSD procedure (**Figure 1**) also provided octaploid plantlets of *C. arabica* (explant donor plant with 2C = 2.62 pg, 2n = 4x = 44 chromosomes). These plantlets, with 4C = 5.24 ± 0.028 pg and 2n = 8x = 88 chromosomes (**Figure 4**), were obtained from 0.5 mM/72 h/M3 (27 plantlets—26.5%) and M4 (34 plantlets—18.2%). Thus, we screened a total of 61 (21.1%) plantlets as octaploid for *C. arabica* (**Table 2**). Considering the evolutive origin of this species, which is a true allotetraploid from *C. canephora x C. eugenioides*, and the ploidy level of the plantlets (4C = 5.24 pg and 2n = 8x = 88), these germplasms can be considered as auto-alloctaploid.

No plantlets were recovered from the cellular masses that were not colchicine-treated or from other colchicine treatments. Due to these different *in vitro* responses (friable calli without MCSE and friable calli with MCSE), we evaluated genetic stability (using SSR markers) and global 5-mC% to verify if these differences are associated with DNA sequence (genetic stability) and/or 5-mC% level (epigenetic) changes. Henceforth, the recovered somatic embryos were used as a parameter to define the responsiveness of the cellular masses.

DNA Sequence Stability and 5-Methylcytosine Level

The ten selected SSR primers, which were developed for *C. arabica*, amplified DNA sequences for the two *Coffea* explant donors and for all sampled cellular masses. One to six allelic forms were evidenced from the ten SSR primers. The primer SSRCa006 was monomorphic for the explant donor plants and all cellular masses for two *Coffea* species. The primer SSRCa091 was monomorphic only for *C. arabica*. The primers SSRCa084 and SSRCa091 were monomorphic for the responsive and non-responsive cellular masses treated with colchicine in *C. canephora* and primer SSRCa085 in *C. arabica*. Specifically, for *C. arabica*, only one cellular mass showed a different allelic form for primers SSRCa085, SSRCa088 and SSRCa095.

The primer SSRCa002 was the most polymorphic, evidencing six allelic forms for *C. canephora* and five for *C. arabica*. This primer amplified three allelic forms in not colchicine-treated cellular mass (friable calli of the M1) of *C. canephora*, and five in cellular mass treated with 2.5 mM colchicine/48 h and maintained in M3 and M4. For *C. arabica*, the same primer provided two alleles for not colchicine-treated cellular mass (friable calli of the M1), and three for the other cellular masses. This shows that for this primer, as well as for other primers, some alleles were specific for each *Coffea* species and for different cellular masses. Despite of the observed polymorphisms, there was no significant difference among the DNA sequences of the *Coffea* explant donors and the cellular masses, colchicine-treated or not, and with or without MCSE.

From these SSR polymorphisms, it was possible to verify that some allele forms were more common for the cellular mass with or without MCSE. The association of the found alleles with cellular masses was identified from the exploratory and descriptive correspondence statistical analysis. SSR045 allele 2 (SSR045_2), SSR002_6, SSR087_45, SSR088_2 were correlated to cellular mass with MCSE, and SSR045_3, SSR002_4 and SSR095_2 with cellular mass without MCSE. Therefore, the somaclonal variation occurred at DNA sequence level, which is demonstrated by emergence and disappearance of alleles verified in some sampled cellular masses, especially for the primers SSRCa002 and SSRCa045. However, its rates are not significant and do not explain the different responses of ISE among the cellular masses.

In the comparison of the mean values of global 5-mC% between *C. canephora* and *C. arabica* explant donor plants with the cellular masses, we were able to identify three different groups using Dunnett's test. In the first group, the mean 5-mC% values of the donor explant plants were statistically identical with 18.33% of *C. canephora* and 18.00% of *C. arabica*. In the second group, global methylation levels were slightly

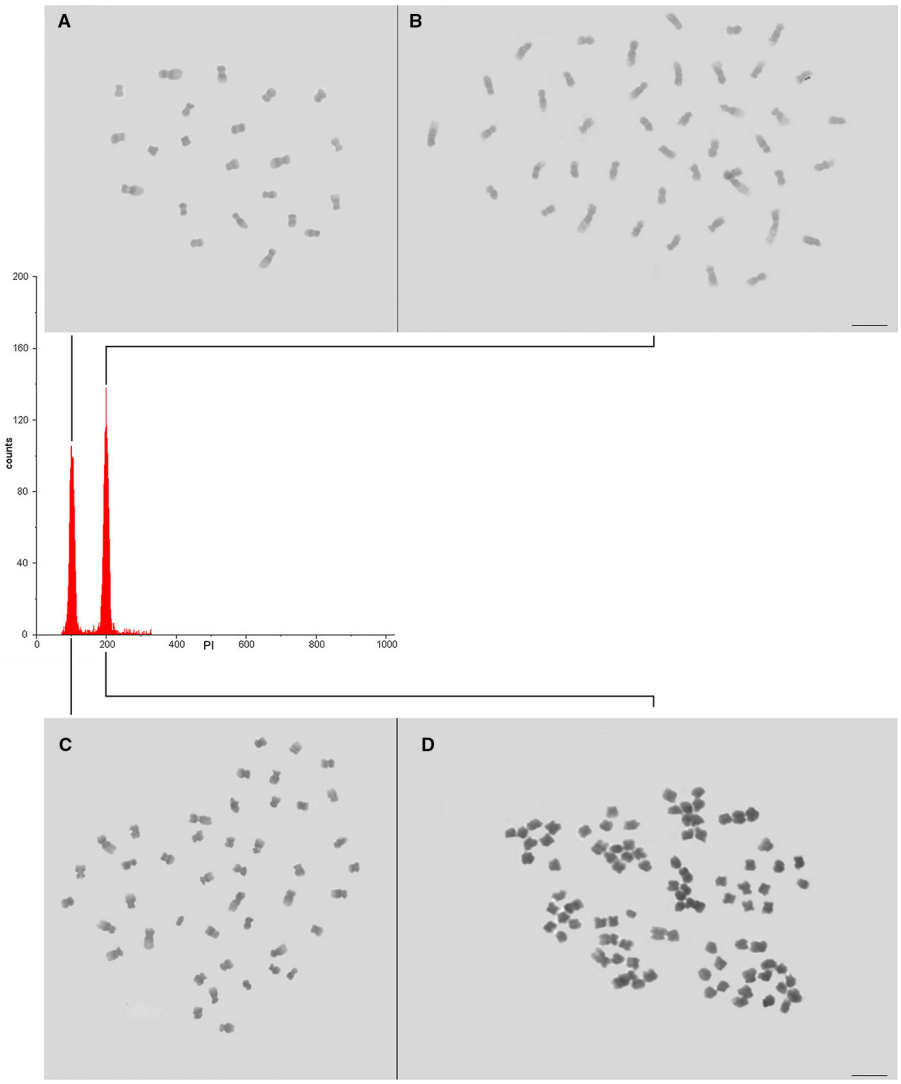

FIGURE 4 | Confirming the CSD of the *Coffea* CAS. **(A, B)** Nuclear DNA content and ploidy level of the diploid (**A**—explant donor plant, 2C = 1.43 pg, 2x = 22 chromosomes) and autotetraploid (**B**—4C = 2.86 pg, 4x = 44 chromosomes) *C. canephora*, and **(C, D)** tetraploid (**C**—explant donor plant, 2C = 2.62 pg, 4x = 44 chromosomes) and auto-alloctaploid (**D** – 4C = 5.24 pg, 8x = 88 chromosomes) *C. arabica*. As the DNA ploidy level determination and nuclear genome size measurement were separately accomplished for *C. canephora* and for *C. arabica*, the channel of the internal standard (explant donor plant) G_0/G_1 nuclei peak was adjusted to 100. So, the G_0/G_1 nuclei peak of the autotetraploid *C. canephora* and auto-alloctaploid *C. arabica* occurred in channel 200.

reduced to 13.41% for *C. canephora* and 14.45% for *C. arabica* in the friable calli developed in M1 medium. In the third group, the global 5-mC% was higher in the cellular masses than in the explant donor plants during somatic embryo recovering in M3 or M4, after colchicine treatments: 23.56% for *C. canephora*/2.5 mM/48 h/M3 or M4 without MCSE, 25.29% for *C. canephora*/2.5 mM/48 h/M3 or M4 with MCSE, 25.24% for *C. arabica*/1.5 mM/72 h/M3 or M4 without MCSE, 26.23% for *C. arabica*/0.5 mM/72 h/M3 with MCSE, and 29.13% for *C. arabica*/0.5 mM/72 h/M4 with MCSE. Among them, the *C. arabica*/0.5 mM/72 h/M4 showed the highest mean value of global 5-mC% (**Table S1**).

DISCUSSION

In this study, a novel CSD procedure was established for two *Coffea* crop species from CAS of the diploid *C. canephora* and the true allotetraploid *C. arabica*, resulting in solid autotetraploid *C. canephora* (2n = 4x) and auto-alloctaploid *C. arabica* (2n = 8x) plantlets. These new germplasms were approximately regenerated within one year: 90 days for friable calli production, 67 days for CAS establishment, 2–4 days for colchicine treatment, 150 days for MCSE regeneration, and 60 days for plantlet recovery. This relative short time represents an

advance in *Coffea* breeding programs, which depend on strategies that demand several crossings, large progeny and a long time to provide new germplasms. For instance, the *in vitro* hybrid selection time (~8 years) is shorter than the hybrid selection time (~25 years) in the traditional breeding program (Etienne et al., 2018). In addition, the new germplasms were formed from a simple and small leave fragment of the selected plants. *Coffea* leave cells showed a higher degree of plasticity, allowing them to reprogram and to form the somatic embryo and the plantlet from the following ISE *in vitro* morphogenic pathway: differentiated → dedifferentiated → redifferentiated.

To induce the polyploidization in liquid system, we explored the high proliferation of the *Coffea* CAS (van Boxtel and Berthouly, 1996; Clarindo et al., 2012). CAS are maintained in constant orbital agitation, allowing that more cells have direct contact with the compounds of the medium, mainly the growth regulators that promote cell proliferation (2,4-D in our study) and the antitubulin (here, the colchicine) during the CSD procedure, than in semisolid system (Loyola-Vargas and Vázquez-Flota, 2006; Dutt et al., 2010). In semisolid system, there is, for example, a reduction in the rate of nutrient diffusion due to the gelatinous consistency of the medium (Loyola-Vargas and Vázquez-Flota, 2006).

The key issue of the procedure (**Figure 1**) was the antitubulin treatment applied to proliferating CAS in liquid *in vitro* system. Again, the chemical and physical conditions of the tissue culture environment were effective, giving rise to friable calli (first step—dedifferentiation) and CAS with proliferative cells (second step), MCSE (third step) and plantlets with different ploidy level (fourth step). This ISE pathway has been extensively exploited for *Coffea* plantlet regeneration since van Boxtel and Berthouly (1996). In addition, it was reproducible for different species and genotypes of the same species (Samson et al., 2006; Almeida et al., 2008; Ibrahim et al., 2015), such as the diploid *C. canephora* and *C. eugenioides*, allotriploid "Híbrido de Timor" "CIFC 4106" and true allotetraploid *C. arabica* (Sanglard et al., 2019). Therefore, the obtained new *Coffea* germplasms are a statement that van Boxtel and Berthouly's (1996) *in vitro* conditions are the base to establish tissue culture protocols for this genus.

Coffea MCSE recovery occurred after removing the exogenous auxin 2,4-D and supplementing the tissue culture medium with activated charcoal. Thus, the elimination of the main chemical component responsible for keeping the cells in totipotent condition was required, as recommended by Rose et al. (2010) and Nic-Can and Loyola-Vargas (2016). Activated charcoal is added to tissue culture because of its adsorption capacity of exogenous 2,4-D residues (Pan and van Staden, 1998) and by adsorbing medium-inhibiting substances or toxic products released by cells, helping to promote the somatic embryo regeneration, conversion and maturation to mature cotyledonary. The osmotic control also is fundamental for somatic embryo recovery. For friable calli induction, CAS establishment and proliferation, a relatively high osmotic potential was necessary. On the order hand, the decrease of osmotic potential is fundamental for somatic embryo regeneration, mimetizing the seed environment during the zygotic embryo development (Dutt et al., 2010).

As summarized in **Figure 5**, the principle to promote the CSD was to polyploidize as many cells as possible of the *Coffea* CAS using the antitubulin colchicine, which may be replaced by another compound with the same and specific effect. The very specific (Planchais et al., 2000) and cytotoxic (Dutt et al., 2010; Acanda et al., 2015) antitubulin compounds hinder mitotic fuse formation, which is fundamental for chromatid segregation during anaphase and for cytokinesis. Considering this as well as the ploidy level, the number of chromosome sets in both *Coffea* species was duplicated due to the action of the anaphase promoting complex (APC)-activated separase pathway and prevention of cytokinesis. Separase (Esp1), a cysteine protease, cleaves the SCC1 subunit of the cohesin (Orr-Weaver, 1999; Tanaka et al., 1999), a protein complex constituted by conserved polypeptides SMC1, SMC3, SCC1 and SCC3 (Cai et al., 2003). This protein keeps the sister chromatids together (Orr-Weaver, 1999; Tanaka et al., 1999) from S-phase (interphase) to initial anaphase. As the polyploidization occurred in *Coffea* CAS, we concluded that the cohesin was cleaved, doubling the chromosome set number as in a normal anaphase. However, these chromosomes remained in the cell as a result of the absence of the mitotic fuse and, consequently, cytokinesis non-occurrence. The nuclear membrane reorganization around these chromosomes happens in telophase, and daughter cells were formed with one nucleus in polyploid condition in comparison to the *Coffea* donor plant.

The colchicine application on the seventh day after the subculture also contributed to the generation of 36 (34.9%) autotetraploid *C. canephora* and 61 (21.1%) auto-alloctaploid *C. arabica*. For *Citrus*, Dutt et al. (2010) also treated the suspension cells with colchicine after seven days in the third subculture. Our results, as well as Dutt et al. (2010) and Zhang et al. (2007), highlight that the moment for antitubulin treatment should be chosen according to the number of cells in S-phase (interphase). This can be checked for each plant species using flow cytometry, for *Coffea*, this cell cycle phase occurs after 7 to 9 days (van Boxtel and Berthouly, 1996). In addition, this previous data increases experimental control and, consequently, the number of polyploids because it allows to attest that the cells are proliferative, to choose a day before mitosis and cytokinesis, and to conduct colchicine treatment in a shorter time.

Another impact of the CSD procedure was the recovery of pure polyploids of the both *Coffea* species. This result corroborates with the ISE possibility of regeneration of the somatic embryo and, consequently, a plantlet from only one cell of the friable calli (semisolid system, (Wu and Mooney, 2002; Petersen et al., 2003; Zhang et al., 2007; Sanglard et al., 2017) or CAS (liquid system, Dutt et al., 2010; Acanda et al., 2015). Moreover, CSD using proembryogenic cells of friable calli or CAS reduced or nulled the regeneration of mixoploid plantlets (Dutt et al., 2010; Acanda et al., 2015; Sanglard et al., 2017). Besides, CAS are considered meristematic cells, becoming a successful source for CSD and

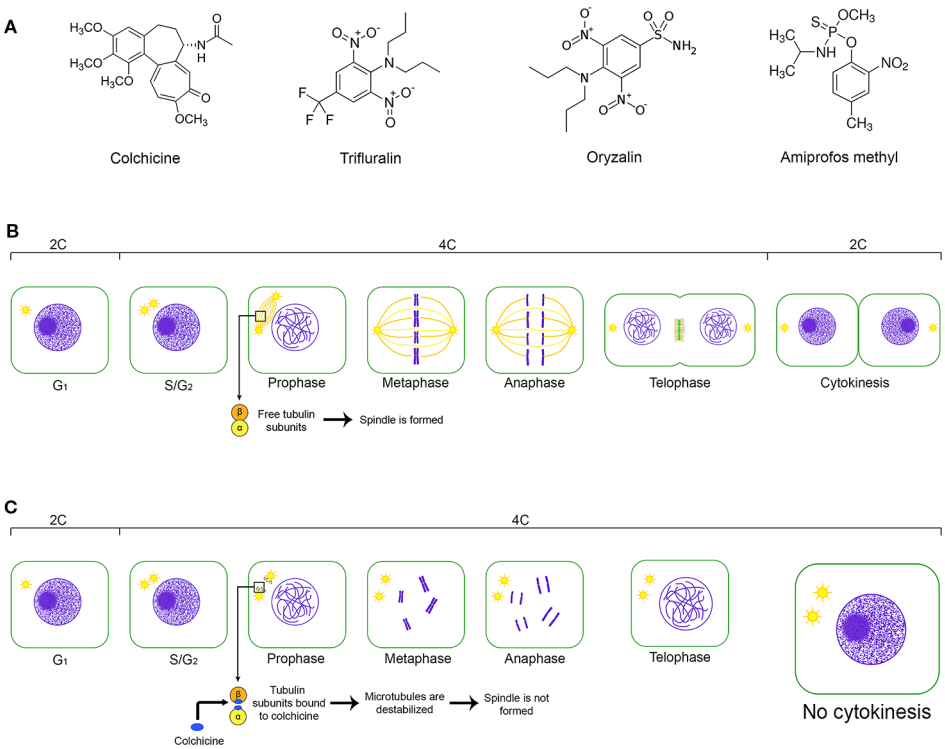

FIGURE 5 | The key issues of the CSD from a cell of the friable calli. **(A)** Chemical structure of the main antitubulin compounds used for CSD: colchicine—(S)-*N*-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo[a]heptalen-7-yl)acetamide; oryzalin—3,5-dinitro-N^4,N^4-dipropylsulfanilamide; trifluralin α,α,α-trifluoro-2,6-dinitro-*N*,*N*,-dipropyl-*p*-toluidine; and amiprofos methyl—O-methyl O-(2-nitro-*p*-tolyl) N-isopropylphosphoramidothionate. **(B)** Representation of the cell cycle without the interference of an antitubulinic agent, its cellular events and the changes of the relative DNA content (2C, 4C) considering a diploid cell in G_1. **(C)** The effect of the antitubulin agent on the cell cycle and, consequently, on the relative DNA content. Antitubulin agents prevent the mitotic fusion formation, avoiding the chromatide segregation and cytokynesis occurence. So, the chromosome set is duplicated in the same cell nuclei, which shows relative DNA content equivalent to 4C in G_1.

providing solid polyploids. Therefore, the CAS can replace the shoot meristems in buds generally used for CSD (Dhooghe et al., 2011). CSD from the shoot apical meristem demands the polyploidization of all, or at least most, cells that constituted the periphery, central and medullar zones (L1, L2, and L3 layers). Therefore, mixoploids generated from this explant are result of the failure of the CSD.

Although the ISE is an advantageous system for CSD (Wu and Mooney, 2002; Petersen et al., 2003; Zhang et al., 2007; Sanglard et al., 2017), we also recorded cellular masses without somatic embryos and another with continuous somatic embryo regeneration, conversion and maturation. The first hypothesis to explain these ISE response divergences is the cytotoxic effect of the colchicine, but the non-colchicine treated cellular masses did not show somatic embryos. Besides, the responsive cellular masses of *C. canephora* were treated with 2.5 mM colchicine, exactly the highest concentration of this compound. Acanda et al. (2015) related that the colchicine exposure reduced the embryogenic potential in comparison to the control. The same has been reported by several authors independently for biological material used for *in vitro* CSD.

Another possible explanation is the occurrence of somaclonal variation (SV), which is a common phenomenon in cell culture caused by genetic and epigenetic changes in the nuclear genome, as well as genetic changes in organelle genomes (Kaeppler et al., 2000; Wang and Wang, 2012). Therefore, the term SV describes the variability produced by *in vitro* propagation due to physical and chemical conditions, time, and employed propagation system (liquid or semisolid). To test the hypothesis that SV occurred, we used SSR DNA markers to monitor cellular masses. We observed clonal fidelity in relation to the *Coffea* explant donors and used molecular markers.

Global 5-mC% divergences were identified between the cellular masses in dependence of MCSE occurrence. *In vitro* plant cells were categorized in a genetic and evolutive context (Wang and Wang, 2012) according to the level and effect of the SV: true cells, neutral cells, deleterious cells and beneficial cells. The term true cell is designated for the *in vitro* cell without SV, but with the possibility of epigenetic modifications essential for the morphogenic process. A neutral cell is defined as the *in vitro* cell with SV, but having the same phenotype as the ancestral cell without changes in fitness. Neutral cells also include cells with deleterious SV but suppressed by other genome changes or by suppressor genes. Deleterious cells are defined as the *in vitro* cells containing SV, which leads to a decrease in the fitness relative to

ancestral cell. Eventually, the beneficial cell is a cell exhibiting SV that increases the fitness value compared to ancestral cells. Based on these concepts by Wang and Wangs' (2012) and in our results, we concluded that the cellular masses of both *Coffea* species showed true and neutral cells. However, other aspects of the genome (as other molecular markers), epigenome (as the histone chemical change and 5-mC% of the genes), transcriptome and metabolome should be evaluated in future studies in order to understand the factors that have hindered the somatic embryo generation.

CONCLUSION

Our results suggest that the CSD using ISE and the antitubulin treatment of the CAS is a successful procedure to produce solid polyploid plants of *Coffea* and potentially for other species as well. We generated new *Coffea* germplasm, autotetraploid *C. canephora* and auto-alloctaploid *C. arabica*, that constituted a diversification of the *in vitro* germplasm of this genus. These individuals can be used as explant donor for other tissue culture procedures or be acclimatized for morphologic, physiologic and reproductive evaluations. Besides, the epigenetic modulation of the chromatin was associated to somatic embryo regeneration and, consequently, to plantlet recovery. Therefore, the tissue culture conditions should promote this typical change related to cellular redifferentiation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

The authors LV, MM and WC conducted the tissue culture experiments and *in vitro* chromosome set doubling. WC carried out the cytogenetic and flow cytometry analyses. LV, PA-S and TS executed the SSR molecular analyses. LV, PA-S, and AP conducted epigenetic analyzes. LV, GC and AF did the statistical analysis. All authors equally contributed for manuscript editing and revision and approved the final manuscript for submission.

ACKNOWLEDGMENTS

We would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília—DF, Brazil, grant: 443801/2014-2, 306870/2017-7, 312048/2018-1) and Fundação de Amparo à Pesquisa do Espírito Santo (FAPES, Vitória—ES, Brazil, grants: 65942604/2014 and 82/2017). This study was also financed in part by the Coordernação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES)—Finance Code 001.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00154/full#supplementary-material

TABLE S1 | Experimental design for DNA sequence variability and global DNA methylation level of individual cellular mass, and statistical comparison among the mean global 5-mC%.

FIGURE S1 | Number of regenerated MCSE for *C. canephora* and *C. arabica* after 90, 120 and 150 days.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 17 March 2020
doi: 10.3389/fpls.2020.00295

Effects of Colchicine on *Populus canescens* Ectexine Structure and 2n Pollen Production

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *21 October 2019*
Accepted: *27 February 2020*
Published: *17 March 2020*

Citation:
Zhou Q, Wu J, Sang Y, Zhao Z, Zhang P and Liu M (2020) Effects of Colchicine on Populus canescens Ectexine Structure and 2n Pollen Production. Front. Plant Sci. 11:295. doi: 10.3389/fpls.2020.00295

Triploid breeding is a central way to improve growth traits, timber quality, and stress resistance in *Populus*. In the present study, the morphology and viability of colchicine-induced 2n pollen, triploid production by crossing induced 2n pollen, and identification of genetic constitution of colchicine-induced 2n pollen were conducted in *Populus canescens* based on optimizing technology for inducing chromosome doubling in pollen. We found that the meiotic stage, injection time, and the interaction between the meiotic stage and injection time had highly significant effects on the 2n pollen production rate. The most effective treatment for inducing 2n pollen was to give 11 injections of 0.5% colchicine solution when pollen mother cells (PMCs) were at the pachytene stage. The highest 2n pollen production rate was 30.27 ± 8.69%. Colchicine occasionally affected ectexine deposition, and some narrow furrows were detected in the ectexine structure. However, no significant difference was observed in the pollen germination rate between natural 2n pollen and colchicine-induced 2n pollen. Moreover, 5 triploids derived from FDR-type 2n pollen were generated by crossing induced 2n pollen, suggesting that colchicine does not eliminate the function of colchicine-induced 2n pollen. However, slower growth of 2n pollen tubes was responsible for a lower triploid production rate.

Keywords: ***Populus canescens*, colchicine, chromosome doubling, 2n pollen viability, triploid**

INTRODUCTION

Populus canescens (section *Populus,* family Salicaceae, genus *Populus*) grows in the Irtysh River Basin, Xinjiang Uygur Autonomous Region, in northwest China. Due to its high ornamental value, resistance to disease, insects, and drought, it is widely used for ecological protection in northwest China (Xu, 2008). However, the annual growth rate of *P. canescens* is lower owing to its shorter growing season. A long-term breeding program was developed by Beijing Forestry University to improve *P. canescens* growth. In 1989, a cross-breed between *Populus tomentosa* × *Populus bolleana* and *P. canescens* was first reported by Li and Zhu (1989), and six superior hybrid clones were developed. In a subsequent study, Duan and Yang (1998) evaluated the cold and drought resistance of these superior hybrids by the anatomical structure of the leaves, water loss rate through the

leaves, and electroconductivity of the shoots. Tian J. et al., 2018 systematically studied the abnormal behavior of chromosomes during meiosis of pollen mother cells (PMCs), pollen morphology and pollen abortion in some male hybrids between *P. tomentosa* × *P. bolleana* and *P. canescens*.

Triploid breeding is one of most powerful approaches to improve the genus *Populus*. The first discovered triploid (2n = 3x = 57) individual *Populus* was a giant form of *Populus tremula* from a natural population in Sweden, which was characterized by extremely large leaves and rapid growth (Nilsson-Ehle, 1936; Müntzing, 1936). Zhu et al. (1998) reported that genetic gains in the stem volume of 9-year-old natural *P. tomentosa* triploid clones was higher (56.4%) than that of the diploid counterparts. In another study, Zhu et al. (1995) documented that the stem volume of the allotriploid clone B301 was 3.5 times higher that of *P. tomentosa* diploids. Subsequently, Zhang et al. (2014a) reported that the fiber length of triploid white poplar hybrid clones was 20.6% larger than that of diploids. The holocellulose of triploid white poplar hybrid clones was higher 1.3% than that of its diploid counterparts. However, the lignin content of triploid white poplar hybrid clones was shown to be 21.7% lower than that of diploid individuals (Zhang et al., 2014b). Therefore, triploid breeding has not only improved the growth rate but also the wood quality of *Populus* (Zhang et al., 2012, 2013, 2015a,b).

Few studies have focused on *P. canescens* triploid breeding. Only Tian M. et al. (2018) reported 2n pollen production induced by high-temperature exposure and 42 triploids were obtained. Subsequently, the 2n pollen *P. canescens* high-temperature-induced was demonstrated to have originated from second division restitution (SDR) during the meiosis of PMCs. The lower heterozygosity transmitted from the male parent provides SDR-type 2n gametes a lower breeding value than first division restitution (FDR)-type 2n gametes (Veilleux, 1985; Dong et al., 2015). Thus, crossing FDR-type 2n gametes with haploid gametes to create *P. canescens* triploids is a promising way to increase growth rate.

The objective of this study was to produce FDR-type 2n pollen by colchicine induction and generate a triploid *P. canescens* germplasm. To optimize the pollen chromosome doubling technology to produce 2n pollen using colchicine, pollen morphology, 2n pollen germination *in vitro,* and genetic constitution identification were conducted, followed by triploid production using colchicine-induced 2n pollen. Our findings will lay the foundation for the triploid breeding of *P. canescens*.

MATERIALS AND METHODS

Plant Materials

Populus canescens and *Populus hopeiensis* are native tree species in northwestern China. *Populus alba* × *Populus glandulosa*, a hybrid with good fertility under normal conditions, was introduced from Korea in 1984. Male floral branches of *P. canescens* "YHY1" were collected from a natural population in Aletai, Xinjiang Uygur Autonomous Region. Female branches of *P. hopeiensis* "HBY1" were collected from the Baotou, Inner Mongolia Autonomous Region, and female branches of *P. alba* × *P. glandulosa* "YXY1" were collected from the Guan Country Nursery, Shandong Province. All sampled floral branches were trimmed and cultured in tap water at the Beijing Forestry University greenhouse (10–20°C). No additional nutrition was added.

2n Pollen Induction With 0.5% Colchicine Solution

Every 2 h, 2–3 flower buds were randomly collected and fixed in Carnoy's solution (ethanol: acetic acid, 3:1). The anthers were dissected from the fixed buds using forceps and were crushed in a droplet of aceto-carmine solution (2%) onto a microscope slide to observe meiosis. When the dominant meiotic stage of the PMCs were the leptotene, pachytene, diplotene, diakinesis, and metaphase I stages. The male buds were injected with 0.5% colchicine solution 3, 5, 7, 9, and 11 times, respectively. The interval between neighboring injections was 2 h. Each bud was injected with 10 μl colchicine solution, and 15–20 flower buds with each treatment were injected. Untreated male buds were taken as a control group. The treated and the control floral branches were hydroponically cultured in a greenhouse for 4 weeks until the catkins matured, then pollen samples were collected and stored in a centrifuge tube with allochronic silica gel at −20°C.

The diameter of the 2n pollen grain is larger than that of haploid pollen grain because of its chromosome doubling in higher plants (De Storme et al., 2012; Zhang and Kang, 2013; Yang et al., 2015). Therefore, we can determine 2n pollen from haploid pollen by the diameter of pollen grains. Thus, the frequency of 2n pollen was estimated according to the method described by Zhang and Kang (2013). The diameters of 200–300 pollen grains per sample were measured.

Morphology of Colchicine-Induced 2n Pollen Grains

For the scanning electron microscopic analysis, pollen samples were sputter-coated with gold for 1 min, using a Hitachi E-010 ion sputter coater (Tokyo, Japan). The pollen grains were observed under a Hitachi S-3400N microscope at an accelerating voltage of 5 kV. The details of ectexine structure and deposition were observed and photographed for each pollen sample.

Pollen Germination *in vitro*

The *in vitro* pollen germination medium was prepared according to the method described by Zhao et al. (2017). The medium was composed of 0.7% agar, 50 mg l^{-1} calcium chloride, and 120 mg l^{-1} boric acid. The pH value of the medium was 6.0. Some fresh colchicine-induced 2n pollen grains were sampled and spread on slides containing the medium. Then, the slides were placed in 120 mm Petri dishes with wet filter paper, and the pollen was germinated in a climate chamber at 26°C. After a 4 h culture, the pollen grains were washed in a liquid germination medium, collected in a 1.5 ml centrifuge tube, and fixed in Carnoy's solution for 10 min after being centrifuged for 5 min at 1,000 rpm. The germination rate and the length of each sample

were calculated using an eyepiece micrometer. A total of 20–30 pollen tubes was assessed in each sample.

Triploid Production by Crossing Colchicine-Induced 2n Pollen

The female flower buds were pollinated with fresh colchicine-induced *P. canescens* pollen when the stigmas of the female flower buds were receptive. After pollination, the female flower branches were hydroponically cultured. Seeds were collected after 4 weeks and sown into 54 × 28 × 10 cm nutrition plates at a depth of 5 cm to promote growth, then placed in a greenhouse.

Ploidy Analysis via Flow Cytometry and Somatic Chromosome Counting

A flow cytometry analysis was conducted by flow cytometer (BD FACSCalibur; Becton Dickinson, Brea, CA, United States). Approximately 0.5 g of chopped young leaves were put into a 55 mm Petri dish with 1 ml of nuclear extraction solution (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate, 1% (v/v) Triton X-100, pH = 7.0), and the nuclei suspension was filtered through a 40 μm nylon mesh. The nuclei were stained with 50 μl of 4′,6-diamidino-2-phenylindole (10 mg/ml) for 5 min. At least 2,000 nuclei were analyzed, and three samples were collected per plant. Leaves sampled from a known diploid plant of *P. tomentosa* (2n = 2x = 38) were taken as a control. The plant DNA *C*-value of *Populus* is 0.46 pg (Kew *C* – value database). The standard peak was set to appear at about channel 50 relative fluorescent intensity. Therefore, when the peaks appeared at channel 75 relative fluorescent intensity, they were considered putative triploids.

We confirmed the ploidy level of each plantlet by somatic chromosome counting. Stem tips were collected and pretreated in a saturated solution of paradichlorobenzene for 2–3 h. Then, the pretreated samples were washed once and fixed in Carnoy's solution at 4°C for at least 24 h. Subsequently, the fixed stem tips were hydrolyzed in 38% HCl for 25 min at room temperature and then rinsed three times for 10 min each with distilled water. The hydrolyzed samples were stained with Carbol fuchsin, crushed with a coverslip, and observed at 100 × oil lens magnification in an Olympus BX51 microscope (Olympus Inc., Tokyo, Japan).

Identification of Genetic Constitutions of Colchicine-Induced 2n Pollen

According to the manufacturer's protocol, DNA was extracted from a 300 mg stored leaf sample of each plant, including the parental poplar lines and their triploid hybrids using the DNeasy Plant Mini Kit (Tiangen Biotech)[1]. The fluorescently labeled TP-M13-SSR method (Schuelke, 2000) was employed in the present study. A forward primer at the 5′ end was attached with a universal primer M13 tail (5′-TGTAAAACGACGGCCAGT-3′) and labeled with four fluorescent substances (6-carboxy-X-rhodamine, 6-carboxy-fluorescein, tetramethyl-6-carboxyrhodamine, or 5-hexachloro-fluorescein). All primers were synthesized by Sangon Inc.[2] The PCR amplification protocol was as follows: 5 min at 94°C; 10 cycles of 30 s at 94°C, 30 s at the optimal annealing temperature for each SSR marker, and 30 s at 72°C; 25 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C; and a final extension of 8 min at 72°C. Then the PCR products were used for capillary electrophoresis fluorescence-based SSR analyses using the ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, United States), and fragment sizes and peak areas analyzed using GeneMarker software V1.75 (Hulce et al., 2011).

We screened SSR primers from the SSR database[3] (beginning with "GCPM," "ORPM", and "PMGC") released by the International *Populus* Genome Consortium (IPGC). BLAST analysis in *Populus trichocarpa* v3.0 (Phytozome v12.0)[4] was done to determinate the location of the SSR loci.

Statistical Analysis

We analyzed the frequency of colchicine-induced 2n pollen using a univariate general linear model (GLM) to reveal the differences among the meiotic stages, injection times, and the interaction between the meiotic stage and injection time. The data were transformed before analysis of variance and multiple comparisons to account for the heterogeneity of variance. All statistical analyses were performed with SPSS software (version 18.0; SPSS Inc., Chicago, IL, United States).

RESULTS

2n Pollen Production Using 0.5% Colchicine Solution

After the sampled male buds were injected with 0.5% colchicine solution at leptotene (**Figure 1A**), pachytene (**Figure 1B**), diplotene (**Figure 1C**), diakinesis (**Figure 1D**), or metaphase I (**Figure 1E**), the treated male *P. canescens* flower buds were hydroponically cultured in a greenhouse until the catkins matured. As the pollen was released, colchicine-induced 2n pollen was collected from the treated male buds (**Figure 1F**). A few naturally occurring 2n pollen grains were found in the control group (**Figure 1G**). The frequency of colchicine-induced 2n pollen ranged from 2.75 to 30.27% (**Table 1**). We found that the dominant meiotic stage ($F = 4.801$, $P = 0.009$), injection time ($F = 12.449$, $P = 0.000$), and the interaction between the dominant meiotic stage and injection time ($F = 3.006$, $P = 0.002$) had highly significant effects on the frequency of colchicine-induced 2n pollen according to the univariate GLM analysis. Further LSD multiple-comparison tests indicated that the differences in colchicine-induced 2n pollen frequency were significantly higher at pachytene than those at the leptotene, diplotene, or metaphase I stages ($p < 0.05$). The differences in the frequency of colchicine-induced 2n pollen for samples given 11 injections was also significantly higher than that of those given 3, 5, 7, and 9 injections. Hence, the optimal protocol for inducing 2n pollen via

[1]http://www.tiangen.com/en/?app.html

[2]http://www.life-biotech.com/sangonEn.html

[3]http://popgenie.org

[4]https://phytozome.jgi.doe.gov/pz/portal.html

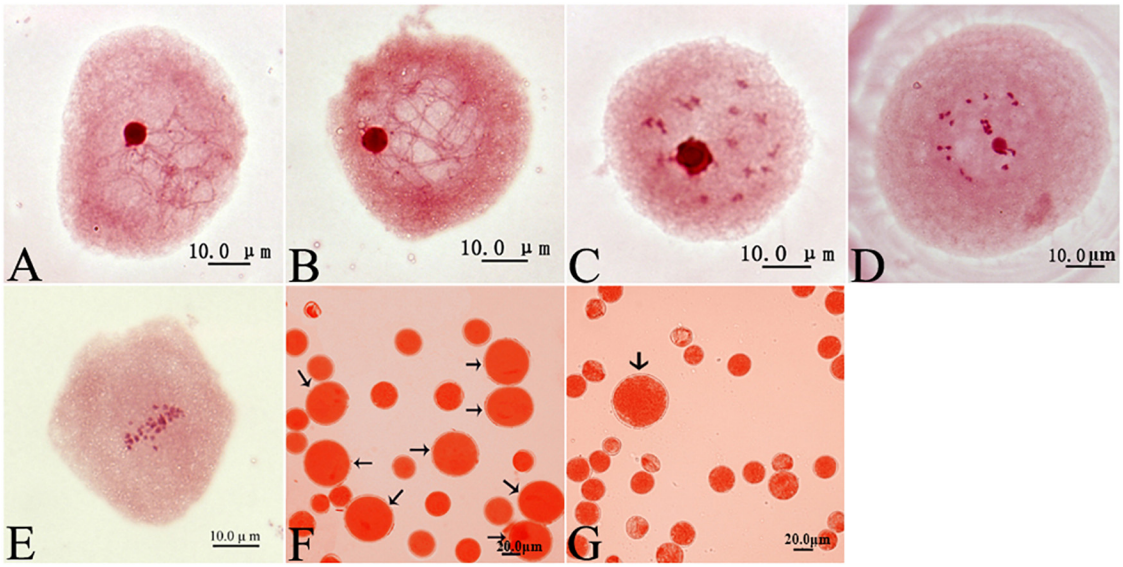

FIGURE 1 | Cytological observations of PMCs, colchicine-induced 2n pollen (arrow) and naturally occurring 2n pollen in *Populus canescens*. **(A)** Leptotene. **(B)** Pachytene. **(C)** Diplotene. **(D)** Diakinesis. **(E)** Metaphase I. **(F)** Colchicine-induced 2n pollen derived from the treatment given 11 injections at pachytene in *P. canescens* (arrow). **(G)** Natural 2n pollen derived from the control group in *P. canescens* (arrow). Scale bar = 10.0 μm **(A–E)** and 20.0 μm **(F,G)**.

colchicine was to give 11 injections of 0.5% colchicine solution at the pachytene stage of the PMCs.

The Effects of Colchicine Treatment on Pollen Morphology

The morphology of the pollen was examined via scanning electron microscopy (**Figure 2**). The pollen grains were non-spherical, with few corrugations and granulated decorations on the surface. 2n pollen was observed in the control group (**Figure 2A**), the 5 injections group (**Figure 2B**), and the 11 injections group at pachytene (**Figure 2C**). The haploid pollen was homogenous, with granulated decorations on the surface, and no aperture was observed in the control or treatment groups (**Figures 2D–I**). No significant differences were observed in the ectexine structure of haploid pollen between the control and treatment groups because haploid pollen grains were not the outcome of colchicine treatment. However, compared with natural 2n pollen in the control group (**Figure 2J**), the morphology of the colchicine-induced 2n pollen (**Figures 2K,L**) showed altered ectexine deposition and some narrow furrows in the ectexine structure.

Triploid Production by Crossing Colchicine-Induced 2n Pollen

A total of 6,741 mature seeds were collected from 2 cross-breeding combinations of colchicine-induced 2n pollen and the control group. All seeds were sown and became 4955 young seedlings. Among them, 76 seedlings were derived from the cross between *P. hopeiensis* and *P. canescens*, and 4,879 seedlings were derived from the cross between *P. alba* × *P. glandulosa* and *P. canescens*. The ploidy level of all offspring was examined by flow cytometry, and five putative triploids were detected (**Figure 3A**). Subsequently, the five putative triploids were confirmed by somatic chromosome counts. The number of chromosomes in the diploid was 38 (2n = 2x = 38, **Figure 3B**), and the number of chromosomes in the triploid was 57 (2n = 3x = 57, **Figure 3C**), indicating that the five putative triploids were real triploids. Among the five triploids, two came from *P. hopeiensis* × *P. canescens*, and three were derived from (*P. alba* × *P. glandulosa*) × *P. canescens*.

The number of hybrid offspring and the triploid production rates in the different crosses are shown in **Table 2**. All triploids were derived from crossing the colchicine-induced 2n pollen with haploid eggs of *P. hopeiensis* or *P. alba* × *P. glandulosa*. No triploid was detected in most of the cross combinations. The highest triploid production rate (16.67%) was observed in the cross between *P. hopeiensis* and *P. canescens*. The average production rate of triploids was 2.35%, which was significantly lower than the frequency of colchicine-induced 2n pollen. These results suggest that the competitive ability of colchicine-induced 2n pollen was significantly weaker than that of haploid pollen.

Pollen Germination *in vitro*

The induced pollen of each treatment contained 2n and haploid pollen because the meiosis of PMCs in *P. canescens* is an asynchronous process (Tian M. et al., 2018). We conducted pollen germination experiments *in vitro* to evaluate the effect of colchicine on induced 2n pollen viability. The fresh colchicine-induced pollen grains derived from the 11-injections treatment and the fresh pollen from the control group were used for the germination test on medium containing 50 mg/L calcium chloride and 120 mg/L boric acid. After a 4 h culture, some of the colchicine-induced pollen grains had germinated (**Figure 4A**).

TABLE 1 | Induction of 2n pollen via a 0.5% colchicine solution in *P. canescens*.

Dominant meiotic stage of PMCs	No. of colchicine injections times	Frequency of colchicine-induced 2n pollen (%)
Leptotene	3	4.79 ± 1.46
	5	6.42 ± 2.13
	7	9.40 ± 1.32
	9	8.19 ± 0.99
	11	10.44 ± 4.41
Pachytene	3	5.46 ± 0.90
	5	6.22 ± 0.89
	7	9.04 ± 2.62
	9	16.70 ± 3.95
	11	30.27 ± 8.69
Diplotene	3	4.18 ± 1.52
	5	5.79 ± 0.60
	7	8.76 ± 0.45
	9	9.48 ± 1.31
	11	9.68 ± 1.76
Diakinesis	3	6.99 ± 1.31
	5	7.81 ± 0.74
	7	8.17 ± 0.56
	9	10.53 ± 0.82
	11	20.36 ± 1.49
Metaphase I	3	2.75 ± 0.43
	5	3.91 ± 1.53
	7	4.34 ± 1.08
	9	11.03 ± 1.23
	11	15.11 ± 4.99
Control		2.08 ± 0.40

The average pollen germination rate was 27.1%. Some germinated colchicine-induced 2n pollen grains (**Figure 4B**) and a few germinated natural 2n pollen grains (**Figure 4C**) were found. The average germination rates of natural 2n pollen and induced 2n pollen were 23.9 and 22.6%, respectively. However, no significant differences were observed in the germination rates between induced 2n pollen and natural 2n pollen (**Figure 5**). The length of the pollen tube in the induced 2n pollen was 61.9 ± 9.0 μm, which was significantly shorter than that of haploid pollen (279.3 ± 10.9 μm) (**Figure 6**), suggesting that the slow-growing pollen tube of the colchicine-induced 2n pollen is responsible for the low triploid production rate in *P. canescens*.

Identification the Genetic Composition of Colchicine-Induced 2n Pollen

To identify the genetic composition of colchicine-induced 2n pollen, two pairs of polymorphic SSR primers, GCPM_1158 and GCPM_124, were screened. **Table 3** presents the detailed information of the two pairs of SSR primers. The GCPM_124 and the GCPM_1158 loci are located on chromosomes 1 (Chr 01) and 2 (Chr 02), respectively. The capillary electrophoresis results showed that the allelic configuration was, at the GCPM_1158 locus, homozygous in the female parent "HBY1" (225.0 bp, for "aa"), and heterozygous in the male parent "YHY1" (240.8 bp and 253.0 bp, for "b" and "c") (**Figure 7A**). The resulting genetic constitution of T1 and T2 triploid hybrids was proven to be "abc." Therefore, the triploid progeny T1 and T2 could be from FDR-type 2n pollen. Similar to the GCPM_1158 locus, the allelic configuration was, at the GCPM_124 locus, homozygous in female parent "YXY1" (212.3 bp for "aa"), and heterozygous in the male parent "YHY1" (200.6 bp and 206.3 bp, for "b" and "c") (**Figure 7B**). The genetic constitution of triploid hybrids T3, T4, and T5, was found to be "abc." Consequently, triploid progeny T3, T4, and T5 could be inferred to derive from FDR-type 2n pollen.

DISCUSSION

Colchicine is considered an effective chemical mutagen to induce diploid gametes in plants. It inhibits microtubule polymerization by binding to tubulin, and thus it inhibits the mitotic spindle formation resulting in the development of a polyploid cell. Colchicine has been successfully used to induce 2n female gametes by treating embryo sacs, zygotes, and somatic cells, furthermore, a large number of triploids and tetraploids have been generated in *Populus* (Li et al., 2001; Kang et al., 2004; Wang et al., 2010a,b; Cai and Kang, 2011; Xu et al., 2016), *Robinia pseudoacacia* (Ewald et al., 2009), *Eucommia ulmoides* (Zhang et al., 2008), and *Eucalyptus urophylla* (Yang et al., 2018). Colchicine has also been applied to induce 2n pollen production in *Populus* (Li et al., 2014; Zhao et al., 2017, 2019), *Eucommia ulmoides* (Gao et al., 2004), and *Eucalyptus* (Yang et al., 2016). Several triploids have been created by crossing colchicine-induced 2n pollen in *Populus* (Zhao et al., 2017). Therefore, triploid breeding is a promising way to improve *Populus*.

Applying colchicine to PMCs at a suitable stage is vital for inducing 2n pollen (Kang, 2016). The most suitable stage is species dependent. Kang et al. (1999) reported that the most suitable stage for inducing 2n pollen via colchicine varies from leptotene to diplotene in *P. tomentosa* × *P. bolleana*. The leptotene to pachytene stage is the most effective for inducing 2n pollen by colchicine in *P. alba* (Li et al., 2014). In our study, the most effective stage for inducing 2n pollen was pachytene during microsporogenesis of *P. canescens*, which was consistent with the findings reported by Zhao et al. (2019) for *P. deltoid*.

The number of colchicine injections is key when inducing 2n pollen in *Populus*. According to previous reports, the suitable number of colchicine injections varies from 3 to 5 in *P. tomentosa* × *P. bolleana* for the highest frequency of induced 2n pollen (88%) (Kang et al., 1999). Zhao et al. (2017) documented that 56.5% of induced 2n pollen is achieved when the male *P. tomentosa* buds are given 7 injections of colchicine. In the present study, 11 colchicine injections to the male buds of *P. canescens* at pachytene were used. The highest frequency of induced 2n pollen was 30.27%, which was significantly lower than that of previous studies (Kang et al., 1999; Zhao et al., 2017). At least two aspects are responsible for the lower frequency of induced 2n pollen. The meiotic process of the PMCs in different anthers inside the same flower bud is asynchronous (Tian M. et al., 2018). When the meiotic stage of PMCs in *P. canescens* was

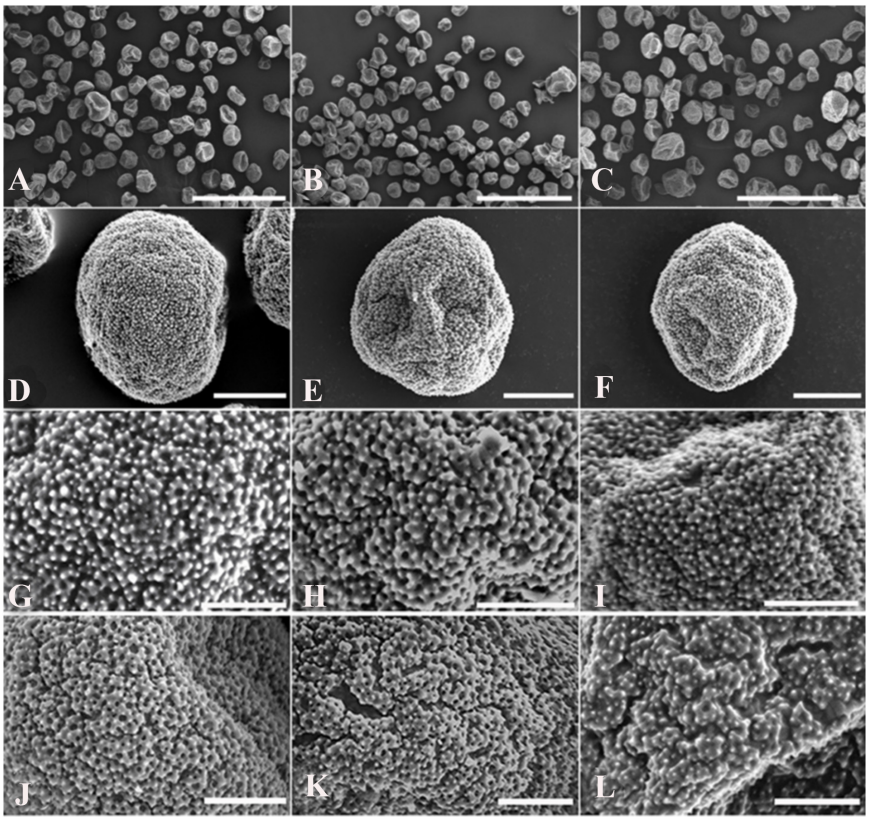

FIGURE 2 | Scanning electron micrographs of *P. canescens* pollen grains derived from untreated and treated male flower buds with 0.5% colchicine at pachytene. **(A)** Morphology of the pollen grains in the control group and natural 2n pollen grains. **(B)** Morphology of the pollen grains derived from the treatment given 5 injections at pachytene and induced 2n pollen grains. **(C)** Morphology of the pollen grains derived from the treatment given 11 injections at pachytene and induced 2n pollen grains. **(D–F)** Ectexine deposition; **(D)** haploid pollen grains in the control group; **(E)** haploid pollen grains derived from the 5 injections treatment at pachytene; **(F)** haploid pollen grains derived from the 11 injections treatment given at pachytene. **(G–L)** Details of the ectexine structure; **(G)** haploid pollen grains in the control group; **(H)** haploid pollen grains derived from the treatment given 5 injections at pachytene; **(I)** haploid pollen grains derived from the treatment given 11 injections at pachytene; **(J)** natural 2n pollen grains; **(K)** 2n pollen grains derived from the treatment given 5 injections at pachytene; **(L)** 2n pollen grains derived from the treatment given 11 injections at pachytene. Scale bar = 100 μm **(A–C)**, 10 μm **(D–F)**, 5 μm **(G–L)**.

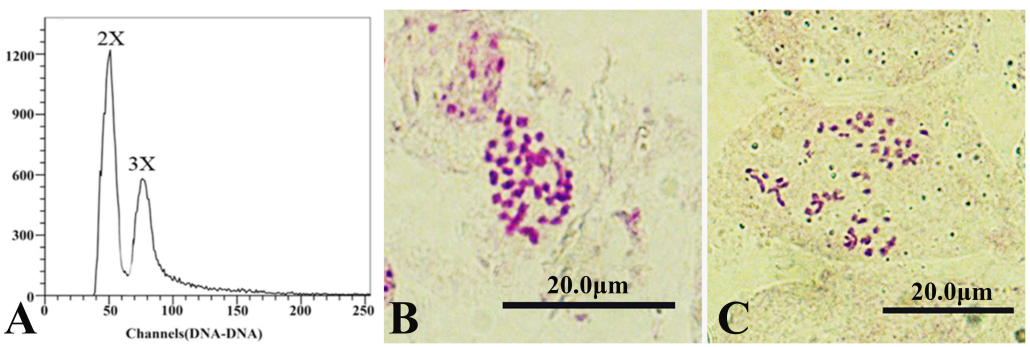

FIGURE 3 | Flow cytometric analysis and somatic chromosome counting of the offspring derived from pollen chromosome doubling induced by colchicine in *P. canescens* (scale bar = 20 μm). **(A)** Flow cytometric analysis of the mixed simple of a diploid plant and a triploid plant; **(B)** Chromosome number of the diploid (2n = 2x = 38); **(C)** Chromosome number of the triploid (2n = 3x = 57).

at pachytene, the catkin had emerged from the bract scales. The effect of colchicine treatment decreased because the colchicine overflowed and evaporated (**Figure 8**).

Heterozygosity within a 2n pollen grain depends on the cytological mechanism governing 2n pollen formation. These mechanisms are subdivided into first division restitution (FDR),

TABLE 2 | Triploid production by crossing colchicine-induced *P. canescens* 2n pollen with haploid female gametes in *P. hopeiensis* and *P. alba* × *P. glandulosa*.

Female parents	Frequency of induced 2n pollen (%)	Number of seeds	Number of seedlings	Number of triploids	Triploid production rates (%)
P. hopeiensis	30.27	38	6	1	16.67
	20.36	33	9	1	11.11
	16.70	64	21	0	0
	15.11	31	16	0	0
	11.03	52	20	0	0
	10.53	27	4	0	0
P. alba × *P. glandulosa*	9.05	912	610	2	0.33
	8.76	1027	864	1	0.12
	6.22	704	563	0	0
	5.79	975	623	0	0
	5.46	1217	964	0	0
	4.34	694	552	0	0
	2.08 (control)	967	703	0	0
	total	6741	4955	5	

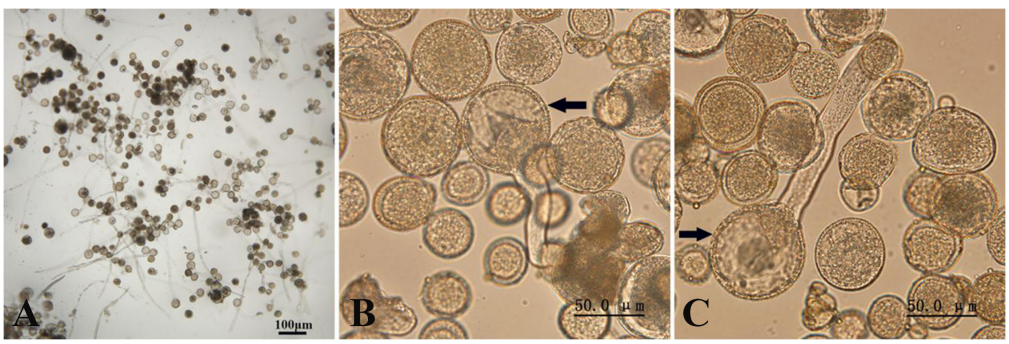

FIGURE 4 | Fresh colchicine-induced pollen of *P. canescens* derived from the treatment given 11 injection times germinated on media. **(A)** Germinated fresh colchicine-induced pollen derived from the treatment given 11 injection times. **(B)** Germinated 2n pollen induced by colchicine (arrow). **(C)** Germinated natural 2n pollen (arrow). Scale bar = 100.0 **(A)** and 50.0 μm **(B,C)**.

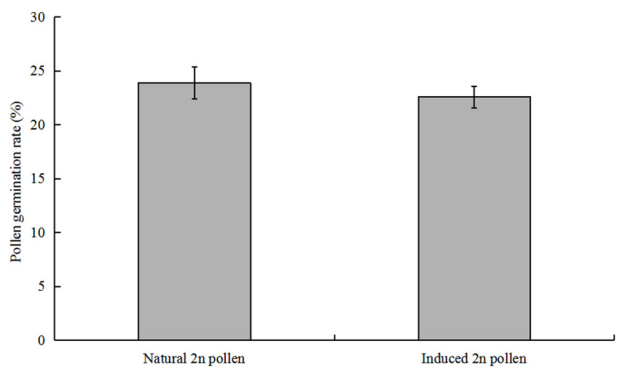

FIGURE 5 | Germination rates of natural 2n pollen derived from the control group and colchicine-induced 2n pollen derived from the treatment given 11 injections with 0.5% colchicine at pachytene in *P. canescens*.

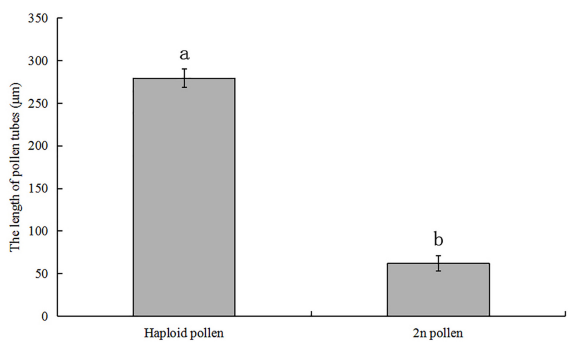

FIGURE 6 | Lengths of the pollen tubes of haploid pollen and colchicine-induced 2n pollen derived from the treatment given 11 injections with 0.5% colchicine at pachytene in *P. canescens*. Lowercase letters represent significant differences at ($P < 0.05$).

second division restitution (SDR) (Ramanna and Jacobsen, 2003), and indeterminate meiotic restitution (IMR) (Lim et al., 2001). Pairing and separation of the homologous chromosomes does not occur at meiosis I during FDR, whereas the second division occurs normally with the two sister chromatids of each chromosome moving to opposite poles. Thus, except for

TABLE 3 | The detailed information of the two pairs of polymorphic SSR primers and annealing temperature (AT).

ID of SSR primer	Chromosome	SSR primer sequence		AT(°C)
		Forward	Reverse	
GCPM_124	Chr 01	TTTGAGCACTTCAACTACCA	TGTCTTCCCTTAGTCACCAC	53
GCPM_1158	Chr 02	ATGCACTTCCTTCCAAATTA	ATCAGTTCCTTCAGCTTCAA	53

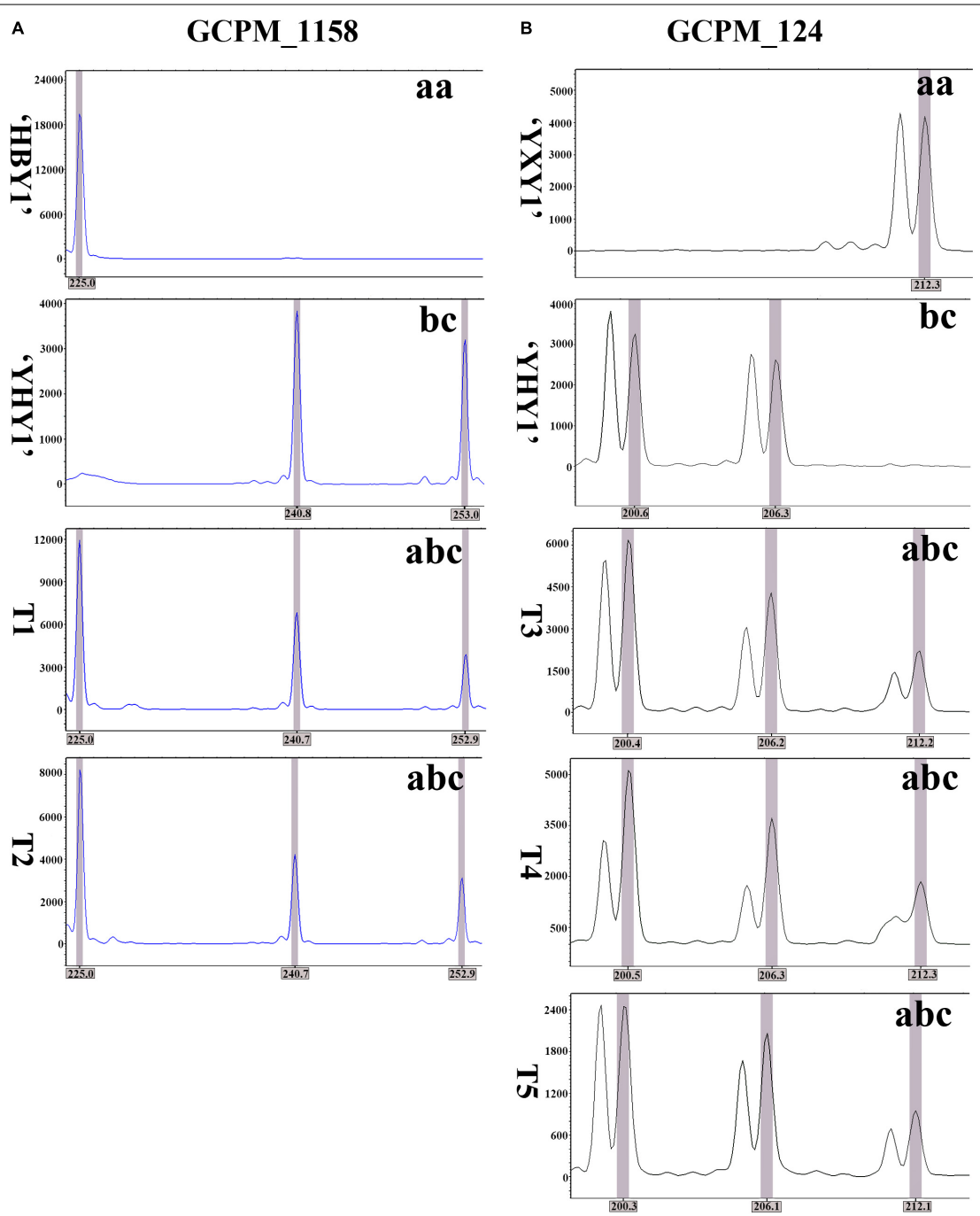

FIGURE 7 | Genetic constitutions of the colchicine-induced 2n pollen in *P. canescens* were revealed by capillary electrophoresis of the loci of primers GCPM_1158 and GCPM_124. **(A)** Allelic configuration in triploid progeny derived from *P. hopeiensis* × *P. canescens* and their parents. T1 and T2 indicate hybrid triploids. **(B)** Allelic configuration in triploid progeny derived from (*P. alba* × *P. glandulosa*) × *P. canescens* and their parents. T3, T4, and T5 also indicate three hybrid triploids.

FIGURE 8 | Male flower buds of *P. canescens* after treatment with 0.5% colchicine solution. Scale bar = 2.0 mm.

cross-over segments, FDR-type 2n pollen retain all homologous parental chromosomes. FDR-type 2n pollen is important when creating heterozygous hybrids due to the highly heterozygous 2n gametes formed (Bretagnolle and Thompson, 1995). In SDR, the pairing and separation of the homologous chromosomes proceed normally during meiosis. In meiosis II, the centromeres of the half-bivalents divide, but the chromatids do not migrate to the poles. Therefore, heterozygosity within SDR-type 2n pollen is lower than that within FDR-type 2n pollen because SDR-type 2n gametes only contain random combinations of sister chromatids. Another restitution mechanism of 2n pollen is IMR. In IMR, unequal distribution of the centromeres of the parental genomes occurs at metaphase I, where some univalents are divided equationally during the first meiotic process, whereas the remaining bivalents are separated before the end of meiosis giving rise to 2n-gametes that cannot be categorized as either FDR or SDR. The IMR mechanism is the process of meiotic restitution to produce fully heterozygous 2n gametes.

Several studies have reported the mechanisms of the formation of 2n gametes in *Populus*. Both FDR-type and SDR-type 2n megaspores are obtained by treating female flower buds with high temperature during megasporogenesis in *P. pseudo-simonii* × *P. nigra* "Zheyin #3" (Wang et al., 2012), *P. adenopoda* (Lu et al., 2013), and *P. tomentosa* (Min et al., 2017). Dong et al. (2015) demonstrated that 74.8% of parental heterozygosity was transmitted by FDR-type 2n female gametes, and 39.6% of parental heterozygosity was transmitted by SDR-type 2n female gametes in *P. pseudo-simonii* × *P. nigra* "Zheyin #3." However, Lim et al. (2001) and Barba-Gonzalez et al. (2005) reported that IMR-type 2n gametes were detected in interspecific lily hybrids by GISH and FISH. SDR- and PMD -type (post-meiotic genome doubling) 2n gametes were also detected by cytological observation and SSR makers in "Eureka" lemon (Xie et al., 2019). In the present study, the allelic configuration, at the GCPM_1158 and GCPM_124 loci, revealed that the five triploids originated from FDR-type induced 2n pollen. Therefore, the colchicine-induced 2n pollen grains were FDR-type when colchicine treatment occurred before metaphase I.

High-temperature is one of most effective physical mutagenic agents and is widely used to induce 2n gametes in higher plants (Pécrix et al., 2011; Tian M. et al., 2018). Many studies have reported changes in pollen wall structure (Porch and Jahn, 2001; Koti et al., 2005) and a decrease in pollen viability (Porch and Jahn, 2001). For example, 36°C exposure during early meiosis of PMCs leads to a decrease in pollen viability and pollen ectexine defects in induced 2n pollen of *Rosa* (Pécrix et al., 2011). Tian M. et al. (2018) showed a decrease in the pollen production per male flower bud when *P. canescens* buds are exposed to 38°C for 6 h, and the morphology of the induced 2n pollen revealed narrow furrows in the ectexine structure. However, the germination rate of induced 2n pollen was not significantly affected.

It is unknown whether colchicine treatment affects the pollen wall structure and induced 2n pollen viability in *Populus*. Liu et al. (2019) reported that the ectexine structure of *P. tomentosa* induced 2n pollen by giving 3 or 7 colchicine injections at diakinesis of PMCs was similar to that of the natural 2n pollen. In the present study, the morphology of induced 2n pollen was observed by scanning electron microscopy to clarify whether colchicine affected induced 2n pollen viability in *P. canescens*. Some narrow furrows were found in the ectexine structure of colchicine-induced 2n pollen. However, no significant difference in germination rates was detected between colchicine-induced 2n pollen and natural 2n pollen, and five triploids were detected by flow cytometry and somatic chromosome counts, suggesting that colchicine treatment will not result in dysfunction of induced 2n pollen.

In this study, the triploid production rates were significantly lower than the frequency of colchicine-induced 2n pollen, indicating that 2n pollen weakly competed with haploid pollen during fertilization. The *in vitro* pollen germination experiments revealed that the reason the induced 2n pollen was weakly competitive during fertilization was because the pollen tubes of colchicine-induced 2n pollen grew slower than those of haploid pollen. Our findings are consistent with Zhao et al. (2017) in male *P. tomentosa* clone 5088. In a future study, we will focus on revealing the precise reason pollen-tubes of 2n pollen grow so slowly in *P. canescens*.

CONCLUSION

The meiotic stage, injection time, and the interaction between the meiotic stage and injection time had highly significant effects on 2n pollen production rates in *P. canescens*.

The most effective treatment for inducing 2n pollen was to give 11 injections of 0.5% colchicine solution when PMCs were at the pachytene stage. Colchicine occasionally affected ectexine deposition, and some narrow furrows were detected in the ectexine structure. However, no significant difference in pollen germination rates was observed between natural 2n pollen and colchicine-induced 2n pollen, and five triploids derived from FDR-type induced 2n pollen were generated by crossing colchicine-induced 2n pollen, suggesting that colchicine will not result in dysfunction of colchicine-induced 2n pollen. Slower growth of 2n pollen tubes was responsible for the lower triploid production rate.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

PZ designed the experiments and edited the language of the manuscript. QZ, JW, YS, and ZZ performed the experiments. QZ performed the data analysis and wrote the manuscript. ML contributed the tools for analysis. All authors read and approved the final manuscript.

FUNDING

This study was mainly supported by the National Key Research and Development Program of China (2016YFD0600403).

ACKNOWLEDGMENTS

We thank technician Junna Shi for her assistance in scanning electron microscopic analysis.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 26 March 2020
doi: 10.3389/fpls.2020.00292

Induction of Synthetic Polyploids and Assessment of Genomic Stability in *Lippia alba*

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Polyploidy is widely recognized as a major evolutionary force in plants and has been reported in the genus *Lippia* (Verbenaceae). *Lippia alba*, the most studied species, has been documented as a polyploid complex involving at least four ploidal levels. *L. alba* presents remarkable chemical and genetic variation and represents a model for understanding genome organization. Although the economic and medicinal importance of the species has been widely described, no established polyploid induction protocol has been reported so far. Here, we describe the production of synthetic polyploid plants of *L. alba* using colchicine. The ploidal levels were estimated by flow cytometry and chromosome counting. In addition, FISH and molecular markers approaches were used to confirm the stability of the synthetic polyploids. The major component of the essential oils was estimated by GCMS to compare with the natural individuals. Tetraploids and triploids were produced providing new opportunities for investigating medicinal, pharmacological, and economic applications as well as addressing intrinsic questions involved in the polyploidization process in tropical plants.

Keywords: artificial polyploidy, colchicine, essential oil, FISH, flow cytometry, genomic instability, medicinal plants, SSR and ISSR markers

OPEN ACCESS

Edited by:
Jen-Tsung Chen, National University of Kaohsiung, Taiwan

Reviewed by:
Joan Vallès, University of Barcelona, Spain
Brian Arnold, Harvard University, United States

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *05 December 2019*
Accepted: *26 February 2020*
Published: *26 March 2020*

Citation:
Julião SA, Ribeiro CdV, Lopes JML, Matos EMd, Reis AC, Peixoto PHP, Machado MA, Azevedo ALS, Grazul RM, Campos JMSd and Viccini LF (2020) Induction of Synthetic Polyploids and Assessment of Genomic Stability in Lippia alba. Front. Plant Sci. 11:292. doi: 10.3389/fpls.2020.00292

INTRODUCTION

Polyploidy (whole-genome multipication) is widely recognized as a major evolutionary force in plants (Stebbins, 1971; Grant, 1981; Otto and Whitton, 2000; Soltis and Soltis, 2009, 2012; Aversano et al., 2012; Wendel, 2015; Wendel et al., 2016). It is estimated that polyploidy events occurred in all angiosperms (Jiao et al., 2011; Albert et al., 2013). Changes in ploidal level may result in broad phenotypic modifications (e.g., Ramsey and Schemske, 2002; Soltis et al., 2003; Adams and Wendel, 2005). These changes may provide polyploids with short-term adaptive potential (Van de Peer et al., 2017) and the opportunity to exploit new niches (e.g., Marchant et al., 2016). In addition, synthetic polyploids have been largely employed to increase agronomic traits mainly due to its higher physiological and morphological fitness compared to their natural diploids (Chung et al., 2017; Cui et al., 2017; Salma et al., 2017; Wei et al., 2018).

Polyploidy events have been documented in the genus *Lippia* (Verbenaceae) (Viccini et al., 2005; Campos et al., 2011; Pierre et al., 2011). *Lippia alba* (Mill.) N. Brown (Linnaeus), the most studied species, has been described as a polyploid complex involving at least four ploidal levels ($2n$ = 30, 45, 60, and 90) (Pierre et al., 2011; Reis et al., 2014). An interesting association between ploidal level and chemical profile of essential oil was also reported. Diploid and tetraploid accessions are citral producers while triploid accessions are linalool producers (Viccini et al., 2014). Genetic distance by molecular markers and phylogenetic analysis showed that natural accessions grouped by ploidal level and only one origin of triploids (Lopes et al., 2019, in press).

Lippia alba is a tropical species widely distributed throughout the Americas. In Brazil, the species is found all over the country (Reis et al., 2014). *L. alba* has been characterized by its remarkable morphological, phytochemical and genetic variations (Manica-Cattani et al., 2009; Jezler et al., 2013; Reis et al., 2014), as well as its economic importance and medicinal properties (Hennebelle et al., 2008). Biological characteristics such as toxicity repellency, antifungal, antibacterial, and antioxidant properties have been identified in essential oils or extracts of *L. alba* that are widely used in folk medicine (Glamočlija et al., 2011; Chies et al., 2013; Peixoto et al., 2015).

Considering the medicinal importance of *L. alba* and the extraordinary chemical and genetic variation, the species represents a model for understanding genome organization, the origin of a natural polyploid, and its association with chemical profile variation. Thus, the production of synthetic polyploids constitutes a great opportunity for replicating the natural polyploidization process in the *L. alba* complex (Münzbergová, 2017; Pavlíková et al., 2017; Castro et al., 2018), opening a window for understanding polyploidization in the tropics as well as for increasing the production of essential oil of economic interest.

Genome doubling is usually induced by compounds that interfere with cell division and are either applied to *ex vitro* or *in vitro* plants (Dhooghe et al., 2011). Colchicine is the oldest and most widely used compound for polyploidization induction (Nebel, 1937; Niel and Scherrmann, 2006; Dhooghe et al., 2011).

Although many studies provide polyploidization protocols (Rêgo et al., 2011; Gomes et al., 2014; Tavan et al., 2015; Widoretno, 2016; Salma et al., 2017; Denaeghe et al., 2018), they generally demonstrate that the optimal procedures are rather species specific. The development of a proper method for polyploidization requires the conduction of several tests to obtain the most suitable combination of antimitotic agent, concentration, exposure time, type of explants, exposure method, and the regeneration mode employed (Allum et al., 2007; Dhooghe et al., 2011; Yang et al., 2014; Sattler et al., 2016). Therefore, the success of induction depends on the way each procedure is performed in each phase and the correct interaction of each step.

In spite of the economic and medicinal importance of *L. alba* (Hennebelle et al., 2008; Reis et al., 2014; Viccini et al., 2014; Lopes et al., 2019, in press) no established polyploidization protocol for the species has been reported so far. Here, we describe the production of synthetic polyploid plants of *L. alba*. We also tried to broadly understand the consequence of the polyploidization process, addressing the following questions about the new plants obtained: (1) Are the ploidal levels and chromosome numbers the same in all synthetic plants? (2) Do synthetic plants remain stable over time after polyploidy induction? (3) Do synthetic plants have the same chemical profile as the natural ones? We hope our study of these synthetic polyploids provides an opportunity for industry exploration, to discover new biological activities as well as to disclose the evolutionary process immediately following polyploidization, which may be involved in the formation of natural polyploids.

MATERIALS AND METHODS

Plant Material and *in vitro* Propagation

A diploid accession of *L. alba* ($2n = 2\times = 30$) was used for chromosome duplication. The accession belongs to the *L. alba* collection of the Universidade Federal de Juiz de Fora, Minas Gerais, Brazil (voucher number 48374, deposited at Herbarium Leopoldo Krieger CESJ-UFJF). *In vitro* plantlets were maintained in test tubes containing 15 mL of MS-based medium devoid of growth regulators and subcultivated at intervals of approximately 40 days. The culture was kept at 25 ± 1°C under a light regime of 16/8 h (hours) (light/dark) cycle of 35 μmol m^{-2} s^{-1} illumination provided by cool, white fluorescent tubes.

Polyploidy Induction and Acclimatization of Plantlets

A pilot experiment was performed to determine the most efficient treatment for polyploidy induction (**Supplementary Table S1**). As a result, two concentrations of colchicine were chosen (0.2% and 0.02%) during 4 h and 72 h of exposure. A colchicine-free MS medium was used as control.

Both for the pilot and the final experiment, nodal segments of *L. alba* were inoculated in colchicine MS medium. At the end of the exposure time, the explants were washed three times with autoclaved distilled water, and then inoculated in a colchicine-free MS medium. For each treatment, one hundred explants were inoculated in a completely randomized design. The treatments were composed of four replicates containing twenty-five explants each.

All plants that survived were *in vitro* propagated for 40 days. Individuals from 3 to 7 cm high were acclimated. Approximately 30 days later, the plants were transplanted into 10 L vessels containing soil and substrate mixture BioPlant (3:1).

Determination of Ploidal Levels

We evaluated the ploidal level of regenerated plantlets in two moments. At first, flow cytometry was performed using *in vitro* plantlets after 40 days of culture. The second evaluation was done when the plants reached 18 months, in the greenhouse.

For DNA content estimation, around 25 mg of leaves and roots were chopped in a Petri dish containing 1 mL of cold ice LB01 buffer (Galbraith et al., 1983). The suspension of nuclei was filtered and stained with 25 μl of propidium iodide (10 mg L^{-1}) (Sigma). 2.5 μl of RNA seq (20 mg L^{-1}) (Sigma) was

added to each sample (Dolezel et al., 2007). At least 10,000 nuclei were analyzed per sample using a FACSCanto II flow cytometer (Becton Dickinson). FACS software Diva 6.1.3 was used to build the histograms that were analyzed using Flowing software 2.5.1 (available at http://www.flowingsoftware.com/).The ploidal level was checked taking as reference the position of the G1 peak of the diploid plant. Three estimates were made for each plant.

The chromosome counting, 45S rDNA mapping, molecular and chemical analyses were performed when the plants were established in the greenhouse.

Chromosome Counting and 45S rDNA Mapping

In order to verify the chromosome number and confirm the ploidal level of synthetic plantlets, root tips were pretreated with 0.003M of 8-hydroxyquinoline (Sigma) at 4°C for 9 h and fixed in ethanol: acetic acid (3:1). The radicular meristems were submitted to enzymatic maceration in 20% pectinase (Sigma) and 2% cellulase (Serva-Onozuka R-10) at 37°C for 6 h. Cytological preparations were performed according to Carvalho and Saraiva (1993).

The fluorescence *in situ* hybridization (FISH) was performed as described by Jiang et al. (1995). The hybridization mixture was denatured at 85°C for 10 min and immediately transferred to an icebox. The slides were denatured at 85°C for 1 min and treated with a series of alcohol washes (70, 90, and 100% ethanol for 5 min each). The hybridization mixture was then added to the slides and the chromosomes allowed to hybridize at 37°C for 18 h in a humidified chamber. Post-hybridization washes were carried out using 2 × SSC buffer (0.3 mol L^{-1} sodium citrate, 0.03 mol L^{-1} sodium chloride, pH 7) and 1 × PBS buffer (0.136 mol L^{-1} sodium chloride, 0.27 mol L^{-1} potassium chloride, 0.1 mol L^{-1} dibasic sodium phosphate, 0.2 mol L^{-1} monobasic potassium phosphate, pH 7.4). Probes were detected with anti-DIG conjugate with rhodamine (Sigma) and post-detection washes were performed using 1 × TNT buffer (0.1 mol L^{-1} Tris, 0.15 mol L^{-1} sodium chloride, 0.05% Tween-20) and 1 × PBS at room temperature. Chromosomes were counterstained with 2 μg mL^{-1} of DAPI (Sigma). The slides were mounted in Vectashield (Vector, Burlingame, CA, United States). Signal visualization was performed under Olympus BX53 fluorescence microscopy and the images were photographed with an Olympus DP72 camera attached to the microscope.

DNA Extraction, Amplification, and Data Analysis

Total genomic DNA from the leaves plants established in the greenhouse was extracted using the CTAB method (Doyle and Doyle, 1990) with a minor modification (double extraction with chloroform-isoamyl alcohol). After extraction, the DNA of the samples were solubilized and quantified using a Nanodrop Spectrophotometer (ThermoFisher Scientific, Inc., Wilmington, DE, United States), diluted to 25 ng μl^{-1}, and kept at −20°C for subsequent use.

Eleven ISSR primers (**Supplementary Table S2**) and seven SSR primers (**Supplementary Table S3**) were selected to estimate the genetic profile of diploid and synthetic polyploid plants. The SSR primers were synthesized with M13-tailed forward primers (**Supplementary Table S3**). PCR was carried out in a DNA Thermal Cycler Mastercycler® (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) following the specifications given in **Supplementary Tables S2**, **S3**.

For ISSR analysis the amplification fragments were analyzed on 2% agarose gels. The products of PCR amplification were recorded as a binary matrix, in which the presence or absence of similarly sized fragments were marked 1 or 0, respectively. Only consistent and reproducible bands between 200 and 800 bp of size were included in the analysis.

The analysis of allelic polymorphisms was performed comparing polymorphic bands between the mother plant and synthetic polyploids. SSR fluorescent products were detected by capillary electrophoresis using a MegaBACE1000 sequencer (GE Healthcare, Buckinghamshire, United Kingdom). The SSR fragment size was measured using the Fragment Profile program (GE Healthcare, Buckinghamshire, United Kingdom). All individuals were scored for the presence or absence of SSR alleles at each of the seven loci. The data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the allele, and this data matrix was subjected to genetic distance analysis.

The Jaccard and Dice coefficients were used to generate dendrograms for ISSR and SSR data using the Unweighted Pair Group Method with Arithmetic Means (UPGMA) and to estimate the similarity values among accessions. The genetic distance and bootstrap analyses of the data were performed with 1000 repetitions using Ntsys v2.11 software (Rohlf, 2000) and PAST (Hammer et al., 2001). As the ISSR and SSR dendrograms showed the same result, they were performed together.

The genetic profile was investigated using Bayesian inference clustering as implemented in Structure 2.3.4[1] (Pritchard et al., 2000), and final plots were produced in Structure Plots v2.0 (Ramasamy et al., 2014). We analyzed 12 accessions as dominant data, coded as presence/absence, using the admixture model with uncorrelated allele frequencies (Stift et al., 2019). The Monte Carlo Markov Chain was run for 100,000 steps, following a burn-in of 10,000 steps. Simulations were performed for the number of groups (K) varying from 1 to 10. We used Structure Harvester (Earl and VonHoldt, 2012) to calculate ΔKm (Evanno et al., 2005).

Essential Oil Microextraction

Leaf tissue (approximately 300 mg) from each individual was frozen in glass vials at −18°C for 24 h. After freezing, 0.5 mL of hexane and 0.25 mL of methanol were added to each sample. The samples were kept in a 70 KHz ultrasonic bath (Thornton-INPEC) at room temperature for 1 h to accelerate the extraction process. Subsequently, the supernatant was filtered through a sterile cotton swab. From the clear solution of extracted oils 1 μL was analyzed on a mass spectrometer gas chromatograph (GCMS-QP2010 Plus; Shimadzu). A 30 m × 0.25 mm Rtx-5MS® (Restek) column was

[1]http://pritchardlab.stanford.edu/structure.html

used. The oven temperature programming started at an initial temperature of 70°C, maintained for 3 min, followed by an increase of 6°C min^{-1} to 300°C. The injector was operated in split mode (1:10), at 240°C, an interface and the mass detector operated at 300°C. Helium was used as a carrier gas, with a flow of 1.53 mL min^{-1}. A standard mixture of linear hydrocarbons was injected under the same conditions of use. Component identification was performed by comparing mass spectra with data from the NIST 9.0 database (correlation > 97%) and confirmed by retention index (Kováts Index) being calculated for each component and compared to data in the literature (Adams, 2007).

RESULTS

Polyploidy Induction and Ploidal Stability

Based on the pilot experiment (**Supplementary Table S1**), we decided to evaluate two concentrations of colchicine, 0.2% and 0.02% during 4 h and 72 h of exposure. Forty days after culture, the ploidal level of regenerated plantlets were assessed. Mixoploids and tetraploids were identified (**Figures 1B,C**). The treatment of 0.2% colchicine showed the highest effect on the modification of the ploidal level of surviving plants. Five mixoploids were obtained when 0.2% colchicine was applied for 4 h, and one tetraploid was observed when 0.2% colchicine for 72 h was used. Using 0.02% colchicine during 4 h, three mixoploid plants were regenerated (**Table 1**). For ploidy determination, the value of the DNA content of the diploid plant (2.56 ± 0.06 pg) was used as a reference (**Figure 1A**). The tetraploids showed the double of DNA content observed for diploids (2C = 5.14 ± 0.03 pg). The mixoploids showed two peaks, corresponding to diploid and tetraploid cells (**Figure 1B**). Synthetic mixoploids and tetraploids were *in vitro* cultivated for 3 months.

After that, the plants were transferred to the greenhouse and their ploidal levels were reassessed. The two peaks previously identified for mixoploids were replaced and only one peak was revealed that showed the same C-value of natural triploids (2C = 3.97 ± 0.03 pg) (**Figure 1D**). In other words, the mixoploids became triploids. The tetraploids maintained the ploidal level initially estimated (**Figure 1E**). We assessed a total of five synthetic tetraploids and six synthetic triploids. The flow cytometry analysis confirmed the stability of the ploidal level in two tissues (leaf and root) over 5 years. No chimeras were found during this period (**Supplementary Figure S1**).

Chromosome counts confirmed $2n = 30$ for the diploid (mother plant), $2n = 60$ for tetraploids and $2n = 45$ for triploids (**Figure 2**). In addition, some aneuploid cells also were observed in emergent triploids. Their chromosome numbers ranged from $2n = 30$ to $2n = 60$. FISH mapping of ribosomal genes revealed six terminal sites for diploid, nine for triploids and 12 for tetraploids (**Figure 2**).

Molecular Profile

Fifty-five ISSR loci with an average of five loci per primer were observed. The number of loci ranged from three (UBC-859) to seven (UBC-826 and UBC-857). The fragment size ranged from 200 to 800 bp (**Supplementary Table S2**). The analysis of allelic polymorphisms was performed comparing the synthetic polyploids (triploids and tetraploids) with the mother plant. The number of polymorphic bands between the mother plant and synthetic triploids ranged from 24 to 31, while between the mother plant and synthetic tetraploids it varied from one to three. Four out of six synthetic triploid plants showed polymorphisms for all primers. The tetraploid plants showed polymorphism for only one or two primers. A high percentage of polymorphism (43.63 to 56.36%) was detected between the mother plant and synthetic triploids. The comparison between the mother plant and the synthetic tetraploids showed lower polymorphism rate, ranging from 1.81 to 5.45%. These polymorphisms are mainly due to the presence of fragments in synthetic polyploid plants that were not observed in the mother plant.

The analysis of SSR loci revealed 25 alleles, with an average of 3.6 alleles per primer. The size of the alleles ranged from 101 to 193 base pairs. The analysis of the allelic polymorphisms was performed as for ISSR markers. The majority of new alleles were observed in synthetic triploids. On average, up to 77% of alleles detected in triploid plants correspond to new alleles, while tetraploid plants revealed 18 to 36% of new alleles.

The analysis of genetic similarity was done separately for ISSR and SSR (data not shown) and showed similar results. Therefore, the analysis was performed together. The similarity coefficients ranged from 0.35 to 0.98 (average of 0.68). Based on the similarity index, two clusters were formed: one with the mother plant and synthetic tetraploids, and another with only synthetic triploid plants. The similarity index among the mother diploid plant and the synthetic tetraploids varied from 0.84 to 0.98 (average of 0.93). Regarding the triploids, the coefficient of similarity among them ranged from 0.85 to 0.98 (average of 0.95). Considering all plants analyzed, the lowest similarity value (0.346) was observed between tetraploid and triploid plants, and the highest (0.982) was observed among triploid plants (**Figure 3A**).

The Structure analysis using ΔKm (Evanno et al., 2005) method indicated that the best number of groups is $K = 3$. This analysis revealed the genetic structure among the natural diploid and synthetic plants (**Figure 3B**). The triploid plants seem to be distinct from the other ploidal levels while the tetraploids showed a genomic structure similar to the mother plant (**Figure 3B**). These results are similar to those observed using the similarity analysis (**Figure 3A**).

Essential Oil Profile

Using gas chromatography coupled to a mass spectrometer, it was possible to identify the components of the essential oil of synthetic polyploid plants of *L. alba*. The main constituents detected were citral (neral and geranial) and linalool (**Figure 4**). The citral was the major component essential oil of the natural diploid (77.51%) and synthetic tetraploids (from 52.71 to 77.04%). The linalool constituent was detected as a major component in all synthetic triploid plants, ranging from 20.3 to 54.13% (**Figure 4**).

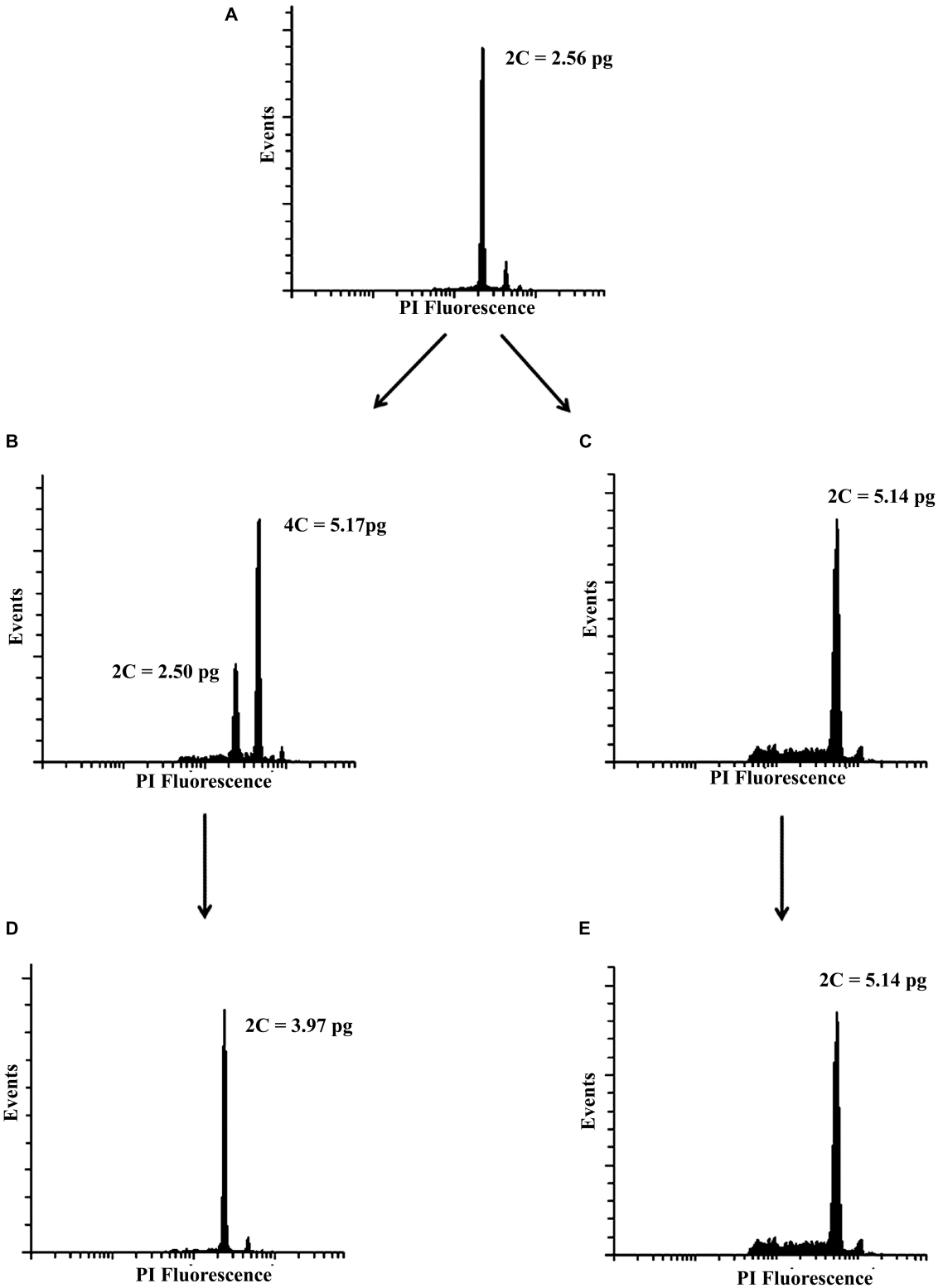

FIGURE 1 | Representative histograms of the mother plant and synthetic polyploids. **(A)** mother plant (diploid), **(B)** mixoploid, **(C)** tetraploid 40 days after polyploidy induction, **(D)** triploid, and **(E)** tetraploid acclimatized in the greenhouse.

TABLE 1 | Survival rate and ploidal level of *Lippia alba* treated with colchicine at different concentrations and exposure times.

		Explants exposed	Surviving	Tetraploid	Mixoploid
4 h	Control	100	78	–	–
	0.02%	100	58	–	3
	0.20%	100	38	–	5
72 h	Control	100	64	–	–
	0.02%	100	44	–	–
	0.20%	100	11	1	–

DISCUSSION

The induction of polyploids has been widely used as a strategy to investigate the effects of artificial genomic duplication on several plant species. Morphological, histological, physiological, agronomic, and genomic traits have been evaluated in different studies (Adams and Wendel, 2005; Buggs et al., 2008; Rêgo et al., 2011; Hegarty et al., 2013; Gomes et al., 2014; Tavan et al., 2015; Gao et al., 2016; Iannicelli et al., 2016; Yan et al., 2016; Sadat-Noori et al., 2017; Salma et al., 2017; Zhou et al., 2017).

Although polyploidy induction has been widely recognized as an important strategy for chromosome duplication in plants, the protocols are still associated with low efficiency. Here we reported the first attempt to produce synthetic polyploids in *L. alba*. The pilot experiment sought to screen interactions between colchicine concentrations and exposure times. We obtained the higher survival rates when lower colchicine concentrations and shorter exposure times were employed. The same effect was previously reported for other species, and was mainly attributed to the toxic effect of colchicine (Niel and Scherrmann, 2006; Lehrer et al., 2008; Sajjad et al., 2013).

The number of polyploid plants here obtained suggests that the 0.2% colchicine concentration combined with 4 h exposure time was the most successful treatment. This opens good perspectives for new trials on testing the procedure using plants at different stages of development as well different ploidy, in particular if we consider that *L. alba* has naturally at least four ploidal levels which have been described (Reis et al., 2014).

The ploidal level of synthetic polyploid plants should be periodically checked to ensure the maintenance of the ploidal level (Väinölä, 2000; Harbard et al., 2012; Blasco et al., 2015). The analyses of flow cytometry, chromosome counting, genetic and chemical diversity are important for identifying possible phenotypic and genotypic variations in synthetic polyploids. Here we observed that tetraploids kept their ploidal level, while mixoploids did not. Stable synthetic tetraploids have been reported in *Eriobotrya japonica* (Thunb.) Lindl (Blasco et al., 2015) and in *Rhododendron* L (Väinölä, 2000). On the other hand, instability of synthetic tetraploid plants was observed in *Acacia mangium* Willd. Two tetraploid plants were reclassified as diploids and two as mixoploid 16 months later, when the plants were transferred from the greenhouse to the field (Harbard et al., 2012).

These results reveal that the response to the duplication process may vary among species, according to the methodology employed and the maintenance of the synthetic plants. Vanstechelman et al. (2010) suggest that synthetic polyploid plants should be reanalyzed after the *in vitro* micropropagation procedure. According to the authors, many sectoral chimeras are not detected in the first analysis by flow cytometry. Plants of *Spathiphyllum wallisii* Regel, for instance, that were initially classified as tetraploids showed roots with diploid and/or mixoploid cells (Vanstechelman et al., 2010). Here, the synthetic tetraploids of *L. alba* presented sixty chromosomes in the metaphases confirming the ploidal level indicated by the flow cytometry analysis. Chromosome mapping of tetraploids showed that the number of 45S rDNA increases proportionally, revealing that the protocol was able to duplicate the genome with no evidence of chromosomal rearrangements after the duplication. The synthetic tetraploids grouped with the mother diploid plant with high genetic similarity. Similar results were observed when a larger number of natural diploid and tetraploid accessions were studied together using microsatellites and phylogenetic inferences (Lopes et al., 2019, in press). On the other hand, FISH mapping of ribosomal genes in natural tetraploids of *L. alba* previously revealed only eight 45S sites (Reis et al., 2014). The difference between natural and synthetic tetraploids regarding the number of 45S markers can be attributed to the structural alterations and genome downsizing, frequently reported in natural polyploids (Hegarty et al., 2013; Doyle and Coate, 2019).

Although synthetic tetraploids showed a similar karyotype compared to the natural diploid, the mixoploid individuals revealed a different scenario. Interestingly, when the DNA content of mixoploids was reassessed, we realized that the plants presented an intermediary DNA amount between diploid and tetraploid, that was equivalent to the natural triploids. Besides, the metaphases of synthetic triploids had $2n = 45$ chromosomes, as observed for natural triploids of *L. alba*, suggesting that some chromosomes were lost in tetraploid cells. Losses of whole chromosomes can occur in regenerated mixoploids in attempt to stabilize the genomic constitution after polyploidy induction in the short-term, but the emergence of a new ploidal level seems to be rare (Dodsworth et al., 2016; Regalado et al., 2017; Salma et al., 2017; Doyle and Coate, 2019). In addition we could not cannot discard the possibility that some cells $2n = 45$ could be present in small quantity in mixoploids and this ploidal level increased in detriment of the others after the acclimatization in greenhouse. More studies need to be done for a better understanding of how the emergence of the synthetic triploids occurs.

Commonly, the polyploid induction may result in chimeras, that typically became stable at one ploidal level. However, some individuals maintain the mixoploidy state (Harbard et al., 2012; Eng and Ho, 2019). In *L. alba*, one natural mixoploid individual was described previously, but chromosome losses were not notified in this accession and the mixoploidy was stable over time (Pierre et al., 2011). Curiously, this mixoploid had cells with chromosome number ranging from $2n = 12$ to 60, being 44, 45, and

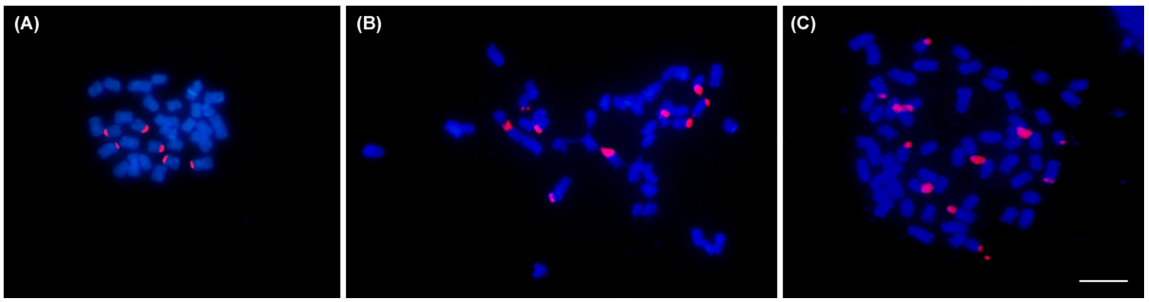

FIGURE 2 | Representative metaphases of three *Lippia alba* cytptypes: **(A)** diploid (2*n* = 30), **(B)** triploid (2*n* = 45), and **(C)** tetraploid (2*n* = 60) individuals. Chromosomes were counterstained with DAPI (blue), 45S rDNA marker was stained with rhodamine (red). Bar = 5μm.

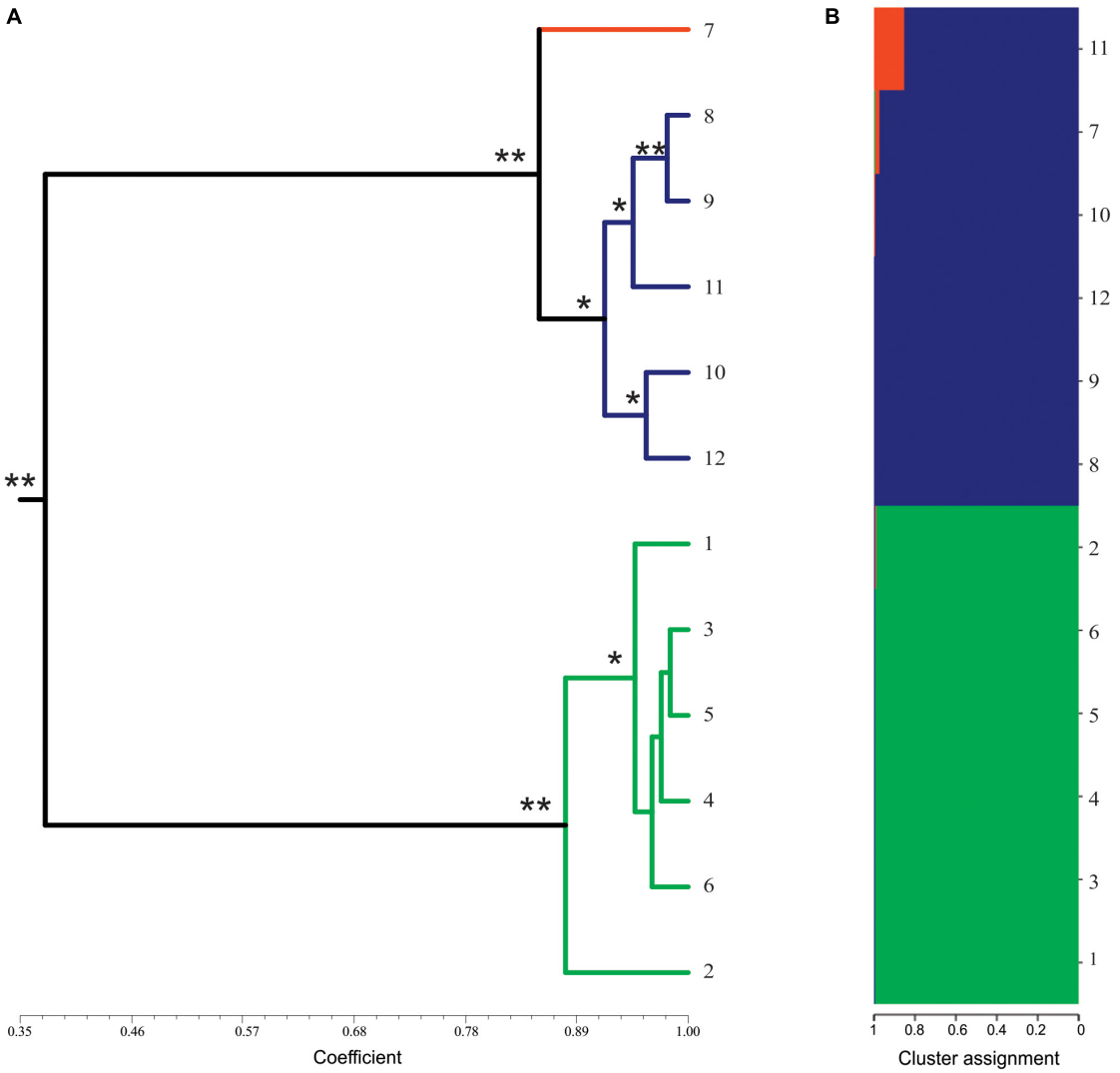

FIGURE 3 | Molecular profile of synthetics plants of *Lippia alba*. **(A)** Dendrogram of genetic similarity by UPGMA of presence/absence of alleles using combined data (ISSR and SSR makers) from 12 plants of *L. alba*. The colors of the branches represent different ploidal levels: diploid in orange, triploid in green, tetraploid in blue. * represents bootstrap values above 50% and ** represents bootstrap values above 90%. Dendrograms with JACCARD and DICE coefficients were identical. **(B)** Bayesian analysis of the genetic structure of 12 plants of *L. alba*. The colors represent the proportion of the genome shared for each individual. Similar genomes are represented by the same color.

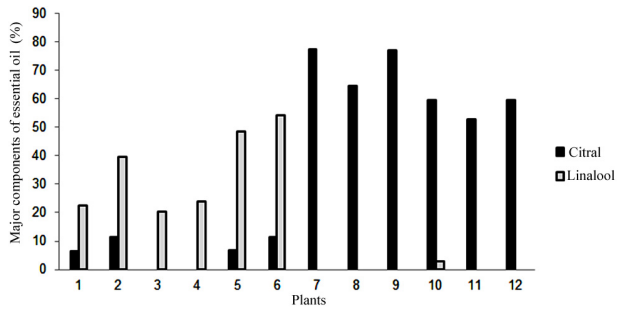

FIGURE 4 | Percentage of the major essential oil content detected by gas chromatography. Mother diploid plant (7), synthetic triploid plants (1–6) and synthetic tetraploid plants (8–12). Gray bars, Citral; Black bars, Linalool.

46 the most frequent numbers of chromosomes observed (Pierre et al., 2011).

Here we observed the majority of aneuploid cells in synthetic triploids varing from $2n = 38$ to 47, but they were restricted to a few metaphases. These cells may be remnants of the previous mixoploidy state. This fact might be linked to a putative process of karyotype uniformity and these cells possibly have some advantage over diploid cells. Although it can be considered a rare event, the emergence of triploid plants using a protocol to produce synthetic tetraploids has been previously described. In *Pyrus communis* L., triploid plants were obtained after *in vitro* treatment of leaf explants with colchicine (Sun et al., 2009). The treatment of apical meristems with colchicine generated triploid plants of poplar (Ewald et al., 2009). The germinated seedlings of trifluralin-treated *Rosa chinensis* Jacq. yielded triploid ($2n = 3\times$) and aneuploid ($2n = 3\times-1$) plants (Zlesak et al., 2005). None of the authors above explained the emergence of triploid plants obtained during tetraploid induction treatments.

In addition to the numerical variations, structural rearrangements are frequently reported in recently formed polyploids and such alterations can originate new allelic polymorphisms among individuals (Tayalé and Parisod, 2013). The triploids showed the highest polymorphism rates and new alleles when compared to the mother diploid. The DNA elimination and a genomic shocking (Buggs et al., 2008) might explain these results. The genomic reorganization detected by molecular markers has also been identified in *Chrysanthemum lavandulifolium* (Fisch. ex Trautv.) Makino, *Paspalum notatum* Flüggé, *Citrullus lanatus* (Thunb.) Mansf., and *Eragrostis curvula* (Schrad.) Nees (Martelotto et al., 2007; Mecchia et al., 2007; Wang et al., 2009). On the other hand, the new synthetic tetraploids showed similar molecular profile when compared to the mother plant (diploid), corroborating the cytogenetic data. Similar results have been observed in *Solanum commersonii* Dunal, *Solanum bulbocastanum* Dunal, and in *Citrus limonia* (L.) Osbeck (Allario et al., 2011; Aversano et al., 2013, 2015). The distinctiveness of the triploids and the similarity of the tetraploids comparing with the diploid was also detected by genetic similarity analysis. Interestingly, the same scenario was previously observed for natural triploids when the molecular profile, the essential oils production, and the morphology were assessed. Triploids seem to be particularly different comparing with other ploidal levels (Viccini et al., 2014; Lopes et al., 2019 in press).

Regarding the essential oil production, the synthetic tetraploids produced the same major component of the essential oil that was produced by the mother plant, while the triploids changed the major component to linalool. The synthetic polyploids showed the same profile observed for the natural polyploids (Viccini et al., 2014). The analysis of the metabolism of autopolyploid plants suggests that polyploidy may cause both qualitative and quantitative changes in the essential oil, due to changes in the mechanisms that regulate its biosynthesis (Fasano et al., 2016; Iannicelli et al., 2020). Vieira et al. (2016) suggested that synthesis of citral, geraniol and other compounds prevailed in diploids, while non-oxygenated monoterpenes were the major ones in polyploids of *Citrus limonia*. Hannweg et al. (2016) also identified changes in major chemical components of essential oils due to the polyploidy induction in *Tetradenia riparia* (Hochst.) Codd (Lamiaceae), a close related family of Verbenaceae. The metabolic activity may be increased due to alteration of gene expression or changes in the concentration of the secondary metabolites (Fasano et al., 2016; Iannicelli et al., 2020). Evidence of the genetic duplication affecting metabolic profiles of different plant species has been widely reported (Caruso et al., 2011; Dehghan et al., 2012; Trojak-Goluch and Skomra, 2013; Gomes et al., 2014; Xu et al., 2014; Tavan et al., 2015; Iannicelli et al., 2016) which reinforces the application of genome duplication protocols for manipulating the biosynthesis of compounds of economic interest.

CONCLUSION

The polyploidization protocol was able to produce stable polyploids in *L.alba*. The synthetic tetraploid showed the same ploidal level over time with similar molecular, karyotipic, and chemical profiles. Interestingly, the mixoploids underwent karyotype uniformity, and the majority of their cells showed 45 chromosomes. Besides that, the emergence of alleles and changes on the major component of essential oil were observed.

The production of synthetic polyploids enables comparison with the natural polyploidization process. Considering all loci are potentially homozygous it is possible to infer how the increase in genome size affects the phenotype.

Taking into account that the species is an aromatic shrub with pharmacological and economic applications, those synthetic plants may open a new scenario for manipulating the genome, regarding the gene expression profile or the production of secondary metabolites of commercial interest (Castro et al., 2018; Long et al., 2019).

Few examples of polyploid complexes from the tropics have been documented (Rice et al., 2019), which makes *L. alba* a potential species for studying the polyploidization process in non-model plants.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are available on request to the corresponding author without restriction.

AUTHOR CONTRIBUTIONS

SJ, CR, JC, and LV conceived and designed the experiments. SJ, CR, and PP performed the *in vitro* tissue culture and chromosome doubling experiments. EM and JC carried out the flow cytometry analyses. AR performed the cytogenetic analyses. JL, MM, and AA performed the molecular markers analyses. RG analyzed the essential oil profile. SJ, EM, JL, AR, and LV contributed to the writing of the manuscript. LV revised the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved the manuscript.

FUNDING

Funding was provided by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG).

ACKNOWLEDGMENTS

We greatly appreciate the efforts of the journal editors and authors.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00292/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 21 April 2020
doi: 10.3389/fpls.2020.00330

Triploid Citrus Genotypes Have a Better Tolerance to Natural Chilling Conditions of Photosynthetic Capacities and Specific Leaf Volatile Organic Compounds

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OPEN ACCESS

Edited by:
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Reviewed by:
Marek Zivcak, Slovak University of Agriculture, Slovakia
Junmin Li, Taizhou University, China

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: 16 December 2019
Accepted: 05 March 2020
Published: 21 April 2020

Citation:
Lourkisti R, Froelicher Y, Herbette S, Morillon R, Tomi F, Gibernau M, Giannettini J, Berti L and Santini J (2020) Triploid Citrus Genotypes Have a Better Tolerance to Natural Chilling Conditions of Photosynthetic Capacities and Specific Leaf Volatile Organic Compounds. Front. Plant Sci. 11:330. doi: 10.3389/fpls.2020.00330

Low temperatures during winter are one of the main constraints for citrus crop. Polyploid rootstocks can be used for improving tolerance to abiotic stresses, such as cold stress. Because the produced fruit are seedless, using triploid scions is one of the most promising approaches to satisfy consumer expectations. In this study, we evaluated how the triploidy of new citrus varieties influences their sensitivity to natural chilling temperatures. We compared their behavior to that of diploid citrus, their parents (Fortune mandarin and Ellendale tangor), and one diploid clementine tree, as reference, focusing on photosynthesis parameters, oxidative metabolism, and volatile organic compounds (VOC) in leaves. Triploid varieties appeared to be more tolerant than diploid ones to natural low temperatures, as evidenced by better photosynthetic properties (P_{net}, g_s, F_v/F_m, ETR/P_{net} ratio), without relying on a better antioxidant system. The VOC levels were not influenced by chilling temperatures; however, they were affected by the ploidy level and atypical chemotypes were found in triploid varieties, with the highest proportions of *E*-β-ocimene and linalool. Such compounds may contribute to better stress adaptation.

Keywords: polyploidy, photosynthesis, chlorophyll fluorescence, oxidative metabolism, volatile organic compounds, cold stress

INTRODUCTION

Citrus is one of the main fruit crops in the world and it has nutritional, economic, and medicinal importance. The Mediterranean region is the largest exporter of fresh market citrus fruits (FAO, 2017). This market is highly competitive, with a large volume of clementines and oranges produced in Spain, Morocco, or Italy, with a marked seasonality. In order to develop market opportunities, citrus breeding programs have focused mainly on innovative small citrus which produce seedless fruit with a different maturity period and interesting pomological, agronomical, and organoleptic traits. Alongside these new consumer preferences, climate changes have triggered the development

of new varieties and highlighted the need to assess plant adaptation to abiotic constraints. Abiotic stresses, like drought or extremes temperatures, adversely affect the quality of citrus production, yield, and growth. In this context, the selection of new citrus varieties, with traits that improve adaptation to abiotic stresses, can contribute significantly to the development of the citrus fresh fruit market. Among Mediterranean countries, France stands out for its clementine production, with 97% produced in Corsica (30,000 tons per year). The Corsican production is characterized by endemic products with a great organoleptic quality thanks to the island producers and climate, resulting in it being given protected geographical indication (PGI). The Corsican clementine is mainly produced from October to January while Corsican pomelo production begins from April to June. Thus, it would be beneficial to boost the citrus fresh fruit market by extending the production period with small innovative seedless citrus from January to April.

Triploidy could play an important role in the coming decades by improving fruit traits, biomass, and abiotic stress tolerance resulting in commercial benefits (Esen and Soost, 1971; Aleza et al., 2010; Costa et al., 2019). Most citrus species are diploid, with a basic chromosome number x = 9 (2x = 18) (Krug, 1943). However, spontaneous autotetraploid have been found among citrus cultivars, while a single triploid formation has occurred in 'Tahiti' lime (Lee, 1988). Triploidy in citrus can occur by sexual hybridization between diploid parents (Esen and Soost, 1971) or between diploid and tetraploid parents (Esen et al., 1978). Triploid hybrids arising from diploid × diploid hybridizations are produced through the formation of unreduced gametes, usually by the female parent, with a relatively low frequency which varies among different genotypes. For example, Navarro et al. (2015) showed that the 'Fortune' mandarin – when used as a female parent – produced a greater frequency of triploid hybrids per fruit than clementines. In citrus cultivars, triploid hybrids have been successfully used to produce seedless fruits (Aleza et al., 2012; Rouiss et al., 2018). Despite their desirable characteristics, such as larger fruit and higher yield (Hoshino et al., 2011), triploid plants are unusual because of their inviable seeds. Triploid citrus are heterozygous and each hybrid is unique in specific allelic combination. Thus, phenotypic traits of triploid hybrids compared to diploid hybrids may be conferred by the expression of specific alleles from parents or by ploidy. This can lead to high gene dosage, gene expression pattern ranging from additive expression from each allele to expression dominance by a single allele, to biases toward a specific parental genome (Madlung, 2013). Polyploidy in citrus is associated with an increase in stomata size and decrease in density (Allario et al., 2011). Although maximal gas exchange had been related to low stomatal size and high density (Franks and Beerling, 2009), some authors concluded that polyploidy *per se* may not necessarily have a reduced gas exchange capacity and can also increase adaptive response to stressful environmental conditions (Ramsey, 2011; Madlung, 2013). Moreover, it has been suggested that hybridization results in great genomic changes that contribute to better and faster adaptation to a novel environment (Wang et al., 2006; Chen, 2007). To our knowledge, no study has been conducted on triploid tolerance to adverse environmental conditions. Yet, the use of tetraploidy, and more generally polyploidy, appears to be a relevant alternative pathway for developing plants that are more tolerant to biotic and abiotic stresses, without impacting fruit yield and quality (Aleza et al., 2012). Several studies revealed that citrus tetraploid seedlings, or used when grafted, were more tolerant to abiotic stresses (salt stress, water deficiency, nutrient disruption, and chilling stress) than diploid ones (Saleh et al., 2008; Allario et al., 2013; Ruiz et al., 2016; Vieira et al., 2016; Oustric et al., 2019).

Among abiotic stresses, chilling stress is a critical factor for citrus fruit production. Chilling stress decreases the CO_2 assimilation and the stomatal conductance leading to disruption of photosynthesis and electron transport through the thylakoid membrane, resulting in cellular damage (Allen and Ort, 2001; Hussain et al., 2018). Indeed, the decline in photosynthesis leads to excess energy in photosystem II and I (PSII and PSI) and, consequently, to photoinhibition (Baker, 2008). The excess energy, which is not safely dissipated, can induce the overproduction of reactive oxygen species (ROS), like superoxide anion, hydroxyl radical, or hydrogen peroxide, leading to severe oxidative damage (Gill and Tuteja, 2010). Chilling stress causes a disruption in cell membrane structure that can lead to cellular electrolyte leakage (Oustric et al., 2017; Wang et al., 2017). Plants have developed a complex defense system that includes enzymes and antioxidant molecules to avoid or reduce chilling injuries. The key role of these antioxidant mechanisms that provide plants with their cold tolerance has been largely described (Santini et al., 2013; Oustric et al., 2017; Agurla et al., 2018; Hussain et al., 2018). Abiotic stresses also modify the biosynthesis and emission of volatile organic compounds (VOCs) (Loreto and Schnitzler, 2010; Peñuelas and Staudt, 2010; Vieira et al., 2016). VOCs include a wide variety of chemical compounds, with terpenes being the largest and most diverse family (Tholl, 2015). Metabolic pathways and emissions of VOCs were assumed to be temperature-dependent (Copolovici et al., 2012) but their emission significantly increases in stressed plants (Guenther et al., 2006). Some studies revealed that VOCs (sabinene, *E*-β-ocimene, linalool) are involved in biotic and abiotic stress responses including huanglongbing-associated bacterium, *Candidatus liberibacter asiaticus* (Hijaz et al., 2016), drought stress (Vieira et al., 2016), winter flooding, and salinity (Velikova et al., 2012). Some authors argued that terpenes may act as signal molecules and antioxidants (Peñuelas and Staudt, 2010; Possell and Loreto, 2013). Vickers et al. (2009) reported that terpenes can also act indirectly as membrane stabilizers reducing lipid peroxidation and, thus, the cell's oxidative state. However, to our knowledge, no study has examined the impact of ploidy combined with temperature variations on the VOC profile.

VOCs have been widely studied in several citrus species during the chemical make-up of leaf essential oils (EO) (Lota et al., 2001; Fanciullino et al., 2006; Espina et al., 2011; Santos et al., 2015). Considering all the aspects mentioned previously, triploidy could be a promising way to both improve abiotic stress tolerance and produce seedless fruits. To take into account consumer expectations, hybridization between the Fortune mandarin and the Ellendale tangor was performed several years ago and the generated hybrids were grown in an experimental orchard on

the island of Corsica. The Fortune mandarin parent was selected for its pomological traits and, used as a female, it results in a high proportion of triploid hybrids, while the Ellendale tangor parent was chosen for its organoleptic quality and its later fruit production (April).

The first aim of our study was to assess triploid varieties for the tolerance of their photosynthetic activities to chilling stress, in comparison with diploid ones obtained from the same cross. Then, we analyzed the biochemical responses of the different lines under chilling conditions in order to identify markers related to this tolerance in 3x varieties. We compared triploid and diploid hybrid citrus behavior with both parents and the diploid clementine tree. To evaluate the response of the selected varieties between a cold period and a warm one, we analyzed some properties related to their photosynthesis activity [net photosynthesis (P_{net}), stomatal conductance (g_s), electron transpiration rate (ETR), maximal quantum efficiency of PSII within dark-adapted leaves (F_v/F_m), and effective quantum efficiency of PSII within light-adapted leaves (Φ_{PSII})], their oxidative metabolism (hydrogen peroxide, malondialdehyde and antioxidant enzymes and compounds) and their changes in leaf VOC levels.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The experiment was carried out on 16-year-old diploid (2x) and triploid (3x) hybrid citrus trees. Scion hybrids were the result of hybridization between the Fortune mandarin (*Citrus reticulata Blanco*) and the Ellendale tangor [*Citrus reticulata Blanco x Citrus sinensis (L) Osb.]*. Diploid and triploid scions were grafted onto C-35 Citrange rootstocks (*Citrus sinensis 'Ruby Blood' x Poncirus trifoliata)*. C-35 was chosen for its tolerance to biotic (Tristeza, phytophtora) and abiotic (cold, drought) stress. C-35 seedlings used for the experiment were strictly chosen in the nursery to eliminate off-types. All trees were grown in an experimental orchard with the same South orientation and a similar height above ground. The orchard is located in San Giuliano (INRA-CIRAD), Corsica, France (42°17′05″ N, 9°31′26″ E) and is composed of 40 triploid hybrids and 40 diploid hybrids. In order to select genotypes with contrasting behavior within this population, these 80 genotypes were screened first using malondialdehyde (MDA) as a marker of stress tolerance in leaves sampled in September 2017 (see **Supplementary Figure S1**) in which the minimal and maximal mean temperatures of the month were 17.6°C and 26.6°C, respectively. Based on this screen, 2x and 3x hybrids with the highest MDA values (D1-2x, T1-3x, and T3-3x) and others with the lowest values (D39-2x, T38-3x, and T40-3x) were selected for further experiments. In addition, both parents (Fortune mandarin and Ellendale tangor) and a common clementine (*Citrus clementina* Hort. Ex Tan; SRA 92) were included in the experimental plot. Using the nine selected genotypes, physiological measurements and samplings were carried out in February 2018 (cold period) and in September 2018 (warm period) to decipher the impact of cold on trait tolerance. To that aim, we focused on the coldest and the hottest sunny days during these two periods. Meteorological data were collected throughout the sampling period (**Table 1**).

Gas Exchange and Chlorophyll Fluorescence Measurements

The main photosynthetic traits were measured on the same leaves. Twelve fully expanded mature leaves per tree were used (12 independent biological replicates). Fully expanded leaves were selected on 1-year old branches subjected to the same light exposure (South). Each parameter was measured between 9 am and 11 am.

Net photosynthesis rate (P_{net}) and stomatal conductance (g_s) were measured using an LC-PRO-SD portable infra-red gas analyzer (ADC, BioScientific Ltd., Hoddeston, United Kingdom). During the experiment, photosynthetically active radiation (PAR) was applied at the leaf surface and fixed at 1400 $\mu mol.m^{-2}.s^{-1}$ (Santini et al., 2013; Oustric et al., 2017; Ruiz et al., 2018). Leaf temperature was set at 25°C and ambient carbon dioxide concentration (CO_2) was used (390 $\mu mol.\ mol^{-1}$).

The maximum quantum efficiency of PSII (F_v/F_m), the effective quantum yield of PSII (Φ_{PSII}), and the electron transport rate (ETR) were monitored using an OS1p chlorophyll fluorimeter (Opti-Sciences, Inc. Hudson, United States). F_v/F_m was monitored on dark-adapted leaves using clips through the thylakoid membrane for 30 min (Oustric et al., 2017). For the fluorescence measurements in the light, the fluorimeter was equipped with an open leaf-clip suitable for measurements on light-adapted leaves. Φ_{PSII} was evaluated as described by Genty et al. (1989) and ETR (also known as J) was expressed according to Krall and Edwards (1992).

Biochemical Assays

Four samples were collected for each genotype and for each period (four independent biological replicates). Each biochemical sample was obtained by pooling 15 fully expanded leaves selected from one individual tree for each variety and for each

TABLE 1 | Meteorological data of cold and warm periods at the experimental plot.

Sampling period	Sampling day			Sampling month		
	Minimum temperature (°C)	Maximal temperature (°C)	Mean Temperature (°C)	Minimum temperature (°C)	Maximal temperature (°C)	Mean temperature (°C)
Cold period	0.1	7.0	2.3	5.7	12.8	7.3
Warm period	21.6	30.5	25.0	19.8	28.5	24.2

period. Harvested samples were immediately immersed in liquid nitrogen, ground to a fine powder, and then stored at −80°C.

Malondialdehyde content was assayed according to Hodges et al. (1999) and adapted to citrus samples as described by Santini et al. (2013). Eighty milligrams of leaf powder were homogenized in 2 mL of 80% ethanol (v/v). Homogenates were centrifuged at 3000 × g at 4°C for 10 min. Absorbance was determined at 440, 535, and 600 nm against a blank.

Measurements of hydrogen peroxide content were carried out using a PeroxiDetect Kit (Sigma Aldrich, St. Louis, MO, United States) according to Jiang et al. (1991). The reaction is based on the oxidization of Fe^{2+} to Fe^{3+} ions by hydrogen peroxide in aqueous solutions with an acidic pH. Leaf powder (150 mg) was homogenized in 300 μL of distilled water and centrifuged at 21,000 × g for 15 min at 4°C. Twenty μL of homogenates were added to each well of a 96-well microplate. A working color reagent was prepared by mixing 1 volume of 25 mM ammonium sulfate in 2.5 M sulfuric acid with 100 volumes of 125 μM xylenol orange (Sigma-Aldrich) in 100 mM sorbitol. A sample of 200 μL of the working color reagent was added to each well, and then the microplate was incubated for 1 h at room temperature. Absorbance was read at 560 nm with a microplate reader (MULTISKAN FC™, Thermo Scientific, Waltham, MA, United States). The hydrogen peroxide concentration was determined from a standard curve.

Ascorbic acid content was determined as described by Stevens et al. (2008). Leaf powder (150 mg) was homogenized in 600 μL of 6% ice-cold trichloroacetic acid (w/v). Homogenates were centrifuged at 13,000 × g at 4°C for 15 min. Absorbance was read at 550 nm with a microplate reader (MULTISKAN FC™, Thermo Scientific, Waltham, MA, United States). Total and reduced ascorbic acid content were determined using a standard curve.

Proline content was assayed according to Carillo and Gibon (2011). Forty milligrams of leaf powder were homogenized in 70% ethanol (v/v). Homogenates were centrifuged at 15,000 × *g* at 4°C for 15 min. The absorbance was read at 520 nm with a microplate reader (MULTISKAN FC™, Thermo Scientific, Waltham, MA, United States). Proline content was determined using a standard curve.

For antioxidant enzymatic activities, leaf powder (54 mg) was homogenized in 2 mL of extraction buffer (0.1 M potassium phosphate, pH 7.5) and the homogenates were centrifuged at 13,000 × *g* for 30 min at 4°C. The supernatant was collected and was used for all enzymatic assays and for protein determination (Bradford, 1976). Superoxide dismutase, catalase, ascorbate peroxidase, and dehydroascorbate reductase assays were performed as described by Santini et al. (2013). Time-course measurements were monitored using a V-630 spectrophotometer (Jasco Inc., Tokyo, Japan).

Leaf Essential Oils Extraction and Terpenes Analysis

One hundred grams of fully expanded leaves per genotype (three independent biological replicates) with the same southern exposure were harvested on each sampling day and then quickly hydrodistilled. EOs were extracted from fresh leaves by hydrodistillation and then were analyzed using gas chromatography with flame ionization detector (GC-FID) and gas chromatography/mass spectrometry (GC/MS).

Gas chromatography analysis was performed on a PerkinElmer Clarus 500 gas chromatograph (FID) equipped with two fused silica gel capillary columns (50 m × 0.22 mm, film thickness 0.25 μm), BP-1 (polydimethylsiloxane), and BP-20 (polyethylene glycol). The oven temperature was programmed to increase from 60 to 220°C at 2°C/min and then held at 220°C for 20 min. Injector temperature and detector temperature were set at 250°C with split of 1/60. Hydrogen (0.8 mL/min) was used as carrier gas. The relative proportions of the oil constituents were expressed as percentages obtained by peak area normalization, without using correcting factors. Retention indices (RIs) were determined relative to the retention times of a series of *n*-alkanes with linear interpolation ('Target Compounds' software from PerkinElmer).

For mass spectrometry, the EOs were analyzed with a PerkinElmer TurboMass detector (quadrupole), directly coupled to a PerkinElmer Autosystem XL, equipped with a fused silica gel capillary column (50 m × 0.22 mm i.d., film thickness 0.25 μm), BP-1 (dimethylpolysiloxane). The oven temperature was programmed to increase from 60 to 220°C at 2°C/min and then held at 220°C for 20 min. Injector temperature and detector temperature were set at 250°C with split of 1/60. Helium (0.8 mL/min) was used as carrier gas. The ion source temperature of the mass spectrometer was set at 250°C with an ionization energy of 70 eV. Electron ionization mass spectra were acquired over the mass range 40–400 Da.

Statistical Analysis

Data were expressed as mean values ± SE and analyzed with R statistical software[1]. Two-way ANOVAs and multiple mean comparisons were carried out with the least significant difference (LSD) test at $P < 0.05$. Data were normalized and used in principal component analysis (PCA) and hierarchical clustering classification using FactomineR package (Le et al., 2008). PCA was conducted to define a relationship between physiological, biochemical, and chemical parameters and genotypes during the cold and warm period. PCA and hierarchical clustering classification helps us to better understand the similarity between variables and individuals. For chemical data, cluster analysis was conducted using Ward's method in normalized data to obtain a hierarchical distribution of varieties and optimal number of clusters.

RESULTS

Warm Period *vs*. Cold Period

Principal component analysis was performed with the parameters collected during cold and warm periods for the nine selected varieties to highlight the potential differences between both periods (**Figure 1**). The first two components explained 49.83%

[1]http://www.R-project.org

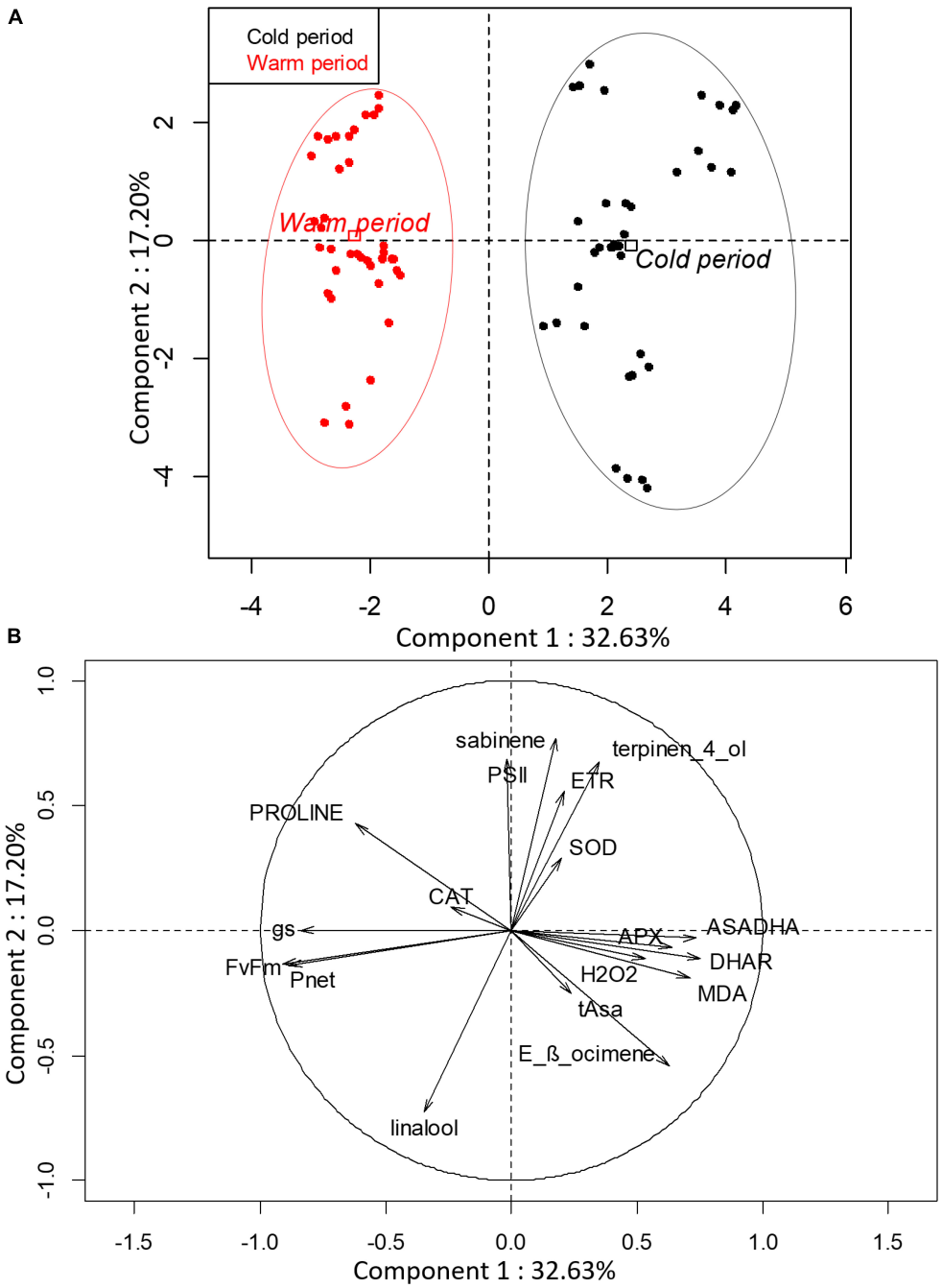

FIGURE 1 | Biplot obtained from PCA performed on leaves of nine citrus varieties during cold and warm period. **(A)** Repartition of varieties on the two first axes and **(B)** contribution of investigated parameters to the two first axes of PCA. (PSII: Φ_{PSII}).

of the total variance of the population. Component 1 was positively correlated with oxidative metabolism parameters (Asa/DHA, DHAR, APX, MDA, and H_2O_2) and negatively correlated with photosynthesis parameters (F_v/F_m, g_s and P_{net}) and proline content. Component 2 was positively correlated with Φ_{PSII}, ETR, SOD, sabinene, and terpinen-4-ol, and negatively correlated with linalool and *E*-β-ocimene. Interestingly, the oxidative markers (H_2O_2 and MDA) were negatively correlated with proline content. PCA clearly splits varieties into two distinct groups according to the cold and warm period.

Changes in Photosynthetic Parameters

During the warm period, all varieties had very high net photosynthesis rates (P_{net}) and stomatal conductance (g_s) (**Figure 2**).

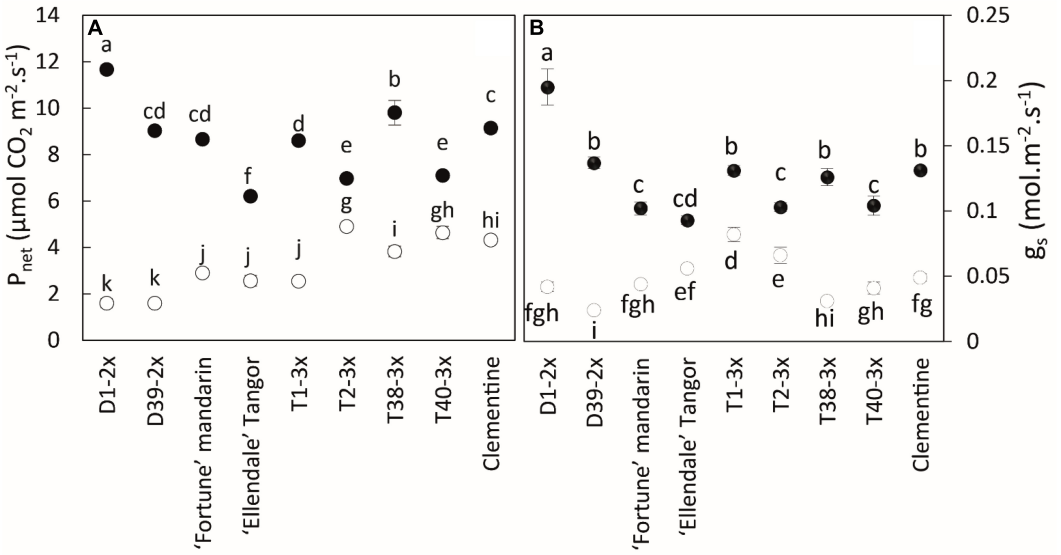

FIGURE 2 | Comparison of **(A)** net photosynthetic rate (P_{net}), **(B)** stomatal conductance (g_s) between nine Citrus varieties during warm period (black point) and cold period (white point). All data are mean values (±SE) of 12 independent biological replicates for each genotype (n = 12). Data were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Different lowercase letters indicate significant differences between the varieties and the sampling periods.

During the cold period, all varieties had lower values of P_{net}, g_s, and F_v/F_m, although the triploid varieties were less affected by chilling conditions than the diploid ones (**Figures 2**, **3A**). T2-3x had high rates of P_{net} and g_s during the cold period while the lowest values were found in the diploid varieties (D2-2x, D39-2x and parents), except for clementine. The highest values of F_v/F_m (**Figure 3A**) were also found in T2-3x while the diploid hybrids had the lowest values. No significant difference was found between clementine, both parents, and some triploid varieties (T1-3x and T38-3x).

During the cold period, the highest values of Φ_{PSII} were found in T1-3x and some diploid varieties (D1-2x, D39-2x) (**Figure 3B**). ETR was lower in some triploid varieties (T2-3x, T38-3x, and T40-3x) during the cold period (**Figure 3C**). The highest value was found in Ellendale tangor, no matter the period. During the cold period, clementine had the lowest value for Φ_{PSII} and ETR.

As a whole during the cold period, triploid varieties, except T38-3x, and clementine had the lowest ETR/P_{net} values (∼4) (**Figure 3D**).

Differences in Oxidative Metabolism

H_2O_2 and MDA contents were higher during the cold period in all varieties (**Figure 4**). Significant differences were found between diploid and triploid varieties, but there were also contrasting values in triploid varieties. T38-3x had the lowest values for both parameters during the cold period.

During the cold period, total ascorbate (tAsa) was increased in all varieties except for D1-2x (**Figure 5A**). The content was high in Fortune mandarin, clementine, and T38-3x while no significant difference was found between the other diploid and triploid varieties. Increased values of Asa/DHA ratio were observed during the cold period (**Figure 5B**).

Low temperatures induced a significant decrease in proline content except for Ellendale tangor, in which no significant variation was observed between both periods (**Figure 5C**).

Overall, low temperatures increased the activity of antioxidant enzymes in all genotypes (**Figure 6**). No clear difference was found between triploid and other genotypes, whereas significant differences were found between some lines. The components of antioxidant systems were highly variable between the different varieties. Diploid and triploid lines could not be distinguished on these parameters.

PCA in Leaves of Citrus Varieties Under Chilling Temperatures

To understand the differences between diploid and triploid lines during the cold period, PCA was performed with all parameters analyzed under this stressful condition (**Figure 7**). The first two principal components described 54.93% of the total variance in the population. Genotypes had clear differences in response to natural low temperatures. Antioxidant enzymes (SOD) and chlorophyll fluorescence parameters (ETR and Φ_{PSII}) were the main parameters included in component 1. This component was also positively correlated with antioxidant molecules (Asa/DHA ratio and proline) and negatively correlated with P_{net} and tAsa content. Component 2 was positively correlated with oxidative markers (MDA and H_2O_2) while it was negatively correlated with APX and g_s (**Figure 7B**). Four clusters were clearly identified (**Figure 7A**). Component 1 separated cluster 1 (Fortune mandarin, T1-3X, T2-3X, T38-3x, and T40-3x) from cluster 2 (Ellendale tangor and D39-2x) and 3 (D1-2x), while component 2 separated cluster 1 and 2 from cluster 3 and 4 (clementine). Thus, Fortune mandarin and the four triploid hybrids were differentiated from diploid

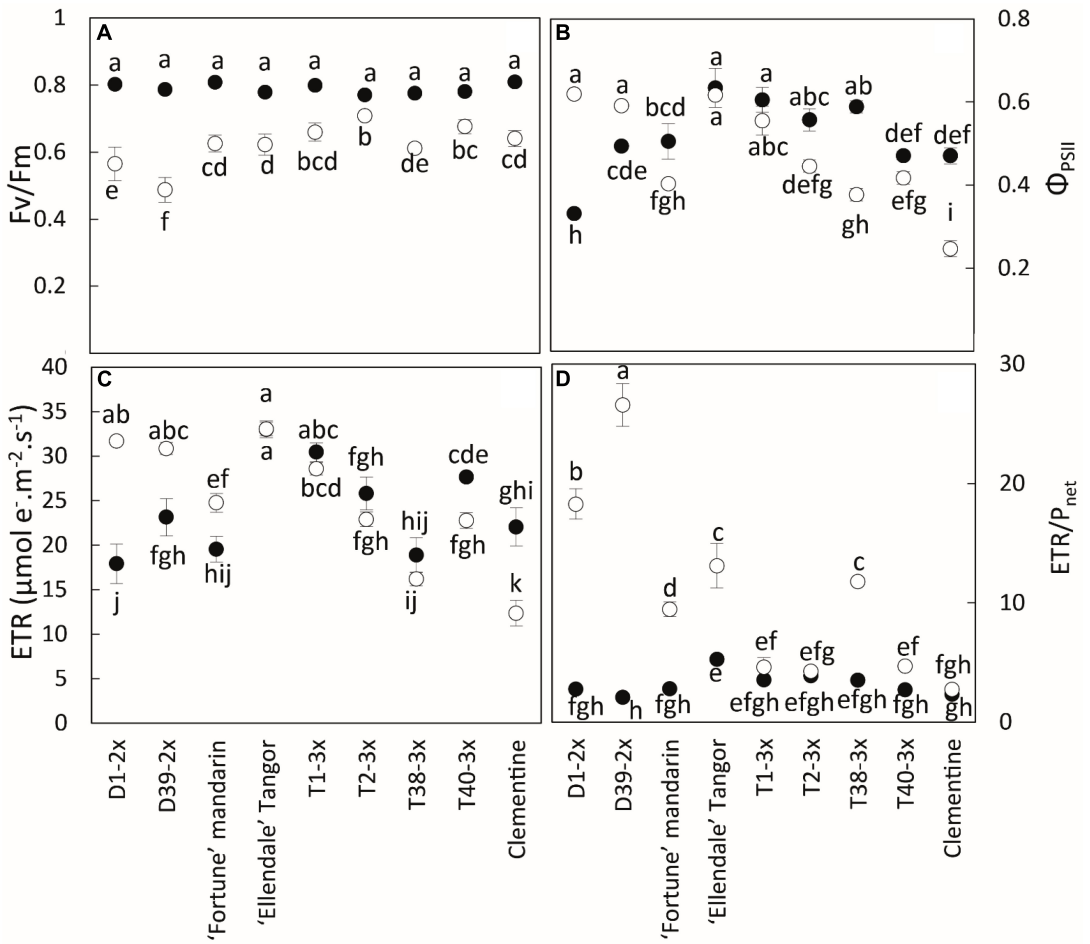

FIGURE 3 | Comparison of **(A)** chlorophyll fluorescence (F_v/F_m), **(B)** quantum yield of electron transfer at PSII (Φ_{PSII}), **(C)** electron transport rate (ETR), and **(D)** ETR/P_{net} ratio between nine citrus varieties during warm period (black point) and cold period (white point). All data are mean values (±SE) of 12 independent biological replicates for each genotype (n = 12). Data were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Different lowercase letters indicate significant difference between the varieties and the sampling periods.

varieties by their enhanced photosynthetic capacities (high P_{net}, g_s and F_v/F_m ratio). Triploid hybrids were also characterized by enhanced antioxidant enzymatic defenses (APX, DHAR and CAT) and higher tAsa content than diploid hybrids (clusters 2 and 3). Interestingly, D1-2x (cluster 3) was differentiated from Clementine (cluster 4) by higher SOD activity, ETR, and Φ_{PSII}, while both clusters had increased oxidative marker accumulation.

Differences in Leaf EO Composition in Citrus Varieties

Oil samples were submitted to GC-FID and GC/MS analyses. In total, 59 compounds were identified (**Supplementary Table S1**), including various monoterpenes accounting for 97.2–100% of the total EO chemical composition. Samples from the nine varieties harvested in both periods were submitted to statistical analyses along with 15 terpenes that exhibited a mean value higher than 0.1%. The data obtained for the EO chemical composition was submitted to a centered and scaled PCA. The coordinates of the individuals were analyzed by discriminant analysis in order to identify a structure among the genotypes based on their chemical composition. Hierarchical clustering dendrogram (**Figure 8A**) suggested the existence of two major groups (**Supplementary Figure S2**). These two main groups can each be subdivided into two sub-groups resulting in a total of four clusters based on the number of major components (**Figure 8**). Group I was characterized by 3x varieties except T1-3x while group II contained both parents, 2x varieties, clementine, and T1-3x variety.

To confirm the cluster analysis, the major compounds found in the varieties were included in a PCA analysis associated to the cluster analysis (**Figure 8A**). Four compounds were identified as being the most discriminant based on their contribution on the two discriminant axes: linalool, *E*-β-ocimene, sabinene, and terpinen-4-ol. Thus, PCA analysis representing 81.28% of the data variance showed four discriminant clusters (**Figure 8A**). The first axis separated the two principal groups defined by the hierarchical clustering: group 1 (composed of clusters 1 and 2)

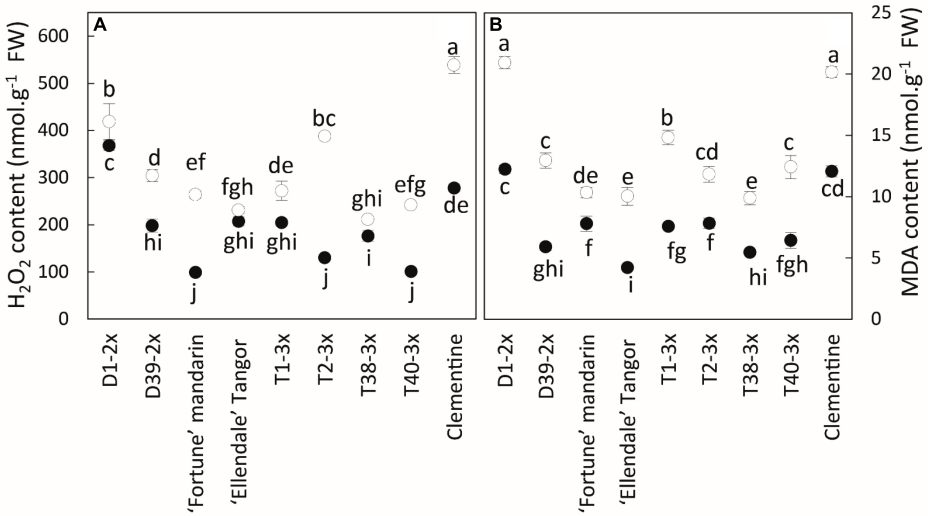

FIGURE 4 | Comparison of **(A)** hydrogen peroxide (H_2O_2) and **(B)** malondialdehyde (MDA) contents between nine citrus varieties during warm period (black point) and cold period (white point). All data are mean values (±SE) of 4 independent biological replicates for each genotype (n = 4) obtained by pooling 15 fully expanded leaves. Data were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Different lowercase letters indicate significant differences between the varieties and the sampling period.

from group 2 (composed of clusters 3 and 4). The second axis separated the two principal groups into two sub-groups (cluster 1 *vs*. 2 and 3 *vs*. 4, respectively). No clear differences were observed between cold and warm periods, but discriminant analysis revealed differences between varieties. Cluster 1 was composed of only one triploid hybrid (T40-3x) and was characterized by an *E*-β-ocimene/linalool chemotype, exhibiting the highest amount of linalool (54.7%) and the lowest amount of sabinene (1.3%) which distinguished it from other clusters (**Figure 8B**). Cluster 2 was characterized by a linalool/sabinene chemotype while clusters 3 and 4 were characterized by a sabinene/linalool chemotype. No specific compounds discriminated the other clusters, but their overall chemical profiles differed with the proportion of major compounds. Cluster 2 contained T2-3x and T38-3x (warm period) and was characterized by a high amount of linalool (40.5%). A greater proportion of sabinene was found in clusters 3 and 4 (39.4 and 49.4% respectively). These clusters contained 2x varieties, both parents. T1-3x and T38-3x (cold period) were also found in these clusters.

DISCUSSION

Comparison of Photosynthetic Responses to Natural Chilling Conditions Between 3x and 2x Varieties

The cold period was characterized by high levels of oxidative stress, antioxidant defenses, and lower photosynthetic activity. It has been reported that stomatal closure, illustrated by the decline in g_s, was the first step followed by a decrease in Rubisco activity and then reduction of photosynthetic rate (Baker et al., 2007; Chaves et al., 2009). The higher values of P_{net} and g_s in 3x varieties argue for their lower sensitivity to low temperatures than 2x varieties. Polyploidization is known to increase stomatal size and decrease stomatal density (Masterson, 1994). Although Franks and Beerling (2009) showed that great density and small stomata were the only way to obtain the highest g_s maximum values, our results suggested that polyploidy may not necessarily induce reduced gas exchange and could be a good alternative to limiting the decrease in gas exchange, and thus, enhance tolerance to abiotic stresses. The chlorophyll fluorescence parameters provide insights on the photosynthesis properties under environmental constraints. Under optimal conditions, the F_v/F_m ratio reaches values about 0.83 in most C3 plants, as do our citrus trees during the warm period (Urban et al., 2017). The decrease in F_v/F_m we observed during the cold period is usually interpreted as being related to photo-inhibition (Genty et al., 1989). However, this decrease can also be due to the development of slowly relaxing quenching process, also known as non-photochemical quenching (Baker and Rosenqvist, 2004). Adams et al. (1995) also argue that induction of the photoprotective energy dissipation process can quite possibly account for the photoinhibition observed during winter stress. Thus, a decrease in F_v/F_m could be also interpreted in terms of a mechanism of photoprotection (Urban et al., 2017). Recently, several studies have monitored other indicators of photosynthetic performance, such as Φ_{PSII} or electron transport rate (ETR), providing rapid information on PSII operating efficiency. Indeed, these parameters depend both on the efficiency of the absorbed energy donated to reaction centers and the rate of open reaction centers undergoing charge separation (Krall and Edwards, 1992; Baker et al., 2007). The natural chilling conditions (**Figure 3A**) seem to induce photo-inhibition of the photosynthetic apparatus (Baker et al., 2007), as expected (Allen and Ort, 2001; Santini et al., 2013; Oustric et al., 2017), but the 3x varieties would be less affected. Unfortunately, there is

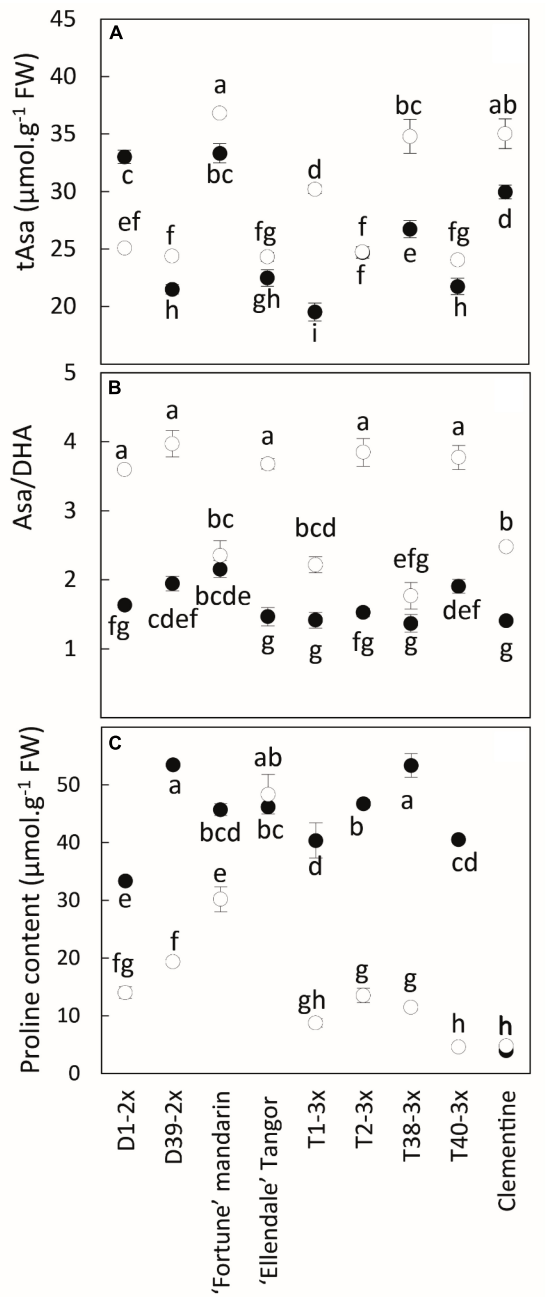

FIGURE 5 | Comparison of **(A)** total ascorbate content (tAsa), **(B)** redox status (Asa/DHA) and **(C)** proline content between nine citrus varieties during warm period (black point) and cold period (white point). All data are mean values (±SE) of 4 independent biological replicates for each genotype (n = 4) obtained by pooling 15 fully expanded leaves. Data were analyzed using ANOVA and Fisher LSD tests. Different lowercase letters indicate significant differences between the varieties and the sampling periods.

little information regarding the adaptation of 3x genotypes to abiotic stresses (Lu et al., 2009) while the better tolerance of tetraploid genotypes has often been observed, particularly among Citrus species (Hussain et al., 2012; Ruiz et al., 2016; Vieira et al., 2016; Oustric et al., 2019). Oustric et al. (2017) reported that the common clementine grafted on tetraploid rootstock had better photosynthetic capacity during natural chilling stress. Allario et al. (2013) concluded that tetraploid Rangpur lime rootstock conferred increased photosynthetic capacity to the diploid sweet orange scion when subjected to water deficit. In our study, 3x hybrids and clementine had different photochemical behaviors in response to low temperatures. The slow decline in Φ_{PSII} and ETR associated with a weaker photo-inhibition suggests that electron flux through PSII was probably less affected. Triploid hybrids (except T1-3x) and clementine lose their excess energy as heat instead of being used to drive photosynthesis (Ribeiro et al., 2009). The consistent ETR/P_{net} ratio (close to the reference value ∼4) in 3x hybrids may suggest photoprotective energy dissipation activity by heat associated to NPQ (Yamori, 2016). Moreover, Azzabi et al. (2012) reported that thermal dissipation within photosystems acts as a safety strategy for cutting down on excess light energy and limiting ROS generation. Machado et al. (2013) also argued that NPQ increase was effective at preventing excessive energy pressure at PSII in Valencia sweet orange grafted on Swingle citrumelo subjected to night-time chilling temperatures. Some studies reported a negative correlation between Φ_{PSII} and NPQ. Thus, a decrease in Φ_{PSII} could mean an increase in thermal dissipation (Maxwell and Johnson, 2000; Ribeiro et al., 2009). The NPQ mechanism is characterized by the pH-dependent xanthophyll cycle which provides effective protection of reaction centers at low temperatures and high light (Baker et al., 2007). High values of Φ_{PSII} and ETR associated with the sharp decline in F_v/F_m in 2x hybrids suggest an involvement of electron sinks other than CO_2. ETR/P_{net} has been largely recognized as an indicator of increased photorespiration (Flexas et al., 1999; Ribeiro et al., 2009; Machado et al., 2013). The high values of ETR/P_{net} (more than the theoretical value ∼4) for 2x hybrids support the contribution of alternative pathways. According to Baker et al. (2007), the proportion of electrons driven to the alternative pathway increases under stress conditions. Thus, photorespiration and the Mehler reaction are the main alternative sinks during environmental constraints (Fryer et al., 1998; Allen and Ort, 2001). Since photorespiration is sensitive to low temperatures, the Mehler reaction would be the main way to dissipate excess energy (Fryer et al., 1998; Flexas et al., 1999). Increases in alternative electron sinks have already been reported in 2x citrus (Medina et al., 2002; Ribeiro et al., 2009; Machado et al., 2010; Santos et al., 2011). Machado et al. (2013) have found a decline in photosynthetic parameters (P_{net}, g_s, Φ_{PSII}) associated with an increase in ETR/P_{net} ratio in Valencia sweet orange scion, under cold conditions. The alternative pathways would not be sufficient for preventing photo-inhibition in 2x hybrids. The involvement of O_2-consuming processes induced ROS production, followed by a higher up-regulation of antioxidant mechanisms.

Relevant Traits Discriminating 2x and 3x Varieties for Chilling Sensitivity

The 3x varieties and their parent Fortune mandarin appear to have less marked photo-inhibition, as supported by their great

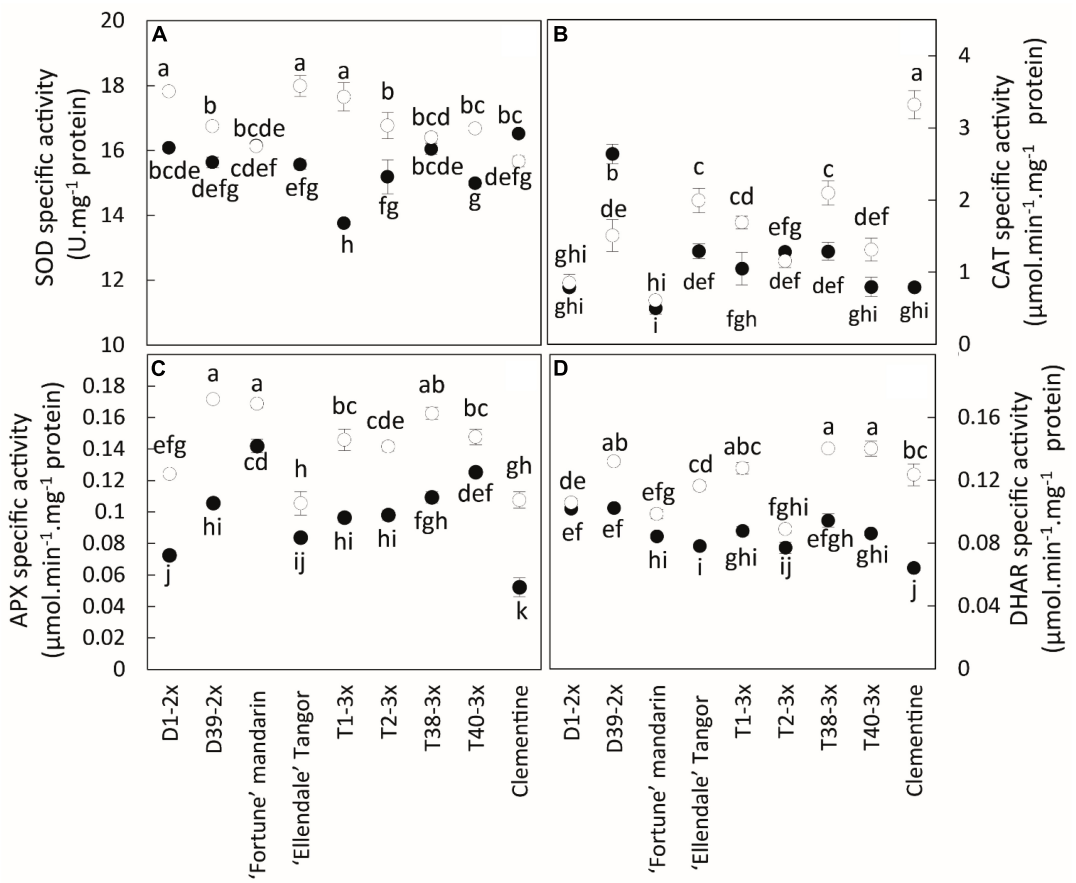

FIGURE 6 | Comparison of antioxidant activities between the nine citrus varieties during warm period (black point) and cold period (white point). The specific activities were assayed for **(A)** SOD, **(B)** CAT, **(C)** APX, and **(D)** DHAR. Data are mean values ± (SE) of 4 independent biological replicates for each genotype (n = 4) obtained by pooling 15 fully expanded leaves. Data were analyzed using ANOVA and Fisher LSD testes (P < 0.05). Different lowercase letters indicate significant differences between the varieties and the sampling periods.

photosynthetic parameters (P_{net}, g_s and F_v/F_m) (**Figure 7B**). The ROS induced by photoprotective processes can be scavenged by effective antioxidant defenses. In our study, specific activities of SOD, APX, and DHAR were enhanced at low temperatures, indicating that an effective antioxidant response is implemented to limit oxidative damage (**Figure 6**). The greater SOD and APX activities under low temperatures contribute to protecting the cells by removing superoxide anion and H_2O_2 (Mittler, 2002). High SOD and APX specific activities were also found in clementine grafted in tetraploid citrus seedlings under natural chilling stress (Oustric et al., 2019) and in citrumelo and Rangpur lime citrus rootstock grafted with sweet orange during low temperatures (Machado et al., 2013). The increased DHAR activity may be helpful for ascorbate regeneration resulting from an increase in the Asa/DHA ratio in response to low temperatures. The differences in antioxidants between 3x varieties suggest different responses between the lines and may be related to the subcellular location of the enzymes. Identifying the respective enzyme isoforms will provide information on their location and, thus, on the antioxidant mechanism. The high total ascorbate (tAsa) content in certain triploid lines, mandarin Fortune, and clementine (**Figure 5A**) evidence *de novo* ascorbate synthesis under natural low temperatures, in addition to the regeneration of reduced ascorbate by DHAR. Previous studies have reported that ascorbate can play a significant role in thermal dissipation of excess energy, acting as a co-factor for violaxanthin de-epoxidase to produce zeaxanthin (Saga et al., 2010; Smirnoff, 2018). Jahns et al. (2009) also argued that the increase in ascorbate results in an increase in NPQ and zeaxanthin formation. Therefore, the ascorbate would help in heat dissipation and, thus, in limiting excess electron flux at PSII in 3x lines (**Figures 3B,D**).

Cluster 2 contained D39-2x and its parent Ellendale tangor while the D1-2x variety belonged to cluster 3. Both clusters were differentiated from the other clusters mainly by ETR and Φ_{PSII}. Associated with low values in photosynthetic parameters, it suggests the involvement of an alternative electron sink leading to ROS generation, such as superoxide anion (Genty et al., 1989; Baker, 2008). Consistent with these data, low temperatures induced accumulation of H_2O_2 (**Figure 4A**) and increased SOD activity (**Figure 6A**). Cluster 3 was also differentiated from cluster 2 by high oxidative damage (**Figure 7**), indicating that D1-2x

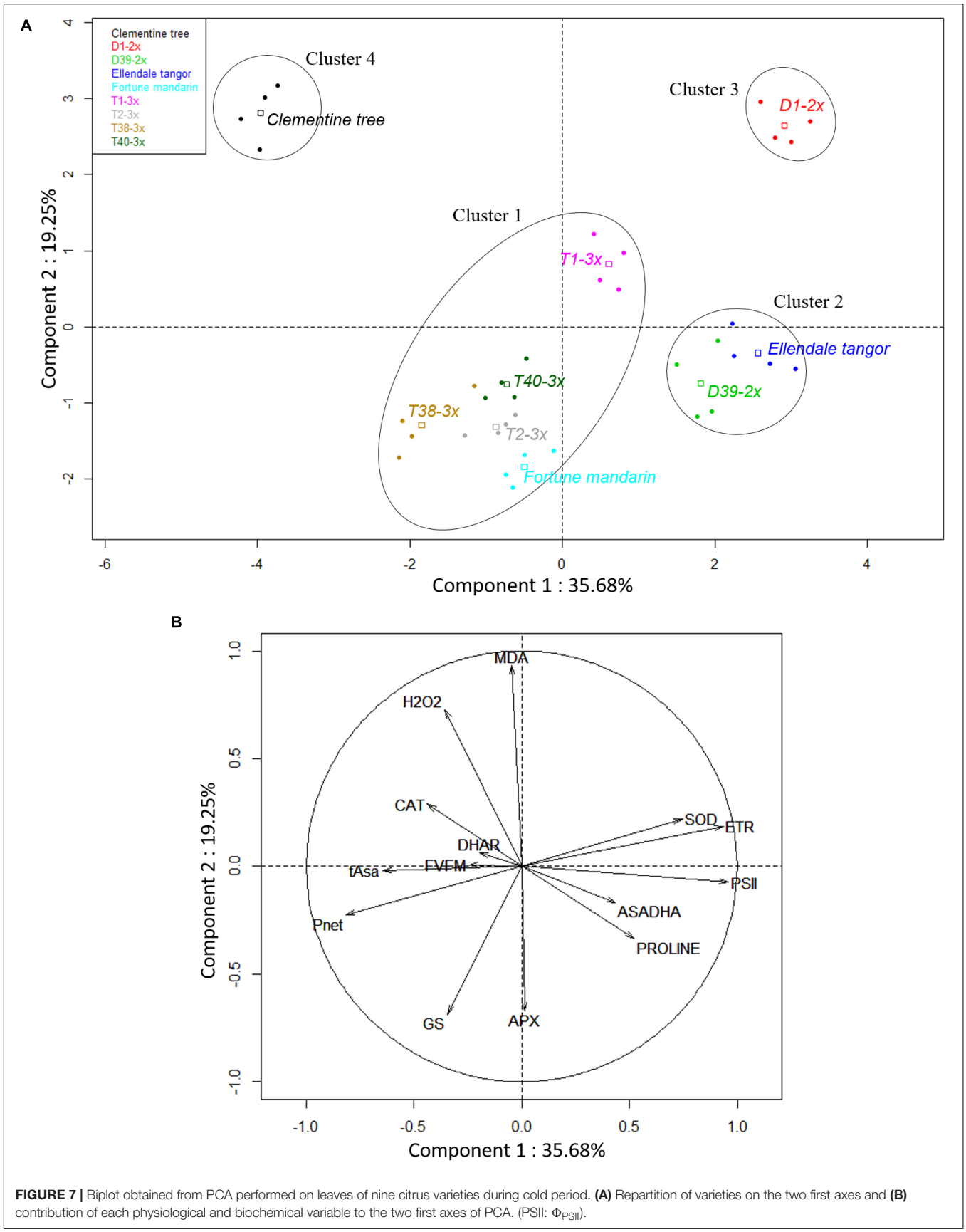

FIGURE 7 | Biplot obtained from PCA performed on leaves of nine citrus varieties during cold period. **(A)** Repartition of varieties on the two first axes and **(B)** contribution of each physiological and biochemical variable to the two first axes of PCA. (PSII: Φ_{PSII}).

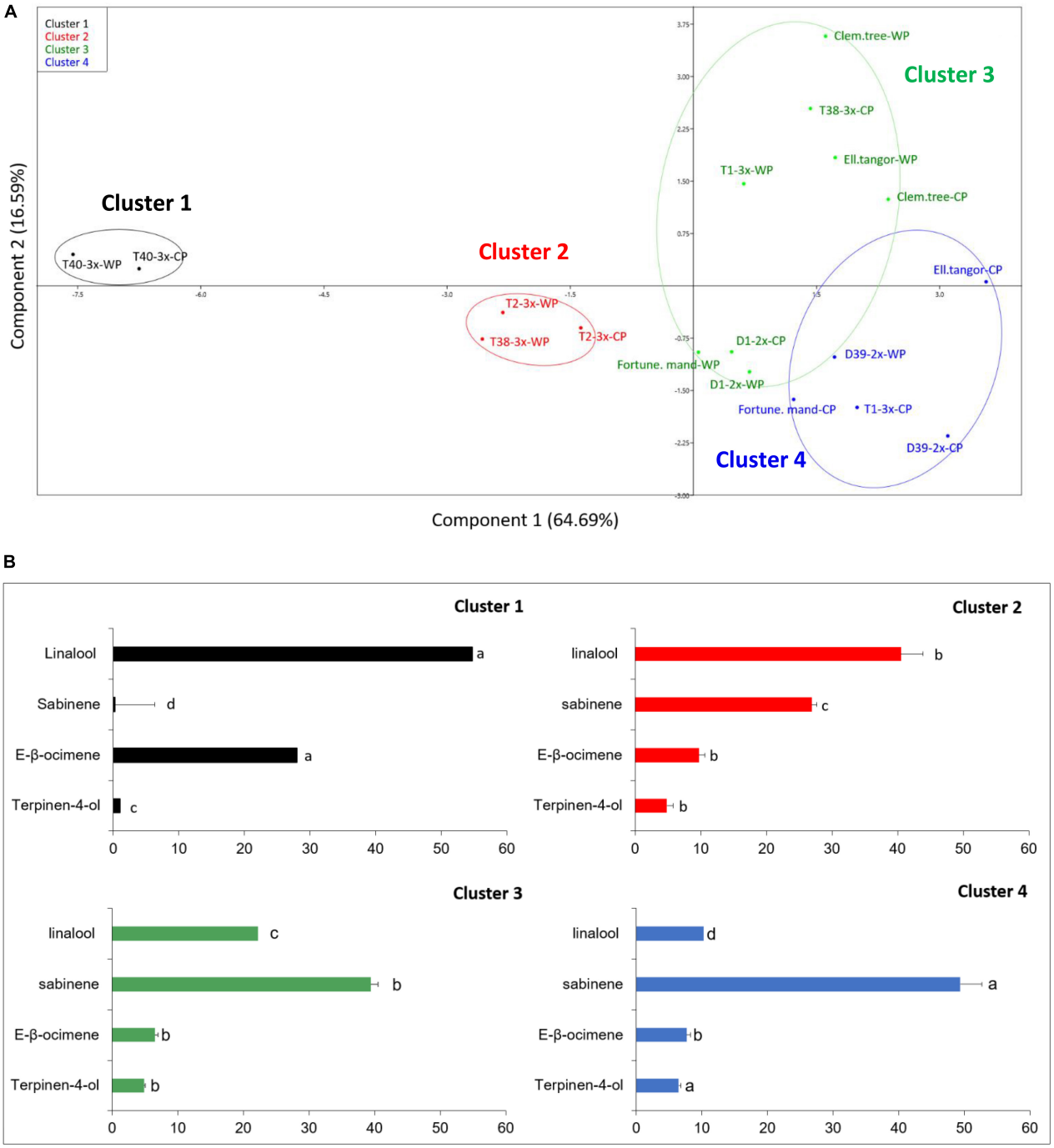

FIGURE 8 | (A) Principal component analysis based on the chemical composition of leaves of nine citrus varieties during cold period (CP) and warm period (WP). **(B)** Comparison of chemotype from each cluster. Data are percentage of the total major compounds levels obtained from 3 independent biological replicates for each genotype (n = 3). The relative percentage of four major compounds was presented for each cluster. Different lowercase letters indicate significant differences between the clusters for each major compound.

was more sensitive to chilling temperatures than D39-2x. These findings were consistent with the previous ranking by MDA levels (**Supplementary Figure S1**).

Clementine (cluster 4) exhibited contrasting behaviors compared to the other varieties. It was differentiated by higher oxidative marker accumulation (H_2O_2 and MDA) than clusters 1 and 2 (**Figure 7A**). Despite the higher photosynthetic rate and CAT and DHAR activities, it appeared to be more sensitive to low temperatures than 3x hybrids (**Figure 4**). Some studies have already reported that cold temperatures

disturb photosynthesis performance and increase oxidative damage (Machado et al., 2013; Santini et al., 2013; Oustric et al., 2019). Proline accumulates in cell plants in response to many environmental stresses, like osmotic stress and drought. Some studies have reported that proline accumulation was associated with H_2O_2, MDA decline, or lipid peroxidation (de Campos et al., 2011). This negative correlation was also observed in our study (**Figure 1**). Our results showed that triploidy enhance photosynthetic activities likely by limiting photo-inhibition, whereas the oxidative metabolism would not be sufficient to clearly discriminate triploid from diploid lines under stressful conditions. The complexity of oxidative metabolism and its cellular compartmentalization hinders the understanding of its contribution under stress conditions.

Citrus species are a major source of VOCs and their variations are mostly temperature dependent (Fares et al., 2012). However, in our orchard conditions, the chemical composition of the varieties was not significantly different between the study periods. In plants, VOCs are involved in various functions, such as defense and reproduction. Few studies reported changes in VOC content in Citrus varieties as a consequence of environmental stresses. Some terpenes (sabinene, linalool, *E*-β-ocimene) were shown to be involved in plant adaptation to drought (Vieira et al., 2016), winter flooding, and salinity (Velikova et al., 2012). Loreto et al. (1998) described the protective roles of VOCs, especially mono- and sesquiterpenes (sabinene, *E*-β-ocimene), under heat stress. Thus, while β-ocimene appeared to be insensitive to high temperatures, increased emission in other monoterpenes such as sabinene were reported in response to high temperatures. The authors also suggested a relationship between monoterpene emission, photosynthetic performance, and oxidative stress. It was discovered that monoterpene fumigation stimulates photosynthesis and decreases ROS levels and cell damage (Loreto and Schnitzler, 2010). Monoterpene emission had been observed to vary under other abiotic stresses. For example, inhibition of linalool emission was reported during drought stress (Gouinguené and Turlings, 2002).

The chemical composition of leaf EOs have already been studied in several citrus species, underlying a great variability among the citrus cultivars (Lota et al., 2001; Fanciullino et al., 2006; Espina et al., 2011; Santos et al., 2015). The most common chemotypes were sabinene/linalool and γ-terpinene/linalool (Lota et al., 2001). The varieties we studied can be distinguished into four clusters for leaf EO (**Figure 8A**). It is interesting to note the T40-3x variety had the greatest proportion of *E*-β-ocimene among all leaf EOs of the Citrus species studied (Lota et al., 2001), confirming the wide diversity of VOCs profiles among Citrus species. Chen et al. (2008) revealed that β-ocimene emission was also stimulated under oxidative stress induced by nitrogen deficiency. Even if no variation of *E*-β-ocimene was found for the T40-3x variety between warm and cold periods, we assume that its high content could help to limit oxidative stress as indicated by the low cellular damage in this variety (**Figure 4**). The large linalool proportion found in 3x varieties (**Figure 8B**) was previously found in mandarin leaf EOs from Citrus *reticulata* Blanco (Lota et al., 2001). Overall, our results suggested the VOC profile was mainly influenced by the ploidy level.

More globally, it is interesting to note that up or downregulation of interest genes that occur only in polyploid lines could be beneficial for its stress tolerance. For example, Del Pozo and Ramirez-Parra (2014) found that gene expression of some transcription factors (TFs) are over-represented between the tetraploidy target genes such as WRKY, DREB, and ERF transcription factors, which appear to play a crucial role in tolerance to cold, drought, and salt stress (Chen et al., 2012; Wang et al., 2017). Higher expression of transcripts of genes encoding antioxidant enzymes (CAT, APX and glutathione reductase) had also been reported in tetraploid lines under water deficit, compared to the diploid counterparts (Yan et al., 2019).

CONCLUSION

This study was the first to look at the impact of natural chilling temperatures combined with triploidy level in new citrus species. Our analysis suggested that triploidy may improve photosynthetic performances under chilling temperatures. The small decrease in PSII photochemistry and the maintenance of ETR/P_{net} ratio indicated effective photo-protective mechanisms to counteract toxic effects induced by low temperatures, suggesting 3x varieties have a high tolerance to low temperatures. The antioxidant response does not allow us to discriminate triploid varieties from others and explain enhanced photosynthetic performances in 3x varieties. Chemical analysis uncovered atypical leaf VOC profiles that can be related to ploidy level. Triploid varieties had the highest proportions of *E*-β-ocimene, linalool, and terpenes involved in oxidative stress protection. To deepen our study, it would be interesting to collect biomass data, including investigation on fruit yield and nutrient uptake behavior. The use of triploidy appear to be a relevant breeding strategy to improve the fresh citrus fruit market and to develop new seedless citrus commercial varieties that have enhanced abiotic stress tolerance.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

RL collected the test data, performed the statistical analyses, interpreted the results, and drafted the manuscript. JS interpreted the results and drafted the manuscript. FT and MG collected the chemical data. MG helped to perform statistical analyses. LB, JG, SH, YF, and RM helped draft the manuscript.

FUNDING

RL was subsidized by the "Collectivité Territoriale de Corse" (CTC). This work was funded by the "Collectivité de Corse" as part of the research project "Innov'agrumes" (FEDER).

ACKNOWLEDGMENTS

We are grateful to Noémie Gonzalez and Jean-Dominique Grossi for technical assistance in the grinding of leaves.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00330/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 May 2020
doi: 10.3389/fpls.2020.00558

Unreduced Male Gamete Formation in *Cymbidium* and Its Use for Developing Sexual Polyploid Cultivars

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: 05 December 2019
Accepted: 14 April 2020
Published: 15 May 2020

Citation:
Zeng R-Z, Zhu J, Xu S-Y, Du G-H, Guo H-R, Chen J, Zhang Z-S and Xie L (2020) Unreduced Male Gamete Formation in Cymbidium and Its Use for Developing Sexual Polyploid Cultivars. Front. Plant Sci. 11:558. doi: 10.3389/fpls.2020.00558

Polyploidy plays an important role in crop improvement. Polyploid plants, particularly those produced through unreduced gametes (2*n* gametes), show increased organ size, improved buffering capacity for deleterious mutations, and enhanced heterozygosity and heterosis. Induced polyploidy has been widely used for improving floriculture crops, however, there are few reported sexual polyploid plants in the floriculture industry. This study evaluated nine cultivars of *Cymbidium* Swartz and discovered that 2*n* male gametes occurred in this important orchid. Depending on cultivars, 2*n* male gamete formation frequencies varied from 0.15 to 4.03%. Interspecific hybrids generally produced more 2*n* male gametes than traditional cultivars. To generate sexual polyploid plants, seven pairs of crosses were made, which produced five triploid and two tetraploid hybrids. Two triploid hybrids were evaluated for *in vitro* regeneration and growth characteristics. Compared to the diploid parents, the triploids were more easily regenerated through rhizomes or protocorms, and regenerated plants had improved survival rates after transplanting to the greenhouse. Furthermore, the sexual polyploid plants had more compact growth style, produced fragrant flowers, and demonstrated heterosis in plant growth. Through this study, a reliable protocol for selection of appropriate parents for 2*n* gamete production, ploidy level evaluation, *in vitro* culture of polyploid progenies, and development of new polyploid cultivars was established. Our study with *Cymbidium* suggests that the use of 2*n* gametes is a viable approach for improving floriculture crops.

Keywords: 2*n* gametes, *Cymbidium*, floriculture crops, micropropagation, sexual polyploidization

INTRODUCTION

Cymbidium Swartz, or boat orchid, is a genus of evergreen flowering plants in the family Orchidaceae. It is mainly native to China and northern Asia and is one of the most important orchids produced commercially as cut flowers and potted flowering plants around the world (Xie et al., 2017). There are 44 recognized species (Griffiths and Huxley, 1992), which have a diploid number of chromosomes of 40. Among them, terrestrial *Cymbidium sinense*, *C. ensifolium*, *C. faberi*, *C. kanran*,

and *C. goeringii* are the most popular and economically significant ornamental plants in China. These species have been cultivated since the time of Confucius (551–479 BC) because of their graceful leaves, erect inflorescences, and sweet-scented flowers (Liu Y. et al., 2017). Today, interest in cymbidiums has shifted from cultivars with small flowers to those possessing large and round flowers with long-lasting inflorescences and fragrance and more robust stems and leaves.

Polyploid plants generally show increased organ size and improved tolerance to stressful environmental conditions (Baduel et al., 2018; Pham et al., 2019). Polyploidization has been used to develop hybrids with floral and growth characteristics unobtainable from diploid forms, such as *Platanus acerifolia* (Liu et al., 2007), *Miscanthus* (Głowacka et al., 2010; Miguel and Leonhardt, 2011), *Acacia senegal* (Diallo et al., 2016), *Plectranthus esculgentusare* (Hannweg et al., 2016), and poinsettia (Pan et al., 2019). Ornamental plants are valued for their aesthetic appearance, including plant canopy height and architecture; leaf shape, texture, and thickness; flower form and color (Henny and Chen, 2003). Through polyploidization, ornamental plants with larger and heavier flowers and prolonged flowering period have been developed (Głowacka et al., 2010; Sattler et al., 2016; Marasek-Ciolakowska et al., 2018; Manzoor et al., 2019). Polyploidization also resulted in plants with thicker leaves with increased width-to-length ratio, robust stems, deeper green leaves, more compact growth form (Eeckhaut et al., 2004; Liu et al., 2007; Zhang et al., 2008) as well as delayed flowering time (Väinölä, 2000; Eeckhaut et al., 2004; Vichiato et al., 2014). In fact, many popular cultivars of orchids are polyploid (Zhu et al., 2006; Chen et al., 2009; Liao et al., 2012; Xie et al., 2014). Polyploid orchids usually have more sturdy stems and thicker leaves, larger flowers with more intense color, and rounder conformation (MacLeod, 1947; Kamemoto and Kam, 1980; Griesbach, 1985; Wimber et al., 1987; Watrous and Wimber, 1988; Chen et al., 2009; Miguel and Leonhardt, 2011; Vichiato et al., 2014), thus exhibiting improved ornamental value.

Polyploidy occurs asexually through somatic chromosome doubling and sexually through the formation of unreduced gametes or 2*n* gametes (Ramanna et al., 2003; Gallo et al., 2007; Fakhri et al., 2016) and polyspermy (Tekleyohans and Groß-Hardt, 2019; Toda and Okamoto, 2019). Mitotic chromosome doubling has been achieved using antimitotic agents, such as colchicine and oryzalin. These agents inhibit mitosis during metaphase by interfering with the function of microtubules and lead to the production of polyploid plants. This technique has been intensively used for inducing polyploid cymbidiums (Menninger, 1963; Wimber and Van Cott, 1966; Kim et al., 1997; Wang et al., 2010, 2011; Yin et al., 2010; Ji et al., 2011; Xie et al., 2017). Unreduced gamete is an important pathway of generating polyploid plants and is a driving force behind the formation of polyploids in nature (Otto and Whitton, 2000; Hegarty and Hiscock, 2008; Soltis et al., 2009; Dewitte et al., 2011; Mason and Pires, 2015; Kreiner et al., 2017a). Compared to somatic polyploidization, sexual polyploidization can be of advantageous in plant breeding due to the resultant genetic diversity and heterosis (Ogburia et al., 2002; Ramanna and Jacobsen, 2003; Brownfield and Köhler, 2010; Khan et al., 2010; Dewitte et al., 2011; Younis et al., 2014; Lai et al., 2015). Sexual polyploidization had been used to develop polyploid ornamental plants including lily (Barba-Gonzalez et al., 2004; Zhou et al., 2008; Khan et al., 2010), tulip (Marasek-Ciolakowska et al., 2014), and *Phalaenopsis* (Zhou et al., 2009). However, there has been little available information on 2*n* gamete production in *Cymbidium* and its use in crop improvement. Polyspermy is referred to as an egg cell fertilized by more than one sperm cell, generating viable progeny in flowering plants (Chaudhary et al., 2020). The potential of polyspermy in plant breeding is yet to be exploited (Mao et al., 2020).

The objectives of this study were to explore 2*n* gamete occurrence in cymbidium cultivars and establish a protocol for 2*n* gamete identification, production of polyploid plants through hybridization, and assessment of ornamental value through morphological evaluation. Our effort has resulted in the discovery of 2*n* gamete formation in cymbidium and development a reliable protocol for improving cymbidium via sexual polyploidization.

MATERIALS AND METHODS

Plant Materials

A total of nine cultivars were used in this study (**Table 1** and **Supplementary Figure S1**). Five of them were traditional cultivars of *C. sinense*. The remaining "Dafeng," "Yunv," "45–17," and "45–32" were hybrids. "Dafeng" and "Yunv" were developed from the cross of *C. sinense* with *C.* "Sleeping Beauty" and *C. sinense* with *C.* "King Arthur," respectively. Whereas "45–17" and "45–32" were developed from interspecific crosses between *C. sinense* and *C. lancifolium*. Plants were grown in black plastic planting bags (2.6 L) filled with a mixture of pine bark (2–4 cm in length) with granite (0.5–1 cm in length) in 3 to 1 ratio based on volume in a shaded greenhouse at the Experimental Farm of South China Agricultural University, Guangzhou, China. Photosynthetically active radiation ranged from 300 to 400 μmol m^{-2} s^{-1}. After plant establishment, 4 g of a slow-release fertilizer (N–P_2O_5–K_2O; 20–20–20) was applied in each planting bag in March and September, respectively each year, and a solution containing 0.1% KH_2PO_4 (w/v) was also sprayed every month during the growing season. Plants started flowering in the middle of December, and the flowering lasted for 4 months.

Determination of Unreduced Male Gametes

To examine the occurrence of unreduced male gametes, pollinia of a blooming flower from different plants were transferred to a slide, crushed and stained with a drop of improved carbolfuchsin solution (Verhoeff, 1912) for 3–5 min or a drop of 4,6-diamidino-2-phenylindole (DAPI) [2 μg.ml^{-1} DAPI, 1% Triton X-100 (v/v), and 1% sucrose (w/v)] in the dark for 10 min. After a cover glass was placed on each slide, the cover glass was squeezed with pencil eraser. The slides were observed under light or UV illumination with ZEISS microscope. Photographs were taken under binocular light microscope with image-forming system. The number of dyads, triads, and tetrads in light vision field of microscope was counted, respectively. Ten vision fields were

TABLE 1 | *Cymbidium* cultivars used for evaluation of 2*n* gamete formation.

Species	Cultivar	Description
C. *sinsense*	Qijianbaimo	A traditional cultivar developed through germplasm introduction and enhancement, which has long and dark green leaves, small, white-jade and perfume flower, blooming in February and March, flowering lasted 15–20 days
C. sinense	Damo	A traditional cultivar developed through germplasm introduction and enhancement. It has short and thick leaves with distortion in the middle, purplish and perfume flower, blooming in February and March, flowering lasted 15–20 days
C. sinense	Hezhihua	A traditional cultivar developed through germplasm introduction and enhancement, which has wide leaves with line art, perfume and reddish-brown flower
C. sinense	Xiaoxiang	A traditional cultivar developed through germplasm introduction and enhancement. It has wide and dark green leaves, reddish brown flower with heavy perfume
C. sinense	Taipingyang	A traditional non-hybrid cultivar, semi-erect leaves with line art, perfume and purple-red flower.
C. ×	Dafeng	A hybrid developed from a cross of *C. sinense* and *C.* × Sleeping Beauty. It has long and semi-erect leaves, large and yellow flower with heavy perfume, blooming in January and February, flowering lasted about 50 days
C. ×	Yunv	A hybrid of *C. sinense* and *C.* × King Arthur, long and erect leaves, large and light yellowish-green flower with gently perfume, blooming in January and February, flowering lasted about 50 days
C. ×	45–17	A hybrid of *C. sinense* and *C. lancifolium*, light green and graceful leaves, purplish red and perfume flower, blooming in December and January, flowering lasted 40 days
C. ×	45–32	A hybrid of *C. sinense* and *C. lancifolium*, light green and graceful leaves, purplish red and perfume flower, blooming in December and January, flowering lasted 40 days

recorded for each slide, which was regarded as a replicate; each sample replicated three times. The frequency for unreduced male gamete occurrence (F_{2n}) was calculated based on the equation F_{2n} (%) = (2Dy + Tr)/(2Dy + 3Tr + 4Te) × 100, where Dy, Tr, and Te were the number of dyads, triads, tetrads, respectively. This experiment was carried out for successive 3 years.

Hybridization

After determination of the frequency of 2*n* gamete formation, healthy plants with blooming flowers were chosen for hybridization, which included "Dafeng" × "Hezhihua," "Yunv" × "Qijianbaimo," "Qijianbaimo" × "Damo," "Yunv" × "Xiaoxiang," "Yunv" × "Taipingyang," "45–32" × "45–17," and "45–32" × "45–32" (**Table 2** and **Supplementary Table S1**). Flower lip and pollinia were removed from a blooming flower of female parent, the stigma was pollinated with pollen from a male parent, i.e., a tooth stick was inserted into male pollinium, and the pollinium was placed into the stigmatic chamber of female flower. A label with date for each cross and their parents was attached to each pollinated flower. The number of pollinated flowers and mature capsules in each cross combination were recorded individually, and the success rate of crosses was calculated.

Hybrid Seed Germination and Propagation

Mature capsules that resulted from the crosses were collected. After removing the fruit stalk and remaining column, the capsules were washed in running tap water, surface sterilized with a 75% (v/v) ethanol solution for 8–10 min and rinsed with sterilized distilled water three times. The sterilized capsules were longitudinally cut, released seeds were cultured on half-strength MS (Murashige and Skoog, 1962) medium supplemented with 0.5 mg·L^{-1} 6-BA (6-benzylaminopurine), 0.2 mg·L^{-1} NAA (naphthaleneacetic acid), 30 g·L^{-1} sucrose, 7.5 g·L^{-1} agar, 100 ml·L^{-1} coconut water (CW), and 0.5 g·L^{-1} active carbon (AC). The pH of the medium was adjusted to 5.8 with 1 *M* KOH or 1 *M* HCl prior to autoclaving for 20 min at 121°C. Seed germination took place in a controlled environment in the dark at 26°C ± 1°C. Seeds produced small, round, and green protocorm or rhizome.

A small piece of rhizome produced from individual seeds was cultured on half-strength MS medium supplemented with 1.0 mg·L^{-1} 6-BA, 0.2 mg·L^{-1} NAA, 30 g·L^{-1} sucrose, 7.5 g·L^{-1} agar, and 0.5 g·L^{-1} AC. They were maintained in a culture room under a light intensity of 40 μmol·m^{-2}·s^{-1} for 12 h and a temperature of 26°C ± 1°C. In order to produce a large number of propagules from each seed, rhizomes derived from the seed were sub-cultured five times, 50 days each. Subsequently, the propagules were cultured on half-strength MS medium containing 2.0 mg·L^{-1} 6-BA, 0.2 mg·L^{-1} NAA, 30 g·L^{-1} sucrose, and 7.5 g·L^{-1} agar, and 0.5 g·L^{-1} AC for shoot induction. When the shoots reached 4 cm in height, they were transferred to half-strength MS medium supplemented with 0.2 mg·L^{-1} 6-BA, 0.5 mg·L^{-1} NAA, 20 g·L^{-1} sucrose, 7.5 g·L^{-1} agar, and 0.5 g·L^{-1} AC for rooting. Plantlets derived from each seed of a cross were a considered to be a breeding line. Based on morphological characteristics, mainly increased stem diameter and leaf size and thickness as well as shorter and thicker roots of the plantlets, putative polyploid lines were selected.

Chromosome Counts

A total of 30 plantlets from each putative polyploid line were randomly selected for counting chromosome numbers of root tip using the squash method (Wang et al., 2010). Briefly, about 0.2 cm of the root-tip was removed from a seedling and pretreated in 2.0 mM 8-hydroxyquinoline solution at 18°C in the dark for 4 h.

TABLE 2 | Ploidy evaluation of *Cymbidium* hybrids resulted from different crosses.

Cross combination (♀ × ♂)	Total no. of hybrid produced	No. of diploid	No. of triploid (Occurrence frequency of triploid/%)	No. of tetraploid (Occurrence frequency of tetraploid/%)
"Dafeng" × "Hezhihua"	830	829	1 (0.12)	0
"Yunv" × "Qijianbaimo"	279	279	0	0
"Qijianbaimo" × "Damo"	200	200	0	0
"Yunv" × "Xiaoxiang"	300	299	1 (0.33)	0
"Yunv" × "Taipingyang"	532	529	1 (0.18)	1 (0.18)
"45–32" × "45–17"	58	56	2 (3.45)	0
"45–32" × "45–32"	54	53	0	1 (1.85)

The root-tips were fixed in Carnoy's solution [a 3:1 (v/v) ratio of 95% (v/v) ethanol and glacial acetic acid] at 4°C for 24 h. After washing with distilled water three times, they were subjected to the acidolysis in 1 *M* HCl solution at 60°C for 12 min. After being soaked in distilled water for 30 min, the fixed apex was stained with improved carbolfuchsin (Verhoeff, 1912) by squeezing them with a needle on a slide. Debris and excess stain were removed and the sample was covered with a cover-slip and observed at 100 × magnification using a photomicroscope (Olympus-IX71, Japan), a digital camera system (Nikon) were used for photography. For each plantlet, at least 20 cells were observed. If more than 90% of the cells had a constant chromosome number, the chromosome number of the plantlet was confirmed.

Flow Cytometric Analysis

The relative DNA content of the polyploid plantlets and their counterparts was measured using flow cytometry (Cui et al., 2009; Corneillie et al., 2019) with a slight modification. Approximately 0.5 cm^2 of leaves was collected and chopped into small pieces with a sharp razor blade in a 55 mm plastic Petri dish containing 0.4 mL of Partec HR-A (Partec GmbH, Münster, Germany), followed by adding 1.6 mL of Partec HR-B with DAPI as DNA staining agent. The nuclei suspension was subsequently filtered through a disposable filter with 30 μm mesh size (Partec, Celltrics, Germany), and kept on ice in the dark for 3–5 min. Samples were analyzed using a Partec PA-II flow cytometer. Collected data were analyzed simultaneously using CyView 8.5 software (Partec GmbH, Münster, Germany) and presented as DNA histograms. Five plants from each line were analyzed. Based on the peaks obtained in the histograms, ploidy levels were estimated as diploid, triploid, and tetraploid.

Plant Production

To produce mature plants, identified polyploid plantlets, along with their diploid counterparts at a height of 8 cm were rinsed with tap water and briefly air dried, and then transplanted into small black plastic planting bags (100 mL) filled with a substrate comprised of small pine bark (1 cm in length) and peat in a 3:1 ratio based on volume, one plantlet per bag. Plants were grown in a shaded greenhouse under a light intensity of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$, temperature ranging from 15°C to 30°C, and relative humidity varying from 70 to 80%. The plants were fertigated with a Hyponex ($N–P_2O_5–K_2O$; 20–20–20) solution every 10 d. After 6 months, they were transplanted into large black plastic planting bags (2.6 L) filled with a substrate comprised of pine bark (2–4 cm in length) and granite (0.5–1 cm in length) in a 3:1 ratio based on volume and cultivated in another shaded greenhouse under a light intensity of 300–400 $\mu mol\ m^{-2}\ s^{-1}$, 3–4 g of a slow-release fertilizer ($N–P_2O_5–K_2O$; 20–20–20) was applied to each planting bag on March and September, respectively, and a solution containing 0.1% KH_2PO_4 (w/v) was sprayed every month during the growing season.

Evaluation of Ornamental Characteristics

Two polyploid cultivars, Yutao and Huanghe along with their diploid counterparts XY and DH were evaluated for their ornamental characteristics. Ten plants were randomly selected from each cultivar. Main morphological characteristics were recorded according to guidelines for testing the distinctness, uniformity, and stability of *Cymbidium* (International Union for the Protection of New Varieties of Plants [UPOV], 1999). Leaf and flower numbers were recorded, the diameter of pseudobulb and inflorescence stalk and the thickness of leaves, sepal and petal were measured using an automatic caliper, and other traits, such as plant height and leaf length and width, were measured using a ruler.

Assessment of Regeneration Capacity

Two triploid cultivars: Yutao and Huanghe and a diploid DH were used for evaluating regeneration capacity. Shoot tips were excised from 8 cm plantlets, cut into 1 mm in length, and cultured in glass culture vessels (height: 10 cm, diameter: 6.5 cm, and volume: 330 mL) containing MS medium. The medium was supplemented with 1.0 $mg \cdot L^{-1}$ 6-BA, 0.2 $mg \cdot L^{-1}$ NAA, 50 ml L^{-1} CW, 30 $g \cdot L^{-1}$ sucrose, 7·g L^{-1} agar, and 0.5 $g \cdot L^{-1}$ AC with a pH of 5.8. The cultures were maintained in darkness at 26°C for 40 days. There were three explants per culture vessel, 30 vessels per treatment. Rhizome induction rates were calculated using the formula: induction rate (%) = $(N_{rhi}/3) \times 100$, where N_{rhi} represented the number of explants forming rhizomes in each culture vessel.

To determine the proliferation of rhizomes, about 2 g of rhizome, each 1 cm in length were inoculated in a culture vessel containing 30 mL MS medium supplemented with 1.0 $mg \cdot L^{-1}$

6-BA, 0.2 mg·L^{-1} NAA, 30 g·L^{-1} sucrose, 7 g·L^{-1} agar, and 0.3 g·L^{-1} AC with a pH at 5.8. There were 15 culture vessels per cultivar, which were cultured under a 12 h photoperiod with a light intensity of 20 μmol m^{-2} s^{-1} at 26°C for 40 days. The fresh weight of rhizomes in each vessel was measured using an electronic balance. The proliferation coefficient was calculated based on the weight of the rhizomes in a vessel after 40 days of culture divided by the initial weight of the rhizomes in the same vessel.

To evaluate shoot regeneration from rhizomes, 10 rhizomes, each in a length of about 1 cm were inoculated in a culture vessel containing 30 ml MS medium supplemented with 1.0 mg·L^{-1} 6-BA, 0.2 mg·L^{-1} NAA, 30 g L^{-1} sucrose, 7 g·L^{-1} agar, and 0.02 g·L^{-1} AC with a pH at 5.8. There were 15 vessels per cultivar, and they were maintained under a 12 h photoperiod with a light intensity of 40 μmol m^{-2}s^{-1} at 26°C for 40 days. The numbers of shoots regenerated from rhizomes per vessel were recorded. The shoot regeneration rate was calculated by the formula: shoot regeneration rate (%) = (the number of rhizomes regenerating shoots in a vessel/10) × 100.

The regenerated shoots were cultured on half-strength MS medium containing 0.1 mg·L^{-1} 6-BA, 0.5 mg·L^{-1} NAA, 20 g·L^{-1} sucrose, and 0.5 g·L^{-1} AC under a light intensity of 40 μmol m^{-2}s^{-1} at 26°C for 50 days. Plantlets at a height of 8 cm were washed with tap water and transplanted to the aforementioned substrate. The plants were grown in a shaded greenhouse as described above for producing mature plants. A total of 120 plantlets were transplanted per cultivar, which were arranged as randomized complete block design with three blocks, 40 plantlets per block. After 60 days, the number of surviving seedlings were recorded using the formula: survival rate (%) = (the number of seedling survived in a replicate/40) × 100.

Statistical Analysis

Collected data were subjected to analysis of variance (ANOVA) using statistical program SPSS 22.0 (IBM Corporation, Somers, NY). When significance occurred, means were separated by Duncan's multiple range test at $P < 0.05$ level.

RESULTS

Occurrence in Unreduced Male Gamete

All nine parental cultivars produced unreduced male gametes, which were either dyad or triad (**Figure 1A** and **Supplementary Table S1**). Cultivars, however, varied significantly in 2*n* male gamete formation frequency, ranging from 0.15% in "Xiaoxiang" to 4.03% in "47–17" (**Figure 1B**). Generally, hybrid, particularly those interspecific ones had higher 2*n* gamete formation frequencies than traditional cultivars.

Hybrid Seeds and *in vitro* Propagation

Seven pairs of crosses were made using the nine parental cultivars. All the crosses were compatible, resulting in 100% success rate (**Supplementary Table S2**). The resultant capsules matured at different times, varying from 210 days for the cross of "Yunv" × "Taipingyang" to 310 days for the cross of "Dafeng" × "Hezhihua." The numbers of hybrid seeds produced from the crosses differed greatly, and the cross of "Dafeng" × "Hezhihua" produced more hybrid seeds than the cross of "45–32" × "45–32" (**Table 2**).

To ensure appropriate germination of the hybrid seeds, they were propagated through *in vitro* culture (**Supplementary Figure S2**). The seeds required at least 150 days to germinate and germination rate differed from 30% to 90% (**Supplementary Table S2**). The germination rates of seeds derived from crosses of "Dafeng" × "Hezhihua," "Yunv" × "Taipingyang," and "Qijianbaimo" × "Damo" were estimated to be 70%, which were higher than those derived from interspecific crosses "45–32" × "45–17" or self-cross of "45–32" which were only 30%.

Evaluation of Ploidy Levels of Hybrids

Ploidy levels of *in vitro* propagated seedlings were examined through morphological evaluation, chromosome counting, and flow cytometric analysis. The results showed that majority of the hybrids were diploid, but few were triploid or tetraploid (**Table 2**). The cross of "Dafeng" × "Hezhihua" produced a triploid plant "Huanghe," which had $2n = 3x = 60$ compared to its diploid counterpart of "DH" with $2n = 2x = 40$ (**Figure 2**). Flow cytometric analysis also confirmed that "Huanghe" was a triploid. Morphologically, "Huanghe" had shorter but thicker roots and was more robust, its leaves were darker green compared with "DH" (**Figure 2**). A triploid called "Yutao" was also identified from the cross of "Yunv" × "Xiaoxiang" (**Figure 3**). Both chromosome counting and flow cytometric analysis confirmed that it was a triploid with $2n = 3x = 60$. One triploid and one tetraploid were found from the cross of "Yunv" × "Taipingyang" (**Supplementary Figure S3**). Two triploid plants were identified from the cross of "45–32" × "45–17" (**Supplementary Figure S4**). Additionally, one tetraploid was found from self-pollinated progenies called "45–32" (**Supplementary Figure S5**). The chromosome counting results were further confirmed by flow cytometric analysis and morphological evaluation.

Ornamental Traits of Polyploid Plants

Ornamental characteristics were comparatively evaluated between selected triploid and the diploid counterpart. Results showed sexual polyploidization caused significant changes in morphological traits. For example, plant canopy width and lip width significantly increased, but flower numbers and peduncle length noticeably decreased in triploid "Huanghe" compared to the diploid "DH," but other parameters were not significantly altered (**Table 3** and **Figures 4A–C**). On the other hand, canopy height; pseudobulb diameter; leaf length, width, and thickness; peduncle thickness; sepal length, width, and thickness; petal width and thickness as well as lip width of triploid "Yutao" were significantly greater than the diploid "XY" (**Table 3** and **Figures 4D–F**). Aesthetically, sexual polyploidization increased the width and thickness of sepal, petal, and lip. Flowers became much rounder in shape and produced fragrance, thus ornamental value was greatly improved (**Figures 4B,E**). Additionally, triploids "Yutao" and "Huanghe" were much sturdier and more robust compared with their counterparts (**Figures 4A,D**). However, there were no

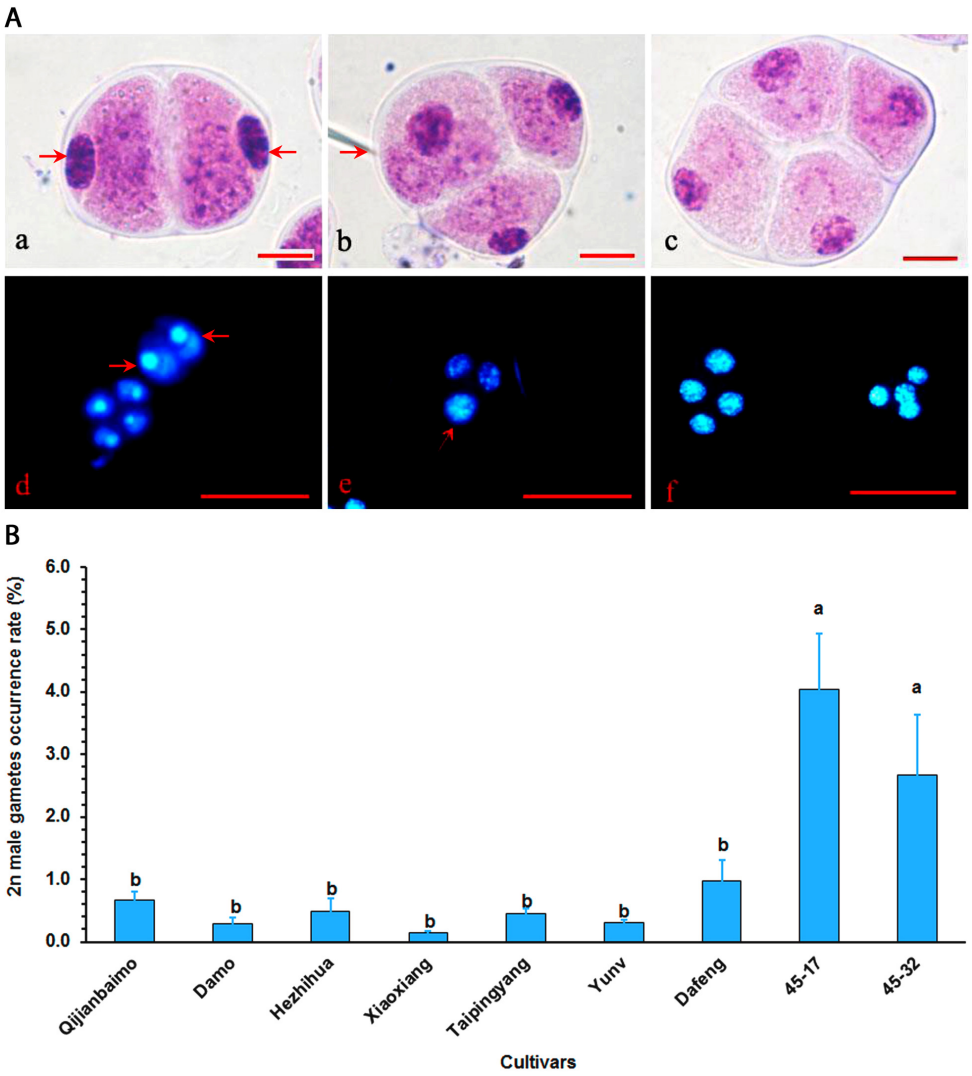

FIGURE 1 | Analysis of unreduced male gametes in *Cymbidium* cultivars using the squash method. **(A)** Mature pollens were stained with carbolfuchsin, and dyad (a), triad (b), and tetrad (c) gametes were identified by the squash method. Bar = 10 μm. The dyad with 2*n* gametes (arrow) (d), triads with one unreduced gamete (arrow) and two reduced gametes (e), and tetrad with four reduced gametes (f) were stained with 4',6-diamidino-2-phenylindole (DAPI). Bar = 50 μm. **(B)** 2*n* gamete formation frequencies (means of 3-year data, **Supplementary Table S1**) among nine cultivars, bars represent standard error (n = 30). Different letters on the top of bars indicate significant cultivar difference in 2*n* gamete formation frequencies analyzed by Duncan's multiple range test at $P < 0.05$ level.

significant differences in the time of flowering between triploids and diploid counterparts.

Regeneration of Polyploid Plants

Regeneration capacity of polyploid plants was evaluated *in vitro*. The rhizome induction rate of triploid "Huanghe" were significantly higher than triploid "Yutao" and diploid "DH" (**Figures 5A,B**). The rhizome proliferation coefficient of "Huanghe" was the highest compared to "Yutao" and "DH," but "Yutao" was significantly higher than "DH" (**Figures 5C,D**). There was significant difference in shoot regeneration between "Huanghe" and "Yutao," but the regeneration rate between "Yutao" and "DH" was not statistically significant (**Figures 5E,F**). However, seedling survival rates of plantlets grown in a shade greenhouse differed significantly. Triploid "Huanghe" and "Yutao" had 96% survival rate compared to 84% of "DH" (**Figure 6**).

DISCUSSION

Cymbidium is known as the King of Orchids due to its fragrant flowers and multitudes of colors which can remain in bloom for up to 3 months. To improve its ornamental value, many hybrids have been developed from the original 44 species (Obara-Okeyo and Kako, 1998). Additionally, polyploid cymbidiums have been explored asexually through somatic cell chromosome doubling (Wimber and Van Cott, 1966; Kim et al., 1997; Yin et al., 2010;

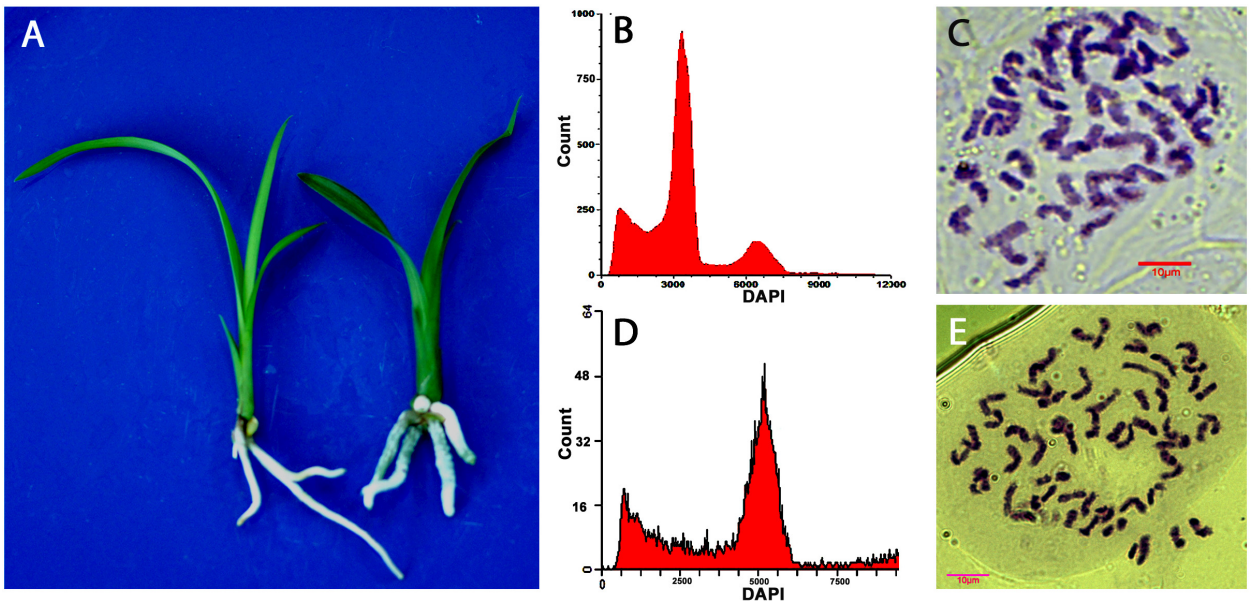

FIGURE 2 | Evaluation of ploidy levels of *Cymbidium* hybrids of "DH" and "Huanghe" resulted from cross of "Dafeng" × "Hezhihua." **(A)** Morphology of "DH" (left) and "Huanghe" (right). **(B)** Flow cytometric DNA histograms of "DH" and **(C)** chromosome numbers of root tip cell of "DH" ($2n = 2x = 40$). **(D)** Flow cytometric DNA histograms of "Huanghe" and **(E)** chromosome numbers of root tip cell of "Huanghe" ($2n = 3x = 60$).

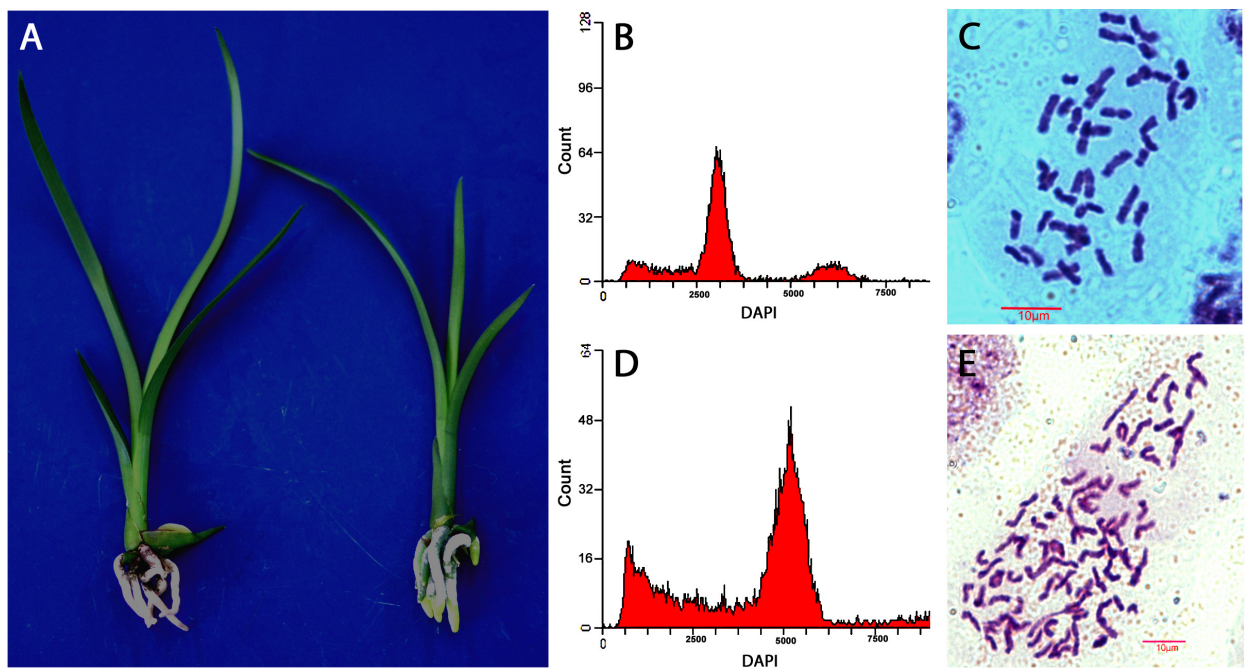

FIGURE 3 | Evaluation of ploidy levels of *Cymbidium* hybrids of "XY" and "Yutao" developed from cross of "Yunv" × "Xiaoxiang." **(A)** Morphology of "YX" (left) and "Yutao" (right). **(B)** Flow cytometric DNA histograms of "YX" and **(C)** chromosome numbers of root tip cell of "YX" ($2n = 2x = 40$). **(D)** Flow cytometric DNA histograms of "Yutao" and **(E)** chromosome numbers of root tip cell of "Yutao" ($2n = 3x = 60$).

Ji et al., 2011; Wang et al., 2011; Xie et al., 2017). However, sexual polyploidization through unreduced gametes has not been reported in *Cymbidium*. Sexual polyploidization through $2n$ gametes can be of immense significance because it can combine genetic effects of polyploidy with meiotic recombination and sexual hybridization and produce tremendous genetic variation and heterosis (Ogburia et al., 2002; Ramanna and Jacobsen, 2003; Brownfield and Köhler, 2010; Khan et al., 2010; Lai et al., 2015).

TABLE 3 | The effect of sexual polyploidization on morphological characteristics of *Cymbidium* cultivars.

Character	"Huanghe"	"DH"	"Yutao"	"YX"
Plant height (cm)	35.0 ± 4.62a	41.8 ± 0.29a	51.2 ± 1.70a	37.1 ± 3.43b
Plant width (cm)	76.0 ± 6.43a	53.7 ± 3.75b	50.9 ± 2.72a	46.7 ± 4.50a
No. of leaf	6.3 ± 0.17a	5.3 ± 0.33a	6.7 ± 0.38a	5.7 ± 0.33a
Pseudobulb diameter (cm)	2.8 ± 0.53a	3.1 ± 0.01a	2.8 ± 0.06a	1.8 ± 0.03b
Leaf Length (cm)	64.9 ± 5.80a	58.5 ± 6.09a	60.3 ± 1.16a	47.6 ± 2.00b
Leaf width (cm)	2.2 ± 0.15a	2.6 ± 0.09a	2.2 ± 0.05a	1.2 ± 0.03b
Leaf thickness (mm)	0.8 ± 0.02a	0.7 ± 0.04a	0.7 ± 0.02a	0.6 ± 0.01b
No. of flowers	7.0 ± 0.00a	10.3 ± 0.69b	7.5 ± 0.31a	6.5 ± 0.29a
Peduncle length (cm)	54.1 ± 0.22a	72.8 ± 4.29b	49.8 ± 2.96a	45.6 ± 0.07a
Peduncle thickness (cm)	0.7 ± 0.02a	0.7 ± 0.04a	0.7 ± 0.02a	0.4 ± 0.00b
Flower width (cm)	7.0 ± 0.36a	7.3 ± 0.11a	4.9 ± 0.19a	5.7 ± 0.38a
Flower Length (cm)	7.6 ± 0.35a	7.8 ± 0.23a	5.3 ± 0.07a	6.1 ± 0.32a
Sepal length (cm)	4.6 ± 0.02a	4.5 ± 0.07a	4.0 ± 0.06a	4.2 ± 0.04b
Sepal width (cm)	1.9 ± 0.06a	1.6 ± 0.10a	1.7 ± 0.02a	0.8 ± 0.00b
Sepal thickness (mm)	0.8 ± 0.02a	0.7 ± 0.03a	0.9 ± 0.01a	0.5 ± 0.01b
Petal length (cm)	4.1 ± 0.02a	4.1 ± 0.11a	3.5 ± 0.04a	3.5 ± 0.03a
Petal width (cm)	2.1 ± 0.17a	1.8 ± 0.15a	1.4 ± 0.02a	0.9 ± 0.02b
Petal thickness (mm)	0.8 ± 0.01a	0.8 ± 0.05a	0.8 ± 0.01a	0.5 ± 0.01b
Lip length (cm)	3.8 ± 0.01a	3.8 ± 0.04a	3.0 ± 0.03a	3.1 ± 0.04a
Lip width (cm)	3.6 ± 0.07a	2.9 ± 0.02b	2.5 ± 0.03a	1.6 ± 0.04b

"Yutao" (3x) and "YX" (2x) come from the cross of "Yunv" × "Xiaoxiang," "Huanghe" (3x) and "DH" (2x) come from the cross of "Dafeng" × "Hezhihua." Values represent mean ± standard error (n = 3). Different letters in the same row of "Yutao" and "YX" or "Huanghe" and "DH" indicate significant difference based on Duncan's multiple range test at P < 0.05 level.

FIGURE 4 | Morphological characteristics of polyploid *Cymbidium* cultivars with their diploid counterparts. **(A)** Mature plants of "DH" (left) and "Huanghe" (right). **(B)** Flowers of "DH" (left) and "Huanghe" (right). **(C)** Sepals (left), petals (middle), and lips (right) of "DH" (above) and "Huanghe" (below). **(D)** Mature plants of "XY" (left) and "Yutao" (right); **(E)** Flowers of "YX" (left) and "Yutao" (right); **(F)** Sepals (left), petals (middle), and lips (right) of "YX" (above) and "Yutao" (below).

The occurrence of 2*n* gametes has been reported in some orchids, such as *Calanthe veratrifolia* Lindl., *Plocoglottis javanica* B., and *Spathoglottis plicata* Bl. (Teoh, 1984), but not in *Cymbidium*. The present study as the first to document 2*n* gamete occurrence in *Cymbidium* and production of polyploid cymbidiums sexually. Furthermore, this study established a reliable protocol for 2*n* gamete identification, polyploid plant generation through hybridization, *in vitro* culture of polyploid plants, and evaluation of polyploid plants for new cultivar development. Using this protocol, five triploid and two tetraploid plants were developed, and some evaluated triploids exhibited heterosis in plant growth and improved ornamental value.

The first step of this protocol is to identify cultivars that have potential to produce 2*n* gametes. This was a challenging

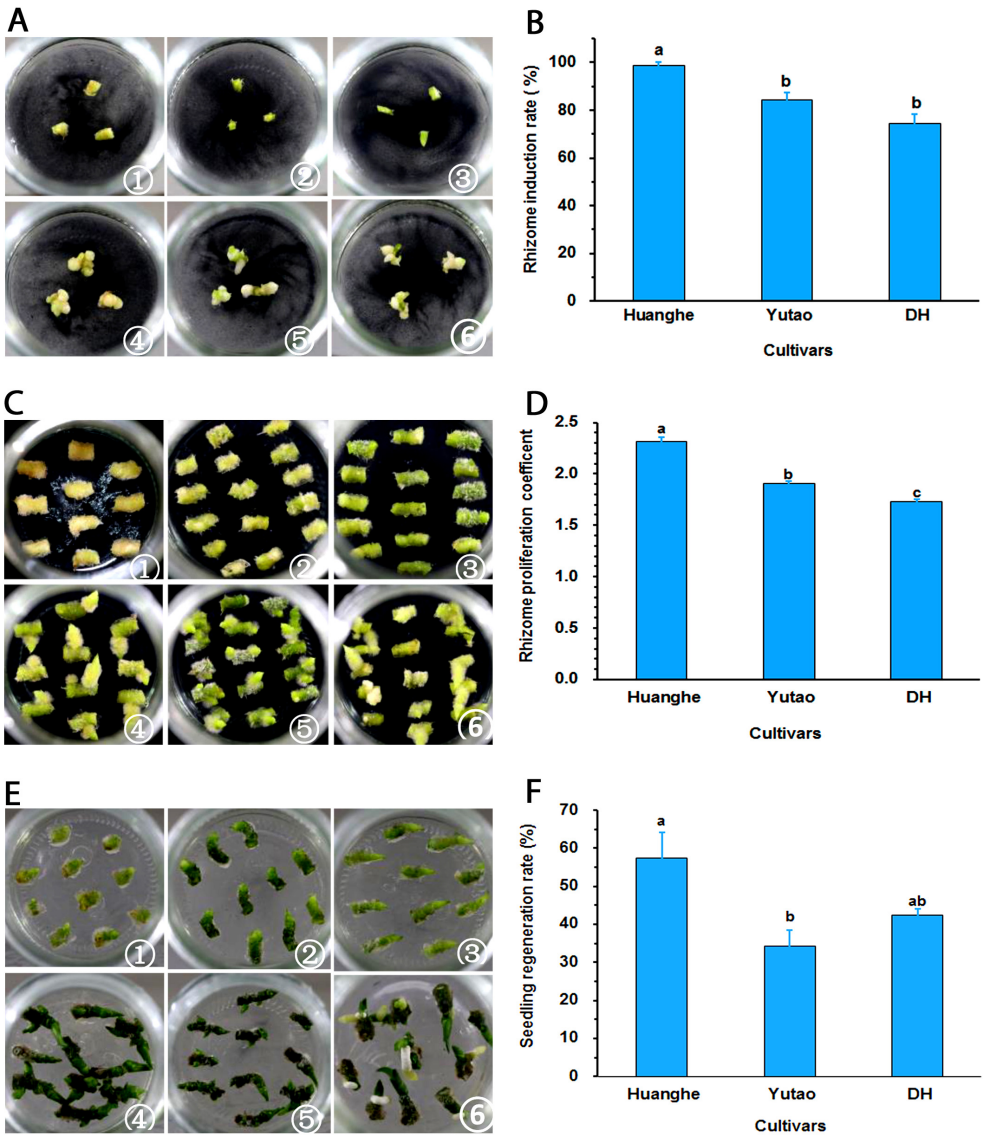

FIGURE 5 | Regeneration ability of polyploid *Cymbidium* cultivars developed from sexual polyploidization. **(A)** Induction of rhizomes from shoot tips: ① Shoot tip of "Huanghe" on day one; ② Shoot tip of "Yutao" on day one; ③ Shoot tip of "DH" on day one; ④ Shoot tip of "Huanghe" on day 40; ⑤. Shoot tip of "Yutao" on day 40; ⑥ Shoot tip of "DH" on day 40. **(B)** Rhizome induction frequencies (%) of the three cultivars. **(C)** Rhizome proliferation: ① Rhizomes of "Huanghe" on day one; ② Rhizomes of "Yutao" on day one; ③ Rhizomes of "DH" on day one; ④ Rhizomes of "Huanghe" on day 40; ⑤ Rhizomes of "Yutao" on day 40; ⑥ Rhizomes of "DH" on day 40. **(D)** Rhizome proliferation coefficient of three cultivars. **(E)** Shoot regeneration from rhizomes: ① Shoot induction of "Huanghe" on day one; ② shoot induction of "Yutao" on day one; ③ shoot induction of "DH" on day one; ④ Shoot induction of "Huanghe" on day 40; ⑤ Shoot induction of "Yutao" on day 40; ⑥ Shoot induction of "DH" on day 40. **(F)** Shoot production rate of three cultivars from cultured rhizomes. Bars represent standard error, and different letters on the top of bars indicate significant cultivar difference for individual traits analyzed by Duncan's multiple range test at $P < 0.05$ level.

task as there had been no report of 2*n* gamete formation in *Cymbidium*. In theory, however, the occurrence of 2*n* gametes in a natural plant population is largely due to the dysfunction of meiosis, therefore, all plants propagated sexually could produce 2*n* gametes. Additionally, 2*n* gametes have negative effects on male fitness in diploid population (Kreiner et al., 2017b); thus, the frequency of 2*n* gamete formation could be relatively higher in asexual species because of the existence of an alternative propagation route and also in interspecific or intergeneric hybrids due to the instability of chromosome pairing. Cymbidiums can be propagated by seeds and division. As a result, we selected seven traditional cultivars and two interspecific hybrids "45–17" and "45–32" for evaluation. Common methods for 2*n* gamete identification include microscopic observation of pollen during maturation and flow cytometric analysis (Kreiner et al., 2017b). Using the two methods, we found that the frequency of 2*n* male gamete formation in traditional cultivars ranged from 0.15 to 1.0% but 2.5–4.03% in interspecific hybrids (**Figure 1**). "47–17"

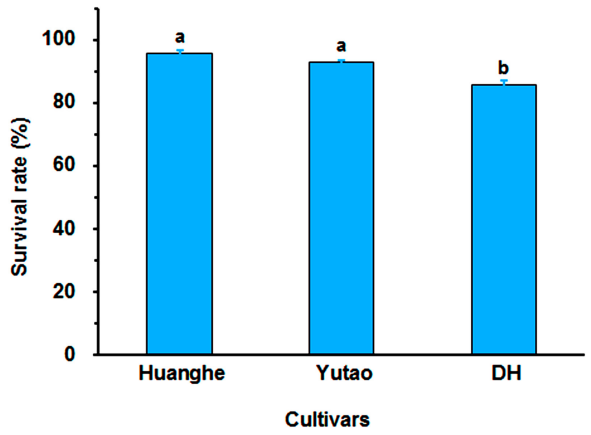

FIGURE 6 | The survival rate of "Huanghe," "Yutao," and "DH" plantlets after transplanting into soilless substrate grown in a shaded greenhouse. Bars represent standard error, and different letters on the top of bars indicate significant cultivar difference in survival rates analyzed by Duncan's multiple range test at $P < 0.05$ level.

as an interspecific hybrid selected from a cross between *C. sinense* and *C. lancifolium* had a frequency of 4.03%, while "Xiaoxiang" was a traditional variety had a frequency of 0.15%. These 2n gamete formation frequencies are comparable to those reported in 60 populations across 24 species of Brassicaceae where most individuals produced less than 2% of 2*n* male gametes (Kreiner et al., 2017b). Our results clearly indicated that interspecific hybrids produced more 2*n* gametes than traditional cultivars, which concur with several reports from other crops (Ramsey and Schemske, 1998; Fakhri et al., 2016; Kreiner et al., 2017b; Liu Z. et al., 2017). Common pathways for 2*n* gamete formation include first division restriction (FDR) and second division restriction (SDR) (Brownfield and Köhler, 2010). In the FDR, 2*n* gametes are produced from a direct equational division of univalent chromosomes at anaphase I or called pseudo-homotypic division. Whereas in the SDR, 2*n* gametes are resulted from the omission of the second meiotic division following chromosome doubling after anaphase I (Peloquin et al., 2008). The exact mechanisms underlying the formation of dyads and triads in this study (**Figure 1A**) have not been well elucidated at this time. Our preliminary data suggested that the dyads could be resulted from either FDR or SDR depending on cultivars. The triads could be caused by cell plate abnormality. Further investigation is needed to confirm the propositions.

The next step of this protocol is to produce sexual polyploid plants through hybridization. The occurrence of polyploid progenies could heavily rely on parental plants used for hybridization. We assumed that parents with high 2*n* gamete formation frequencies should produce more polyploid progenies. Meanwhile, polyploid progenies should have desirable ornamental characteristics, which are also dependent on parental combinations. Considering these two factors, we made crosses outlined in **Table 2**. More hybrid seeds were produced from crosses using traditional cultivars as parents compared to the cross of two interspecific hybrid and the self-cross of "45–32."

Ensuring maximum germination of hybrid seeds produced from each cross and accurately identifying polyploid individuals from the progenies are critically important for polyploidy breeding. In this step, we propagated seeds *in vitro* to ensure that seed germination took place in appropriate conditions. Geminated seeds were then *in vitro* propagated through rhizome to increase plantlet numbers for ploidy identification and for greenhouse production. Methods used for the ploidy discrimination include squash root tip cells (Lai et al., 2015), guard cell measurements (Miguel and Leonhardt, 2011), genomic *in situ* hybridization (Liu Z. et al., 2017), and flow cytometric analysis (Khan et al., 2010). Our results indicated that the squash method, along with flow cytometry, accurately identified ploidy levels of *Cymbidium* hybrids. Furthermore, we found that seedlings of polyploid progenies had distinguishable morphological characteristics compared to diploid counterparts. Roots of polyploid seedlings were shorter and thicker with much greater diameter in contrast to the roots of diploid seedlings. Additionally, stem diameter and leaves were also thicker (**Figures 2A**, **3A** and **Supplementary Figures S3D,G, S4D,G, S5D**). Such morphological characteristics substantially assisted in the identification of sexual polyploid individuals from each cross population.

Results from the ploidy evaluation showed that our assumption was partially correct. The hybridization of "45–32" with "45–17," both had higher 2*n* gamete formation frequencies, produced two triploid plants (**Table 2** and **Supplementary Figure S4**). Additionally, a self-pollination of "45–32" produced one tetraploid (**Table 2** and **Supplementary Figure S5**), suggesting that "45–32" might produce 2*n* eggs. However, the cross between "Yunv" and "Xiaoxiang," two parents with rather low frequencies of 2*n* gamete formation, produced one triploid progeny (**Table 2** and **Figure 3**). The cross between "Yunv" and "Taipingyang" produced one triploid and one tetraploid (**Table 2** and **Supplementary Figure S3**), which also suggest that "Yunv" could produce 2*n* eggs, whereas, no polyploid progenies were produced from the cross of "Yunv" and "Qijianbaimo" as well as the cross between "Qijianbaimo" and "Damo." Nevertheless, it appears that parents with high frequencies of 2*n* gamete formation, particularly those interspecific hybrids, have a higher probability to produce polyploid progenies. On the other hand, polyploid progenies could also be produced by parents with relatively low 2*n* male gamete formation frequencies.

The identified polyploid individuals were propagated through *in vitro* culture, which served dual purposes: One was to produce enough plant materials for phenotypic evaluation of ornamental value. The other was to establish reliable methods for increased propagation of the desirable progenies for commercial production. In breeding of field crops, triploid plants are usually sterile due to unbalanced meiotic chromosome segregation and endosperm imbalance (Köhler et al., 2010; Wang et al., 2017). In breeding of ornamental crops, however, a key objective is to improve aesthetic value, not for seed production. Desirable triploids can be effectively propagated asexually using tissue culture, to immediately increase the number of plants for commercial production. Thus, micropropagation plays an important role in the development ornamental

plant industry (Chen and Henny, 2008). Micropropagation has been the main method of propagating orchids (Chugh et al., 2009). Somatic polyploidization has been shown to significantly reduce regeneration ability of orchids (Xie et al., 2017; Pham et al., 2019). For example, colchicine induced triploid *Cymbidium hybridum* plants had decreased protocorm-like body (rhizome) proliferation and shoot regeneration (Xie et al., 2017). However, the present study showed that rhizome induction rate and proliferation coefficient, shoot regeneration, and seedling survival rates of triploid "Huanghe" and "Yutao" were either significantly higher than or equal to those of diploid "DH" (**Figures 5**, **6**), suggesting that sexual polyploidization actually improved micropropagation efficiency in *Cymbidium*. The cause of the improved regeneration efficiency is unclear, but it could be implicated by at least two factors. One is the genetic background of two female parents "Dafeng" and "Yunv" used for developing the triploid hybrids "Huanghe" and "Yutao," respectively. Both "Dafeng" and "Yutao" are relatively easy for regeneration. The other factor could be the meiotic recombination and sexual hybridization resultant genetic epistasis, which requires further investigation.

The final step of this established protocol is to evaluate the performance of sexual polyploidized progenies for developing new cultivars. Morphological novelty, mechanical robustness, increased plant growth, and improved tolerance to stressful factors are highly sought-after traits in ornamental plant breeding. Polyploidization generally increases plant organs, called gigas effect (Sattler et al., 2016). In the genus *Dendrobium*, somatic polyploidization resulted in increased petal and/or sepal sizes and leaf thickness, prolonged durability of flowering but decreased growth rates and flower numbers per pseudobulb (Chaicharoen and Saejew, 1981; McConnell and Kamemoto, 1993; Ketsa et al., 2001; Vichiato et al., 2014). Our results showed that sexual polyploidization significantly increased plant height, pseudobulb diameter, leaf length, width, and thickness, peduncle thickness, sepal width and thickness, petal width and thickness, and lip width of triploid "Yutao" compared to diploid "YX" (**Table 3**). On the other hand, morphological characteristics of triploid "Huanghe" only showed significant increase in plant width and lip width but decrease in flower numbers and peduncle length compared to diploid "DH" (**Table 3**). Additionally, both "Yutao" and "Huanghe" had rounder shaped flowers (**Figures 4B,E**) with much more robustness compared to respective diploid counterparts. Furthermore, triploid "Yutao" grew much faster, exhibiting heterosis. On the contrary, the growth of triploid "Huanghe" was largely comparable to diploid "DH," exhibiting limited heterosis. Our speculation is that 2*n* gametes produced from parent "Xiaoxiang" could be resulted from the process of FDR, whereas 2*n* gametes from parent "Hezhihua" might be produced through the SDR. In the process of FDR, meiosis I fails to occur, i.e., there are no chromosome pairing and recombination; instead, chromosomes undergo directly to meiosis II, an equational division. Parental heterozygosity and epistasis are fully retained (De Storme and Geelen, 2013). As a result, polyploid hybrids derived from FDR 2*n* gametes possess great genetic diversity and heterosis. Thus, "Yutao" showed increased heterosis in plant growth. On the other hand, chromosomes in SDR have normal pairing and recombination, i.e., normal meiosis I, but an omission of meiosis II. 2*n* gametes resulted from this process generally have reduced level of heterozygosity and shows a substantial loss of parental epistasis (Peloquin et al., 2008). Therefore, "Huanghe" showed less heterosis, but it did show improved efficiency in regeneration. Further research is warranted to confirm these speculations, specifically morphological characteristics of triploid plants produced through sexual polyploidization should be compared with those autoploid plants. Nevertheless, compared to the diploid parents, triploid cultivars produced from this study show improved ornamental value, increased regeneration capacity during *in vitro* culture or enhanced growth. In contrast to these results, somatic polyploidization usually shows reduced regeneration ability and decreased plant growth rates (Chaicharoen and Saejew, 1981; Vichiato et al., 2007). Our results with cymbidium provide additional evidence supporting the advantages of sexual polyploidization in crop improvement.

CONCLUSION

The present study as the first to document 2*n* gamete occurrence in cultivated cymbidiums. Depending on cultivars, 2*n* male gamete formation frequencies varied from 0.15 to 4.03%. Hybrid cultivars, especially those interspecific ones have relatively higher frequencies of 2*n* male gamete formation. Hybridization using parents with higher 2*n* male gamete formation frequencies generally have a high probability to produce polyploid progenies. Five triploid and two tetraploid progenies were produced in this study. Triploid sterility is not an obstacle to ornamental plants as they are valued for the aesthetic appearance, not for seed production. Triploid plants can be propagated through tissue culture to increase plant numbers for commercial production. Characterization of two triploid plants showed that they exhibited improved ornamental value, i.e., rounder flowers with wider sepal, petals and lips. The sexual triploids had higher regeneration capacity during *in vitro* culture or displayed increased plant growth. Our results demonstrate that sexual polyploidization through unreduced gametes is a viable way for improving cymbidium.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

Z-SZ and R-ZZ designed the study. JZ and G-HD performed determination of unreduced male gamete in parents, hybrid combination making, seedling production, and polyploid identification. S-YX and H-RG cultivated the hybrid and completed the effects of sexual polyploidization on micropropagation characteristics and ornamental traits. LX, R-ZZ, and Z-SZ carried out and analyzed the data. R-ZZ wrote a

first draft of the manuscript that was further critically reviewed by JC, Z-SZ, LX, JZ, S-YX, H-RG, and G-HD.

FUNDING

This work was supported by the National Natural Science Foundation of China (31401899), Special Project of Public Interest Research and Capacity-Building of Guangdong Province, China (2015A020209101 and 2017B020201010), Special Project of Agriculture Science Independent Innovation of Guangzhou Agricultural Bureau, China (1810593 and 19100210) and Project of Guangdong Provincial Key Laboratory of Plant Molecular Breeding (GPKLPMB201902), and China Scholarship Council.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00558/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 29 May 2020
doi: 10.3389/fpls.2020.00622

Artificial Chromosome Doubling in Allotetraploid *Calendula officinalis*

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Calendula officinalis L. is known as an ornamental plant as well as a source of biochemical compounds used in cosmetics and industry. *C. officinalis* has a complex karyotype. Published chromosome numbers differ between $2n = 4x = 28$ or 32. We have estimated genome sizes in nine commercial cultivars and evaluated the ploidy level by karyotyping and fluorescent *in situ* hybridization (FISH) using 5S and 45S rDNA loci. The detection of chromosome sets of two rather than four homologues would suggest that *C. officinalis* has an allotetraploid background. In addition, four signals for 45S but only two for 5S were found by using FISH. Artificial chromosome doubling is a common technique in plant breeding, as polyploidization results in several consequences for plant growth and development. Especially the suggested allotetraploid background in *C. officinalis* is interesting when examining the effect of chromosome doubling on the plant phenotype. Here we describe chromosome doubling of three allotetraploid cultivars of *C. officinalis*, 'Nova,' 'WUR 1553-7' and 'Orange Beauty'. Three antimitotic agents – colchicine, oryzalin and trifluralin - were used in different concentrations to find the combination of the best agent and the best dosage to obtain octaploids. For all three cultivars a few octaploids were obtained. A concentration of 200 and 400 ppm of colchicine was most efficient for chromosome doubling in 'Nova' and 'Orange Beauty,' respectively. For 'WUR 1553-7' the treatment with 20 ppm oryzalin was also effective. Cell numbers and first observations of the phenotype in the chromosome doubled plants show thicker leaves and bigger cells, as commonly observed after ploidy doubling. Due to the low number of chromosome doubled plants obtained more elaborate phenotyping will be performed on following generations cultivated under field conditions.

Keywords: colchicine, fluorescent *in situ* hybridization, FISH, oryzalin, polyploidy, trifluralin

OPEN ACCESS

Edited by:
Jen-Tsung Chen,
National University of Kaohsiung, Taiwan

Reviewed by:
Margarida L. R. Aguiar-Perecin, University of São Paulo, Brazil
Jianjun Chen, University of Florida, United States

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *04 December 2019*
Accepted: *22 April 2020*
Published: *29 May 2020*

Citation:
Esmaeili G, Van Laere K, Muylle H and Leus L (2020) Artificial Chromosome Doubling in Allotetraploid Calendula officinalis. Front. Plant Sci. 11:622. doi: 10.3389/fpls.2020.00622

INTRODUCTION

The genus *Calendula* is native to the Mediterranean region, Macronesia and Southwest Asia. It is a taxonomically complex genus, with 10–27 species being reported depending on the author. *C. officinalis* L. is an important annual medicinal and ornamental plant native to Europe and North Africa (Ao, 2007). Several classes of biochemical compounds were identified in *C. officinalis* inflorescences including essential oil, carotenoids, flavonoids, terpenoids, coumarins, quinones, amino acids, lipids, and carbohydrates. These compounds can be used for various pharmaceutical

and medicinal purposes: as an anti-oxidant, anti-inflammatory, anti-bacterial, anti-fungal, anti-cancer, anti-HIV, to promote wound healing, and more (Khalid and da Silva, 2012; Jan and John, 2017; Cruceriu et al., 2018; Verma et al., 2018; Givol et al., 2019).

Interest in *C. officinalis* is growing as a good source of bio-compounds. It is known that within *C. officinalis* the content of biochemical compounds varies widely, depending on the color of the inflorescence and the rate of ligulate florets (Khalid and da Silva, 2012). The performance of existing cultivars is therefore being screened with the aim of selecting higher yielding cultivars. In addition, (artificial) polyploidization is a successful method to improve plant traits. Polyploidy has an effect on biosynthesis pathways and gene expression (Dhooghe et al., 2010; Sattler et al., 2016; Salma et al., 2017; Zhang et al., 2018). Polyploids may have bigger inflorescences, larger and thicker leaves and stems, larger pollen, a higher biomass, higher concentration of secondary metabolites, and better tolerance to environmental stress (Mori et al., 2016; Li et al., 2018; Luo et al., 2018; Eng and Ho, 2019). Polyploidy has led to an increase in secondary metabolites in medicinal plants in two ways: first, by the increase of biomass in chromosome doubled plants, and second, by the increase in number of gene copies related to the pathways of the secondary metabolites. Examples of higher levels of secondary metabolites after chromosome doubling are the increase of alkaloids in *Datura stramonium* (Berkov and Philipov, 2002) and *Atropa belladonna* (Huang et al., 2010), essential oils and chamazoline in *Matricaria chamomile* (Gosztola et al., 2006), terpenoids and flavonoids in *Salvia miltiorrhiza* (Gao et al., 1996) and morphine content in *Papaver somniferum* (Mishra et al., 2010).

Calendula species show a large variation in chromosome numbers. Basic chromosome numbers are 7, 8, 9, 11, and 15 (Rice et al., 2015). *C. officinalis* is mostly described as tetraploid, but different chromosome numbers have been published, namely $2n = 28$ or $2n = 32$ [Chromosome Counts Database (CCDB) (Rice et al., 2015)]. Higher chromosome numbers are found in *C. arvenisis* ($2n = 4x = 44$) and in *C. palaestina* and *C. pachysperma* ($2n = \pm 85$), which are probably autopolyploids of *C. arvensis* (Heyn et al., 1974). The differences in chromosome number, karyotype, genome size and ploidy level are the result of high levels of hybridization, chromosome losses and dysploidy (Nora et al., 2013), which makes taxonomy in *Calendula* very difficult. Nora et al. (2013) used genome sizes and chromosome numbers for the evaluation of evolutionary relationships and taxonomy in *Calendula*. They conclude that *C. officinalis* is a tetraploid plant with 32 chromosomes ($2n = 4x = 32$) with a genome size of 2.97 ± 0.08 pg/2C.

In our study we aimed to evaluate and confirm the ploidy level and genome size of nine *C. officinalis* cultivars using flow cytometry and fluorescent *in situ* hybridization (FISH) analysis. FISH using 45S and 5S rDNA is a valuable method to study plant evolution and ploidy levels (Kirov et al., 2017) and has been used in species such as *Chrysanthemum* (Kondo and Abd El-Twab, 2002; Abd El-Twab and Kondo, 2013), *Alium fistulosum* (Kirov et al., 2017), *Gossypium hirsutum* L. (Andres and Kuraparthy, 2013), *Prunus* species (Schmöllerl, 2009) for the identification of chromosome sets, chromosome numbers, copies and ploidy level. Recently, 45S and 5S rDNA FISH was used to assess the cytogenetic variability of *C. officinalis* after chemical mutagenesis (Samatadze et al., 2019).

The second aim of our study was to perform chromosome doubling in *C. officinalis* cultivars establishing a protocol for artificial ploidy doubling. In a seed treatment three antimitotic agents: colchcine, trifluralin and oryzalin, were tested each in three different concentrations in search for an optimal product-dosage combination. First results on differences in cell numbers are given. Next generations of the chromosome doubled plants will be phenotyped with the aim of evaluating them for possible increased yield of biochemical compounds.

MATERIALS AND METHODS

Plant Material

Nine cultivars were used: 'Orange Beauty,' 'Neon,' 'Apricot Beauty,' 'Cream Beauty,' 'Lemon Beauty,' 'Yellow Gem,' 'Orange Porcupine,' and 'Nova' obtained from Vreeken's Zaden (Dordrecht, Netherlands) and 'WUR 1553-7' obtained from Wageningen University.

Genome Size of *Calendula* Cultivars

The 2C value of nine *C. officinalis* cultivars (**Table 1**) was measured using a PASIII flow cytometer (Partec, Germany) equipped with a 20 mW 488 nm laser. The CyStain PI kit (Sysmex, Germany) was used for sample preparation according to the manufacturer's protocol with minor modifications. One piece (± 0.5 cm^2) of fresh young leaf tissue was chopped together with fresh leaf tissue of the internal standard, *Pisum sativum* 'Ctirad' 9.09 pg/2C (Doležel et al., 1998), in 500 µl nuclei extraction buffer (CyStain PI kit). Samples were filtrated through a 50 µl CellTrics filter (Sysmex, Münster, Germany) and subsequently 1200 µl staining solution (CyStain PI kit) with propidium iodide and RNase A stock solution (both CyStain PI kit) were added. Samples were incubated at 4°C for 30 min before measurement. All histograms were analyzed using FloMax software (Quantum Analysis, Germany). For every cultivar two biological replicates were analyzed. The biological replicates were analyzed on different dates and samples were taken from different plants of the same cultivar. For every biological replicate the analysis was repeated (two technical replicates per biological replicate). Mean values and standard deviations were calculated based on the four histograms obtained.

Calendula Karyotyping Using 45S and 5S rDNA FISH

Chromosome slides were made for *C. officinalis* 'Nova,' 'WUR 1553-7,' and 'Orange Beauty' according to the 'SteamDrop' method (Kirov et al., 2014). Briefly, young root tips were pretreated for 3 h in a solution containing 0.1% colchicine and 8-hydroxyquinoline. Root fixation was done in Carnoy solution 3:1 (ethanol:acetic acid) for 1 h at room temperature. Cell suspensions were made after digestion using 0.6% enzyme solution (mixture of cellulase, pectolyase and cytohelicase)

TABLE 1 | 2C-value (pg) of various *C. officinalis* cultivars.

Cultivar	'Nova'	'Apricot Beauty'	'Cream Beauty'	'Lemon Beauty'	'Yellow Gem'	'Orange Porcupine'	'Neon'	'Orange Beauty'	'WUR 1553-7'
2C value (pg)	2.79 ± 0.04	2.76 ± 0.07	2.72 ± 0.04	2.90 ± 0.06	2.76 ± 0.09	2.82 ± 0.05	2.68 ± 0.08	2.80 ± 0.08	2.81 ± 0.06

Given values represent the mean genome size ± SD of four analyses (two biological replicates with each time a technical replicate).

incubation at 37°C for 50 min. During slide preparation 2:1 ethanol:acetic acid and 1:1 ethanol:acetic acid were used as fixative 1 and fixative two, respectively.

Plasmids containing 5S rRNA genes of rye (pSCT7, Lawrence and Appels, 1986) and 45S rRNA genes of wheat (pTA71, Gerlach and Bedbrook, 1979) were labeled by Digoxigenin- and Biotin- Nick Translation Mix (Roche, Germany), respectively, according to the manufacturer's protocol. For FISH we used the protocol described in Heslop-Harrison et al. (1991) with some modifications. Briefly, slides were incubated overnight at 37°C. Chromosomes were pretreated with 4% paraformaldehyde in 2 × SSC for 8 min at RT and dehydrated in ethanol (70, 90, and 100%). The hybridization mixture consisted of 50% (v/v) deionized formamide, 10% (w/v) dextran sulfate, 2 × SSC, 0.25% sodium dodecyl sulfate, and 2 ng/μl probe DNA. The mixture was denatured at 75°C for 10 min, placed on ice for 5 min, and 80 μl was applied to each slide. Slides were denatured at 80°C for 5 min and incubated overnight at 37°C in a humid chamber. For stringency washing 0.1 × SSC was used at 48°C for 30 min. Biotin and digoxigenin labeled probes were detected with streptavidin-Cy3 (Sigma-Aldrich, United States) and anti-dig-FITC (Roche, Germany), respectively. Slides were counterstained with DAPI [0.2 μl DAPI + 20 μl Vectashield (Labconsult, Brussels, Belgium)] and analyzed using a fluorescence microscope, Zeiss AxioImager M2 (Carl Zeiss MicroImaging, Belgium), equipped with an Axiocam MRm camera and ZEN-software (Carl Zeiss MicroImaging, Belgium). Chromosome and signal analysis was done in DRAWID software version 0.26 (Kirov et al., 2017) on five well-spread metaphases of each cultivar. Chromosome classification and calculation of arm ratios was done according to Levan et al. (1964).

Chromosome Doubling

Chromosome doubling was performed on *C. officinalis* 'Nova,' 'Orange Beauty,' and 'WUR 1553-7'. Seeds were stored under low humidity in a seed storage room at 4°C before use. Dry seeds (about 1000 seeds per cultivar) were primed in 150 ppm GA_3 for 72 h at room temperature. Per treatment 90 seeds were immersed in different antimitotic agents: 200, 400, 800 ppm colchicine (Duchefa, Netherlands); 20, 40, 80 ppm trifluralin (Sigma-Aldrich, Belgium); 20, 40, 80 ppm oryzalin (Duchefa, Netherlands) and a control treatment using water, during 24 h at room temperature. Stocks of the antimitotic agents were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Belgium). After treatment the seeds were washed thoroughly with tap water (three times for 5 min). Subsequently for every treatment seeds were germinated on filter paper in Petri dishes. The 90 seeds per treatment were put in three Petri dishes each containing 30 seeds and incubated in the germinator (GC10, Flohr Instruments, Netherlands) with a daylight period of 12 h at 22°C. For the 90 seeds per treatment seed germination was recorded during 14 days. Germinated seeds were transplanted in a plant tray containing a Saniflor peat mixture [(Van Israel, Geraardsbergen, Belgium) 1.5 kg/m^3; fertilizer: 12N:14P:24 K including trace elements, pH 5.0-6.5, EC 0.450 mS/cm] and placed in the greenhouse (greenhouse conditions: light 16 h in case of shorter day period, day/night: ventilation temperature: 18/22°C, heating temperature 15/18°C etc.). Both germination rate (%) after 14 days and survival rate (%) 3, 6, and 9 weeks after transplanting were calculated.

The ploidy level of the treated seedlings was determined 3, 6, and 9 weeks after transplant using a Cyflow Space flow cytometer (Partec, Germany) equipped with a UV-LED. Plants that were considered chromosome doubled were reanalyzed 6 months later. The protocol used is based on Otto (1990). Approximately 0.5 cm^2 of young leaf tissue was chopped in a 500 μL extraction buffer containing 0.1 M citric acid monohydrate and 0.5% Tween-20. Samples were filtered through a 50 μm CellTrics filter to eliminate cell debris. Then 750 μL of staining buffer containing 0.4 M $Na_2HPO.12HO$, 2 mg/L 4′, 6-diamidino-2-phenyllndole (DAPI), 0.1% polyvinylpyrrolidone (PVP) was added. All histograms were analyzed using FloMax software.

Cell Number and Size

The cell number ratio between the original and chromosome doubled plants was determined using flow cytometry according to Hias et al. (2017). In the flow cytometric histograms the number of nuclei in the different phases of the cell cycle (G1, S, and G2/M) can be determined using the true volumetric cell counting option of the flow cytometer Cyflow Space (Flomax Software). Sample preparation was done as described above for flow cytometric ploidy analysis. Leaf disks (4 mm diameter) were cut from tetraploid and octaploid plants using a cork borer. The fresh weight (FW) of the leaf disks was determined on a balance (XS104 Mettler-Toledo, Zaventem). In a pooled sample a leaf disk of the tetraploid plant was co-chopped together with a leaf disk of an octaploid plant. Both leaf disks are put on top of each other for chopping. The histogram of the pooled sample with the tetraploid (4x) and octaploid (8x) leaf disks showed three peaks: in the first peak are the $G1_{4x}$ nuclei, a second peaks shows nuclei in $G2_{4x}$ + $G1_{8x}$ (overlapping peaks) and the third peak are the $G2_{8x}$ nuclei. The signal of the nuclei in the S-phase for the tetraploid and octaploid leaf disk is found in between the $G1_{4x}$ and $G2_{4x}$ peaks and $G1_{8x}$ and $G2_{8x}$, respectively. From this histogram the ratio of tetraploid nuclei/g FW over octaploid nuclei/g FW can be determined. To be able to differentiate the overlapping $G2_{4x}$ and $G1_{8x}$ peaks, the percentage of dividing nuclei in the tetraploid leaf is taken into account. Therefore a separate analysis is performed using a leaf disk of the tetraploid plant. From the resulting histogram the percentage of nuclei in

division was calculated. A mean ratio was calculated bases on four repetitions.

For histological visualization a modified protocol of Habarugira et al. (2015) was used. Slides were prepared from control and chromosome doubled plant material. Each time leaves and petals were sampled from three different plants of 'Nova'. Pieces (±1 cm^2) of full grown leaves and petal pieces (±1 cm length) were harvested and fixated in formalin alcohol acetic acid (FAA) solution (10:7:2:1 EtOH 99%:demineralized water:formalin:glacial acetic acid) in glass tubes and put under vacuum during 20 min. Subsequently the FAA solution on the samples was renewed and stored overnight at 4°C. Samples were rinsed twice with 50% ethanol and preserved at −20°C in 50% ethanol until use. Dehydration was performed by increasing series of ethanol concentrations (70, 85, and 100% during 2 h each time). For the infiltration step 1:1 99% EtOH:glycol methacrylate (Technovit 7100, Heraeus Kuzler, Germany) + 1 g hardener was used. The dehydration was performed in 1.5 ml Eppendorf tubes under vacuum during 20 min. The samples remained for 2 h in the infiltration solution at 4°C. All liquid was removed and replaced by 100% Technovit infiltration solution and kept at 4°C until embedding. For embedding the samples were transferred to 15:1 Technovit:hardener two and kept on ice. The samples were positioned upright in the Eppendorf tube and kept at 4°C for 1 h. Polymerization was performed at 37°C overnight. The samples in the blocks were sectioned at 5 µm using a HM360 Microtome (Thermo Fisher Scientific, Merelbeke, Belgium). Slides were stained for 10 min with 1% Toluidine Blue O (Acros Organics, Geel, Belgium) and rinsed twice with demineralized water. Slides were covered and sealed with DPX mounting medium (Merck KGaA, Darmstadt, Germany). Microscopy was performed using bright field microscopy and pictures were taken (Zeiss AxioImager M2 (Carl Zeiss MicroImaging, Belgium), equipped with an Axiocam MRm camera) and ZEN-software (Carl Zeiss MicroImaging, Belgium).

Statistical Analysis

The data on seed germination were compared using a one-way ANOVA. If the *F*-test was significant ($p < 0.05$), a Scheffé's *post hoc* test was performed.

For the data on cell number, a Student's *t*-test was used. Differences were considered significant when $p < 0.001$.

RESULTS

Genome Size and Chromosome Karyotype Analyses on *C. officinalis*

To confirm the genome size and ploidy level of the *C. officinalis* cultivars, flow cytometry and karyotype analysis were performed. The mean genome size value (2C) of nine *C. officinalis* cultivars ranged between 2.68 ± 0.08 and 2.90 ± 0.06 pg/2C (**Table 1**). Since there is only a small variation in *C. officinalis* genome sizes, we can expect that all our *C. officinalis* cultivars have the same ploidy level and chromosome count.

Chromosome analysis for *C. officinalis* 'Nova', 'WUR 1553-7' and 'Orange Beauty' revealed 32 chromosomes ($2n = 32$). The chromosome length ranges between 1.87 ± 0.37 µm and 3.98 ± 0.53 µm (**Figure 1** and **Table 2**). The karyotype contains two sets of 16 homoeologous chromosomes showing a karyotype formula 2(8M + 8SM), with chromosomes 2, 3, 4, 6, 7, 10, 12, and 16 metacentric (M) chromosomes while the rest were submetacentric (SM) (**Table 2** and **Figure 1**).

FISH analysis with the 45S rDNA probe revealed four signals located at the terminal region of the chromosome pairs 1 and 10 (**Figures 2A,C**). Two bright signals of the 5S rDNA probe hybridization were observed in the pericentromere region of chromosome pair 7 (**Figures 2B,C**). Multicolor FISH analysis using both 45S and 5S rDNA probes confirmed the four 45S rDNA and two 5S rDNA signals (**Figure 2C**). The signals were observed in all analyzed metaphase cells. The karyotype indicates an allotetraploid background in *C. officinalis*.

Polyploidization in *C. officinalis*

Seed Germination After Polyploidization

Chromosome doubling using antimitotic agents was assessed on seeds of three allotetraploid *C. officinalis* cultivars with the aim to obtain octaploids. The effect of the antimitotic agents on seed germination is shown in **Table 3**. Seed germination was affected by the use of antimitotic agents in all three cultivars, as

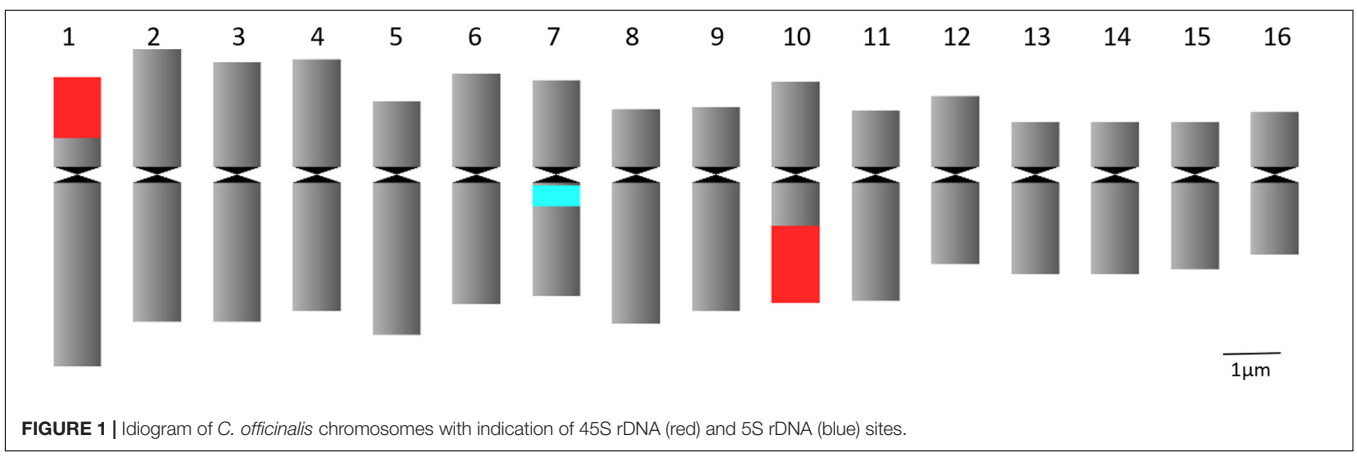

FIGURE 1 | Idiogram of *C. officinalis* chromosomes with indication of 45S rDNA (red) and 5S rDNA (blue) sites.

TABLE 2 | Chromosome characteristics of *Calendula officinalis*.

Chromosome name	Centromere index (%)[a] ± SD	Chromosome length (μm) ± SD	Short arm length (μm) ± SD	Long arm length (μm) ± SD	Arm ratio	Chromosome designation[b]
1	32.03 ± 4.02	3.99 ± 0.53	1.26 ± 0.39	2.72 ± 0.31	2.38 ± 0.88	SM
2	46.02 ± 3.30	3.82 ± 0.56	1.75 ± 0.27	2.06 ± 0.35	1.18 ± 0.17	M
3	42.68 ± 3.85	3.57 ± 0.65	1.52 ± 0.29	2.05 ± 0.42	1.26 ± 0.36	M
4	45.62 ± 3.47	3.48 ± 0.74	1.59 ± 0.36	1.89 ± 0.42	1.21 ± 0.19	M
5	29.83 ± 3.98	3.21 ± 0.54	0.94 ± 0.25	2.26 ± 0.40	2.57 ± 0.41	SM
6	43.33 ± 2.93	3.16 ± 0.52	1.36 ± 0.22	1.79 ± 0.34	1.30 ± 0.16	M
7	43.3. ± 3.86	2.96 ± 0.47	1.29 ± 0.26	1.68 ± 0.26	1.32 ± 0.21	M
8	29.14 ± 3.16	2.93 ± 0.51	0.85 ± 0.13	2.09 ± 0.42	2.47 ± 0.40	SM
9	31.45 ± 2.65	2.77 ± 0.26	0.87 ± 0.11	1.90 ± 0.19	2.20 ± 0.31	SM
10	45.24 ± 3.55	2.77 ± 0.53	1.21 ± 0.23	1.56 ± 0.36	1.30 ± 0.25	M
11	31.80 ± 2.73	2.58 ± 0.35	0.82 ± 0.13	1.76 ± 0.25	2.17 ± 0.31	SM
12	46.01 ± 3.30	2.24 ± 0.51	1.04 ± 0.26	1.21 ± 0.26	1.18 ± 0.16	M
13	32.99 ± 4.44	2.03 ± 0.46	0.67 ± 0.17	1.37 ± 0.33	2.09 ± 0.47	SM
14	32.80 ± 3.00	2.00 ± 0.28	0.65 ± 0.11	1.34 ± 0.20	2.07 ± 0.29	SM
15	34.05 ± 3.00	1.95 ± 0.39	0.66 ± 0.14	1.29 ± 0.27	1.96 ± 0.27	SM
16	43.23 ± 4.76	1.87 ± 0.37	0.75 ± 0.27	1.12 ± 0.18	1.72 ± 0.91	M

[a]*Values are means (n = 4) ± SD;* [b]*M, Metacentric and SM, Sub-metacentric.*

shown by the one-way ANOVA ['Nova' $F = 9.12$, $p = 0.00002$; 'Orange Beauty' $F = 4.79$, $p = 0.0017$; 'WUR 1553-7' $F = 2.93$, $p = 0.026$]. Although in all three cultivars and for every treatment a lower number of seeds germinated when compared to the control, the Scheffé's *post hoc* test showed that the negative effect of the application of colchicine is only significant at the higher doses of 400 and 800 ppm, and not at 200 ppm. The negative impact on seed germination was more pronounced when higher concentrations of colchicine were applied. When compared to the control treatment, seed germination in 'Nova' significantly decreased for all oryzalin and trifluralin concentrations applied, but for 'Orange Beauty' and 'WUR 1553-7' the decrease in seed germination is less severe and differs significantly in only a few treatments when compared to the control treatment. For oryzalin and trifluralin no dose response is observed as no significant differences are found between concentrations used. However, high concentrations of antimitotic agents resulted in a delay in root emergence and abnormal seedlings were observed that had compact roots without a root tip and with fragile cotyledons.

Ploidy Analysis

For all three cultivars antimitotic agents had a negative effect on seedling survival. Nevertheless octaploids and mixoploids were obtained for all three antimitotic agents, but the efficiency depended on the *Calendula* genotype (**Tables 4**–**6**). A high rate of reversion to the tetraploid level was observed. In total 168 'Nova' seedlings were transplanted after treatment. The highest seedling mortality was observed between 3 and 6 weeks after transplant when only 23 seedlings were still alive. After 9 weeks 21 seedlings survived. In 'Nova' the highest number of stable tetraploid plants was obtained using 200 ppm colchicine and oryzalin or trifluralin at a concentration of 20 ppm (**Table 4**). For 'Orange Beauty' the number of surviving seedlings also dropped most between 3 and 6 weeks after transplanting, from 455 transplanted to 313 after 3 weeks and 43 seedlings after

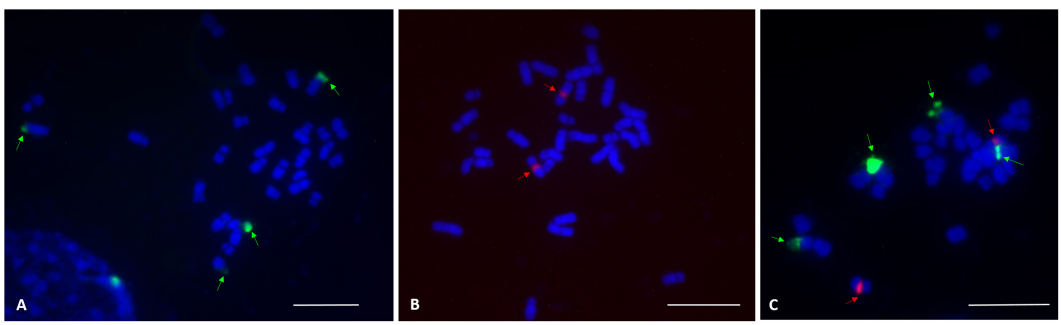

FIGURE 2 | FISH localization of 45S rDNA and 5S rDNA loci (indicated by arrows) on mitotic metaphase chromosomes of *C. officinalis* 'Nova.' **(A)**, four 45S rDNA signals were identified in the terminal regions of chromosome pair 1 and 10. **(B)**, Two 5S rDNA signals were detected in the centromeric region of chromosome pair 7. **(C)**, 45S rDNA (green) and 5S rDNA (red) signals. Bar = 10 μm.

TABLE 3 | Seed germination for three *C. officinalis* cultivars after treatment with antimitotic agent treatments in different concentrations.

Antimitotic agent	Concentration (ppm)	% Seed Germination		
		'Nova'*	'Orange Beauty'	'WUR 1553-7'
		% ± SD	% ± SD	% ± SD
Control	0	73.3 ± 3.8^{a}	85.3 ± 1.9^{a,b}	81.3 ± 12.4^{a}
Colchicine	200	42.2 ± 12.3^{a,b}	68.9 ± 7.9^{a,b}	63.3 ± 7.2^{a,b}
	400	25.6 ± 11.3^{b}	66.7 ± 11.9^{a,b,c}	46.7 ± 4.7^{a,b}
	800	8.9 ± 4.7^{b}	45.6 ± 4.2^{b,c}	40.0 ± 9.4^{b}
Oryzalin	20	18.9 ± 6.3^{b}	60.0 ± 2.7^{a,b,c}	44.4 ± 3.1^{a,b}
	40	15.6 ± 5.7^{b}	61.1 ± 12.3^{a,b,c}	40.0 ± 5.4^{b}
	80	15.6 ± 1.6^{b}	46.7 ± 9.4^{b,c}	48.9 ± 15.0^{a,b}
Trifluralin	20	15.6 ± 4.3^{b}	60.0 ± 9.8^{a,b,c}	57.8 ± 5.7^{a,b}
	40	27.8 ± 8.7^{b}	32.2 ± 3.1^{c}	46.7 ± 5.4^{a,b}
	80	14.4 ± 8.3^{b}	58.9 ± 6.3^{a,b,c}	48.9 ± 4.2^{a,b}

*For each treatment mean values (%) and standard deviations (±SD) are calculated based on the three Petri dishes with 30 seeds in each Petri dish. *within one genotype different letters exhibit statistically significant differences (Scheffé's test; $p < 0.05$).*

TABLE 4 | The number of surviving seedlings and ploidy level of 'Nova' treated with various antimitotic agents.

'Nova'		Number of transplanted seedlings	Number of surviving seedlings			Tetraploid plants			Octaploid plants			Mixoploid plants		
Antimitotic agent	Conc. (ppm)		Weeks after transplanting											
			3	6	9	3	6	9	3	6	9	3	6	9
Colchicine	200	38	33	6	5	26	3	3	4	3	2	3	0	0
	400	22	11	3	3	9	1	3	1	1	0	1	1	0
	800	9	5	3	3	4	0	2	1	1	1	0	2	0
Oryzalin	20	17	16	2	2	15	1	1	1	1	1	0	0	0
	40	14	8	0	0	5	0	0	0	0	0	3	0	0
	80	14	5	2	2	1	0	1	1	1	0	3	1	1
Trifluralin	20	16	16	2	2	14	1	1	1	1	1	1	0	0
	40	25	10	1	1	8	1	1	1	0	0	1	0	0
	80	13	11	4	3	7	2	2	0	0	0	4	2	1
Total		168	115	23	21	89	9	14	10	8	5	16	6	2

Each treatment contains three Petri dishes with 30 seeds in each Petri dish. Numbers are given on the total of 90 seeds per treatment.

6 weeks (**Table 5**). Three weeks after transplanting, 32 octaploid plants were found, while only 2 octaploid seedlings were left after 6 weeks and only one after 9 weeks. A high number of mixoploid seedlings (75 seedlings) was detected 3 weeks after transplanting, but again after 9 weeks, the number decreased to only 10 mixoploid seedlings (**Table 5**). Most mixoploids either died or reverted back to the tetraploid level. Also in the other cultivars it is observed that the number of tetraploids increases over time because octaploids or mixoploids return to the tetraploid level. After germination, 393 'WUR 1553-7' seedlings were transplanted (**Table 6**). For this cultivar as well, most of the seedlings died between 3 and 6 weeks after transplant. In total 37 octaploid plants were detected 3 weeks after transplant and only 16% of them (6 seedlings) remained octaploid after 9 weeks. Most octaploid plants were obtained after colchicine treatments and 80 ppm oryzalin (**Table 6**). The ploidy analysis on the chromosome doubled plant 6 months later confirmed the stability of these octaploids.

For all three cultivars, octaploid plants exhibited a slower growth rate compared to tetraploid control plants in the first weeks after transplantation. However, after 3 weeks the remaining octaploid plants showed increased growth vigor compared to the tetraploid control plants. Flowering was observed in both the tetraploid and the octaploid plants (**Figures 3A,B**).

Cell Number

A significant difference (*t*-test; $p < 0.001$) was found when the weight of the 4 mm diameter punches of the control and the chromosome doubled leaves were compared. The weight of the control leaf disks was 4.4 ± 0.2 mg while it was 5.8 ± 0.6 mg for the chromosome doubled leaf disks.

When the number of nuclei in the original and the chromosome doubled leaf disks was calculated, the number of nuclei per gram is significantly lower in the chromosome doubled plants ($p < 0.001$; *t*-test). When the ratio is calculated for every combined analysis of undoubled and chromosome doubled leaf

TABLE 5 | The number of surviving seedlings and ploidy level of 'Orange Beauty' treated with various antimitotic agents.

'Orange Beauty'		Number of transplanted seedlings	Number of surviving seedlings			Tetraploid plants			Octaploid plants			Mixoploid plants		
Antimitotic agent	**Conc. (ppm)**		**Weeks after transplanting**											
			3	**6**	**9**	**3**	**6**	**9**	**3**	**6**	**9**	**3**	**6**	**9**
Colchicine	200	62	34	3	3	26	2	2	1	0	0	7	1	1
	400	60	29	5	5	14	1	2	2	1	1	13	2	2
	800	41	9	2	2	3	0	0	1	1	0	5	1	2
Oryzalin	20	54	49	7	7	36	6	5	4	0	0	9	1	2
	40	55	50	2	2	30	1	1	1	0	0	19	1	1
	80	42	13	7	7	3	2	6	3	0	0	7	5	1
Trifluralin	20	59	59	1	1	45	1	1	9	0	0	5	0	0
	40	29	23	7	7	18	6	7	3	0	0	2	1	0
	80	53	47	9	9	31	7	8	8	0	0	8	2	1
Total		455	313	43	43	206	26	32	32	2	1	75	14	10

Each treatment contains three Petri dishes with 30 seeds in each Petri dish. Numbers are given on the total of 90 seeds per treatment.

TABLE 6 | The number of surviving seedlings and ploidy level of 'WUR 1553-7' treated with various antimitotic agents.

'WUR 1553-7'		Number of transplanted seedlings	Number of surviving seedlings			Tetraploid plants			Octaploid plants			Mixoploid plants		
Antimitotic agent	**Conc. (ppm)**		**Weeks after transplanting**											
			3	**6**	**9**	**3**	**6**	**9**	**3**	**6**	**9**	**3**	**6**	**9**
Colchicine	200	57	44	3	3	37	1	2	7	0	1	0	2	0
	400	42	29	8	8	15	2	3	10	1	2	4	5	3
	800	36	10	4	4	5	0	0	2	1	1	3	3	3
Oryzalin	20	40	36	7	5	32	3	4	2	0	0	2	4	1
	40	36	36	5	5	26	2	5	8	0	0	2	2	0
	80	44	21	10	9	12	2	4	3	3	2	6	5	3
Trifluralin	20	52	49	0	0	48	0	0	1	0	0	0	0	1
	40	42	30	4	4	22	4	3	4	0	0	4	0	1
	80	44	21	21	21	19	16	15	0	0	0	2	5	6
Total		393	276	62	59	216	30	36	37	5	6	23	26	18

Each treatment contains three Petri dishes with 30 seeds in each Petri dish. Numbers are given on the total of 90 seeds per treatment.

disks, an average of 3.16 ± 0.91 times more cells were observed in the non-doubled plants.

Leaves and flowers of the chromosome doubled and control plants were prepared as slides. Microscopic images confirmed that the leaves and flowers of chromosome doubled leaves were thicker and both flowers and leaves had bigger cells (**Figures 3C–F**).

DISCUSSION

Despite the number of studies published on the evolution in the genus *Calendula*, some ambiguity remains about chromosome numbers and ploidy levels. In our study, genome sizes between 2.68 ± 0.08 and 2.90 ± 0.05 pg/2C were found for the nine *C. officinalis* cultivars tested, which is a bit lower than the 2.97 ± 0.08 pg mentioned in literature (Nora et al., 2013). Intraspecific variations of 2C values in plants is subject to controversy. It is difficult to separate intraspecific variation in genome size caused by cytolic compounds from various molecular mechanisms including duplications, deletions, chromosomal polymorphisms, the presence of B-chromosomes and/or the presence of repetitive sequences. For example Bilinski et al. (2018) observed differences in genome sizes in maize landraces correlated to abundance of transposable element families and heterochromatic knobs. We observed small but significant differences in genome sizes in *C. officinalis* cultivars between greenhouse grown plants and field grown plants (results not shown). Therefore we assume that the variation in genome sizes between cultivars of *C. officinalis* is due to stoichiometric errors of the flow cytometric analysis and by variations in levels of secondary metabolites (Greilhuber, 1998; Noirot et al., 2000; Huang et al., 2013).

Infra-generic variation in genome size among homoploid species is a common feature in plants (Bennett and Leitch, 2011). In *Calendula* taxa a positive correlation is found between genome size and chromosome number (Nora et al., 2013). But for *C. officinalis* the genome size is lower than expected when

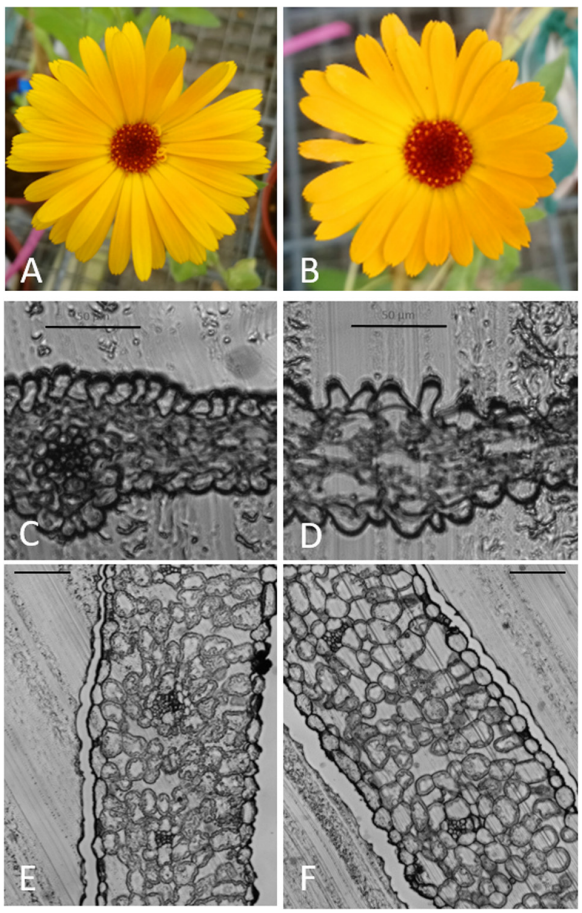

FIGURE 3 | Flowering in tetraploid and octaploid *Calendula officinalis* 'Nova' and cross sections of leaf and petal. **(A,C,E)**: flower, flower cross section and leaf cross section of a control plant. **(B,D,F)**: flower, flower cross section and leaf cross section of a chromosome doubled plant. **(C,D)** bar = 50 μm; **(E,F)** bar = 100 μm.

compared to other *Calendula* species with the same chromosome number. Evaluating different possibilities of interspecific hybridizations and ploidy doubling, Nora et al. (2013) assumed, based on genome sizes, that dysploidy might been involved in the origin of *C. officinalis*. For *C. officinalis* either 28 or 32 chromosomes are mentioned, depending on the author (Rice et al., 2015). Garcia et al. (2010) reported 28 chromosomes while a recent study (Samatadze et al., 2019) reported 32 chromosomes. In the present study, chromosomal slides were prepared and an ideogram was made based on the counted number of 32 chromosomes. In addition, Garcia et al. (2010) observed four 45S rDNA and two 5S rDNA signals after FISH analysis. This was confirmed by Samatadze et al. (2019) who describe four signals of 45S rDNA on chromosome pairs 1, 9, weak (polymorphic) 45S rDNA signals on chromosome 5 and 10 and 2 signals of 5S rDNA on chromosome pair 10. In our study, we also observed four signals for 45S rDNA on chromosome pair number 1 and 10 and 2 signals for 5S rDNA on chromosome pair 7. The differences in chromosome identification between our study and the study of Samatadze et al. (2019) are most probably due to the small and rather uniform chromosomes of *C. officinalis*. When taken together, the following observations strongly indicate that *C. officinalis* is an allotetraploid species: (1) the prevalence of four signals of 45S rDNA on two different chromosome pairs and two signals of 5S rDNA on one chromosome pair taken together with the number of chromosomes, (2) the morphology of the chromosomes illustrating the fact that chromosomes morphologically appear in sets of two. Autopolyploids arise from intra-species whole genome duplication events, while allopolyploids arise from genome duplication events involving inter-specific hybridization (Ramsey and Schemske, 2002). Nora et al. (2013) discuss different hypotheses to explain the chromosome number and the genome size in *C. officinalis*. Both hypotheses, chromosome losses in *C. maroccana* (Heyn and Joel, 1983) or hybridization between *C. stellata* ($2n = 14$) and a species with $2n = 18$ (e.g., C. *maroccana*, *C. eckerleinii*) followed by chromosome doubling (Ohle, 1974) are not well supported when the genome sizes of these species are taken into account. Other techniques like genomic *in situ* hybridization (GISH) using probes of the possible ancestors could be used to explore what species are involved in the genetic background in *C. officinalis* as well as to examine the loss of specific chromosomes. Further, intra-genomic and inter-genomic meiotic pairings can be studied in allo- and autopolyploids to discriminate homologous and homoeologous chromosomes (Lloyd and Bomblies, 2016); this could be interesting to study introgression breeding.

The indications for allopolyploidy in *C. officinalis* and the higher chromosome numbers found in some other *Calendula* species (e.g., $2n = \pm 85$ in *C. palaestina* and *C. pachysperma*) (Heyn et al., 1974) led us to explore chromosome doubling in *C. officinalis*. Genome doubling is often induced by treating seeds with antimitotic agents (Salma et al., 2017). Colchicine is the most common substance for chromosome doubling in plants, but it has several undesirable side effects: it is highly toxic to humans, shows high sensitivity to light, and shows low affinity to plant tubulin in low concentrations (Dhooghe et al., 2010; Eeckhaut et al., 2018; Eng and Ho, 2019; Manzoor et al., 2019). We therefore also included oryzalin and trifluralin in our study. Due to their better affinity for plant tubulin, lower concentrations (one-tenth of colchicine) can be used (Dhooghe et al., 2010; Sattler et al., 2016; Eeckhaut et al., 2018).

A general trend observed in the present study was that as the metaphase inhibitor concentrations increased, seed germination decreased. Among our *C. officinalis* cultivars, 'Orange Beauty' had the highest seed germination compared. Lowest germination rates were found in 'Nova', both as control and in the treatments. Especially for colchicine, higher doses resulted in lower seed germination rates. For oryzalin and trifluralin the decrease in germination was less pronounced and the results were more variable. Our results were in agreement with previous studies that showed a fatal effect of high doses of antimitotic agents on seeds (Luo et al., 2018; Nasirvand et al., 2018). High doses of these substances are toxic to plant cells and modify various plant activities (Pintos et al., 2007; Luo et al., 2018). Moreover, the antimitotic agents were dissolved in DMSO and exposure lasted relatively long (24 h). DMSO increases cell

wall permeability to antimitotic agents and thereby augments effectiveness (Tavan et al., 2015; Eng and Ho, 2019). On the other hand, in high concentrations DMSO is lethal to cells and can lead to a reduction of the root tip mitotic index (Dillé and King, 1983). Deleterious effects of antimitotic agents have been reported in various plants, not only in terms of seed germination but also in treated explants throughout the life cycle (Eng and Ho, 2019; Manzoor et al., 2019). In our study, abnormal seedlings with small and thick roots without root tips and hairs were obtained (especially after high dosages), and these seedlings died after transplant. The same was observed in *Taraxacum* (Luo et al., 2018) and *Onobrychis elata* (Avci et al., 2019). In addition, the octaploid *C. officinalis* plants grew slower than tetraploid seedlings during the first weeks. In this study ploidy levels were estimated using flow cytometry, therefore the occurrence of aneuploidy cannot be excluded and should be checked by chromosome counts in root metaphases after seed propagation.

Analysis of putative polyploid seedlings showed that doses of 200 and 400 ppm of colchicine were most efficient for chromosome doubling in 'Nova' and 'Orange Beauty', respectively, while treatment with 80 ppm oryzalin was also effective for 'WUR 1553-7'. However, the very low number of octaploids makes it difficult to make general conclusions. Other studies have shown that the optimal antimitotic agent and concentration is strongly genotype-dependent (Dhooghe et al., 2010), such as in parsley (Nasirvand et al., 2018), *Onobrychis elata* (Avci et al., 2019), and *Escallonia* (Denaeghel et al., 2018), among many others. Unfortunately most of the octaploid plants detected at 3 weeks after transplant reverted to tetraploid status after 9 weeks. Unstable polyploidization has been observed in other plants, such as *Eriobotrya japonica* (Blasco et al., 2015), *Gerbera jamesonii* (Gantait et al., 2011), etc. Probably reversion is due to the higher mitotic index in tetraploids versus their octaploid counterparts whereby the tetraploid cells multiply faster. Application of oryzalin and trifluralin was less effective due to the low survival rate, especially in 'Nova.' Colchicine is applied in higher concentrations when compared to oryzalin and trifluralin because of its lower affinity for plant tubulin.

Generally, polyploidy is associated with a higher leaf size, thicker leaves and stem diameter, shorter internodes and superior agronomic traits ('giga' effects) as compared to their diploid counterparts (Li et al., 2018; Luo et al., 2018; Eng and Ho, 2019). These changes caused by polyploidization led to the tendency of plant breeders to test chromosome doubling as an efficient breeding tool (Sattler et al., 2016; Corneillie et al., 2019). However, the increase of plant and organ size is not correlated to the polyploidy level. It is known that ploidy has an optimum level. Tetraploids are often bigger than triploids, which are in turn bigger than diploids. But it is also possible that tetraploids have a more compact growth habit as compared to diploids, as shown in *Escallonia* (Denaeghel et al., 2018). Higher ploidy levels are often marked by stunted growth as was shown in *A. thaliana* (Corneillie et al., 2019). Therefore the effect of polyploidization cannot be predicted. In most research on induced polyploidy, morphological characteristics are studied, but usually not with a micro-morphological approach. In our study, the number of leaf cells was shown to decrease in octaploid plants; however, the leaves of the octaploids have a higher weight. Hias et al. (2017) also made this observation after chromosome doubling in diploid apple, where the number of nuclei per leaf area decreased by a factor of ±2,42 after chromosome doubling. We can assume that for *C. officinalis* this decrease in cell number is associated with an increase in cell size. Indeed, first observations in the leaf cross-sections show that leaves are thicker and cell size increases in octaploid plants. This phenomenon has been reported also in other polyploid plants, such as (4x, 6x, and 8x) *Arabidopsis thaliana* (Corneillie et al., 2019), tetraploid plants of *Juncus effucus* (Xu et al., 2010), and *Limonium bellidifolium* (Mori et al., 2016). This affects plant growth and production of secondary metabolites. Therefore we will perform a more thorough study growing the offspring of the plants with different ploidy levels in bigger numbers and under crop-appropriate field conditions to phenotype them.

CONCLUSION

C. officinalis probably has an allotetraploid background. Its economic importance and the occurrence of other *Calendula* species with higher chromosome numbers makes *C. officinalis* an interesting candidate for chromosome doubling. High dosages of antimitotic agents have a toxic effect on seed germination and survival of seedlings. Application of colchicine in low concentrations (about 200–400 ppm) is the most efficient antimitotic agent for chromosome doubling in *Calendula*. The number of octaploids obtained is low (maximum 2.2%), but the plants obtained are valuable for further breeding as well as the study of the agricultural value and metabolite profiles among ploidy levels. Such insights can lead to development of better performing cultivars.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

GE conducted the experimental work in cytogenetics, polyploidization, and flow cytometry. KV contributed to the experimental work on cytogentics. HM contributed to the plant experimental work. LL contributed to the experimental work on polyploidization and flow cytometry. GE, KV, HM, and LL participated in the writing of the manuscript.

FUNDING

This work was funded by VLAIO, project GOGO: Goudsbloem, een gouden kans! HBC.2016.0799.

ACKNOWLEDGMENTS

We thank Nancy Mergan for technical assistance on flow cytometry and Miriam Levenson for language editing.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 16 June 2020
doi: 10.3389/fpls.2020.00851

Meiotic Chromosome Stability and Suppression of Crossover Between Non-homologous Chromosomes in x*Brassicoraphanus*, an Intergeneric Allotetraploid Derived From a Cross Between *Brassica rapa* and *Raphanus sativus*

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OPEN ACCESS

Edited by:
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University of Cambridge, United Kingdom

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: 07 November 2019
Accepted: 27 May 2020
Published: 16 June 2020

Citation:
Park HR, Park JE, Kim JH, Shin H, Yu SH, Son S, Yi G, Lee S-S, Kim HH and Huh JH (2020) Meiotic Chromosome Stability and Suppression of Crossover Between Non-homologous Chromosomes in xBrassicoraphanus, an Intergeneric Allotetraploid Derived From a Cross Between Brassica rapa and Raphanus sativus. Front. Plant Sci. 11:851. doi: 10.3389/fpls.2020.00851

Hybridization and polyploidization are major driving forces in plant evolution. Allopolyploids can be occasionally formed from a cross between distantly related species but often suffer from chromosome instability and infertility. x*Brassicoraphanus* is an intergeneric allotetraploid (AARR; 2n = 38) derived from a cross between *Brassica rapa* (AA; 2n = 20) and *Raphanus sativus* (RR; 2n = 18). x*Brassicoraphanus* is fertile and genetically stable, while retaining complete sets of both *B. rapa* and *R. sativus* chromosomes. Precise control of meiotic recombination is essential for the production of balanced gametes, and crossovers (COs) must occur exclusively between homologous chromosomes. Many interspecific hybrids have problems with meiotic division at early generations, in which interactions between non-homologous chromosomes often bring about aneuploidy and unbalanced gamete formation. We analyzed meiotic chromosome behaviors in pollen mother cells (PMCs) of allotetraploid and allodiploid F1 individuals of newly synthesized x*Brassicoraphanus*. Allotetraploid x*Brassicoraphanus* PMCs showed a normal diploid-like meiotic behavior. By contrast, allodiploid x*Brassicoraphanus* PMCs displayed abnormal segregation of chromosomes mainly due to the absence of homologous pairs. Notably, during early stages of meiosis I many of allodiploid x*Brassicoraphanus* chromosomes behave independently with few interactions between *B. rapa* and *R. sativus* chromosomes, forming many univalent chromosomes before segregation. Chromosomes were randomly assorted at later stages of meiosis, and tetrads with unequal numbers of chromosomes were formed at completion of meiosis. Immunolocalization of HEI10 protein mediating meiotic recombination revealed that COs were more frequent in synthetic allotetraploid x*Brassicoraphanus* than in allodiploid,

but less than in the stabilized line. These findings suggest that structural dissimilarity between *B. rapa* and *R. sativus* chromosomes prevents non-homologous interactions between the parental chromosomes in allotetraploid x*Brassicoraphanus*, allowing normal diploid-like meiosis when homologous pairing partners are present. This study also suggests that CO suppression between non-homologous chromosomes is required for correct meiotic progression in newly synthesized allopolyploids, which is important for the formation of viable gametes and reproductive success in the hybrid progeny.

Keywords: hybrids, polyploidy, meiosis, synapsis, intergeneric hybridization

INTRODUCTION

Hybridization and polyploidization are major driving forces in plant evolution (Van de Peer et al., 2017). It is estimated that most of the extant plant species are polyploids that have undergone whole-genome duplication (WGD) in their evolutionary path (Leebens-Mack et al., 2019). Polyploids can be divided into two classes: ones that undergo the multiplication of a whole set of chromosomes within species (autopolyploids) and the others resulting from hybridization between different species followed by chromosome doubling (allopolyploids). Cytogenetically, autopolyploids have random association among four homologous chromosomes (in tetraploids) leading to tetrasomic segregation during meiosis, whereas allopolyploids have two non-pairing sets of homoeologous chromosomes carrying out disomic segregation (Doyle and Egan, 2010). Allopolyploids can be occasionally formed from a cross between genetically divergent species, for instance, between the individuals that belong to different species or even to different genera. Interspecific hybridization and allopolyploidization are likely to contribute to the emergence of many important crop plants such as oilseed rape (*Brassica napus*), cotton (*Gossypium hirsutum*), tobacco (*Nicotiana tabacum*), wheat (*Triticum aestivum*), sugarcane (*Saccharum officinarum*), and coffee (*Coffea arabica*) (Renny-Byfield and Wendel, 2014). However, many studies report that most synthetic allopolyploids exhibited genetic instability and sterility, the latter of which is mainly caused by meiotic abnormalities during sexual gamete formation (Madlung et al., 2005; Mestiri et al., 2010; Szadkowski et al., 2010, 2011).

Meiosis is the process by which the number of chromosomes in a diploid cell is reduced by half producing haploid gametes that are capable of sexual reproduction. Meiotic cell division consists of two consecutive stages meiosis I and II. In particular, the initial process of meiosis I is important for chromosome assortment and recombination of genetic information. According to a current model, meiotic recombination is initiated by a DNA double-strand break (DSB) and 5′–3′ resection followed by strand invasion to form a displacement loop (D-loop) structure (Hunter, 2015; Mercier et al., 2015; Lambing et al., 2017). Once the D-loop is extended, the second end of DSB can anneal to the displaced strand of the D-loop in a process called second end capture annealing, forming a double Holliday junction (dHJ). Resolution of dHJ can lead to reciprocal recombination through interhomolog strand exchanges known as class I COs. Alternatively, class II COs may occur in a dHJ-independent manner presumably by resolution of D-loops (Hunter, 2015; Mercier et al., 2015). The DSBs that do not produce COs are likely to form noncrossovers (NCOs). NCOs can result either from dissolution of dHJ or from D-loops via synthesis-dependent strand annealing, usually concurrent with gene conversion (Hunter, 2015; Mercier et al., 2015). The process of meiosis is further characterized by synapsis formation, the assembly of synaptonemal complex (SC) and chiasma formation, bringing about genetic diversity during gametogenesis. Particularly, formation of synapsis and crossing-overs between homologous chromosomes are essential for subsequent homologous chromosome co-orientation during meiosis I, producing four haploid gametic cells during meiosis II (Mercier et al., 2015; Lambing et al., 2017). Nondisjunction or failure in bivalent formation impairs reductional segregation, frequently causing aneuploidy in gametes. Lack of chromosome pairing in meiosis of interspecific hybrids is one of the main causes of sterility observed in many synthetic hybrids, which is manifest as a post-zygotic barrier in artificial interspecific hybridization (Dion-Côté and Barbash, 2017). Crossing-over between homologous chromosomes is essential for their co-orientation resulting in proper meiotic chromosome segregation (Stewart and Dawson, 2004).

In early generations of synthetic hybrids non-homologous chromosome pairing, multivalent formation, and chromosome rearrangement are frequently observed, and exert a detrimental effect on the survival of allopolyploid plants (Bomblies et al., 2016; Wendel et al., 2018). Thus, meiosis is critical to the success of sexual reproduction ensuring correct segregation of chromosomes into balanced gametes. During homologous chromosomes are synapsed, the SC is formed at the interface between the chromosomes along the axis. ASYNAPTIC1 (ASY1) and ZIPPER1 (ZYP1) are the lateral and axial elements of meiotic chromosomes, respectively. ASY1 is associated with meiotic chromosomes at early prophase I, and ZYP1 is then deposited at the interface between homologous chromosomes upon synapsis formation (Higgins et al., 2005). HUMAN ENHANCER OF INVASION 10 (HEI10) is a component of ZMM complex (ZIP4, MSH4/5, MER3, MLH1/3) that mediates a meiotic crossover (Chelysheva et al., 2012; Mercier et al., 2015; Gonzalo et al., 2019). The coordinated action of these proteins is crucial for the establishment and progression of synapsis formation and recombination, and abnormal meiosis

often results from the lack of proper configuration of these chromatin components.

The Brassicaceae family contains a number of vegetable crops such as broccoli, cabbage, cauliflower, oilseed rape, turnip and radish. Several *Brassica* species are famous for interspecific hybridization to produce allotetraploid plants. For instance, three diploid species *B. rapa* (AA), *B. nigra* (BB), and *B. oleracea* (CC) can be crossed to each other producing allotetraploid species *B. napus* (AACC), *B. juncea* (AABB) and *B. carinata* (BBCC). Such interspecific cross combinations are epitomized by the model of "U's Triangle," which first proposed the process by which ancestral diploid *Brassica* species are combined to create novel tetraploid species (Nagaharu and Nagaharu, 1935). In the Brassicaceae family, hybridization between different species can be expanded to the intergeneric level. Since 1826 when intergeneric hybridization between *Brassica* and *Raphanus* was first reported (Prakash et al., 2009), the allotetraploid plants have been sporadically generated but failed to survive due to genetic instability and sterility (Karpechenko, 1924; McNaughton, 1979; Dolstra, 1982). The recently developed x*Brassicoraphanus* (AARR; 2n = 4x = 38) is also synthesized from a cross between *B. rapa* (AA; 2n = 2x = 20) and *Raphanus sativus* (RR; 2n = 2x = 18). Unlike other synthetic allopolyploid plants, x*Brassicoraphanus* displays great fertility and genetic uniformity over successive generations (Lee et al., 2011, 2017).

We assumed that exceptional genetic integrity of x*Brassicoraphanus* should require a reliable and precise control of meiosis, which is critical not only to the production of functional gametes but also to the maintenance of fertility in successive offspring. For this, non-homologous interactions between the parental chromosomes of *B. rapa* and *R. sativus* must be inhibited during meiosis in x*Brassicoraphanus*, which would otherwise cause detrimental chromosome rearrangements resulting in unbalanced gamete formation. In this study, we investigated meiotic chromosome behaviors in pollen mother cells (PMCs) of newly synthesized allodiploid (AR) and allotetraploid (AARR) x*Brassicoraphanus*, while providing a mechanistic insight into the chromosome compatibility for the reproductive success of hybrids formed between distantly related species.

MATERIALS AND METHODS

Plant Materials Production

Seeds of *B. rapa* cv. Chiifu-401-42, *R. sativus* cv. WK10039, and x*Brassicoraphanus* cv. BB1 were sown on 1× Murashige and Skoog (MS) medium (Duchefa, The Netherlands) supplemented with 2% sucrose and 0.8% plant agar (w/v) in a growth chamber under 16 h of fluorescent light at 20 ± 10 μmol m^{-2} s^{-1}, at 24°C for 2 weeks. BB1 was derived from microspore culture of a synthetic hybrid of *B. rapa* and *R. sativus*, and maintained for more than ten generations by self-pollination (Lee et al., 2011). The seedlings were vernalized in the 4°C cold chamber for 4 weeks with 16 h of light and 8 h of dark. The plants were transferred to pots in the greenhouse with the same light condition. Synthetic allodiploid x*Brassicoraphanus* were produced by crossing *B. rapa* cv. Chiifu-401-42 as a female parent with *R. sativus* cv. WK10039 as a pollen donor. Floral buds of *B. rapa* prior to anthesis were emasculated and hand-pollinated with *R. sativus* pollen. Thirty-day-old immature hybrid seeds were cultured on MS medium (Duchefa, Netherlands) supplemented with 2% sucrose (w/v) and 0.8% plant agar (w/v). The seeds were vernalized and transferred to the above-described growth conditions. The newly synthesized allodiploid x*Brassicoraphanus* individuals were subjected to chromosome doubling by applying 0.3% colchicine-soaked cotton on the emerging shoot apical meristem for 2 days.

Flow Cytometry Analysis

Flow cytometry was used to verify the ploidy level (Pfosser et al., 1995). Leaves of *B. rapa* cv. Chiifu-401-42, *R. sativus* cv. WK10039, their synthetic allodiploid and allotetraploid F1 hybrids, and x*Brassicoraphanus* cv. BB1 were subjected to ploidy analysis. Approximately 20 mg of leaves were finely chopped with a clean razor blade in 1 mL of ice-cold Tris-$MgCl_2$ buffer (0.2 M Tris, 4 mM $MgCl_2$, 0.5% Triton X-100, pH 7.5) in a glass petri dish on ice (Pfosser et al., 1995). Nuclei were isolated and stained in 50 μg L^{-1} of propidium iodide solution with 50 μg L^{-1} of RNase, filtered through a 40 μm cell strainer, and kept on ice. Flow cytometry was performed on a FACS Canto II flow cytometer (BD Biosciences, United States) system with a medium flow rate according to the manufacturer's protocol. The data were analyzed with the BD FACSDiva software (BD Biosciences, United States). An FL2 detector was used to measure fluorescence, and forward scatter (FSC) and side scatter (SSC) parameters were used for data analysis according to the manufacturer's instruction. Fluorescence of *B. rapa* and *R. sativus* was used as a reference to assess the ploidy level of resynthesized hybrids.

Immunofluorescence of α-Tubulin

For detection of α-tubulin, the method of Wang et al. (2010) was adopted with modifications. Anthers were squashed in SuperFrost Plus™ Adhesion (Thermo Fisher Scientific, United States) slides. The PMCs were incubated with monoclonal anti-α-tubulin IgG (Invitrogen, United States) diluted 1:100 for 2 h at 37°C in a moist chamber. The slides were washed and incubated with a FITC-conjugated anti-mouse IgG (Sigma-Aldrich, United States) diluted 1:50 for 2 h at 37°C in a dark chamber. Subsequently slides were washed again and mounted with a mounting medium with 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, United States). The prepared slides were imaged using a Leica confocal microscope SP8X controlled by Leica LAS X.

Genome *in situ* Hybridization (GISH) Analysis

Inflorescence was fixed in the Carnoy's solution (ethanol : glacial acetic acid, 3:1 v/v) for 24 h and stored in 70% ethanol at −20°C until use. The fixed floral buds with 0.8–1.2 mm in length were rinsed in distilled water and stained with 3% aceto-orcein. Anthers were thoroughly washed with distilled water and treated with the enzyme mixture including

2% Cellulase R-10, 1% Macerozyme R-10 (Duchefa Biochemie, Netherlands), 1% Pectinase, and 0.5% Pectolyase Y23 (Sigma-Aldrich, United States) in 150 mM citrate buffer (pH 4.5) for 60–90 min at 37°C. Treated anthers on the SuperFrost Plus™ Adhesion slides were squashed in 60% acetic acid and air-dried. Genomic DNA was isolated from *B. rapa* and *R. sativus* leaves, fragmented by sonication, separated by agarose-gel electrophoresis, and DNA fragments within the range of 200–500 bp were eluted and purified. The fragmented genomic DNA of *B. rapa* and *R. sativus* was labeled with digoxigenin-11-dUTP and biotin-16-dUTP (Roche, Germany) by nick translation, respectively. For GISH with A- and R- genome probes in x*Brassicoraphanus*, the methods of Kwon and Kim (2009) and Belandres et al. (2015) were adopted with modifications. First, chromosome spreads were incubated with digoxigenin- and biotin-labeled probes along with Fluorescein Avidin DCS (diluted 1:100) (Vector Laboratories, United States) at 37°C for 1 h. After washing three times for 10 min each in 4X SSCT, reactions were performed with rhodamine-conjugated sheep anti-digoxigenin antibody (diluted 1:10) (Roche, Germany) and biotinylated-anti-avidin D antibody (diluted 1:100) (Vector Laboratories, United States) at 37°C for 1 h. In the final reaction, dig-rhodamine and biotin-avidin labeled probes were detected with anti-sheep Texas Red antibody (diluted 1:100) and Fluorescein Avidin DCS (diluted 1:100) (Vector Laboratories, United States), respectively. Chromosomes were counterstained with DAPI in Vectashield reagents (Vector Laboratories, United States). Slides were covered with glass coverslips and examined using Axioskop2 microscope equipped with an Axiocam 506 color CCD camera (Zeiss, Germany).

Immunolocalization of HEI10

The coding sequence of *BrHEI10* gene was amplified from cDNA of young floral buds of *B. rapa* with oligonucleotides 5′-TTAAGAATTCATGAGGTGCAACGCCTGTTGGAGGG and 5′-TTAACTCGAGGAACAGTTGCGGGCGAGAACG, digested with *Eco* RI and *Xho* I, and then cloned into the pET-28a expression vector at corresponding restriction sites (Novagen, United States). The resulting construct was transformed into *Escherichia coli* Rosetta2 (DE3) strains (Novagen, United States). Transformants were grown at 30°C in 1 L of LB medium in the presence of 50 $\mu g\ mL^{-1}$ of kanamycin and 50 $\mu g\ mL^{-1}$ of chloramphenicol until OD_{600} reached 0.4. Protein expression was induced with 1 mM of isopropyl b-D-thiogalactopyranoside (IPTG) at 16°C for 16 h. Cells were harvested by centrifugation at 6,500 rpm for 15 min at 4°C, and the pellet was resuspended in 100 mL of ice-cold column buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol, 0.1 mM PMSF). Cells were lysed by sonication for 5 min in ice (output power, 4; duty cycle, 50%; Branson Sonifer 250, Branson, United States). The lysate was subjected to centrifugation at 9,000 rpm for 25 min at 4°C. Inclusion bodies were collected by centrifugation and dissolved in 4 M urea buffer. Protein concentration was estimated using the Coomassie Brilliant Blue R 250 dye-binding method (Bradford, 1976). The purified BrHEI10 protein was used to produce polyclonal antibodies from rabbits by Youngin Frontier (Korea).

A modified version of the method described by Chelysheva et al. (2013) was used to prepare chromosome spreads, for which the fixed inflorescence was rinsed in distilled water and subsequent procedures were essentially the same. For immunolabeling, anti-HEI10 primary antibody was diluted to 1:250 in PBST-BSA buffer and spread onto slides. The slides were covered with parafilm and incubated at 4°C for 39–48 h in a moist chamber, and then washed with PBST. The secondary antibody solution (Goat anti-rabbit IgG H&L, Alexa Fluor 488) diluted to 1:500 was spread on slides and incubated at 37°C for 1 h in a dark moist chamber. After wash with PBST, slides were mounted with a mounting medium with DAPI (Vector Laboratories, United States) to counterstain chromosomes. Photographs were taken using Axioskop2 microscope equipped with an Axiocam 506 color CCD camera (Zeiss, Germany).

Synteny Analysis

Genome assembly and gene annotation data obtained from the databases of *B. rapa* (Zhang et al., 2018),[1] *B. oleracea* (Liu et al., 2014; Cunningham et al., 2019),[2] and *R. sativus* (Jeong et al., 2016)[3] were subjected to synteny analysis using Synorth (Cheng et al., 2012) with default parameters. The regions containing at least 20 syntenic orthologs were defined as syntenic blocks.

RESULTS

Diploid-Like Meiotic Behavior in Synthetic Allotetraploid x*Brassicoraphanus*

Many synthetic allopolyploid plants display numeric and structural chromosome aberrations typically caused by abnormal meiosis. Therefore, we first investigated and compared meiotic chromosome behaviors in PMCs of *B. rapa*, *R. sativus*, their synthetic allodiploid and allotetraploid F1 hybrids, and x*Brassicoraphanus* cv. BB1 whose ploidy levels were all confirmed by flow cytometry analysis (**Supplementary Figure 1**). Normal chromosome behaviors were observed in the entire course of meiosis of *B. rapa*, *R. sativus*, BB1, and synthetic allotetraploid x*Brassicoraphanus* (**Figure 1**). At leptotene, condensation of meiotic chromosomes was initiated (**Figures 1A,H,O,CC**). The alignments of homologous chromosomes became prominent at zygotene (**Figures 1B,I,P,DD**). At pachytene, all chromosomes were closely juxtaposed, preparing for synapsis formation between homologous chromosomes (**Figures 1C,J,Q,EE**). The chromosomes were condensed into bivalents at diakinesis (**Figures 1D,K,R,FF**). At metaphase I, bivalents were placed at the metaphase plate (**Figures 1E,L,S,GG**). The homologous chromosomes were evenly separated at telophase I (**Figures 1F,M,T,HH**). Finally, four balanced gametes were produced after the second meiotic division in all PMCs (33, 41, 83, and 25 PMCs for *B. rapa*, *R. sativus*, BB1, and allotetraploid x*Brassicoraphanus*, respectively) (**Figures 1G,N,U,II**). These

[1]http://brassicadb.org

[2]http://plants.ensembl.org

[3]http://radish-genome.org

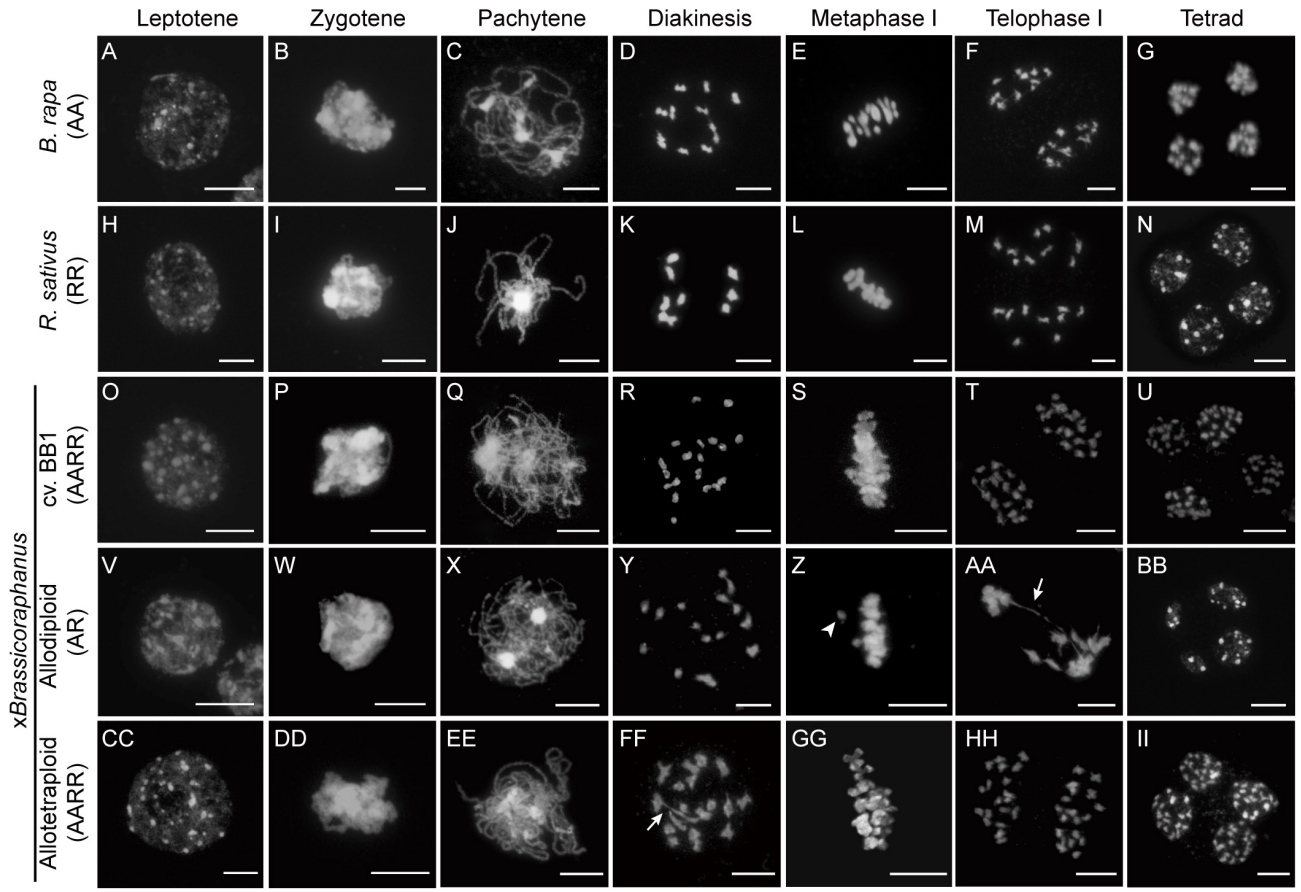

FIGURE 1 | Chromosome behavior during meiosis. The chromosomes in PMCs of *B. rapa* **(A–G)**, *R. sativus* **(H–N)**, BB1 **(O–U)**, and synthetic allodiploid **(V–BB)** and allotetraploid x*Brassicoraphanus* **(CC–II)** were visualized with DAPI staining. Meiotic chromosomes were condensed at leptotene **(A,H,O,V,CC)**, and seen as thin threads at zygotene **(B,I,P,W,DD)**. The synapsis between homologous chromosomes appeared at pachytene **(C,J,Q,EE)**. However, chromosomes were not juxtaposed and only thin chromosome threads were observed at pachytene of synthetic allodiploid x*Brassicoraphanus* **(X)**. Homologous chromosomes were condensed and bivalents were formed at diakinesis **(D,K,R,FF)**. Various numbers of univalents and multivalents were observed in synthetic allodiploid x*Brassicoraphanus* **(Y)**. All chromosomes were aligned on the metaphase plate at metaphase I **(E,L,S,GG)** but unpaired univalent (arrowhead) was sometimes detected in allodiploid x*Brassicoraphanus* **(Z)**. Homologous chromosomes were separated to the opposite poles at telophase I **(F,M,T,AA,HH)**. Chromosome bridges (arrow) were often observed in synthetic allodiploid x*Brassicoraphanus* **(AA)**. Individual chromatids were evenly separated into four microspores at tetrad **(G,N,U,II)**. Unbalanced tetrads were detected in allodiploid x*Brassicoraphanus* **(BB)**. Scale bars = 10 μm.

observations indicate that meiosis occurs normally in synthetic allotetraploid x*Brassicoraphanus* while ensuring faithful chromosome segregation after hybridization between *B. rapa* and *R. sativus*.

Meiotic chromosome behaviors in allodiploid x*Brassicoraphanus* were similar to those of the parents at early stages (**Figures 1V,W**). However, thinner pachytene chromosomes of all allodiploid x*Brassicoraphanus* PMCs indicate unpaired chromosomes, which would probably lead to rare synapsis formation (59 PMCs; **Figure 1X**). At diakinesis, bivalents were detected at low frequency and univalents were more frequently observed in allodiploid x*Brassicoraphanus* (**Figure 1Y**). At metaphase I, two out of 9 PMCs displayed proper alignment of all chromosomes at the metaphase plate, whereas the other seven carried some chromosomes detached from the plate (one isolated chromosome in 3 PMCs, two in 2 PMCs, and three in 2 PMCs) (**Figure 1Z**). Subsequently, meiotic chromosomes were unequally segregated, and in several occasions (13 out of 20 PMCs), chromosome bridges appeared at anaphase I/telophase I (**Figure 1AA**). In tetrad, unbalanced gametes with unequal numbers of chromosomes in each microspore were formed at the end of meiosis (**Figure 1BB**). These observations suggest that meiosis in allodiploid x*Brassicoraphanus* PMCs has a severe defect mostly due to a lack of homologous pairing, albeit some non-homologous interactions still persist as exemplified by bridge formation (**Figure 1AA**).

Microtubule Distribution in x*Brassicoraphanus*

Microtubules are important for the formation of meiotic spindles to support correct segregations of chromosomes. Microtubule dynamics was investigated in *B. rapa*, *R. sativus*, and allodiploid

and allotetraploid x*Brassicoraphanus* through immunostaining of α-tubulin at different stages of meiosis (**Figure 2**). At pachytene, microtubules were organized at the perinuclear zone (**Figures 2A,E,I,M,Q**). At metaphase I, microtubules were arranged into the spindle structure and attached to kinetochores, engaging a typical bipolar fusiform configuration at the metaphase plate (**Figures 2B,F,J,N,R**). At anaphase I, microtubules pushed chromosomes toward the opposite poles (**Figures 2C,G,K,O,S**). Notably, some chromosomes of synthetic allodiploid x*Brassicoraphanus* were not attached to meiotic spindles (**Figure 2O**). Then interzonal microtubules appeared at the equator forming the phragmoplast. At completion of meiosis, microtubules dissolved and dispersed in the cytoplasm in tetrads (**Figures 2D,H,L,P,T**). These observations indicate that microtubules behave normally in x*Brassicoraphanus* PMCs.

Non-homologous Chromosome Associations at Meiosis of Synthetic Allotetraploid x*Brassicoraphanus*

Non-homologous chromosome pairing often induces meiotic chromosome aberrations in many resynthesized allopolyploids (Madlung et al., 2005; Mestiri et al., 2010; Szadkowski et al., 2010, 2011). To investigate non-homologous interactions between A and R chromosomes in x*Brassicoraphanus*, GISH analysis was performed during meiosis (**Figure 3**). Twenty chromosomes

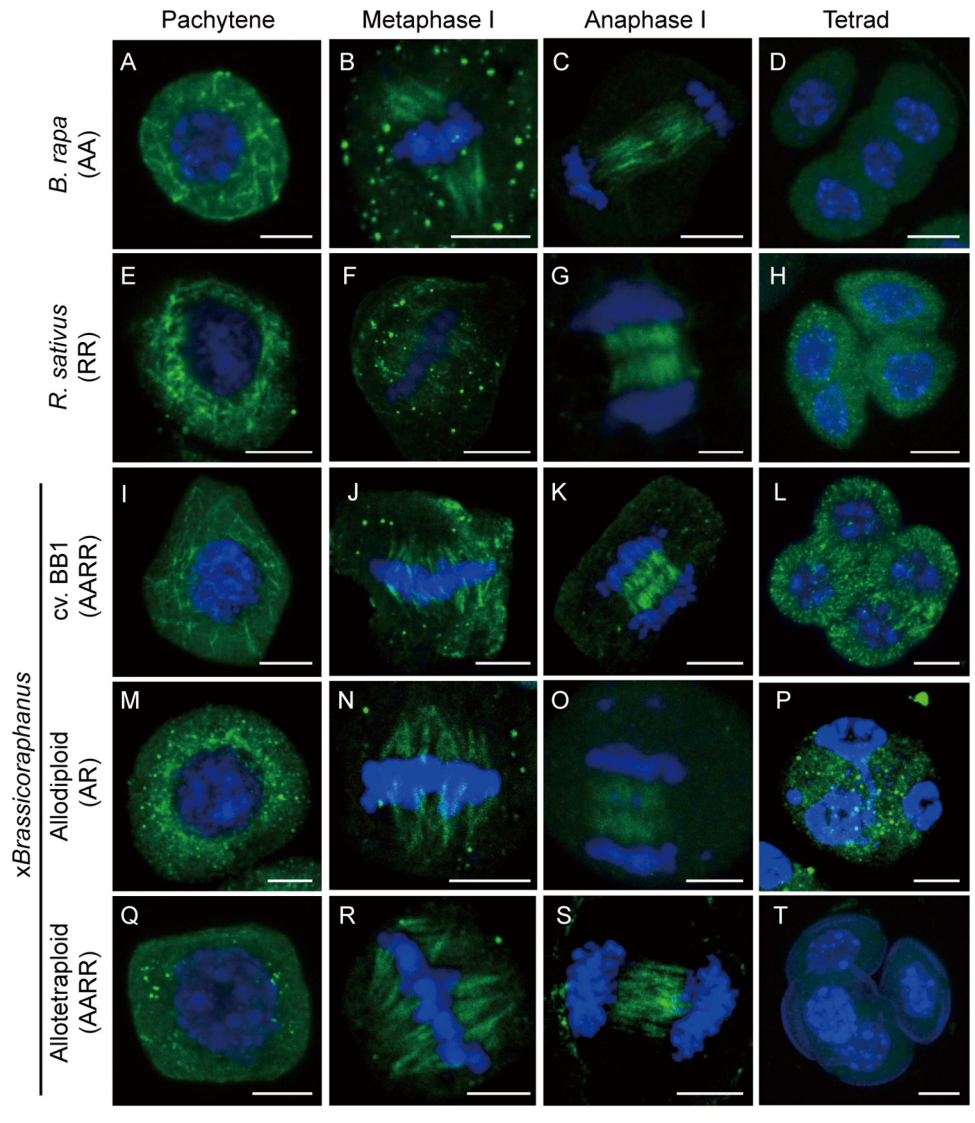

FIGURE 2 | Microtubule distribution during meiosis. Microtubule and chromosome behaviors were observed in PMCs of *B. rapa* **(A–D)**, *R. sativus* **(E–H)**, BB1 **(I–L)**, and synthetic allodiploid **(M–P)** and allotetraploid x*Brassicoraphanus* **(Q–T)**. Microtubules appeared throughout the cytoplasm of the PMC at pachytene **(A,E,I,M,Q)**. Connections between chromosomes and microtubules were displayed at metaphase I **(B,F,J,N,R)**. Phragmoplast microtubules were located between two daughter nuclei in anaphase I **(C,G,K,O,S)**, and four separated daughter nuclei were observed at tetrad **(D,H,L,P,T)**. Microtubules and chromosomes were in green and blue, respectively. Scale bars = 5 μm.

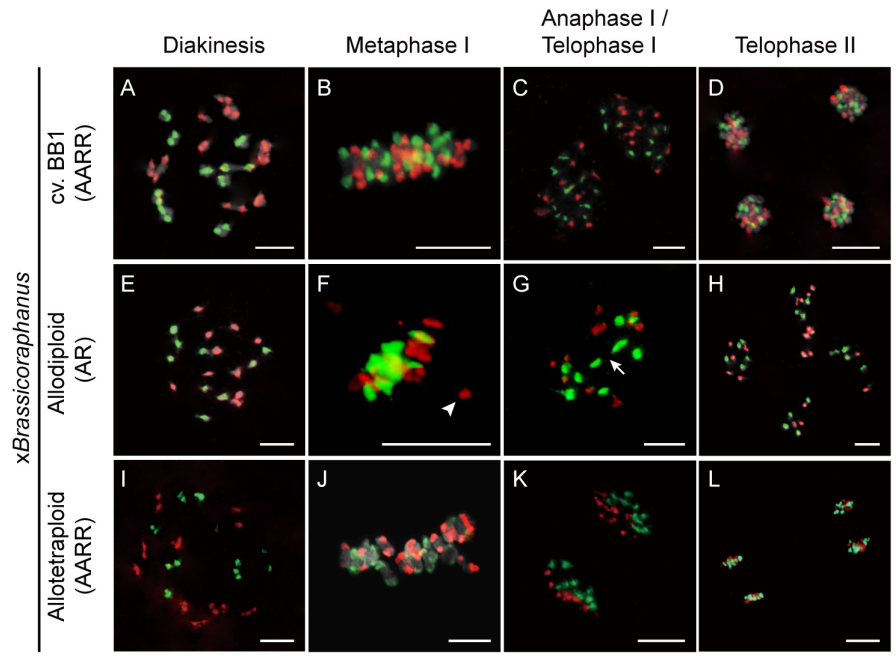

FIGURE 3 | Chromosome identification of x*Brassicoraphanus* by GISH analysis. Distribution of A and R chromosomes were observed in PMCs of BB1 **(A–D)**, and synthetic allodiploid **(E–H)** and allotetraploid x*Brassicoraphanus* **(I–L)**. Nineteen complete bivalents comprising ten from *B. rapa* and nine from *R. sativus* were shown in BB1 and allotetraploid x*Brassicoraphanus*, and homoeologous interactions between A and R chromosomes were scantily detected in allodiploid x*Brassicoraphanus*. The A and R chromosomes are stained in red and green, respectively. Arrow indicates the position of chromosome bridge formed between R chromosomes (**Supplementary Figure 3**). Arrowhead indicates an isolated chromosome. Scale bars = 10 μm.

of *B. rapa* and 18 chromosomes of *R. sativus* existed in BB1 and synthetic allotetraploid x*Brassicoraphanus* at diakinesis (**Figures 3A,I**). At diakinesis and metaphase I, 19 bivalents were present in an autosyndetic (A-A or R-R) form, probably with ten A-A bivalents and nine R-R bivalents in synthetic allotetraploid x*Brassicoraphanus* (**Figures 3A,B,I,J**). At telophase I, chromosomes were correctly segregated, and ten A chromosomes and nine R chromosomes were evenly distributed at each pole in synthetic allotetraploid x*Brassicoraphanus* (**Figures 3C,K**). At telophase II, chromosomes were evenly segregated to tetrads with ten A and nine R haploid chromosomes, respectively (**Figures 3D,L**). A-R chromosome associations were unnoticeable during the entire course of meiosis in synthetic allotetraploid x*Brassicoraphanus*. These observations suggest the absence of non-homologous interactions between A and R chromosomes, or very few, if any, which would prevent chromosome rearrangement and aneuploidy in newly synthesized allotetraploid x*Brassicoraphanus*.

In PMCs of allodiploid x*Brassicoraphanus*, ten chromosomes of *B. rapa* and nine chromosomes of *R. sativus* were present, but they mis-segregated at later stages of meiosis (**Figures 3E–H**). At diakinesis, 0.36 A-A and 0.56 R-R autosyndetic bivalents on average were observed, whereas 1.16 A-R allosyndetic bivalents were present (**Table 1** and **Supplementary Figure 2**). Also, 4.63 A and 3.80 R univalents on average were observed at diakinesis in allodiploid x*Brassicoraphanus* (**Table 1**). A smaller number of multivalents (0.81 trivalent and 0.78 quadrivalent or more on average) were observed with very few autosyndetics (**Table 1**). These observations indicate that non-homologous interactions still persist in allodiploid x*Brassicoraphanus*, albeit only 5.1% of PMCs (n = 78) contained 19 univalents without chromosome pairing (**Figure 3E**). At metaphase I, most univalent chromosomes were placed at the metaphase plate but a few were detached as isolated units (**Figure 3F**). At anaphase I, chromosome bridges were often observed in allodiploid x*Brassicoraphanus* PMCs (**Figure 3G** and **Supplementary Figure 3**). At telophase II, A and R chromosomes were randomly segregated to each microspore (**Figure 3H**). A low frequency of A and R associations suggests that a considerably low level of meiotic recombination likely occur in allodiploid x*Brassicoraphanus*. This also suggests that non-homologous interactions between A and R chromosomes are not preferred during synapsis formation at early stages of meiosis.

Suppression of Crossovers in Synthetic Allodiploid x*Brassicoraphanus*

Formation of COs was investigated by immunolocalization of HEI10 at pachytene of *B. rapa*, *R. sativus*, and synthetic allodiploid and allotetraploid x*Brassicoraphanus*. It is known that HEI10 is essential for transition of early recombination intermediates into final class I COs, which represent the actual sites where strand exchanges and recombination take place (Chelysheva et al., 2012; Gonzalo et al., 2019). To examine intensity and frequency of COs, HEI10 foci were examined in *B. rapa*, *R. sativus*, and synthetic allodiploid and

TABLE 1 | Chromosome associations in PMCs of allodiploids at diakinesis as revealed by GISH.

Lines	Total PMCs	I			II				III	≥IV
		I^A	I^R	Total	II^{AA}	II^{RR}	II^{AR}	Total		
#20	38	5.18 (0–9)	3.96 (2–7)	9.14 (2–15)	0.39 (0–2)	0.57 (0–1)	1.25 (0–4)	2.21 (0–5)	0.71 (0–2)	0.61 (0–2)
#30	16	4.05 (2–7)	3.15 (0–5)	7.20 (2–12)	0.40 (0–2)	0.60 (0–2)	1.50 (0–5)	2.50 (0–7)	0.95 (0–3)	0.85 (0–2)
#43	24	4.67 (0–10)	4.28 (1–9)	8.94 (2–19)	0.28 (0–2)	0.50 (0–2)	0.72 (0–2)	1.50 (0–4)	0.78 (0–3)	0.89 (0–2)
Average		4.63 ± 0.57	3.80 ± 0.58	8.43 ± 1.07	0.36 ± 0.07	0.56 ± 0.05	1.16 ± 0.4	2.07 ± 0.52	0.81 ± 0.12	0.78 ± 0.15

I, univalent; II, bivalent; III, trivalent; ≥ IV, more than quardrivalent. I^A *and* I^R *indicate univalent belonging to the A and R genomes, respectively.* II^{AA} *and* II^{RR} *indicate autosyndetic bivalents and* II^{AR} *indicates allosyndetic bivalents formed between A and R chromosomes.*

allotetraploid x*Brassicoraphanus* (**Figure 4**). The average number of HEI10 foci at pachytene was 17.54 in *B. rapa* (n = 51 PMCs) and 17.33 in *R. sativus* (n = 18 PMCs) (**Figure 5**). In BB1, 30.92 foci on average were observed (n = 13 PMCs), suggesting that an increase in number of COs was attributed to the doubled chromosome number by allopolyploidization. In synthetic allotetraploid x*Brassicoraphanus*, 19.74 HEI10 foci were observed on average (n = 79 PMCs) (**Figure 5**). Interestingly, only 4.38 HEI10 foci on average were detected in allodiploid x*Brassicoraphanus* (n = 34 PMCs) (**Figure 5**), and the HEI10 foci were less conspicuous compared to the parental species and allotetraploid x*Brassicoraphanus* (**Figure 4**). It is reported that the formations of large and bright HEI10 foci occur only in properly synapsed regions (Grandont et al., 2014), and our observations suggest that a faint HEI10 signal is attributed to unstable synapsis between chromosomes in allodiploid x*Brassicoraphanus*. Also, non-homologous recombination is unlikely to occur in x*Brassicoraphanus* owing to few interactions between A and R chromosomes.

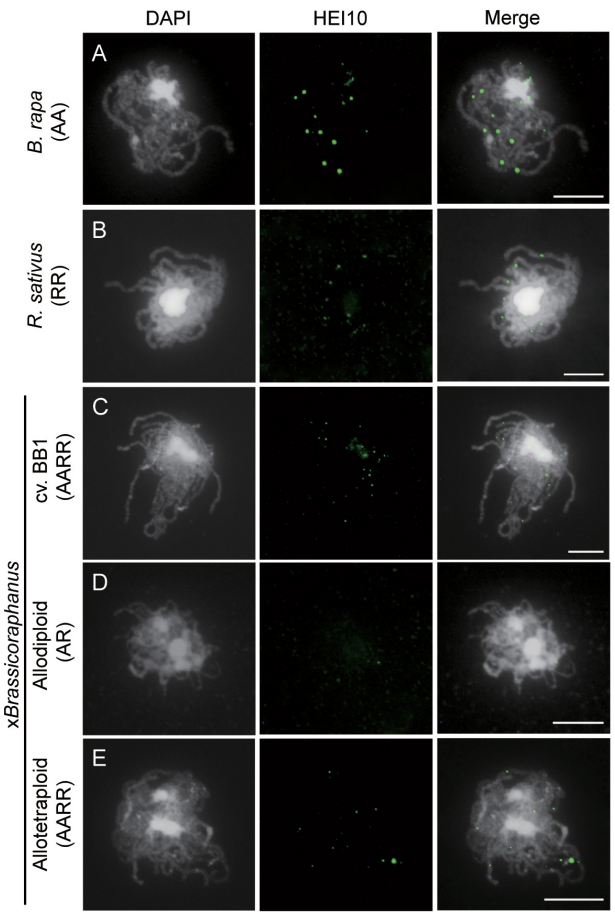

FIGURE 4 | Immunolocalization of HEI10 at pachytene. HEI10 foci were observed in PMCs of *B. rapa* **(A)**, *R. sativus* **(B)**, BB1 **(C)**, synthetic allodiploids **(D)**, and allotetraploid x*Brassicoraphanus* **(E)**. Few HEI10 foci were detected in allodiploid x*Brassicoraphanus*. Chromosomes were labeled with DAPI (white) and HEI10 antibodies (green). The overlay of two signals is shown (Merge). Scale bars = 10 μm.

Structural Divergence of A and R Genomes

Formation of allosyndetics in PMCs of allodiploid x*Brassicoraphanus* suggests that chromosomes of *B. rapa*

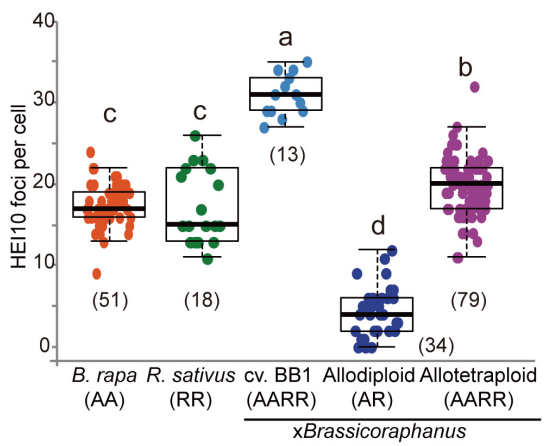

FIGURE 5 | The number of HEI10 foci per PMC at pachytene. The parental species showed similar number of HEI10 foci (17.54 ± 3.48 in *B. rapa* and 17.33 ± 4.51 in *R. sativus*). BB1 represented twice number of HEI10 foci than parental species (30.92 ± 2.53 in BB1) and slightly low number of foci were observed in resynthesized allotetraploid x*Brassicoraphanus* (19.74 ± 3.52). In synthetic allodiploid x*Brassicoraphanus*, only few foci were detected (4.38 ± 2.97). The numbers of observed PMCs were represented in parenthesis. One way analysis of variance (ANOVA) showed differences among treatments ($p < 2.5e^{-18}$) and letters indicate the differences followed by Duncan test ($p < 0.05$).

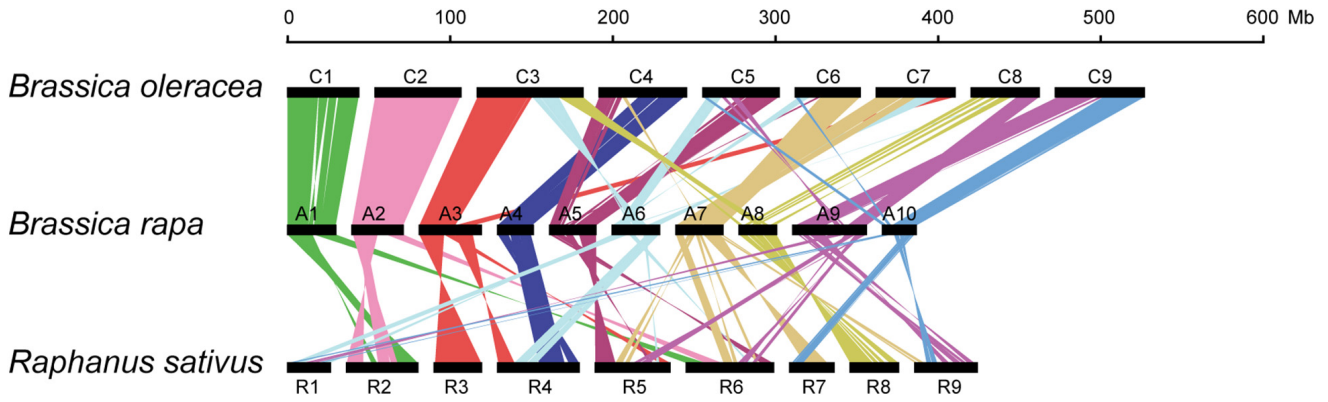

FIGURE 6 | Schematic representation of chromosomal synteny among *B. rapa* (A1–A10), *B. oleracea* (C1–C9) *and R. sativus* (R1–R9). Each pair of syntenic blocks is connected with colored lines.

and *R. sativus* share regions similar enough to allow non-homologous interactions. Thus, we conducted synteny analysis to investigate the degree of genome similarity between the species. We identified a total of 339 synteny blocks consisting of 25,054 orthologous gene pairs between A and C genomes of *B. rapa* and *B. oleracea*, and 324 synteny blocks with 17,918 pairs between A and R genomes of *B. rapa* and *R. sativus*. Comparison of synteny blocks revealed that large portions of A1 (76.9%), A2 (71.9%), and A4 (75.9%) chromosomes of *B. rapa* are highly syntenic to those of C1 (76.6%), C2 (73.8%), and C4 (42.7%) of *B. oleracea*, respectively (**Figure 6** and **Supplementary Table 1**). The A genome of *B. rapa* also shares syntenic regions with R genome of *R. sativus* but the similarity is substantially lower. For instance, A3 (29.2%) and A8 (51.7%) are syntenic to R3 (59.9%) and R8 (51.7%), respectively, but the level of similarity is relatively low in other chromosomes (**Figure 6** and **Supplementary Table 2**). Moreover, the R genome appears to be more fragmented from the A genome compared to the C genome of *B. oleracea* (**Figure 6**). This suggests that the low synteny level, along with structural divergence, is conceivably responsible for the suppression of non-homologous interactions and crossovers between A and R chromosomes in x*Brassicoraphanus*.

DISCUSSION

Hybridization barriers exist in nature to prevent a gene flow between different species, and can be divided into pre- and post-zygotic stages according to the timing of fertilization. Pre-zygotic barriers prevent fertilization between species, whereas post-zygotic barriers are mechanisms engaged after fertilization that reduce the viability or fertility of hybrid offspring. In particular, hybrid sterility is often associated with a failure in meiosis. Normal meiosis requires the formation of COs between homologous chromosome pairs, and when they are abolished or formed between multiple and/or non-homologous chromosomes, the chromosomes segregate abnormally, resulting in unbalanced gamete formation and reduced fertility (Martinez-Perez and Colaiacovo, 2009).

x*Brassicoraphanus* has a full complement of both parental chromosomes. Unlike many other resynthesized allopolyploids, x*Brassicoraphanus* did not show aneuploidy or apparent chromosome rearrangements, suggesting that COs between non-homologous chromosomes rarely occur during meiosis, despite we cannot completely rule out the possibility that NCOs and gene conversion may occur. Indeed, the number of parental chromosome interactions per PMC in synthetic allodiploid x*Brassicoraphanus* (**Table 1**) is significantly lower than that of allodiploid *B. napus* (1.16 vs. 3.45) (Cui et al., 2012). In addition, 55.64 and 88.9% of allodiploid chromosomes of x*Brassicoraphanus* and *B. napus* (Szadkowski et al., 2011), respectively, participated in the formation of bivalents or multivalents at early stages of meiotic prophase I (**Table 1**). We also showed that meiosis in allotetraploid x*Brassicoraphanus* proceeds normally like a diploid cell, albeit A-R chromosome interactions are sporadically observed in allodiploid x*Brassicoraphanus*. This suggests that during meiotic prophase I the chromosome pairing preferentially occurs between the homologous chromosomes of the same progenitor, although non-homologous interactions are also possible when there is no authentic homologous counterpart.

At early stages of meiotic prophase I, homologous chromosomes are aligned in juxtaposition and SCs are formed at the interface between them along the axis, where ASY1, ZYP1 and HEI10 proteins systematically participate in the formation of COs to exchange chromatids. In resynthesized *B. napus*, synapsis is frequently formed between A and C chromosomes (C from *B. oleracea*) via similar segments carried by different chromosomes, and non-homologous recombination results in aneuploidy and interchromosomal rearrangement (Gaeta et al., 2007; Xiong et al., 2011). Such homoeologous regions are still remnant in *B. rapa* and *B. oleracea* genomes although they have diverged several million years ago. For example, A1/C1, A2/C2, and the long arm of A5 and short arm of C4 chromosomes share homology with each other (Parkin et al., 2005). To note, allodiploid x*Brassicoraphanus* formed fewer number of COs (4.38 between A and R; **Figure 5**) than allodiploid *B. napus* (20.3 between A and C; Grandont et al., 2014). This strongly

suggests that interactions between A and R chromosomes are intrinsically inhibited in x*Brassicoraphanus* probably due to a scarcity of homologous regions required for synapsis and recombination.

It is notable that BB1 is fertile producing normal pollen, whereas synthetic allotetraploid x*Brassicoraphanus* F1 is sterile mainly due to aborted pollen formation. This indicates that PMCs of resynthesized allotetraploid x*Brassicoraphanus* are able to perform disomic segregation during meiosis, but the later stage has a developmental defect leading to male sterility. Interestingly, the female gametophyte of resynthesized allotetraploid x*Brassicoraphanus* is functional as it produces viable seeds when pollinated with BB1 as a pollen donor. This suggests that synthetic x*Brassicoraphanus* F1 is mechanistically capable of performing normal meiosis, but its developmental abnormality is manifested only in the male gametophyte. Indeed, the progenitor line of BB1 was initially obtained from a cross between commercial *B. rapa* and *R. sativus* cultivars, and thus has a genetic background different from that of x*Brassicoraphanus* synthesized from *B. rapa* cv. Chiifu-401-42 and *R. sativus* cv. WK10039 whose genome sequences are available. In addition, BB1 was generated by microspore culture in the presence of N-nitroso-N-methylurea (NMU) (Lee et al., 2011), which might have induced mutations of unknown genes that would help escape hybrid incompatibility between different species generally observed in many hybrid individuals (Bomblies and Weigel, 2007). We presume that resynthesized x*Brassicoraphanus* is male-sterile as a default state, possibly caused by incompatibility between the paternal nuclear genome and the maternal cytoplasm, but BB1 has overcome such barriers during the course of artificial hybridization by unknown mechanisms.

Diverse species in the genus *Brassica* are considered to have originated from the same ancestral species after genome triplication, which is approximated to be 9–15 million years ago (Town et al., 2006; Yang et al., 2006; Wang et al., 2011; Cheng et al., 2014). Oilseed rape *B. napus* was formed by hybridization between *B. rapa* and *B. oleracea* approximately 7,500 years ago and supposedly went through abundant homoeologous exchanges (Chalhoub et al., 2014). Recent study also proposed that the genera *Brassica* and *Raphanus* are paraphyletic with a close relationship to each other and predicted that hexaploid progenitor chromosomes were rearranged into nine chromosomes in *R. sativus*, while undergoing differential subgenome fractionation and massive chromosome rearrangement (Jeong et al., 2016). However, according to the genome collinearity, *B. rapa* and *R. sativus* still share numerous syntenic regions across the genome, particularly for chromosomes A3/R3 and A8/R8 (Kitashiba et al., 2014). Despite the presence of syntenic regions between *B. rapa* and *R. sativus* genomes, our observations of crossover suppression in synthetic AR hybrids suggest that rearrangement events have rarely occurred in these chromosomal regions, and thus it is less plausible that they have nearly identical structures or compositions to support non-homologous crossovers. In addition, transposable elements are dispersed throughout the genome, and the frequency and classes greatly vary among species. For instance, it is estimated that *B. rapa* and *R. sativus* have different classes of DNA transposons and retrotransposons differently enriched in their genomes (Mitsui et al., 2015). These transposable elements are expected to have been further diversified and fragmented after speciation, uniquely shaping the genomic landscapes in *B. rapa* and *R. sativus*, even in syntenic regions. Therefore, it is presumed that *B. rapa* and *R. sativus* genomes have gradually lost the similarity in genome structure after speciation and become divergent enough to inhibit A-R chromosome interactions. Such structural differences may allow independent assortment of A and R chromosomes during meiosis, which is conceivably beneficial to the acquisition of meiotic stability in x*Brassicoraphanus*.

Moreover, transposable elements are known to have a strong correlation with meiotic recombination rates in most eukaryotes. In particular, heterochromatic regions that usually contain a high density of transposable elements show strong recombination suppression (Kent et al., 2017). Transposable elements are heavily methylated in general and transcriptionally silenced, and DNA methylation also reinforces genome stability by limiting recombination in higher eukaryotes (Greenberg and Bourc'his, 2019). For example, during meiosis DNA methylation may keep transposable elements-rich regions of the genome from engaging in homology-dependent search and recombination (Zamudio et al., 2015). Therefore, we cannot rule out the possibility that epigenetic factors – particularly DNA methylation – have another profound effect on the inhibition of meiotic recombination between non-homologous but still similar regions of the two progenitor chromosomes.

Interestingly, a newly synthesized F1 allotetraploid of *B. rapa* and *R. sativus* showed a significantly lower recombination rate than genetically stable x*Brassicoraphanus* cv. BB1 (**Figure 5**). This suggests that immediately after hybridization, meiotic recombination is somewhat suppressed probably due to a conflict in recombination machineries between the two parental genomes. Alternatively, abrupt changes in epigenome landscape and chromatin structure after hybridization may interrupt a proper alignment of homologous chromosomes and crossing-over during meiosis. Investigation of meiotic chromosome behavior and recombination in successive generations will give some important clues to transgenerational progression of genome/epigenome stabilization and its effect on the recombination rate in a newly synthesized hybrid. By increasing the number and repertoire of hybridization combinations and performing an in-depth cytological analysis such as BAC FISH, essential features determining the recombination rate will be more clearly understood at the genome and chromosome levels. In addition, immunolocalization of ZYP1 protein will clearly demonstrate whether synapsis is indeed established and SCs properly assembled between A and R chromosomes in newly synthesized x*Brassicoraphanus*.

Eventually, the in-depth genome study on x*Brassicoraphanus* including genome sequencing and annotation, and transcriptome and epigenome profiling will reveal many fundamental aspects

of a hybrid genome resulting from a merger between *B. rapa* and *R. sativus* genomes. This will also facilitate researches on interesting traits unique to the hybrids and its application to the breeding program especially taking advantage of hybrid vigor whose genetic regulatory mechanisms are largely unknown.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

HP and JH designed the study. HP, JP, and JK performed the experiments. HP, JP, JK, HS, SY, and JH analyzed the data and wrote the manuscript. HS conducted the synteny analysis. HP, JP, JK, HS, SY, and GY prepared the plant materials. SS helped with the GISH experiment. S-SL provided the plant materials. HK provided the technical assistance in cytological analysis. All authors contributed to the manuscript and approved the submitted version.

FUNDING

This work was supported by the Next-Generation BioGreen 21 Program (PJ013262) and the National Agricultural Genome Program (PJ013440) by Rural Development Administration (RDA) and the Agri-Bio Industry Technology Development Program (117045-3) from Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (IPET), Ministry of Agriculture, Food and Rural Affairs (MAFRA).

ACKNOWLEDGMENTS

We would like to thank Dr. Jin-Kyung Kwon for the technical assistance with microscopy.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00851/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 26 June 2020
doi: 10.3389/fpls.2020.00954

In Support of Winge's Theory of "Hybridization Followed by Chromosome Doubling"

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Polyploidy—or chromosome doubling—plays a significant role in plant speciation and evolution. Much of the existing evidence indicates that fusion of unreduced (or 2*n*) gametes is the major pathway responsible for polyploid formation. In the early 1900s, a theory was put forward that the mechanism of "hybridization followed by chromosome doubling" would enable the survival and development of the hybrid zygote by providing each chromosome with a homolog with which to pair. However, to date there is only scant empirical evidence supporting this theory. In our previous study, interspecific-interploid crosses between the tetraploid *Hylocereus megalanthus,* as the female parent, and the diploid *H. undatus,* as the male parent, yielded only allopentaploids, allohexaploids, and 5*x*-and 6*x*-aneuploids instead of the expected allotriploids. No viable hybrids were obtained from the reciprocal cross. Since *H. undatus* underwent normal meiosis with regular pairing in the pollen mother cells and only reduced pollen grains were observed, the allohexaploids obtained supported the concept of "chromosome doubling." In this work, we report ploidy level, fruit morphology, and pollen viability and diameter in a group of putative hybrids obtained from an embryo rescue procedure following controlled *H. megalanthus* × *H. undatus* crosses, with the aim to elucidate, for the first time, the timing and developmental stage of the chromosome doubling. As in our previous report, no triploids were obtained, but tetraploids, pentaploids, hexaploids, and 5*x*- and 6*x*-aneuploids were found in the regenerated plants. The tetraploids exhibited the morphological features of the maternal parent and could not be considered true hybrids. Based on our previous studies, we can assume that the pentaploids were a result of a fertilization event between one unreduced (2*n*) female gamete from the tetraploid *H. megalanthus* and a normal (*n*) haploid male gamete from *H. undatus*. All the allohexaploids obtained from the embryo rescue technique where those that regenerated from fertilized ovules 10 days after pollination (at the pro-embryo stage), showing that the chromosome doubling event occurred at a very early development stage, i.e., at the zygote stage or shortly after zygote formation. These allohexaploids thus constitute empirical evidence of "hybridization followed by chromosome doubling."

Keywords: allopolyploidization, flow cytometry, genome duplication, *Hylocereus*, interspecific-interploid crosses, true hybrids, unreduced gametes

OPEN ACCESS

Edited by:
Jeremy Coate,
Reed College, United States

Reviewed by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *06 January 2020*
Accepted: *10 June 2020*
Published: *26 June 2020*

Citation:
Tel-Zur N, Mouyal J, Zurgil U and Mizrahi Y (2020) In Support of Winge's Theory of "Hybridization Followed by Chromosome Doubling". Front. Plant Sci. 11:954. doi: 10.3389/fpls.2020.00954

INTRODUCTION

Genome doubling or polyploidy – the state of having more than two full sets of chromosomes – has played a major role in the diversification and speciation of the plant kingdom, generating the genetic and epigenetic novelty that has contributed significantly to the diversity prevailing today (Stebbins, 1971; Soltis and Soltis, 1993; Osborn et al., 2003; Van de Peer et al., 2017). Autopolyploids arise within a single species and carry homologous chromosomes, while allopolyploids arise from two different taxa and have homoeologous chromosomes (Leitch and Bennett, 1997; Ramsey and Schemske, 1998; Soltis and Soltis, 2000). More specifically, taxonomically, autopolyploids are formed from within a single species, whereas allopolyploids are formed by hybridization between two or more species. Genetically, autopolyploids are plants with random associations among four (in the tetraploid cases) homologous chromosomes, resulting in tetrasomic segregation, whereas allopolyploids have two sets of homoelogous chromosomes that do not typically pair, leading to disomic segregation (Doyle and Egan, 2010; Doyle and Sherman-Broyles, 2017). As such, allopolyploids can, potentially, generate all the enzymes produced by each parent as well as new hybrid enzymes. This genetic redundancy or "enzyme multiplicity" of allopolyploids is considered to be an advantage, contributing to evolutionary success (Soltis and Soltis, 1993; Soltis et al., 2014; Glover et al., 2016; Lloyd et al., 2018) and facilitating speciation when a new hybrid is both fertile and reproductively isolated from its parental species.

The biological significance of allopolyploids thus derives from their origin, establishment, and persistence. Allopolyploid establishment and persistence have been extensively studied over the past few decades (Jenczewski and Alix, 2004; Feldman and Levy, 2009; Steige and Slotte, 2016; and references therein in all these citations), but what do we know about the pathways to the origin and formation of allopolyploids? It was over 100 years ago that Winge (1917) proposed his theory of "hybridization followed by chromosome doubling" as a mechanism enabling the survival and development of the hybrid zygote by providing each chromosome with a homolog with which to pair. According to Winge, hybridization involving two genetically very different gametes would not produce a viable zygote, since the gametes would not be able to pair (as was then solely in the realm of speculation and not based on scientific examinations). He thus suggested that chromosomes would split longitudinally, thereby allowing to generate a pair of homologous chromosomes that would permit the development of the hybrid with double the parental number of chromosomes. However, to date, most empirical studies have not provided evidence to support Winge's theory. Rather, the findings of most studies support one of the following two mechanisms for allopolyploid origin: (1) sexual polyploidization through the fusion of *2n* gametes, where the increase in the chromosome number occurs in the first generation through the union of one or both unreduced gametes (Harlan and deWet, 1975 and references therein, Gao et al., 2019); or (2) chromosome doubling in somatic tissues, i.e., somatic doubling, which is a mitotic rather than a meiotic event. The first report of spontaneous somatic doubling was that for *Primula kewensis*, a first-generation hybrid between *P. floribunda* and *P. verticillata* (Newton and Pellew, 1929). The hybrid diploid plant was sterile, but periodically one of the cuttings produced fertile flowers in branches that were undergoing spontaneous doubling (somatic doubling), a process that apparently restored fertility by providing each chromosome with an identical partner with which to pair (Newton and Darlington, 1929). Today, it is known that somatic doubling in meristem tissues can also be induced by artificial means (Ramsey and Schemske, 1998 and references therein; Sattler et al., 2016 and references therein).

As mentioned above, over the years only scant empirical evidence has been offered in support of Winge's theory of "hybridization followed by chromosome doubling." To the best of our knowledge, the following examples are the only crosses that support his theory: 1) fertile *Nicotiana glutinosa* L. × *N. tabacum* L. tobacco allotetraploids (Clausen and Goodspeed, 1925); 2) two ryegrass genotypes with an unexpected double number of chromosomes from a BC_2 progeny [a cross between the diploid *Lolium multiflorum* and a triploid BC_1 (itself the result of a cross between the tetraploid *Festuca arundinacea* var. glaucescens and a synthetic *L. multiflorum* tetraploid)] (Morgan et al., 2001); and 3) hexaploid and 6*x*-aneuploid pitaya hybrids resulting from a cross between the tetraploid *Hylocereus megalanthus* and the diploid *H. undatus* (Tel-Zur et al., 2003; Tel-Zur et al., 2004). These three examples suggest that the unexpected high ploidy level of the newly formed hybrids was the result of a process that occurred immediately after or soon after fertilization.

Hylocereus species are night-blooming vine cacti native to the tropical and sub-tropical regions of the Americas (Barthlott and Hunt, 1993). These species are characterized by triple-ribbed stems, large flowers, and attractive edible fruits (Mizrahi and Nerd, 1999). Cytological studies show that *Hylocereus* species are diploids (Banerji and Sen, 1955; Spencer, 1955; Lichtenzveig et al., 2000; Tel-Zur et al., 2003; Tel-Zur et al., 2004), with the exception of *H. megalanthus*, which is a tetraploid. The diploid species bear large (250-800 g) flavorless, red-purple fruits, whereas the tetraploid *H. megalanthus* bears sweeter, but smaller (180-250 g), yellow fruits (Tel-Zur et al., 2003; Tel-Zur et al., 2011). With the aim of producing elite cultivars with improved fruit quality, a long-term breeding program was thus initiated about three decades ago at Ben-Gurion University of the Negev. In this framework, interspecific-interploid crosses were performed with the aim to combine the size and attractiveness of the diploids with the fruit quality of the tetraploid species (Tel-Zur et al., 2004). No true hybrids or chimeras were obtained in the sampled *H. megalanthus* × *H. undatus* crosses (Tel-Zur et al., 2003: Tel-Zur et al., 2004), but for the reciprocal cross, tetraploids, pentaploids, hexaploids, and 6*x*-aneuploids, rather than the expected triploids, were obtained (Tel-Zur et al., 2003), thereby indicating a uni-directional gene flow among these species. The tetraploids exhibited the morphological features of the maternal parent and were therefore not considered true hybrids; rather, they were considered to be

either a result of self-pollination due to contamination during the hand-cross pollination process or to be of somatic origin, since polyembryony has been reported in *H. megalanthus* (Cisneros et al., 2011). It was therefore proposed that zygotic or post-zygotic somatic chromosome doubling constituted the mechanism for the formation of the hexaploid and 6*x*-aneuploid hybrids (Tel-Zur et al., 2003; Tel-Zur et al., 2004). The above premise was based on our previous work (Lichtenzveig et al., 2000) showing the formation of both unreduced pollen grains and unbalanced gametes due to irregular chromosome disjunction at anaphase. Anaphase I separations such as 22–22, 23–21, and 24–20 were observed in the tetraploid *H. megalanthus*, suggesting that some degree of aneuploidy could be tolerated, while the diploid species exhibited both the regular chromosome disjunction at anaphase I and a uniform pollen diameter (Lichtenzveig et al., 2000; Tel-Zur et al., 2003). Furthermore, to date, all interspecific diploid *Hylocereus* × *Hylocereus* crosses have produced diploid hybrids, which strongly indicates the negligible production of unreduced gametes by the diploid species (Tel-Zur et al., 2004). Consequently, we can assume that the allohexaploid and 6*x*-aneuploid hybrids obtained from crosses of *H. megalanthus* and *H. undatus* occurred at frequencies that were much higher than the frequency that would be expected for the fusion of unreduced gametes from both egg and pollen donor parents. In addition, no chimeras were obtained, i.e., plants with identical fruit and vegetative morphology were observed in the *H. megalanthus* × *H. undatus* hybrids studied, which indicates a very low likelihood of somatic doubling later in development and provides further support for our theory.

Against the above background, the main goals of this research were to confirm the occurrence of spontaneous chromosome doubling following interspecific-interploid hybridization, i.e., Winge's theory, and to identify the timing or the developmental stage at which the event occurs. We postulated that genome doubling takes place either in the hybrid zygote or shortly after zygote formation, a process that ensures the viability of the new hybrid embryo. The methodology applied involved estimating the ploidy level by measuring the relative DNA content by means of flow cytometry of the putative allopolyploids regenerated by the embryo rescue technique following *H. megalanthus* × *H. undatus* crosses. In addition, the viability and fertility of the resulting true allopolyploids were evaluated in terms of fruit morphology and weight and in terms of the pollen viability and diameter of selected allopolyploids.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The plant material used in this study comprised 38 plants regenerated from crosses between the tetraploid *H. megalanthus* [(Schum. ex Vaupel) Moran] Bauer (accession 90-003) as the female parent and the diploid *H. undatus* (Haw) Br. and Rose (accession 89-024) as the male parent, according to the embryo rescue technique given in Cisneros et al. (2013) and described briefly below. It has previously been shown that the total number of seeds per fruit in *H. megalanthus* is about 200-300, resulting in 20-35% aborted/empty seeds (Cisneros et al., 2011; Tel-Zur et al., 2011). A detailed description of zygote, embryo, and endosperm formation in *H. megalanthus* is provided in Cisneros et al. (2011). In particular, that study reported that an 8-cell pro-embryo was observed in *H. megalanthus* at 11 days after pollination (DAP) and well-developed endosperm was observed 28 DAP. The growing embryo consumed the endosperm, which remained incipient (only a few cells surrounding the embryo) at 42 DAP (Cisneros et al., 2011). Endosperm recovery and/or ploidy determination of endosperm cells is technically very challenging and is thus beyond the scope of our work as discussed below.

The development of the embryo rescue technique in our laboratory started with the rescue of embryos following intraspecific *H. undatus* and *H. megalanthus* crosses. All the resulting offspring showed the expected ploidy, i.e., diploid and tetraploid (unpublished data). In parallel, we applied the embryo rescue technique to fertilized ovules carrying embryos at very early pro-embryonic stage following reciprocal interspecific diploid crosses [*H. undatus* × *H. monacanthus* (syn. *H. polyrhizus*)], which resulted in diploid hybrids alone (Cisneros and Tel-Zur, 2010), thereby showing that the technique did not affect the ploidy level of the resulting hybrids. Briefly, the protocol used for embryo rescue technique included half-strength basal Murashige and Skoog (MS) medium containing 680 μM glutamine, 0.54 μM α-naphthaleneacetic acid, 0.45 μM thidiazuron, and 0.17 M sucrose. The regenerated plants were obtained from fertilized ovules collected from developing fruits at 10, 30, and 47 DAP when the embryos were at the pro-embryo (10 DAP), globular (30 DAP), and heart (47 DAP) stages, respectively, according to Cisneros et al. (2011). The 38 putative allopolyploid plants were planted in January 2013 in 10-L pots held under 50% shade in a greenhouse located on the Bergmann Campus, Ben-Gurion University of the Negev, Beer-Sheva, Israel (31°15'N, 34°48'E). Each plant was watered with 200 L $year^{-1}$ applied *via* a drip system, with a nutrient concentration of 60 mg L^{-1} of N, 18 mg L^{-1} of P and 60 mg L^{-1} of K fertilizer with trace elements (23-7-23 Deshanim, Israel).

Ploidy Identification

Cytological Observations

Developing floral buds were collected from the putative allopolyploid lines and fixed for 24 h in 3:1 ethanol/glacial acetic acid. The buds were stored in 70% ethanol at 4°C until examination. The chromosomes were stained with 2% acetocarmine by using the standard squash method. Pollen mother cells (PMCs) were examined through an Axio ImagerA1 microscope with LED illumination (Zeiss) and photographed with a ZEISS Axiocam 305 color camera and the ZEN imaging software program.

Flow Cytometry Analysis

Tissue was collected from the tips of newly developed branches of 4- to 8-year-old putative hybrids, of the allohexaploid J-42, and of the parental species. Nuclear suspensions were prepared

from this tissue according to the protocol of Li et al. (2017). Samples were analyzed using a iCyt Synergy SY3200 sorter (Sony Biotechnology, San Jose, USA) equipped with a 561-nm laser and a 595/50 band pass filter. Results were analyzed using Winlist 3D software ver 8.0 (Verity Software House). Genome size was assessed by comparing the nuclear DNA content of the tetraploid *H. megalanthus* with that of the relevant putative allopolyploid. The tissue of each putative hybrid was analyzed at least four times to verify the reproducibility of the results. In addition, a range of nuclear DNA contents indicating each ploidy level was determined on the basis of previously reported nuclear DNA contents for the parental species, i.e., *H. megalanthus* accession 90-003 with 8.70 pg/2C (Tel-Zur et al., 2011), *H. undatus* accession 89-024 with 3.86 pg/2C (Tel-Zur et al., 2011), and the allohexaploid J-42 reported to have 66 chromosomes (Tel-Zur et al., 2004) with 13.4 pg/2C (unpublished data); the ranges of nuclear DNA content per ploidy level were determined according to the variance around the means; thus: 8.00 to 9.50, 10.00 to 11.50, and 12.0 to 13.5 pg/2C for 4*x*, 5*x,* and 6*x*, respectively.

Fruit Morphology and Weight

Flowers of the putative allopolyploids were cross pollinated by hand at anthesis with a mixture of fresh pollen collected from diploid *Hylocereus* spp. Pollen grains were applied to the surface of the stigmata with the aid of a brush early in the morning. Fruits were collected at the ripening stage of full color over five consecutive years (2014-2018). Fruits from each allopolyploid were weighed on the harvesting day.

Pollen Grain Stainability and Diameter

Pollen grains from the allopentaploid N-149 and the allohexaploid N-134 were collected at anthesis and stored at 4°C until evaluation. These particular allopolyploids were chosen for this study due to the high number of flowers they produced every year. Pollen grains were stained with 2% acetocarmine (Belling, 1921), since previous studies showed similar outcomes for staining with acetocarmine, fluorescein diacetate, and Alexander's reagent, but acetocarmine can be used to stain both fresh and stored pollen, while the other two reagents are suitable only for fresh pollen. About 300 to 400. and 100 to 150 pollen grains from four different flowers of each line were evaluated for pollen viability and diameter, respectively. Photomicrographs were taken with an Axio ImagerA1 microscope with LED illumination (Zeiss) and photographed with a ZEISS Axiocam 305 color camera and the ZEN imaging software program. Averages ± SE were calculated for pollen diameter and pollen viability. A *t*-test was used to assess the differences in pollen performances between allopentaploids and allohexaploids.

RESULTS

Ploidy Identification

Cytological Observations

In this study, an attempt was first made to determine ploidy of putative hybrids by chromosome counting. However, cytomixis, i.e., the transfer of chromatin material from one cell to another through intercellular channels (cytomictic channels) during meiosis, proved to be an obstacle that obviated direct counting. Migration of chromatin through cytomictic channels and chains of pollen mother cells (PMCs) united by the migrating chromatin were observed in both allopentaploids and allohexaploids (**Figure 1**). Despite our efforts to very carefully count chromosomes in the PMCs, our results for the number of chromosomes in different PMCs isolated from the same flower bud were not consistent, probably due to the cytomixis. Consequently, ploidy was estimated by flow cytometry.

Flow Cytometry Analysis

The estimated ploidy – calculated as described in Materials and Methods – indicated that among the 38 putative hybrids studied, 32 were true hybrids, and 6 were tetraploids (not considered true hybrids) like the female *H. megalanthus* parent (**Table 1** and **Figure 2**). Of the 32 true hybrids, 18 were allopentaploids and 8 allohexaploids. Nuclear DNA content of the allopentaploids ranged from 10.07 to 11.40 pg/2C, and that of the allohexploids ranged from 12.17 to 13.39 pg/2C (**Table 2**). Estimation of the ploidy level of the other 6 hybrids showed that they exhibited an intermediate ploidy and that they were probably aneuploids, i.e., 3 were 4–5*x*, and 3 were 5–6*x*, ranging from 9.55 to 9.78 pg/2C and 11.67 to 11.76 pg/2C, respectively (**Table 2**). No allotriploids were found in the studied hybrids. Average genome sizes ± SE were calculated and are reported for each hybrid line in **Table 2**.

Fruit Morphology and Weight

The putative hybrids developed well under growth conditions similar to those for the parental species. Plant morphology of the putative hybrids, i.e., triple-ribbed stems as well as shape and number of thorns at base of the vegetative buds, was identical to that of the parental lines, indicating that it was impossible to identify true hybrids according to the morphology of the vegetative parts. Fruit morphology of the hybrids was compared with that of the parental species (**Tables 2** and **3**; **Figure 3**). All 32 allopolyploids bore elongated fruits. The fruits were covered by bracts, with thorns at the base of each bract—a trait inherited from the maternal tetraploid species *H. megalanthus*. The spiny peel of all 32 allopolyploids (allopentaploids, allohexaploids, and the probable aneuploids) was yellow-orange in color, with a pink layer between the peel and the flesh. The fruit flesh was white, as in both parental species. All the allopolyploids set both viable and aborted seeds (**Figures 3C, D**).

Average fruit weights ± SE were calculated and are reported for each hybrid line in **Table 2**. A *t*-test was used to assess the difference in fruit weights between allopentaploids and allohexaploids. Statistically significant differences were observed for fruit weight, as determined by an unpaired two-tailed *t*-test (significant at $P = 0.0001$), between the allopentaploids and the allohexaploids, with the allohexaploid lines bearing smaller fruit (87 ± 3 g) than the allopentaploid lines (133.5 ± 3 g).

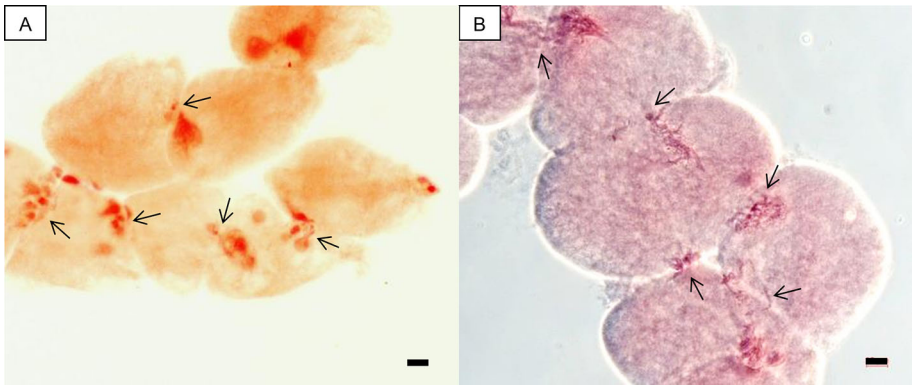

FIGURE 1 | Cytological photographs of groups of PMCs involved in chromatin transfer, i.e., cytomixis (arrow). **(A)** A chain of PMCs united by channels of transferred chromatin in the allopentaploid N-124. **(B)** Migration of chromatin through cytomictic channels in the allohexaploid N-121. Scale bar, 10 µm.

TABLE 1 | Ploidy of the confirmed hybrids* according to cytometric analysis.

Cross combination ♀ × ♂	DAP**	No. of progeny planted	No. of confirmed hybrids	No. of confirmed hybrids according to ploidy				
				3*x*	4–5*x*	5*x*	5–6*x*	6*x*
H.	10	19	18	0	1	8	1	8
megalanthus ×	30	15	13	0	2	10	1	0
H. undatus	47	4	1	0	0	0	1	0

**Total number of putative hybrids studied: 38; true hybrids: 32. **DAP, days after pollination.*

Pollen Grain Stainability and Diameter

The percent of pollen viability ± SE of the allopentaploid N-149 (29.9 ± 0.06%) differed significantly from that of the allohexaploid N-134 (49.5 ± 0.01%), as shown by a *t*-test (significant at P = 0.019). Images showing viable and aborted pollen grains are presented in **Figures 4A, C**. The average ± SE pollen diameters were 129.5 ± 0.9 and 126.8 ± 0.6 µm for the allopentaploid N-149 and the allohexaploid N-134, respectively. No statistically significant differences were observed for pollen diameter, as determined by an unpaired two-tailed *t*-test (significant at P = 0.248) between the allopentaploid and allohexaploid lines. The diameters of most of the stainable pollen grains of the allopentaploid N-149 and allohexaploid N-134 (~67% and ~81%, respectively) lay in the range of 110–139 µm, whereas for both allopolyploids, 7% had a smaller diameter than the average, and the remaining diameters exceeded the average range. Pollen diameter frequency distribution is presented in **Figures 4B, D**.

DISCUSSION

The results of this study strongly support Winge's theory of "hybridization followed by chromosome doubling" by showing high ploidy, i.e., 6*x* instead 3*x*, following interspecific-interploid hybridization. No hybrids were obtained for the *H. undatus* × *H. megalanthus* cross, and in the reciprocal cross only allopentaploids, allohexaploids, and 4-5*x* and 5-6*x* aneuploids were obtained. It has been proposed that the main route to polyploidization is through unreduced gametes and the formation of triploids (Bretagnolle and Thompson, 1995). The "triploid block" acts as a reproductive barrier in the endosperm, preventing backcrossing with the parental species. Triploids were not obtained for the *H. megalanthus* × *H. undatus* crosses, but crosses between the diploid *H. monacanthus* as the maternal species and the tetraploid *H. megalanthus* as the paternal species resulted in triploid and 3*x*-aneuploid hybrids, while the reciprocal cross yield only pentaploids (Tel-Zur et al., 2003; Tel-Zur et al., 2004). These findings suggest that *H. monacanthus* is more closely related to *H. megalanthus* than to *H. undatus*. Moreover, back-crosses, using triploid *H. monacanthus* × *H. megalanthus* hybrids, were indeed obtained (Tel-Zur et al., 2012), thereby showing that the "triploid block" does not exist in *Hylocereus* species.

As mentioned above, we have previously shown that the tetraploid *H. megalanthus* produces both normal (reduced) and unbalanced and unreduced gametes, but the diploid *H. undatus* produces only normally reduced gametes (Lichtenzveig et al., 2000; Tel-Zur et al., 2003). Thus, the presence of allohexaploids and the absence of allotriploids in *H. megalanthus* × *H. undatus* cross combinations provide empirical evidence supporting our hypothesis that chromosome doubling occurred at the zygote formation stage or very soon thereafter. We thus suggest that chromosome doubling occurs as a spontaneous process of mitotic cell division without cytokinesis. The sources of the 4*x*-5*x* aneuploids are probably unbalanced gametes and unbalanced-unreduced gametes, respectively, from the tetraploid *H. megalanthus* (Lichtenzveig et al., 2000; Tel-Zur et al., 2003). The 6*x* aneuploids are probably a result of a fertilization event with one reduced but unbalanced gamete from *H. megalanthus* followed by chromosome doubling. In this context, it is interesting to note that chromosome doubling at the zygote level can be artificially induced. For example, high temperatures induced the formation of synthetic triploids and tetraploids

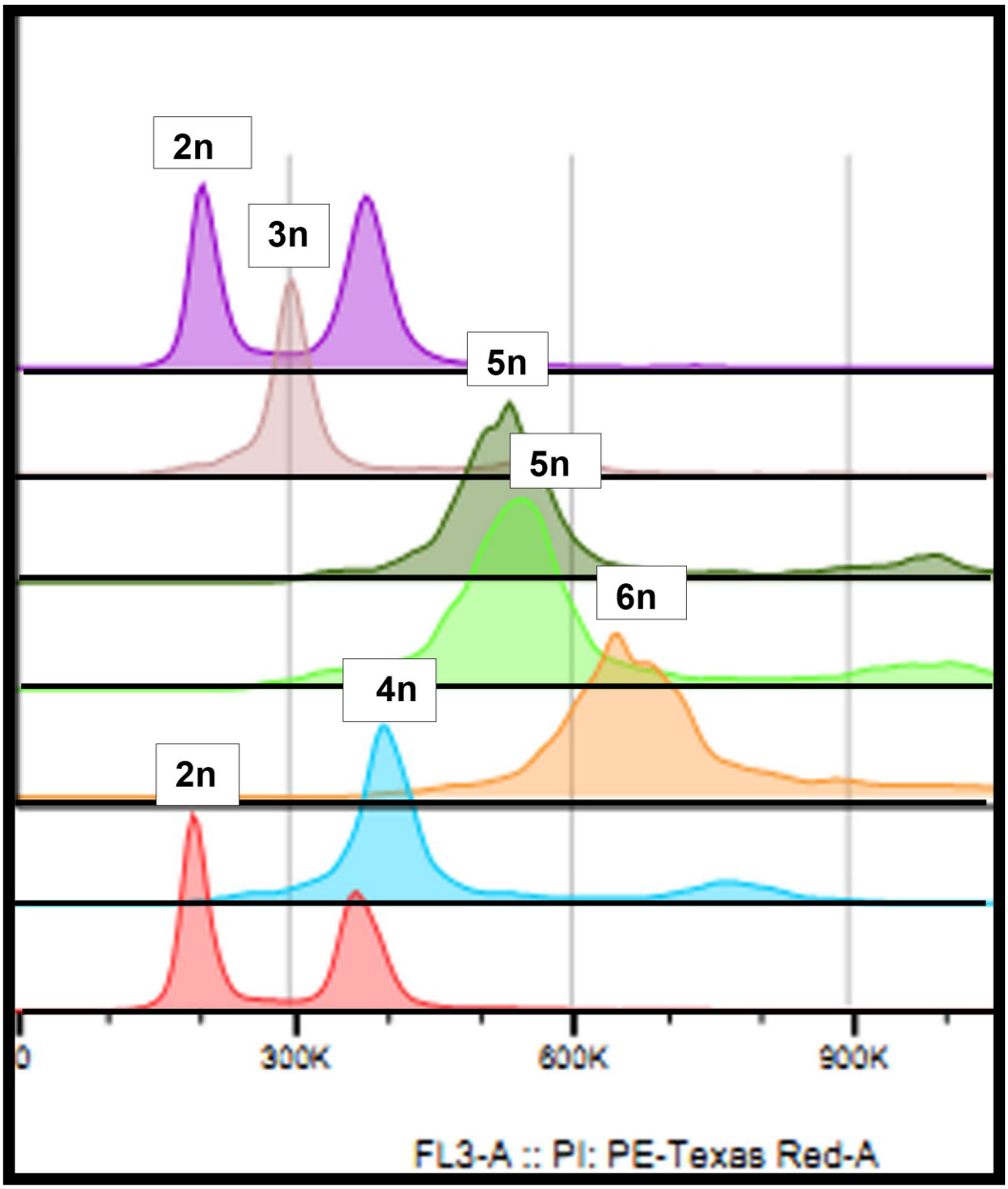

FIGURE 2 | Flow cytometry histogram: violet and dusky pink—the diploid maternal species *H. undatus* at the beginning and the end of the run, respectively; brown—a triploid hybrid (S-75); olive green and light green—two independent runs of the regenerated allopentaploid N-122; orange—the regenerated allohexaploid N-121; and blue—the paternal species, the tetraploid *H. megalanthus*.

through embryo sac and zygote embryo chromosome doubling in *Populus simonii* and its hybrids (Guo et al., 2017). Similarly, corn plants exposed to high temperatures after pollination produced diploid, tetraploid, and octoploid seedlings (Randolph, 1932).

One of the crucial questions in allopolyploidy formation relates to the genetic consequences of combining two genetic systems into a common nucleus (Huxley, 1942; McClintock, 1984; Parisod et al., 2009; Lloyd et al., 2018). Subsequent to the successful formation of a new allopolyploid, its establishment depends on the following factors: 1) the correct control of chromosome pairing, a pre-requisite for the production of viable seeds and pollen grains, 2) epigenetic factors, and 3) 'homoeologous exchanges,' i.e., exchanges of large chromosomal segments between homoeologous chromosomes *via* the meiotic homologous recombination pathway (Jenczewski and Alix, 2004; Feldman and Levy, 2009; Madlung and Wendel, 2013; Glover et al., 2016; Steige and Slotte, 2016; Liang and Schnable, 2018). Natural allopolyploids are stable and well adapted, whereas

TABLE 2 | Ploidy level estimated using flow cytometric analysis and fruit weight in interspecific-interploid hybrids from a cross of *H. megalanthus* ♀ × *H. undatus* ♂ rescued 10, 30, or 47 days after pollination (DAP) by hand.

Hybrid code	DAP	Nuclear DNA content pg/2C ± SE	Ploidy estimated	Fruit weight g ± SE
N-121	10	12.98 ± 0.28	6*x*	112.8 ± 16.5
N-122		10.33 ± 0.26	5*x*	124.0 ± 42.8
N-123		13.20 ± 0.32	6*x*	84.2 ± 9.8
N-124		11.30 ± 0.12	5*x*	92.5 ± 12.4
N-132		11.76 ± 0.89	5-6*x*	70.3 ± 12.4
N-133		11.32 ± 0.43	5*x*	100.7 ± 9.2
N-134		12.17 ± 0.28	6*x*	81.8 ± 3.3
N-135		10.72 ± 0.18	5*x*	123.8 ± 6.1
N-136		11.40 ± 0.43	5*x*	133.6 ± 7.0
N-137		11.18 ± 0.54	5*x*	128.8 ± 7.9
N-138		12.69 ± 0.14	6*x*	90.8 ± 6.0
N-140		10.07 ± 0.33	5*x*	110.9 ± 3.6
N-173		10.23 ± 0.35	5*x*	142.7 ± 8.4
N-174		12.65 ± 0.50	6*x*	72.2 ± 4.4
N-175		12.71 ± 0.38	6*x*	62.0 ± 12.3
N-176		13.39 ± 0.31	6*x*	80.9 ± 6.6
N-177		9.55 ± 0.43	4-5*x*	133.0 ± 15.0
N-178		12.54 ± 0.09	6*x*	93.0 ± 3.8
N-125	30	9.78 ± 0.05	4-5*x*	87.6 ± 11.4
N-126		11.29 ± 0.27	5*x*	152.1 ± 10.5
N-127		10.31 ± 0.37	5*x*	154.6 ± 10.8
N-128		11.67 ± 0.32	5-6*x*	123.9 ± 9.5
N-129		10.74 ± 0.43	5*x*	171.0 ± 13.1
N-130		11.18 ± 0.07	5*x*	171.2 ± 10.5
N-131		10.94 ± 0.94	5*x*	147.2 ± 10.7
N-144		11.19 ± 0.32	5*x*	128.1 ± 8.3
N-145		10.52 ± 0.45	5*x*	105.5 ± 7.0
N-147		9.58 ± 0.55	4-5*x*	121.0 ± 6.0
N-148		10.56 ± 0.51	5*x*	139.3 ± 11.7
N-149		11.15 ± 0.45	5*x*	125.9 ± 10.8
N-150		10.89 ± 0.33	5*x*	133.2 ± 10.4
N-013	47	11.74 ± 0.08	5-6*x*	141.8 ± 13.0

TABLE 3 | Fruit characteristics of the parental species and allopolyploids.

Plant material	Ploidy	Weight, g ± SE	Fruit characteristics		
			Spiny peel	Peel color	Flesh color
Parental species					
*H. megalanthus**	4*x*	267 ± 17	+	Yellow	White
*H. undatus**	2*x*	372 ± 11	–	Red	White
Cross combination ♀ × ♂					
H. megalanthus ×	4-5*x*	ND**	+	Yellow-orange	White
H. undatus	5*x*	133.5 ± 3	+	Yellow-orange	White
	5-6*x*	ND**	+	Yellow-orange	White
	6*x*	87 ± 3	+	Yellow-orange	White

**Data previously reported by Lichtenzveig et al. (2000) and Tel-Zur et al. (2011). **ND, not determined.*

synthetic allopolyploids can exhibit incompatible interactions between parental genomes, i.e., intergenomic incompatibilities (Comai, 2000). Cytomixis, as was observed here in both allopentaploids and allohexaploids (**Figure 1**), can be the consequence of such intergenomic incompatibilities, but in *Hylocereus* species—like in other species—the biological and evolutionary significance of cytomixis is not known (Mursalimov and Deineko, 2018).

In a parallel but opposite scenario to that reported here, reciprocal crosses between the diploid *Arabidopsis thaliana* and the tetraploid *A. arenosa* resulted in aborted seeds or unsuccessful pollen germination, but crosses between the tetraploid *A. thaliana* as the maternal parent and the tetraploid *A. arenosa* restored seed viability (Bushell et al., 2003). Seeds from reciprocal crosses between the diploid *A. lyrata* and *A. arenosa* failed to germinate, due to failure of endosperm cellularization in the *A. lyrata* × *A. arenosa* cross, while in the reciprocal cross the endosperm cellularized precociously (Lafon-Placette et al., 2017). Interestingly, crosses between the tetraploid *A. lyrate* and the diploid *A. arenosa* were fully viable, but crosses between the tetraploid *A. arenosa* and the diploid *A. lyrate* did not produce viable seeds (Lafon-Placette et al., 2017). Endosperm defects can explain the failure of the cross and the cross direction, illustrating very clearly the role of the endosperm in hybridization barriers.

In flowering plants, sexual reproduction is characterized by two separate gametic fusion events that form the embryo and the endosperm. In diploid crosses, these events result in a diploid embryo and most commonly a triploid endosperm, with a ratio of 1:1 and 2:1 of maternal-to-paternal genomes, respectively. A fundamental question regarding embryo survival and seed development following interspecific-interploidy crosses centers on maternal or paternal genomic excess and the cases in which the embryo and the endosperm can develop with different maternal/paternal genome ratios. To address this question, Johnston et al. (1980) put forward the endosperm balance number (EBN) theory, which stated that rather than an absolute numerical ploidy, each species has an effective genome ratio, which must be 2:1 maternal/paternal for normal endosperm development. This oversimplification of the very important biological phenomenon of zygote formation and embryo development was useful for plant breeders for predicting the success of a cross, especially among *Solanum* species (Carputo et al., 1997; Jansky, 2006), but failed to predict crossing success in other plant species.

We note here that ploidy determination in immature embryos was not directly addressed in this study. *Hylocereus* embryos – even completely mature embryos – are very small, and there is thus not enough tissue to provide sufficient material even for a single flow cytometry run. Nonetheless, even though genome duplication can occur spontaneously in somatic tissues, the probability that such an event would occur in all the studied true hybrids is – in our opinion – extremely low. Moreover, there were no differences between the morphologies of the hybrid plants at the vegetative level and, similarly, no differences in the flow cytometry results for tissues collected from the tips of different newly developed branches; both these findings indicate the low probability of chimera events in the new hybrids.

Determination of endosperm ploidy in the allopolyploids was beyond the scope of this study, but we did calculate the expected endosperm ploidy of the allopentaploid and allohexaploid *H. megalanthus* × *H. undatus* crosses described

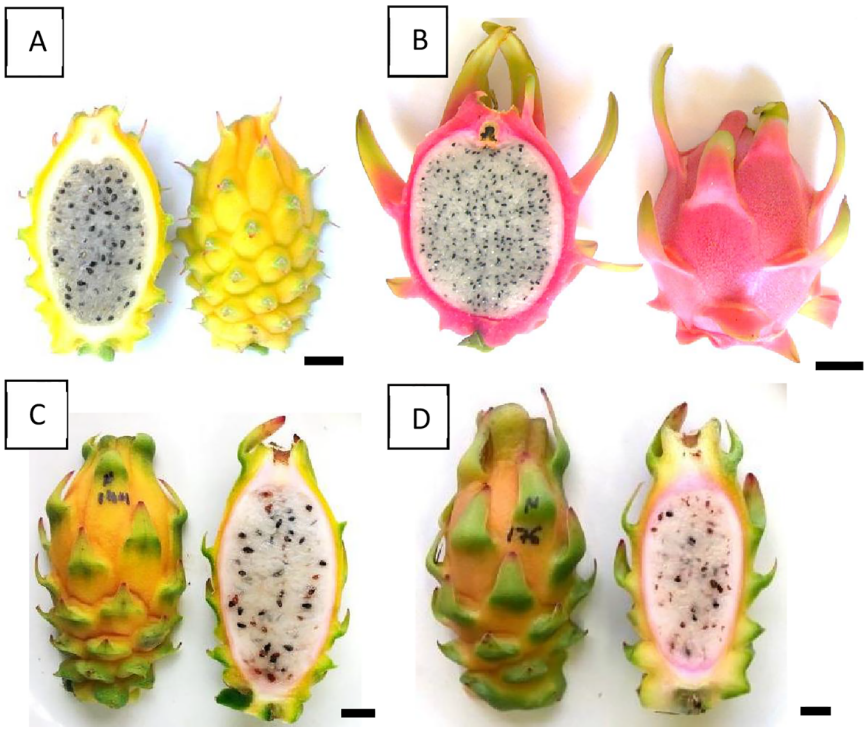

FIGURE 3 | Fruit morphology. **(A)** The maternal species, the tetraploid *H. megalanthus*. **(B)** The paternal species, the diploid *H. undatus*. **(C)** The allopentaploid N-144. **(D)** The allohexaploid N-176. The average fruit weights were 218, 372, 128, and 81 g for *H. megalanthus*, *H. undatus*, allopentaploid N-144 and allohexaploid N-176, respectively.

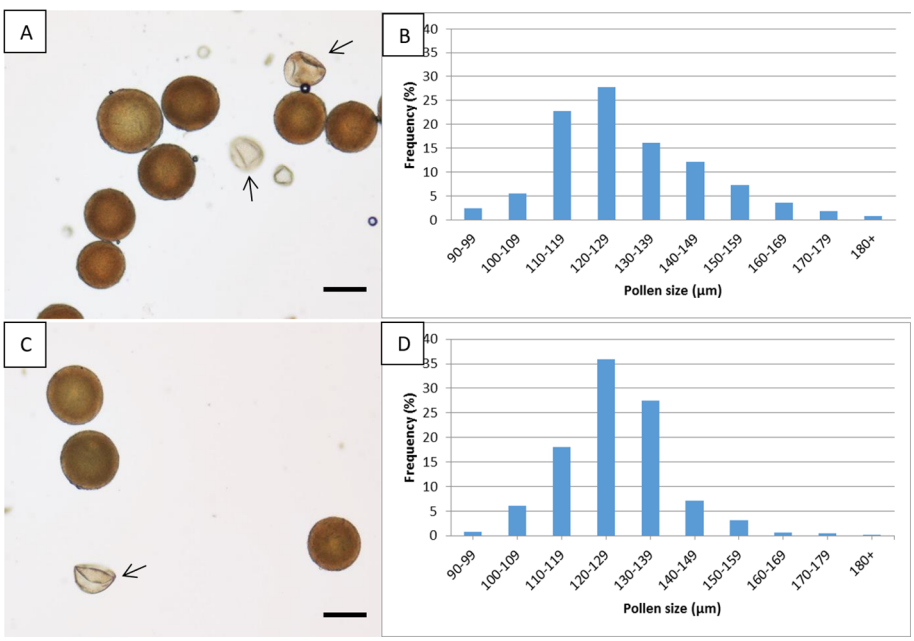

FIGURE 4 | Pollen viability and diameter. Images showing viable and aborted (arrow) pollen grains and pollen diameter distribution in the allopentaploid N-149 **(A, B)** and the allohexaploid N-134 **(C, D)**. Scale bar, 100 µm.

TABLE 4 | Theoretical outcome of interspecific-interploidy crosses following paternal and maternal excess.

	Parental ploidy		Ploidy (m/p)	
	Maternal (m)	**Paternal (p)**	**Predicted endosperm ploidy (m/p)**	**Hybrid ploidy obtained (m/p)**
Paternal excess (p)	2*x*	4*x*	4*x* (2m:2p)	NH[1]
Maternal excess (m)	4*x*	2*x*	**Normal (reduced) embryo sac**	
			5*x* (4m:1p)	NH[2]
			Unreduced embryo sac[3]	
			9*x* (8m:1p)	5*x* (4m:1p)
			Normal (reduced) embryo sac followed by zygote/embryo chromosome doubling[4]	
			5*x* (4m:1p)	6*x* (4m:2p)
			Both endosperm and zygote/ embryo chromosome doubling[5]	
			10*x* (8m:2p)	6*x* (4m:2p)

2x: H. undatus, 4x: H. megalanthus.

[1]NH - No hybrids were obtained from the H. undatus × H. megalanthus cross, even when we used an embryo rescue technique.

[2]NH- No H. megalanthus × H. undatus allotriploids were obtained.

[3]Assuming that all the embryo sac cells (including the egg cell) result from unreduced gamete formation, since megasporogenesis occurs before megagametogenesis.

[4]Ploidy in the embryo sac cells was assumed to be reduced, while the allohexaploids are a result of chromosome doubling.

[5]Chromosome doubling occurs in the endosperm and zygote/embryo, resulting in 10x and 6x, respectively.

above as 9*x* (8m:1p) and 5*x* (4m:1p), respectively, with no production of 3*x* hybrids with an endosperm ploidy of 5*x* (4m:1p) or 10*x* (8m:2p) (**Table 4**). Likewise, no hybrids were obtained in the reciprocal cross *H. undatus* × *H. megalanthus* with an expected endosperm ploidy of 4*x* (2m:2p)—even when the embryo rescue technique was used (**Table 4**). Whole genome duplication (hexaploid rather than triploid) did not change the maternal/paternal ratio in the embryo (**Table 4**), but it did change the maternal-to-endosperm-to-embryo genome ratios by doubling the ploidy of the embryo, which probably allowed normal embryonic development. It therefore seems likely that only the above-described ratios between the endosperm and/or the maternal tissues and/or the embryo permit the survival of the new hybrid embryo in *H. megalanthus* × *H. undatus* crosses. Studies focusing on the nature of zygotic genome activation have shown that the maternal and paternal genomes contribute equally to early plant embryo development and that early embryogenesis is mostly under zygotic control (Nodine and Bartel, 2012). In rice, for example, polyploid zygotes with a maternal excess developed normally, whereas most polyploid zygotes with a paternal excess showed developmental arrest, indicating that paternal and maternal genomes act synergistically to allow zygote development but probably with distinct functions for each (Toda et al., 2018). The findings that only allopentaploid and allohexaploids were obtained in the *H. megalanthus* × *H. undatus* cross combination and the lack of allotriploids in both reciprocal crosses suggest that there are (still) unknown factor (s) preventing the formation and development of a triploid embryo.

Hylocereus species bear large flowers (30-35 cm diameter) with numerous mega- and microspores, which simplify technical manipulations (Nerd and Mizrahi, 1997), such as hand cross pollination and the embryo rescue technique (Cisneros et al., 2013). Thus, from a technical point of view, *Hylocereus* species can be used as good model plants in polyploid studies. Among the 32 true hybrids obtained, all eight (25%) allohexaploids were obtained from embryo rescue at 10 DAP (pro-embryo stage), showing that the chromosome doubling event occurs very early in embryo development. We note here that in a previous report (Cisneros et al., 2013) two plants regenerated from embryo rescue 30 DAP following *H. megalanthus* × *H. undatus* crossing were reported in error as a diploid and a triploid, whereas they were, in fact, tetraploids. Tissues in *Hylocereus* species are rich in polysaccharides, making the flow cytometric analysis a challenging task. Subsequent to that report (Cisneros et al., 2013), and in view of the limitations of the published protocols, we developed an accurate protocol (Li et al., 2017) aimed to solve, simplify, and streamline flow cytometry for ploidy determination in polysaccharide-rich tissues; that procedure was used in this work.

One of the key questions following interspecific and interspecific-interploid hybridization is the fertility of the resulting hybrids. Fruit morphology verifies hybrid origin but also provides the essential information that these hybrids (both allopentaploid and allohexaploid) can grow and can set fruit and seeds. Fruit weights in the allohexaploids were lower than those in the allopentaploids, a finding that is in line with previous reports in auto- and allopolyploid *Hylocereus* species, showing that fruit weight declined as the ploidy level increased (Tel-Zur et al., 2004; Tel-Zur et al., 2012; Cohen et al., 2013). All the allopolyploids set both viable and aborted seeds, and the allopentaploids and allohexaploids produced both viable and aborted pollen grains, showing them to be partially fertile. Pollen viability was about 30% and 50% for the studied allopentaploids and allohexaploids, respectively, with most of the pollen grains presenting – in both cases (67% and 81%, respectively) – a large diameter of 110 to 139 μm. The pollen grains of *H. undatus* exhibited a uniform diameter of 70–80 μm, but those of *H. megalanthus* showed a wide variation in diameter, with values lying between 90 and 190 μm. These differences in diameter represent different ploidy levels in the viable pollen grains and are, most probably, due to meiotic abnormalities, such as the formation of unreduced gametes, unbalanced chromosome segregation, or cytomixis (Ramanna and Jacobsen, 2003; Shamina, 2005; Mursalimov and Deineko, 2018). All the above findings indicate that both allopentaploids and allohexaploids can reproduce by sexual reproduction, since both produce a certain percent of viable male and female gametes. Taken as a whole, our data indicate

that interspecific-interploid hybridization among *Hylocereus* does not culminate in a genetic dead-end in the F_1 generation.

CONCLUSIONS

On the basis of our findings, we concluded that the allohexaploids were produced as a result of "hybridization followed by chromosome doubling" during the very early stage of embryo development, which probably facilitates the survival of hybrids with specific genomic combinations and maternal/paternal ratios in the endosperm.

Interspecific-interploidy *Hylocereus* hybrids constitute a unique model system for the study of polyploidization mechanisms, providing experimental support for somatic chromosome doubling at the zygote or very early embryo development stages, although the nature of the trigger(s) as well as the details of all the process remain unclear. Questions as to the roles of the endosperm in the control of the doubling and in the initial development of the embryo remain open. Further experimental work, including studies on meiotic stability, synapsis, pairing, and recombination, could provide new insights into the possible establishment of these recently produced hybrids with high ploidy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

NT-Z conceived and planned the experiments. JM and UZ performed the experiments and analyzed the data. NT-Z wrote the manuscript and YM performed part of the experiments and revised the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank Ms. Inez Mureinik for editing the manuscript and Dr. Benyamin Rosental and Ms. Orly Gershoni-Yahalom, Faculty of Health Sciences and Regenerative Medicine and Stem Cell Research Center, Ben-Gurion University of the Negev, for providing flow cytometry assistance.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 05 November 2020
doi: 10.3389/fpls.2020.540894

Synthetic Polyploidy in Grafted Crops

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Synthetic polyploids have been extensively studied for breeding in the last decade. However, the use of such genotypes at the agronomical level is still limited. Polyploidization is known to modify certain plant phenotypes, while leaving most of the fundamental characteristics apparently untouched. For this reason, polyploid breeding can be very useful for improving specific traits of crop varieties, such as quality, yield, or environmental adaptation. Nevertheless, the mechanisms that underlie polyploidy-induced novelty remain poorly understood. Ploidy-induced phenotypes might also include some undesired effects that need to be considered. In the case of grafted or composite crops, benefits can be provided both by the rootstock's adaptation to the soil conditions and by the scion's excellent yield and quality. Thus, grafted crops provide an extraordinary opportunity to exploit artificial polyploidy, as the effects can be independently applied and explored at the root and/or scion level, increasing the chances of finding successful combinations. The use of synthetic tetraploid (4x) rootstocks may enhance adaptation to biotic and abiotic stresses in perennial crops such as apple or citrus. However, their use in commercial production is still very limited. Here, we will review the current and prospective use of artificial polyploidy for rootstock and scion improvement and the implications of their combination. The aim is to provide insight into the methods used to generate and select artificial polyploids and their limitations, the effects of polyploidy on crop phenotype (anatomy, function, quality, yield, and adaptation to stresses) and their potential agronomic relevance as scions or rootstocks in the context of climate change.

Keywords: grafting, polyploid, rootstock, scion, stress tolerance

OPEN ACCESS

Edited by:
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University of Florida, United States

Reviewed by:
Gennaro Fazio,
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *06 March 2020*
Accepted: *28 September 2020*
Published: *05 November 2020*

Citation:
Ruiz M, Oustric J, Santini J and Morillon R (2020) Synthetic Polyploidy in Grafted Crops. Front. Plant Sci. 11:540894. doi: 10.3389/fpls.2020.540894

HIGHLIGHTS

- Grafting improves agronomic traits by combining well adapted rootstocks and improved scions.
- Polyploidy induces large changes in anatomical traits in the rootstock as well as in the scion.
- Polyploidy of the rootstocks and scions may contribute to stress adaptation.
- Phenotypic traits in polyploids are often associated with large physiological, biochemical, transcriptomic, and gene expression changes.

INTRODUCTION

Polyploidy is one of the main factors driving evolution in higher plants (Grant, 1981; Soltis and Soltis, 1995, 2009; Wendel and Doyle, 2005; Cui et al., 2006; Chen, 2007, 2010; Husband et al., 2008; Hollister et al., 2012), conferring genotypic plasticity by increasing the number of copies of the genome (autopolyploidy) or adding different genomes (allopolyploidy), thus increasing their potential for adaptation (Leitch and Leitch, 2008) and promoting their selection (Feldman and Levy, 2012). It has been proposed that polyploidy favors adaptive evolution to changing environmental conditions (Ramsey, 2011) through differential expression of duplicate genes (Dong and Adams, 2011; Tan et al., 2015).

Better adaptive plasticity was found in natural polyploid plants allowing successful domestication events for many species under natural growing conditions (Salman-Minkov et al., 2016) such as autotetraploid wheatgrass [*Agropyron desertorum* (Fisch. ex Link) Schult.], potato (*Solanum* spp.), and allopolyploid wheat (*Triticum* spp.; DeWet, 1980; Asay et al., 1986; Liu et al., 2001). However, when considering human-made polyploidy, only a few events have achieved commercial success, like autotriploid watermelon (*Citrullus vulgaris* Schard.), autotriploid sugar beet (*Beta vulgaris* L.), autotetraploid kiwi (*Actinidia chinensis* Planch), auto-allopolyploid apple (*Malus* spp. Mill), banana (*Musa* spp.), and grape (*Vitis* spp.; Yamane and Kurihara, 1980; Crow, 1994; Janick et al., 1996; Motosugi et al., 2002; Wu et al., 2012).

In agriculture, the genomic modifications that take place during polyploidization confer many interesting advantages over the diploid (2x). The most important for crop production are dwarfing effect on trees, increase in organ biomass (leaves, fruit, seeds, roots, etc.), alteration of flowering time, intensification of color (leaves and fruit), such as illustrated in **Figure 1** and detailed in **Table 1**, increased primary and secondary metabolite content and enhanced tolerance or resistance to abiotic and biotic stresses (see Polyploidy Improves Stress Tolerance section). In addition, triploidization can limit gametic fertility due to unbalanced meiosis. Associated with parthenocarpy, this reduced fertility allows the production of seedless fruit, which is a desirable trait for consumers. Polyploidization can also restore fertility in newly created hybrids (Sattler et al., 2016). Thus, the success of polyploidization as a tool is highly dependent on the crop species and is shaped by commercial interests such as biomass production, ornamental crops, or the pharmaceutical industry.

Grafting has been used for centuries to propagate fruit trees. In vegetable crops, the technique has been used mainly since the beginning of the 20th century. Grafting of a scion or crop variety onto a rootstock is effective at providing faster and more regular growth in commercial orchards, facilitating earlier development by shortening juvenility, improving yield and fruit quality, modulating the harvest season (Koepke and Dhingra, 2013), and providing abiotic stress tolerance and resistance to specific biotic stress (Mudge et al., 2009). In some perennial plants, grafting helps to maintain uniformity through clonal propagation of both scion and rootstock and in some cases, it limits the impact of the juvenile phase. Thus, combining grafting impacts with the use of a polyploid rootstock and scion might bring great advantages in cultivated crop.

Climate change is already challenging agriculture (Intergovernmental Panel on Climate Change, IPCC – Fifth Assessment Report 2014; IPCC, 2014; Thornton et al., 2018). It will result in higher temperatures, drought, and increased soil salinity (Korres et al., 2016). As a major force for plant evolution (Chen, 2010), polyploidy promotes better adaptation traits in crops, since polyploid plants are thought to have been selected during evolution because of their phenotypic and genomic plasticity (Leitch and Leitch, 2008). Much larger proportions of polyploid plants have been found in the Arctic (Brochmann et al., 2004) and at mountainous elevations (Schinkel et al., 2016), suggesting that these genotypes are better adapted to severe cold climatic constraints. However, these populations such as paleopolyploids may have experienced large genome changes leading to a loss of their polyploid status.

The information presented in this review regarding the adaptation traits in synthetic polyploids is consistent with that observed in natural polyploid plant populations and underlines the importance of pursuing investigations in synthetic polyploidy for grafted crops. The use of polyploid rootstocks and scions brings up numerous questions on the genetic, transcriptomic, physiological, and agronomic level. Thus, we addressed (i) the methods to create artificial polyploids; (ii) the reproductive biology implications that using polyploid rootstocks and scions may have on crops; (iii) the importance of grafting in agriculture and the implications of using polyploid crops; (iv) the phenotypic variation induced by polyploidy and its effect biomass production and fruit; (v) the implications that polyploidy has in the regulation of genome expression with a focus on fruit quality and stress tolerance; and (vi) the role of polyploidy for enhancing stress tolerance.

METHODS TO GENERATE POLYPLOIDS AND REPRODUCTIVE BIOLOGY IMPLICATIONS

Associated to breeding programs, different methods have been developed to generate polyploids that require having a good knowledge of the reproductive biology of the investigated species. Several advantages and disadvantages have been associated with each one of the approaches.

The oldest method used to generate polyploids is sexual polyploidization. It is based on controlled pollination that involves at least one of the parents providing non-reduced

Abbreviations: NAD-IDH, Isocitrate dehydrogenase; GABA-T, ɣ-aminobutyric acid transaminase; GABP, ɣ-aminobutyric acid permease; ABA, Abscisic acid; ACO2, Assimilation rate; APX, Ascorbate peroxidase; BRs, Brassinosteroids; CAT, Catalase; DHAR, Dehydroascorbate reductase; E, Transpiration rate; GA, Giberellic acid; GPX, Glutathione peroxidase; GR, Glutathione reductase; GS, Stomatal conductance; GSH, Glutathione; GST, Glutathione S-transferase; IAA, Indole acetic acid; Lp, Root hydraulic conductivity; MDHAR, Monodehydroascorbate reductase; POD, Peroxidase; PRX, Peroxiredoxin; QTL, Quantitative trait loci; ROS, Reactive oxygen species; SOD, Superoxide dismutase.

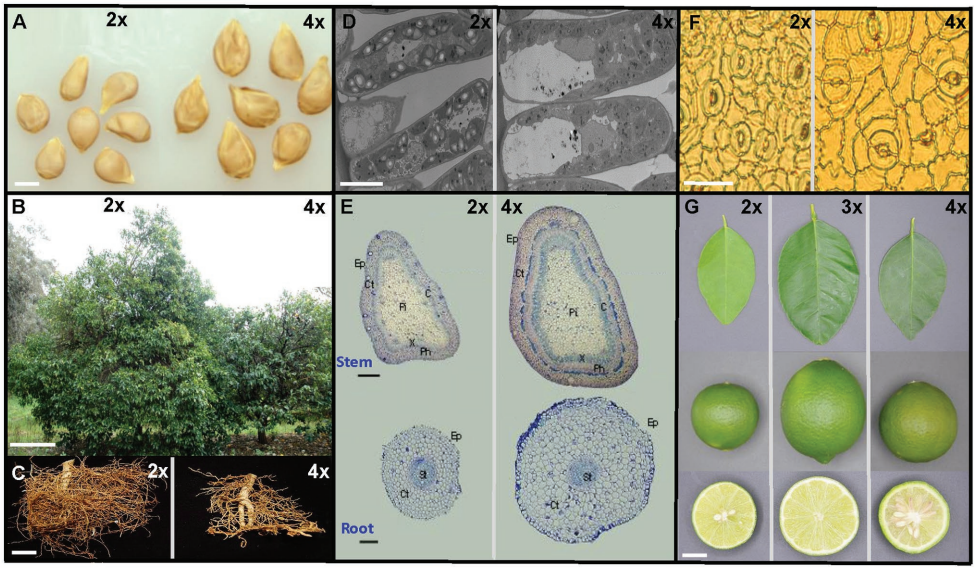

FIGURE 1 | Illustration of the phenotypic differentiation between diploid (2x) and polyploid (triploid, 3x and tetraploid, 4x) citrus at the plant, organ and cellular level. **(A)** Diploid and 4x seeds of Citrumelo (bar = 0.5 cm). **(B)** Diploid and 4x Citrumelo trees planted at the same age at the INRAe – Cirad germplasm of San Giuliano, France (bar = 0.5 m). **(C)** Diploid and 4x Carrizo citrange fibrous roots (bar = 1 cm). **(D)** Scanning electron microscopy pictures of the leaf palisade parenchyma of 2x and 4x Volkamer lemon (bar = 10 μm). **(E)** Light micrographs of cross-sections of internodes and roots of Rangpur lime plants (bar = 25 μm) from Allario et al. (2011). **(F)** Light micrographs of leaf epidermis showing stomata from 2x and 4x Citrumelo (bar = 10 μm). **(G)** Leaf and fruit of Mexican lime (2x), Tahiti lime (3x), and Giant Mexican lime (Autotetraploid; bar = 0.5 cm).

gametes (usually 2n) as a consequence of meiotic aberrations. First-division restitution (FDR) and second division restitution (SDR) are the predominant mechanisms of 2n gamete formation in plants (De Storme and Geelen, 2013). These occur because the first or second meiotic divisions fail, respectively, leading to the formation of restituted nuclei with a somatic chromosome number (Mendiburu and Peloquin, 1976; Park et al., 2007). As a result, FDR and SDR 2n gamete formation mechanisms have different genetic implications that are worth considering for polyploid breeding because it has a direct influence on the genetic constitution of the progeny. FDR 2n gametes contain non-sister chromatids, which in the absence of crossover, maintain the parental heterozygosity. When crossover occurs, the parental heterozygosity restitution (PHR) rates vary from 100% for loci close to the centromere to 60–70% for loci far from the centromere, depending on the level of chromosome interference (Cuenca et al., 2011). For SDR, the 2n gametes contain two sister chromatids, which reduces the parental heterozygosity level (Bastiaanssen et al., 1998; Cuenca et al., 2011; De Storme and Geelen, 2013). In this case, when crossover occurs, the PHR rate varies from 0% for loci close to the centromere to 60–75% for loci far from the centromere, depending on the level of chromosome interference (Cuenca et al., 2011). In the genus *Annona*, which includes edible fruits like cherimoya (*A. cherimola* Mill.) and sugar apple (*A. squamosa* L.), the production of non-reduced gametes has been identified after unusual polyploid progenies were observed (Martin et al., 2019). Also, analyses based on molecular markers can be used to estimate the PHR rates for 2n gametes in polyploid progenies and, therefore, to identify the mechanisms underlying unreduced gamete formation (Cuenca et al., 2011, 2015). The main limitation of sexual polyploidization is that non-reduced gametes are usually produced by plants at a very low rate, when abnormal meiosis is induced by genetic or environmental factors, such as temperature, herbivory, wounding, water deficit, or nutrient shortage (Ramsey and Schemske, 1998). However, unlike somatic methods, sexual polyploidization is effective at preventing somaclonal variation on the progenies.

Another method to generate polyploid plants is interploid sexual hybridization, which involves crossing plants that have different ploidy levels. This is a very common approach to recover triploid (3x) plants by 2x × 4x, 4x × 2x, or 2x × 3x crosses. However, it has the disadvantage of frequent endosperm development failure and hence seed abortion (Birchler, 2014). For this reason, embryo rescue is an indispensable technique for breeding programs based on interploid crosses (Wang et al., 2016); it has been applied extensively in many fruit crops, such as apple (Dantas et al., 2006), citrus (Aleza et al., 2012), or grape (Sun et al., 2011). Li et al. (2015) provided an overview of the factors that may affect its efficiency.

Polyploidization can also be induced by somatic doubling, which involves chromosome duplication in non-germ cells to generate autopolyploidy or allopolyploidy, according to the phylogenomic structure of the initial 2x accession. In this case, duplication arises because of a mitotic failure and can be chemically induced by antimitotic agents, such as colchicine, trifluralin, and oryzalin. However, in crops that have nucellar embryony, such as several citrus and mango (*Mangifera* spp.) species, the spontaneous somatic duplication events occurring in the nucella may result in the natural

TABLE 1 | Selection of polyploid crops that have been reported for their agronomic interest.

Crop	Ploidy	Agronomic traits	References
Apple	3x, 4x	Increased fruit size and dwarfism[1,2]	Janick et al., 1996; Sedov, 2014; Ma et al., 2016
	4x	Drought tolerance	De Baerdemaeker et al., 2018
Birch	4x	Increased biomass and dwarfism	Mu et al., 2012
Black locust	4x	Salinity tolerance[1]	Wang et al., 2013a; Meng et al., 2016; Luo et al., 2017
Blackcurrant	4x	Increased fruit size	Podwyszyńska and Pluta, 2019
Citrus	4x	Drought, salinity, and boron excess tolerance[2,3]; cold, nutrient deprivation and chromium toxicity tolerance	Allario et al., 2013; Podda et al., 2013; Ruiz et al., 2016a; Balal et al., 2017; Oustric et al., 2017, 2018, 2019a; Khalid et al., 2020
	4x	Dwarfism[1,2,3]	Hussain et al., 2012; Grosser et al., 2015; Ruiz et al., 2016a
	3x	Increased fruit quality, size and seedlessness[1,2]	Ollitrault and Navarro, 2012; Tan et al., 2019; Sdiri et al., 2020
Cherimoya	3x	Increased fruit size	Martin et al., 2019
Fig tree	3x	Increased fruit size	Falistocco, 2016
Forest mangrove	3x, 4x	Increased biomass yield and quality	Harbard et al., 2012; Griffin et al., 2015
Grape	3x, 4x	Increased fruit size and seedlessness[1,2]	Yamada and Sato, 2016
Kiwi	4x	Increased fruit size[1]	Wu et al., 2012; Zhong et al., 2012
Loquat	3x, 4x	Increased fruit size	Jiang et al., 2016
Mango	4x	Increased fruit size; enhanced photoprotection	Galan-Sauco et al., 2001; García-García et al., 2020
Mulberry	3x, 4x	Increased fruit size and leaf biomass yield	Dai et al., 2015; Shafiei, 2018
Olive	4x	Increased fruit size	Rugini et al., 2016
Pawlonia	4x	Increased biomass yield and quality; salinity and drought tolerance	Tang et al., 2010; Zhao et al., 2017
Persimmon	6x	Increased fruit size[1]	Yesiloglu et al., 2018
Pomegranate	4x	Increased fruit size	Shao et al., 2003
Sweet cherry	3x	Dwarfism[1,2]	Prassinos et al., 2009
Watermelon	4x	Higher vigor and resistance to the RKN[1,2,3]	Levi et al., 2014
	3x, 4x	Increased fruit size and quality	Jaskani et al., 2004; Zhang et al., 2019
Willow	3x, 4x	Increased biomass yield and quality; dwarfism	Serapiglia et al., 2015; Dudits et al., 2016

[1]*Commercial success.*
[2]*Tested grafted.*
[3]*Tetraploid tested as a rootstock.*

development of tetraploid (4x) embryos that can be selected from seedlings (Galan-Sauco et al., 2001; Aleza et al., 2011). When somatic duplication is induced chemically, success depends largely on the development of an effective protocol that sets the proper explant type, antimitotic agent dose, exposure time, and *in vitro* regeneration conditions. Ploidy chimeras are often a secondary effect induced by chemical treatments. Therefore, this method requires further verification of the ploidy of different organs on each regenerant explant that usually involves morphological examination, karyotyping, or flow cytometry analytical approaches. Somatic doubling has been extensively used for polyploid crop breeding, as reviewed by Sattler et al. (2016).

Lastly, somatic hybridization by protoplast fusion, which was initially developed to overcome crossing barriers between species, allows the whole genomes of two different parents to be combined in a single cell that can be regenerated into a hybrid plant that is usually polyploid. This method allows polyploids that have not been through meiotic recombination events to be generated. Thus, progenies combine the whole genomes of the two parents and, potentially, all the dominant parental traits, irrespective of their heterozygosity and surpassing limitations imposed by reproductive biology. Meanwhile, the probability of combining all the desired traits in a sexual recombinant hybrid is much lower and slower on crops with long juvenile stage (Dambier et al., 2011; Ollitrault and Navarro, 2012). Difficulties in protoplast isolation, culture, and plant regeneration hinder the use of this approach in many crops. However, it is an integrated component of several citrus breeding programs over the world, both for scion and rootstock breeding (Grosser et al., 2000; Dambier et al., 2011; Grosser and Gmitter, 2011).

Once obtained, artificial polyploids can be classified either as breeding material or considered as potential new varieties. Hence, their potential benefits and disadvantages need to be examined relative to their prospective use, in order to maximize the potential of each new developed genotype. Triploids are mainly valuable for having bigger seedless fruit, while their limited fertility usually acts as a dead end for further improvement. The way to add required traits such as disease resistance *a posteriori* into a 3x variety is to resynthesize it using improved parents (de Carvalho-Santos et al., 2019). More importantly, 3x crops need to be able to activate a parthenocarpic fruit set, which can be stimulated by pollination, induced by hormonal treatments or through epigenetic manipulation (Joldersma and Liu, 2018). Fortunately, natural parthenocarpy is very common among cultivated species, especially on trees, plants of hybrid origin, and polyploids (Picarella and Mazzucato, 2019).

Tetraploids (4x) are widely used as progenitors and are often included in germplasm collections to assist in breeding programs. As a genetic resource, 4x can be used to generate 3x varieties through interploidy crosses or to serve as a bridge for genetic transfer between two species when direct crossing is not possible (Liu et al., 2017). According to Sattler et al. (2016), genome duplication can also be induced to restore the fertility of sterile hybrids as can restore meiosis and can buffer the effect of deleterious alleles. In addition to this, 4x genotypes are a valuable resource when used as crops that are cultivated

for their vegetative organs because biomass generally increases with genome duplication. Also, 4x can be very useful as rootstocks because genome duplication can enhance some desired traits like stress resistance or canopy size control. However, delayed flowering and poor fruit quality are recurrent phenotypes observed in autotetraploid and allotetraploid species that hamper their use for fruit production.

Vegetative propagation is usually a requirement for both 3x and 4x varieties, as 4x usually have lower rates of seed production, thus clonal multiplication is needed to maintain the genotype. The enhancement of asexual reproduction is a common consequence of polyploidy in many species (Comai, 2005) and is required for crops that are cultivated grafted. Thus, suitability for clonal propagation might be facilitated at higher ploidies. Another essential point is that polyploidization can be a way to overcome incompatibility and self-incompatibility (Entani et al., 1999), which are barriers for breeding and fruit set, respectively. However, the molecular basis for this response is still unclear.

PHENOTYPIC VARIATION INDUCED BY POLYPLOIDY

Morphological changes after genome duplication can be explained as a series of downstream effects triggered by the increased cell size and shape, which is directly influenced by bulk DNA amount irrespective of genic content. At the cellular level, novel traits can be as simple as a change in surface-to-volume ratio, cell size, nuclear volume, or cell cycle duration (Doyle and Coate, 2019), whereas gene expression of heterozygous loci could also be affected by dose (Finigan et al., 2012). Nevertheless, whether these cellular changes are mechanistically connected to variations affecting the whole organism's phenotype and function remains unknown. Additionally, it is sometimes difficult to distinguish between ploidy driven changes and those induced by other factors affecting artificial polyploids. To obtain, either autopolyploids or allopolyploids, it is necessary to go through processes, like chemical induction for genome duplication, protoplast fusion, *in vitro* regeneration, or the occurrence of genomic shock (Song et al., 1995) that contribute to doubt about ploidy effects and genomic identity between them and its 2x counterparts (Comai, 2005; Münzbergová, 2017).

In general terms, bigger polyploid cells can differ from 2x in their organelle number, size, and distribution, can have a higher water content or can have altered metabolic and development rates. All these modifications may have functional consequences on the plant architecture organ size and composition, physiology of essential processes, like gas exchange, photosynthesis, and water relations, probably driven by alterations of organelles such as chloroplasts and vacuoles (Doyle and Coate, 2019).

Cell and Organ Size Modifications

A wide range of phenotypic changes at the organ level have been reported in plants due to polyploidy (Beest et al., 2012). Thicker and greener leaves, higher leaf water content, thicker and smaller roots as well as a dwarf phenotype are some of the most recurrent novel traits (Cameron and Frost, 1968; Romero-Aranda et al., 1997; Motosugi et al., 2002; Padoan et al., 2013; Ruiz et al., 2016a,b,c; Wang et al., 2016). **Figure 1** illustrates some phenotypic traits induced by polyploidy in citrus and **Table 1** summarizes some traits mentioned in the literature in different polyploid species. Polyploidy may also alter cell wall composition. However, this does not always change the size of the whole plant (Corneillie et al., 2019). Stomatal and epidermal cell frequency per unit leaf is usually decreased with increased ploidy level, while cell area is increased (Jellings and Leech, 1984; Beck et al., 2003; Mouhaya et al., 2010; Zhang X. Y. et al., 2010; Allario et al., 2011; Oustric et al., 2019b). Larger flowers and seeds are usually observed in polyploids such as citrus (Yahmed et al., 2016). Phenotypic changes in polyploids such as specific features of the roots or leaves may be at the origin of better tolerance traits. However, the basis for DNA amount-driven functional differentiation and the potential effects of genomic shock are poorly described, forcing those who use polyploid breeding for abiotic stress tolerance to rely on empirical methods (see Polyploidy Improves Stress Tolerance section).

Quantitative and Qualitative Modifications on Biomass

Regarding biomass production, there is no consensus about ploidy effects on plant growth, since some species may present similar (Niu et al., 2016) or lower (Hennig et al., 2015; Denaeghel et al., 2018) organ size or biomass associated to polyploidy. Independently of plant height, polyploid plants often develop higher biomass that results in thicker tissues and organs and alter plant architecture when compared to the 2x. Several examples can be found in **Table 1**. There is an open discussion on whether genome duplication causes divergent changes between primary and secondary growth of woody species. While these two parameters are generally correlated in 2x species, the regulatory mechanisms coordinating plant organ growth differ between many 2x and autotetraploid trees, where dwarfism but higher biomass has been found on autotetraploid versions when compared to their corresponding 2x (**Table 1**). For this reason, some authors suggest genome duplication as a useful breeding tool to tailor crops for biomass production, as it has been described on the model plant Arabidopsis (*Arabidopsis thaliana* L. Heynh; Corneillie et al., 2019). In this species, polyploid biomass has an altered composition that is easier to saccharify. This property, which has largely remained unexplored for feed and food production, might pave the way for improving the efficiency and sustainability of biomass production. As an example, 3x and 4x Willow (*Salix* spp.) were found to have lower lignin content compared to the 2x parental lines (Serapiglia et al., 2015). Due to the increasing interest in using plants as a source of energy and chemical building blocks, researchers may find it useful to explore polyploidy for biomass quality improvement. Nevertheless, the mechanisms that lead genome duplication to influence plant growth, development, yield, and quality remain unanswered.

Fruit Quality

Triploidy induces the production of seedless fruit and favors vigor (Wang et al., 2016). While triploid breeding for seedlessness in some ligneous plants such as grapevine or citrus has led to the development of outstanding varieties, some triploid apple or citrus lime have seedy fruits with more limited interest. In grape and kiwi, polyploidy increased fruit size (Shengjian et al., 2005; Wu et al., 2012).

Polyploidization can be used as a breeding tool to modify crop quality. The main effects reported are absence of seeds and modifications in fruit size, shape, and organoleptic quality. Bigger fruit size is very important commercially and is often boosted by cultural practices or chemical treatments. Polyploidization offers a natural and environmentally friendly alternative for increasing fruit size. This is a well-known effect, although the mechanisms behind it continue to be debated today. Final fruit size is determined by coordinated progression of cell production and cell expansion and is correlated with cell size, which is bigger in polyploids (Doyle and Coate, 2019). Alternatively, altered transport or signaling of auxins and cytokinins may reduce apical dominance, thereby facilitating fruit proliferation (Malladi and Hirst, 2010). Independent of the mechanism, bigger fruit size is a recurrent effect, at least for lower polyploidy levels (3x, 4x, or 6x). Recently reported examples of ploidy-driven increase in fruit size on species that are cultivated grafted are summarized in **Table 1**. Although, several of these recently induced polyploid forms have not yet attained the required market qualities, they comprise valuable germplasm sources for application in prospective breeding experiments.

Triploid plants are usually both male and female sterile and seeds are not viable or not present. Seedlessness is a highly desirable characteristic for consumers (Ollitrault and Navarro, 2012). Besides, triploidy increases fruit profitability in 2x species with big seeds, because seedlessness provides extra capacity for flesh (Jiang et al., 2016). Tetraploid apple varieties have no commercial value because of their low-quality fruit and low resistance to cold, being mostly used to develop the 3x cultivars (Sedov et al., 2014). The situation is similar for grapes (Yamada and Sato, 2016) and citrus (Ollitrault and Navarro, 2012). Yet, 4x germplasm is a necessary resource to obtain and maintain 3x varieties by interploid crosses. To produce 3x plants, crops like citrus or fig that are vegetatively propagated have an advantage over the ones propagated by seed, such as watermelon, that needs *de novo* interploid crosses to produce each generation.

Polyploid fruits usually have similar organoleptic and nutritional quality than their 2x counterparts. However, some studies report sporadic differences that can be relevant for commercial purposes. For example, 2x "Kinnow" mandarin has 9.5% higher total soluble solids content and 46.1% higher juice content than 4x (Jaskani et al., 2002). Similarly, 4x "Ponkan" mandarin has altered accumulation of primary and secondary metabolites in the fruit. Total acid and ascorbic acid, which are the main components of fruit flavor, are higher in the 4x fruit (Tan et al., 2019). Downregulation of gene expression involved in its transport and uptake in mitochondria like NAD-IDH, GABA-T, and GABP has been observed. Meanwhile, sucrose, fructose, and glucose content are very similar between 2x and 4x fruit, and ripening season does not differ between them, while secondary metabolites such as flavonoids and carotenoids are decreased (Tan et al., 2019). Similar results have been observed in the fruit and seeds of natural allohexaploid pitaya (*Hylocereus* spp.) and natural autotetraploid pear fruit (*Pyrus communis* L.) depending on age and growing conditions (Cohen et al., 2013; Tsukaya et al., 2015). Tetraploid kiwi has reduced flesh firmness and flesh color intensity compared with parental 2x plants (Wu et al., 2013). However, the quality of extra virgin olive (*Olea europaea* L.) oil is similar when extracted from 2x or 4x olives (Rugini et al., 2016). Not surprisingly, 3x fruits have been proven to have excellent organoleptic and nutritional quality, like is the case of mandarins (Sdiri et al., 2020). Thus, 3x is the most common ploidy in polyploid fruit breeding since it combines bigger fruit size, higher yield, and organoleptic quality with seedlessness. Additionally, some variations in specific compounds have been associated with genome duplication. This is the case for 4x watermelon, which has higher β-carotene, lycopene, fructose, and glucose content than 2x fruit (Jaskani et al., 2004). At higher ploidy levels (8x, 10x), wild kiwiberry (*Actinidia* spp.) has high concentrations of certain compounds, like ascorbic acid and some amino acids (Zhang Y. et al., 2017). So far, only a few studies have investigated the occurrence and extent of metabolic alterations following polyploidization. Thus, the effect of ploidy on plant metabolism is still unclear.

POLYPLOID EFFECTS IN COMPOSITE PLANTS

Impact at the Rootstock Level and in Grafted Plants

When used as rootstocks, polyploid plants can provide desirable attributes like vigor reduction or enhanced biotic and abiotic stress tolerance. Vigor reduction or dwarfism is one of the most sought-after phenotypes in rootstocks and is a very common effect of genome duplication on trees (**Table 1**). The decrease in tree volume, height, canopy diameter, and/or circumference reduces the need for pruning in commercial orchards and facilitates phytosanitary interventions. Interestingly, greater vigor was also found in sweet cherry (*P. avium* L.) grafted on 3x "Colt" rootstock (Webster et al., 1996). Tree vigor is known to be affected by numerous factors, including root hydraulic pressure, water uptake efficiency, hormone profile, nutrient uptake, and stomatal conductance (Warschefsky et al., 2016). The ploidy-driven changes in vascular anatomy may be the main factor underpinning the dwarfing effect. Increased cell size, reduced number of cells, changes in cell cycle duration, altered water transport capacity, or higher photosynthesis rates led by changes in leaf function and size have been suggested to trigger this differential growth pattern. These processes are regulated by phytohormones, whose levels have been proven to be altered by genome duplication (Dudits et al., 2016). Some 2x rootstocks can promote dwarfism in apple trees (Pilcher et al., 2008; Fazio et al., 2014). Polyploid rootstocks have been used commercially to reduce tree size, like citrus 4x somatic

hybrid (allotetraploids) and 3x cherry rootstocks (4x *P. cerasus* L. cv. *Schattenmorelle* × 2x *P. canescens* Bois; Grosser and Chandler, 2000; Grosser et al., 2003; Prassinos et al., 2009). This can also be the case when using 4x rootstocks in citrus (Hussain et al., 2012) or tetraploid varieties in apple (Ma et al., 2016). This phenotype is most of the time associated with the translocation of some compounds such as hormones from the root to the grafted scion (Atkinson and Else, 2001), leading to gas exchanges regulation and anatomical changes (Allario et al., 2013) such as in leaf size and thickness or fruit size (**Figure 2**; see also **Table 1**). In 4x apple, decreased brassinosteroid and indole-acetic acid, which play a role in plant elongation, may also impact size reduction (Ma et al., 2016). In citrus, polyploid breeding over the last decades has successfully provided opportunities to select highly marketable dwarfing rootstocks (Grosser and Gmitter, 2011; Ollitrault and Navarro, 2012). Both autotetraploid and allotetraploid citrus often grow slower than their 2x counterparts and can induce dwarfism to the scion (Grosser et al., 2012; Ruiz et al., 2016a, 2018; **Figure 2**). In the end, the induced dwarfism allows high-density plantation and, in turn, facilitates orchard management (**Figure 2**). When used as rootstocks, 4x citrus do not reduce the scion's yield efficiency nor do they have an impact on scion fruit quality parameters such as fruit size, yield, acids, or sugars. However, 4x rootstocks may have an impact on specific fruit compounds like phenolics or flavonoids (Hussain et al., 2012; Ruiz et al., 2016c). The use of 4x rootstock in association with clementine (*C.* × *clementina*) did not impact fruit quality (Hussain et al., 2012). Thus, citrus cultivated on 4x rootstocks produce fruits with excellent organoleptic qualities and can increase the profitability of plantations (Grosser et al., 2015). Sugars, organic acids, fatty acids, carotenoids, and flavonoids are the main drivers of fruit organoleptic quality (Ulrich and Olbricht, 2011), which is also highly influenced by the rootstock (Castle, 1995). Grafting allows us to take advantage of the rootstock's better adaptation to abiotic and biotic stresses, which may improve plant development and yield production. For example, the 4x watermelon rootstock USVL-360 is an alternative to *Cucurbita* spp. rootstocks and provides vigor and resistance to the root knot nematode, while having similar yields to commercially available rootstocks (Levi et al., 2014).

From a breeding perspective, using polyploid genotypes on grafted crops to achieve even better tolerance to biotic stress is questioned. Breeding of grafted crops focuses on finding complementary traits for scions and rootstocks so they can be matched. Furthermore, an important part of the adaptation to variable abiotic and biotic environments is devoted to the rootstock.

It is very difficult to find a rootstock that provides all the desired characteristics required by a crop production system. However, a good combination is important for the cultivation

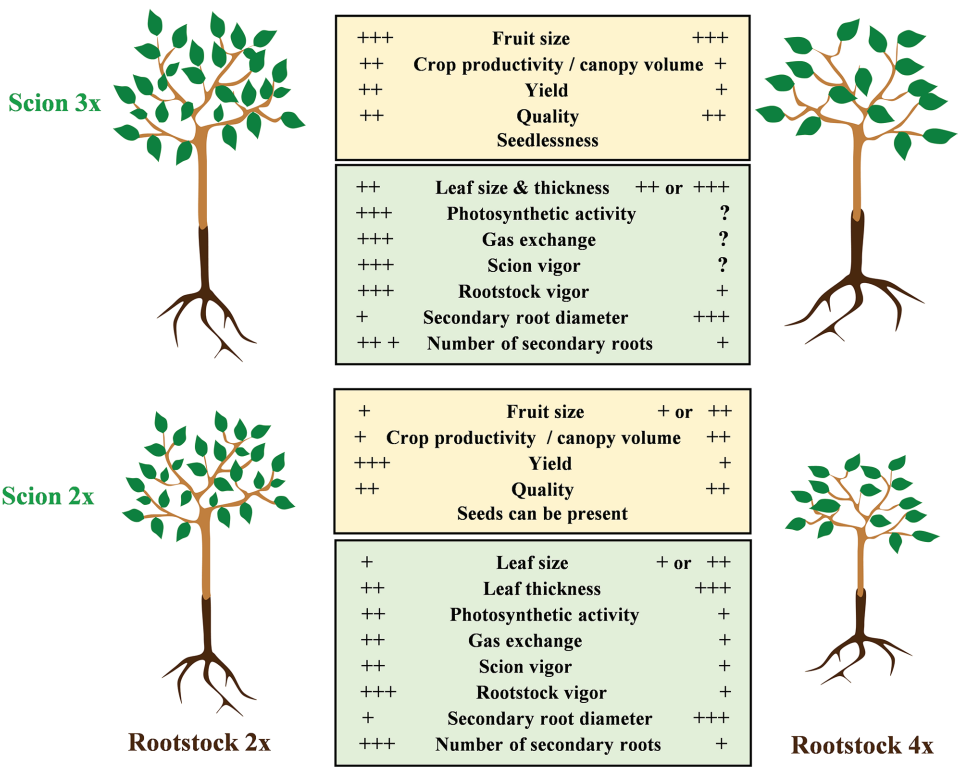

FIGURE 2 | Physiological implication of the polyploidization when associating 2x and 3x scion with 2x or 4x rootstock. On one hand, 4x rootstock confers a more limited vigor than its respective 2x. On the other hand, a 3x scion will induce a greater vigor and larger leaves than in the 2x scion. At root level, tetraploidy will limit the tree vigor but will favor an increase of the size of the secondary roots. At the root and scion level, polyploidy may induce large phenotypical changes.

of a crop in a given available area. In some cases, the existence of a suitable rootstock is the decisive factor for cultivating in areas that are severely affected by environmental constraints (**Figure 3**), plant diseases, and pests (Reig et al., 2019). Moreover, the rootstock choice has long-term implications in tree crop cultivation, as it will strongly determine the profitability of the plantation. It is usually recommended that different suitable rootstocks are combined within each cultivation area to interfere with the spread of diseases and promote resilience (Koepke and Dhingra, 2013). Thus, generating variability and having a collection of rootstocks that is adequately phenotyped for disease resistance and stress tolerance is key for the long-term success of a crop cultivation in a given area.

Grafting Compatibility and Impact of Polyploidy

Several reviews have been devoted to the grafting process and mechanisms (Mudge et al., 2009; Goldschmidt, 2014) and to the impact of the rootstock on the scion (Jensen et al., 2010; Koepke and Dhingra, 2013; Gautier et al., 2019). The grafting behavior of polyploid rootstocks or scions in association with classical 2x plant materials remains unexplored. In citrus, the use of 4x rootstocks with 2x or 3x scions that are compatible with the respective 2x rootstock does not seem to alter graft compatibility (Hussain et al., 2012; Allario et al., 2013). This can be explained by the genetic status of the 4x being identical to the 2x. However, due to the anatomical changes induced by polyploidy, such as increased cell size, an altered cell adhesion process could be expected. Interestingly, studies in tobacco (*Nicotiana* spp.) have shown that the entire nuclear genomes of both the scion and the rootstock can be transferred across the graft junctions to generate a novel 4x species (Fuentes et al., 2014). The plants regenerated by tissue culture from cells taken from the junction are true allotetraploid hybrids, underlying the possible interactions between different ploidy levels at the rootstock/scion intersection.

GENE EXPRESSION CHANGES IN POLYPLOID CROPS AND FUNCTIONAL IMPLICATIONS

Genome doubling driven by polyploidization modifies organization at the genetic and epigenetic levels. In comparison to artificial polyploids, natural polyploids of the same species have acquired novel characteristics thanks to polyploid lineage evolution (Semenuik and Arisumi, 1968; Lignowski and Scott, 1972; Lumaret, 1988; Levin, 2002). Meanwhile, the artificial ones may show superior performances relative to the natural ones, probably due in part to the strong selective pressure that the antimitotics, or other substances used to create the artificial autopolyploids, may impose. For example, the consequences of colchicine treatment may appear in the second generation of artificial polyploid plants (Münzbergová, 2017). Whether natural or artificially induced, genomic shock occurs mainly by the association of distant relatives by allopolyploidization and causes many genetic and/or epigenetic changes on gene expression. These include chromosome rearrangement, sub-functionalization and transposon activation, duplicate gene loss or gain, and gene activation or repression. Epigenetic changes induce variations at the gene expression level without altering the DNA sequence by modifying the DNA compaction

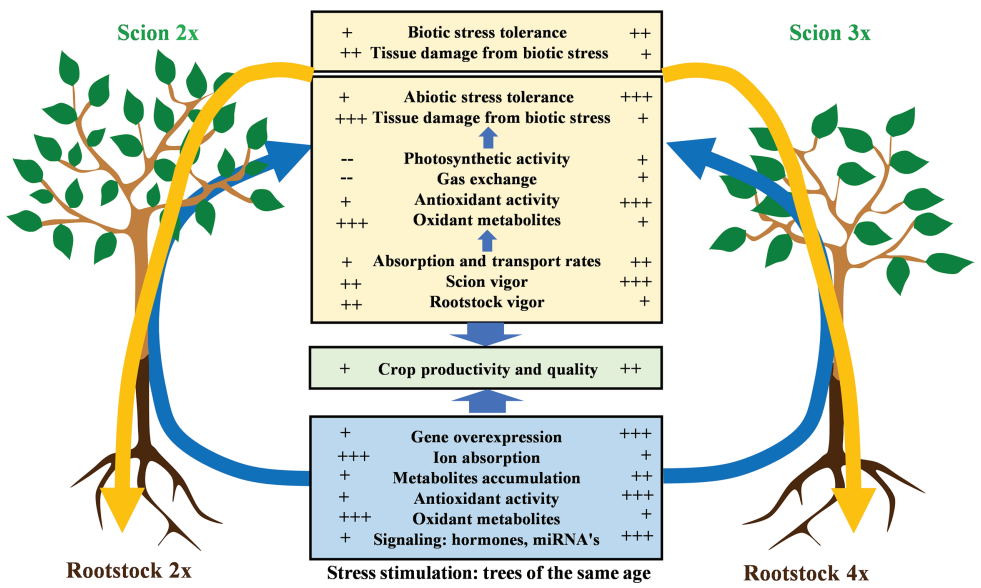

FIGURE 3 | Physiological and molecular implications for stress tolerance when associating a 2x or 3x scion with a 2x or 4x rootstock. Arrows indicate the influence of the different factors at root and scion level depending on the ploidy that may change the physiology or the adaptation of the scion/rootstock association.

or by interfering with RNA function (Comai, 2005; Madlung, 2013; Sattler et al., 2016). Nevertheless, the effect of genome fusion and gene duplication on gene expression at the transcriptome level differs according to whether the genome doubling comes from allopolyploidization or autopolyploidization (Renny-Byfield and Wendel, 2014). It has long been recognized that allopolyploids are more frequently observed in the wild than autopolyploids and that allopolyploids played a more important role in plant evolution. However, some studies have shown a higher incidence of autopolyploids than was previously thought (Soltis and Soltis, 1999; Soltis et al., 2007). The phenomenon of allopolyploidization alters transcription at the genome scale. Changes in gene expression are primarily affected by divergent genome hybridization and possible variation in genetic components rather than by a change in the ploidy level (Osborn et al., 2003; Auger et al., 2005; Wang et al., 2006). Conversely, in autopolyploids, the effect of genome doubling on gene expression is quite limited and does not show a linear relationship between the ploidy level and the transcriptional output. For example, citrus fruit of interspecific somatic allotetraploid showed around 4% transcriptome divergence between its two progenitors (Bassene et al., 2010). The difference reached up to 25% in allotetraploid cotton plants (Adams et al., 2003). Meanwhile, in mature leaves of "Yuzu" lime (*C. junos* Siebold ex Tanaka), "Rangpur" lime (*C.* × *limonia* Osb.), and mulberry (*Morus alba* L.), the differences observed in gene expression between 2x and autotetraploid genotypes are only 0.8, 1.08 and 2.87%, respectively (Allario et al., 2011; Dai et al., 2015; Tan et al., 2015).

Whether the genome doubling comes from allopolyploidization or autopolyploidization, the changes in gene expression affect a variety of biological processes in response to internal and external signals, such as, growth, development, and tolerance/resistance to both abiotic and biotic stresses. In addition, this differential gene expression depends on the organ, development stage, and environmental conditions (Chen and Ni, 2006; Doyle et al., 2008; Zhao et al., 2009).

Modifications of growth, development, and adaptation to abiotic and biotic stress in polyploid plants have been associated with modifications in gene expression, upregulation or downregulation of biosynthesis, transport, reception of primary and secondary metabolites (hormones, carbohydrates, organic acids, amino acids, and proteins, etc.), and enzymes. Polyploidy also changes the amount of signal molecules like small noncoding RNAs (sRNAs). These molecules regulate the expression of transcription and signaling factors related to cellular growth, development, and adaptation by regulating gene expression (Dhawan and Lavania, 1996; Ni et al., 2009; Jackson and Chen, 2010; Kim and Chen, 2011; Ng et al., 2012; Zhou et al., 2015; Javadian et al., 2017; Van Hieu, 2019). All the above mentioned molecules and metabolites circulate between the rootstock and scion through the vascular system, and have an impact either directly as a metabolite or indirectly as a signal to modulate the composite plant's function (Notaguchi et al., 2008, 2015; Martínez-Ballesta et al., 2010; Goldschmidt, 2014; Albacete et al., 2015).

The regulation of phytohormones, such as cytokinin, ethylene, gibberellin (GA), brassinosteroids (BRs), and auxins plays a central role in genetic networks involved in altering plant growth (**Table 1**; Boss and Thomas, 2002; Wang et al., 2013b; Ayano et al., 2014). In polyploid plants, the variation of gene expression in comparison to 2x has been related to differential phytohormone concentrations. For example, the artificial autotetraploid mulberry (*Morus alba* L.) showed that compared to its 2x about 30 of these differentially expressed genes were associated with biosynthesis and transduction of phytohormones, such as cytokinin, GA, ethylene, and auxin involved in phenotypic changes (Dai et al., 2015). The changes in expression of key genes that affect the regulation of phytohormone pathways may lead to plant dwarfism. For instance, in 3- and 5-year-old plants of the artificial autotetraploid apple (*M.* × *domestica*), the altered gene expression modified the content of indoleacetic acid (IAA) and BRs leading to a decrease in tree growth resulting in a dwarfing effect. In addition, the accumulation of miR390 after genome duplication results in upregulation of short-acting apple RNA3 (MdTAS3), which in turn downregulates the expression of MdARF3, an auxin response factor (Ma et al., 2016). The dwarfing effect is also observed in other artificial autotetraploid apple trees prior to flowering; however, growth is restored after the juvenile phase (Xue et al., 2017). Rootstocks play a key role in controlling scion growth and development by the modulation of hormone signaling pathways (Cookson et al., 2013; Gregory et al., 2013; Berdeja et al., 2015; Corso et al., 2016; Adams et al., 2018). Indeed, in grafted plants, cytokines are synthesized in the rootstock and then transported into the apical meristem through the phloem, while ethylene is synthesized in the rootstock xylem. Conversely, auxin is synthesized in the scion's leaves and is then transmitted to the roots *via* phloem (Zhao, 2018). The use of polyploid rootstocks or scions may alter the concentration and balance of phytohormones (ABA, IAA, BRs, auxin, and cytokinin) and impact the tree's phenotype. In "Rough" lemon (*C. jambhiri* Lush.) grafted with autotetraploid "Commune" clementine, Apetala 2 (AP2)/ethylene element binding protein (EREBP) genes that play a role in regulating various developmental processes are repressed (Riechmann and Meyerowitz, 1998; Ninoles et al., 2015). When 2x "Delta Valencia" sweet orange [*C.* × *sinensis* (L.) Osb.] is grafted into 4x "Rangpur" lime, the root-to-shoot signaling, which is mediated by the constitutive expression of many genes, shows a differential pattern when compared to plants grafted into the corresponding 2x rootstock. Overexpression of the CsNCED1 gene, which is involved in regulating abscisic acid (ABA) biosynthesis, increases ABA transfer from the roots to the aerial part and modifies gas exchange at the scion level (Allario et al., 2013). Studies based on autopolyploid Paulownia [*P. fortunei* (Seem) Hemsl.] transcriptome found that many miRNAs are also predicted to participate in ABA signaling (Zhao, 2018; **Figure 3**). However, it is interesting to note that the phytohormone contents fluctuate throughout development, independently of ploidy, but a different pattern may predominate. In autotriploid and autotetraploid watermelon fruit, IAA is higher than in 2x during developmental stages. Meanwhile, no differences in ABA expression have been observed on the 3x watermelons. On the other hand, GA, the main cytokine zeatin riboside (ZR) and BR contents are lower

than IAA and ABA and are gradually reduced during fruit development independently of the ploidy. The low concentrations of ZR and BR are thought to be due to a small impact of ploidy on the expression of related genes (Dou et al., 2017).

Organic acids acting as intermediates in the tricarboxylic acid (TCA) cycle (citric acid, malic acid, fumaric acid, and succinic acid), a central pathway of energy metabolites and anabolic precursors for plant proliferation and survival, are also affected by modifying the gene dosage. The main difference between 2x and autotetraploid Col-0 *Arabidopsis* metabolite accumulation is found in the TCA cycle and γ-amino butyric acid (GABA) shunt. TCA and GABA concentrations respond to a differential expression of their related genes (Vergara et al., 2016). Studies performed on mature leaves of mandarin (*C. reticulata* L.) grafted on 2x and 4x trifoliate orange (*Poncirus trifoliata* L. Raf.) rootstock, show an increase in few TCA compounds including citric acid, but a decrease in other secondary metabolites such as phenylpropanoids and terpenoids. The accumulation of primary metabolites appears to be related to epigenetic modifications rather than genetic variation. Thus, Tan et al. (2017) hypothesized that the decrease in secondary metabolites could indicate that primary metabolism takes priority to relieve the genomic stress encountered in the early stages of genome doubling, probably to allow better vitality and growth. Overall, the impact of polyploidization on metabolites differs depending on the species and specially on their roles as scions, rootstocks, or non-grafted plants (**Figure 3**). This behavior indicates that metabolic changes induced by polyploidization is associated to roostock-scion interactions (Fasano et al., 2016) and further research is needed to decipher stable patterns.

POLYPLOIDY IMPROVES STRESS TOLERANCE

Polyploidy, whether auto or allo is associated with enhanced tolerance to a wide range of stresses, including drought, salinity, cold, heat, nutrient deprivation, or excess light both in wild and cultivated plant species (Doyle and Coate, 2019). According to the abundant research on synthetic autopolyploids, genome doubling can generate variation *per se* in the absence of hybridity. There are widespread recurring patterns associated with genome duplication that lead to enhanced stress tolerance (Doyle and Coate, 2019). Most of the mechanisms described involve substantial changes in morphology but subtle changes in gene expression (Allario et al., 2013). However, altered hormone signaling (del Pozo and Ramirez-Parra, 2014), differential metabolic responses or altered DNA methylation patterns (Yu et al., 2009; Aversano et al., 2013) have also been identified on autopolyploids. These effects can be highly variable between species or even among individuals within the same species. For instance, Denaeghel et al. (2018) reported increased cold tolerance in autotetraploid *Escallonia rubra* (Ruiz and Pav.) Pers. when compared to the 2x. In contrast, autotetraploid *E. rosea* Griseb. does not differ from 2x in its cold tolerance. In artificial allopolyploids, that combine increased ploidy and hybridization effects, differential gene (Zhao et al., 2017) and protein (Yan et al., 2017) expression has been described more often. In this case, research has shown that it is possible to efficiently combine the desired parental phenotypes on the progenies (Grosser and Gmitter, 2011), although genome instability and allelic losses have also been described (Pensabene-Bellavia et al., 2015; Ruiz et al., 2018). Overall, polyploid breeding is progressively carving out its place as a method to improve crops for abiotic stress tolerance, as it opens the possibility of adding functional novelty, while combining genomes that are associated with a well-known and highly valued agronomic behavior. The outcome minimizes the risk that undesired behavior causes economic loss when compared to traditional breeding methods. In the following section, we review the effect of genome duplication on abiotic stress tolerance. Specifically, we will focus on the biochemical, morphological, and physiological modifications underpinning the enhanced tolerance of polyploid crops to a wide range of environmental stresses that have been described lately.

Alterations in ROS Metabolism

Most abiotic stresses have a common impact on plants: they induce the accumulation of reactive oxygen species (ROS), which are by-products of the physiological metabolism. According to the abundant available literature, altered ROS metabolism is a common effect of polyploidization that helps to improve stress tolerance. In 4x *Arabidopsis* subjected to drought stress, ROS homeostasis is altered when compared to 2x, resulting in more efficient adaptation (del Pozo and Ramirez-Parra, 2014). Similarly, several polyploid crops have enhanced stress tolerance responses that are correlated with oxidative metabolism alterations. The increase in ROS is an immediate consequence of salt stress in plants, as they are involved in transcriptional regulation and ion flux alteration to improve the plant's general performance. High levels of salinity generate damaging ROS as a part of the programmed cell death response (Isayenkov and Maathuis, 2019) and are balanced by ROS scavengers. In this sense, faster ROS production response in earlier stress phases and a more efficient ROS scavenging capacity in later stages have been described in Black locust (*Robinia pseudoacacia* L.; Meng et al., 2016; Luo et al., 2017) and different citrus species (Podda et al., 2013; Khalid et al., 2020) as a way to cope with salt stress. This effect has been widely documented in 4x citrus for a variety of stresses. For instance, 4x *P. trifoliata* subjected to drought has a transcriptome enriched in genes coding for enzymes related to antioxidant process, higher peroxidase (POD) and superoxide dismutase (SOD) activity, lower level of ROS, and less tissue damage. This mechanism in combination with osmotic adjustment has been described to enhance tolerance to drought when compared to the 2x (Wei et al., 2019). Furthermore, allotetraploidization might have a stronger effect on stress protection, combining two different genetic pools and the genome duplication effect. The allotetraploid hybrid FlhorAG1 (*C. deliciosa* Tan. + *P. trifoliata*) when compared to its parents and respective autotetraploids, had lower photoinhibition (Fv/Fm) and less accumulation of the oxidative markers malondialdehyde (MDA) and H_2O_2. This was correlated with a greater increase in some antioxidant activities during cold stress (SOD, ascorbate

peroxidase or APX and glutathione reductase or GR) and light stress (SOD, APX, and monodehydroascorbate reductase or MDHAR; Oustric et al., 2018). Later studies on nutrient deprivation showed that the allotetraploid FlhorAG1 and several autotetraploid citrus genotypes are more tolerant to nutrient deficiency than their 2x counterparts. This behavior is related to enhanced photosynthetic capacity and a more favorable balance in their oxidative metabolism (Oustric et al., 2019a). The stress-protective effect driven by polyploidy was found to be graft-transmissible by different studies on citrus varieties grafted on 4x rootstocks. For instance, "Commune" clementine is more tolerant to cold stress when grafted on 4x "Carrizo" citrange (*P. trifoliata* × *C. sinensis*) than in 2x, as photosynthetic machinery stays more active and thus ROS production and damage are limited (Oustric et al., 2017). Lower levels of MDA, less electrolyte leakage and higher specific activities of catalase (CAT), APX, and dehydroascorbate reductase or DHAR were detected on plants grafted on 4x rootstocks, suggesting that a more efficient antioxidant activity promoted by the rootstock plays a role in their enhanced cold tolerance. Another example is the behavior of "Kinnow" mandarin plants grafted on three different rootstocks and subjected to chromium toxicity. In this study, plants grafted on 4x rootstocks were more tolerant than plants grafted on 2x rootstocks (Balal et al., 2017). This was attributed to more efficient accumulation of the metal on the 4x roots, and in turn, decreased transfer to leaves that prevented damage by accumulation. A more active antioxidant system was also identified in 4x roots.

Changes in primary and secondary metabolite expression, such as upregulation of sugars, amino acids, organic acids, and fatty acids driven by genome duplication have been reported to underpin the ROS metabolism alterations (Tan et al., 2015). The reason behind may be that large cells are disproportionately more productive than small cells. This might be driven by surface-to-volume ratio effects or increase in organelle number and size as described by Doyle and Coate (2019). These authors have also highlighted the potential contribution of ploidy-driven changes at the nucleolus, mitochondria, chloroplast, and endoplasmic reticulum on enhanced tolerance to abiotic stress. However, there is still a lack of knowledge in this area.

Enhanced Tolerance to Drought and Salinity

One of the main mechanisms involved in plant drought tolerance is the ability to deal with cavitation is one of the mechanisms favoring plant drought tolerance (Brodersen and McElrone, 2013). In polyploid species, xylem vessels are usually bigger in diameter than in 2x counterparts due to bigger cell size (Soltis et al., 2014), suggesting that polyploidy may increase sensitivity to drought due to an increased vulnerability to water flow instability under tension (Tyree and Ewers, 1991; Maherali et al., 2009). However, there are reports of decreased sensitivity to low soil moisture among polyploids (Maherali et al., 2009; Hao et al., 2013; Zhang W. W. J. et al., 2017). This paradox may be explained going deeper into the complexity of the occurrence and reversion of embolism in plants. Cavitation is not a straightforward process; it is influenced by several other traits that are not commonly evaluated. Pit anatomy (Zhang W. W. J. et al., 2017), vessel wall surface properties, and associated fibers or the whole xylem architecture may play a fundamental role in cavitation propension (Rockwell et al., 2014; Guet et al., 2015). To our knowledge, these traits have not yet been explored on polyploid crops.

Equally important for drought tolerance are the changes in the transpiration rate. Stomata in polyploids are typically bigger and their distribution is less dense than in 2x (Hennig et al., 2015; Hïas et al., 2017; Corneillie et al., 2019), which may force lower stomatal conductance than 2x counterparts (Singh and Sethi, 1995; Syvertsen et al., 2000; Zhang et al., 2007; Allario et al., 2011) and improve photosynthetic efficiency (Warner and Edwards, 1993). In 4x "Hanfu" apple the enhanced photosynthetic capacity has a positive impact on fruit yield and quality (Xue et al., 2017). However, this effect seems to be dependent on scion polyploidy, as 4x rootstocks do not enhance the photosynthetic capacity of 2x scions (Oustric et al., 2017). It has been showed that stomata closure can depend on leaf ABA, even though rootstock may contribute to the production of ABA (McAdam et al., 2016). Polyploidy can lower the gas exchange capacity as seen in 4x strawberries (*Fragaria moupinensis* Cardot; Gao et al., 2017). In this sense, the stomata closure regulatory mechanism, led by root-to-shoot signaling that is triggered by drought and mediated by the plant hormone ABA (Schachtman and Goodger, 2008) is said to be altered by polyploidy. Autotetraploid "Rangpur" lime has higher constitutive production of ABA than the 2x counterpart, associated with increased drought tolerance (Allario et al., 2013). Likewise, stomatal closure in 4x *Arabidopsis* is more responsive to drought and ABA than in 2x (del Pozo and Ramirez-Parra, 2014), but there is also evidence of increased gas exchange capacity in 4x *Arabidopsis* (Monda et al., 2016). These findings suggest that additional ploidy-driven modifications at the root level may have a strong influence on the gas exchange process such as the root's hydraulic conductivity (see next section). Overall, these stomata-related findings suggest that polyploids might provide better functional adaptation to drought stress, either when used as rootstocks or scions.

Furthermore, root hydraulic conductivity (Lp; $m{\cdot}s^{-1}{\cdot}MPa^{-1}$), which determines the root's system water uptake capacity and plays an important role in water use, might be modified by ploidy. The main traits contributing to decrease Lp, both in woody and herbaceous species, are cortex width and the presence of suberin barriers, which manage the radial flow of water and solutes to the stele (Rieger and Litvin, 1999). Polyploids may have thicker root cortex and suberin depositions, determined by their bigger cell size and more active metabolism (Doyle and Coate, 2019), as it is the case of citrus (Syvertsen et al., 2000; Ruiz et al., 2016a,c) or Willow (Dudits et al., 2016), which would result in lower Lp than in 2x roots (Ruiz et al., 2016c). Decreased water uptake conserves the resource in the soil for longer periods and prevents root leakiness or backflow under dry conditions, thus delaying plant mortality. As an example, suberin barrier reinforcement contributes to reducing the transpiration rate, while increasing water-use efficiency in

Arabidopsis (Baxter et al., 2009); this is a well-known adaptation mechanism that helps many annual and woody species overcome water stress (Barrios-Masias et al., 2015). Shorter, thicker and less branched roots that develop earlier and thicker suberin barriers have been identified on different 4x citrus rootstocks (Syvertsen et al., 2000; Ruiz et al., 2016a,c) and have been associated with lower hydraulic conductivity (Huang and Eissenstat, 2000). These modifications operate in "Carrizo" citrange to maintain unaltered leaf hydric status under osmotic stress, allowing gas exchange parameters to be sustained and limiting water consumption, while the 2x is drastically affected (Ruiz et al., 2015, 2016b; Oliveira et al., 2017). Similarly, 4x *Acacia* (*Acacia senegal* L. Willd) grew faster than 2x only under drought stress (Diallo et al., 2016). However, these anatomical root modifications have yet to be described in other polyploid crops.

The mechanisms described above are operative in the early stages of drought stress when water resources are in short supply to plant organs. Meanwhile, alternative local mechanisms may act in later stages to prevent dehydration and tissue damage. Hydraulic capacitance involves the use of tissue water storage to buffer local desiccation, maintain function (Huang et al., 2017; Vogel et al., 2017), or reverse cavitation (Brodersen et al., 2018). In that sense, polyploid plants may have an advantage over the 2x at preserving tissue water content as shown by the autotetraploid *Arabidopsis* detached rosette leaves (del Pozo and Ramirez-Parra, 2014). Similarly, autotetraploid "Gala" apple has the ability to delay drought-induced misperformance by trading hydraulic safety for increased release of capacitively stored water from living tissues (De Baerdemaeker et al., 2018). However, it is unknown whether bigger polyploid cells can accommodate bigger vacuoles increasing their water storage capacity.

Plant responses to salinity are induced by two different components of saline solutions. Initial adjustments are attributed to the water-stress effects triggered by the osmotic component of soil water potential. Ion-specific toxicity occurs later when saline ions accumulate causing tissue damage and abscission. Both effects reduce the plant's photosynthetic capacity, first by dramatically decreasing stomatal and root hydraulic conductance to limit water use and second, by reducing the leaf area. Consequently, the plant must reduce its growth rate (Munns and Tester, 2008). At the later stage, plant mortality appears when toxic damage, poor water status, and starvation overcome tolerance capacity. Several polyploid crops respond more efficiently to soil water depletion aided by the different mechanisms mentioned above. These would also be operative to deal with both toxic and osmotic components of salt stress. In citrus, higher salt tolerance has been described in different 4x rootstocks in association with low water use (Saleh et al., 2008; Ruiz et al., 2016b,c) or low water availability (Mouhaya et al., 2010). The efficient regulation of water use in response to osmotic stress greatly prevents ion intoxication by excessive accumulation when uptake is proportional to water use (Moya et al., 2003). Conversely, this tolerant response could require a trade-off between fruit production and ion avoidance.

Also, important when dealing with salinity is the more balanced potassium-sodium (K^+/Na^+) homeostasis shown by polyploid plants. Maintaining high K^+ uptake and tissue concentration is essential for salt tolerance (Wu et al., 2018), as K^+ competes for similar ion channels, transporters, and active sites as Na^+, preventing its accumulation and resulting functional disruption (Nieves-Cordones et al., 2010; Isayenkov and Maathuis, 2019). For example, polyploid *Arabidopsis* has greater tolerance to salinity, associated with higher K^+ uptake and lower Na^+ accumulation in leaves. Surprisingly, this effect has been shown to rely on rootstock polyploidy rather than on shoot cytotype (Chao et al., 2013). Hence, it is a root-dependent phenotype that could be provided to grafted crops using polyploid rootstocks. Higher K^+ retention when faced with salt stress has been also observed in hexaploid bread wheat (*T. aestivum* L.; Yang et al., 2014), in "Carrizo" citrange (Ruiz et al., 2016b) and in the allohexaploid sweet potato wild relative *Ipomoea trifida* (Kunth) G. Don (Liu et al., 2019). However, the reasons behind this ploidy-driven differential cation regulation are unknown.

Most crops suffer from the effects of salt toxicity, mainly associated with Na^+ leaf accumulation (Munns and Tester, 2008). However, some tree crop species are mostly affected by leaf chloride (Cl^-) accumulation instead, such as avocado (*Persea americana* Mill.; Acosta-Rangel et al., 2019), grape (Henderson et al., 2014), or citrus (Moya et al., 2003). Moreover, independent of which ion is the most damaging, the key for salt tolerance is the exclusion ability that some species have to limit ion uptake and translocation.

On the root apoplastic pathway, barriers to toxic ions are mainly based on suberin depositions at the exodermis and endodermis layers, whose presence/absence patterns are a determining factor for water and ion flow. In 4x citrus, enhanced Cl^- and boron exclusion ability has been related to a less branched and thicker root that develops earlier and thicker suberin barriers that are more restrictive to ion flux than in 2x counterparts (Ruiz et al., 2016a,c). Another essential mechanism used to deal with salinity is excess ion partition. Although this is not well understood, the three main compartments for Na^+ and Cl^- allocation are under discussion. Most data suggest that plants prevent excessive Na^+ and Cl^- accumulation in the cytosol and apoplast, to stop them from reaching concentrations beyond 50–80 mM to avoid hydric, biochemical, and nutritional imbalance (Isayenkov and Maathuis, 2019). In contrast, vacuoles can endure 10-fold higher ion concentrations than the later compartments. In this sense, polyploids would theoretically be at an advantage because of the ability to allocate, exchange, and dilute ions in their bigger vacuoles (Doyle and Coate, 2019). However, whether this mechanism plays a role in enhancing salinity tolerance has not been described on polyploid plants yet.

Does Polyploidy Induce Better Tolerance to Biotic Stress?

In earlier sections of this review, some polyploids were shown to be more tolerant to abiotic stress induced by genome expression regulation leading to changes in physiological traits, hormonal production, or better antioxidant systems. QTL analysis

revealed quantitative resistance for *Phytophthora infestans* and *Tecia solanivora* in 4x potato (Santa et al., 2018). Tetraploid and 3x banana cultivars had little damage due to nematodes and did not significantly reduce their plant height (Dochez et al., 2009). All the cultivated citrus are sensitive to Huanglongbing (HLB), a disease caused by the bacterium Candidatus *Liberibacter* sp. In citrus, HLB leads to an increase of callose synthesis at the sieve plate of the phloem cells, which will cause plugging of the pores (Koh et al., 2012), and thus will stop the symplastic transport between phloem cells. In the short term, the tree's physiology will be greatly affected which will rapidly lead to the tree's death. "Persian" lime (*Citrus* × *latifolia* Tan. ex Jimenez), which is 3x, is the most tolerant variety to HLB. It is thus possible that the better tolerance in "Persian" lime is related to the larger phloem vessels induced by polyploidy, which in turn will contribute to maintaining the flux of the phloem sieve for a longer time.

CONCLUDING REMARKS

We believe that the research into synthetic polyploids in agriculture is still too limited. Overall, the mechanisms that contribute to stress tolerance may also play a role in keeping plant productivity and fruit quality during environmental constraints or intentional water shortage. For this reason, polyploid scions and rootstocks might be a convenient choice for promoting efficient and sustainable agriculture. As an example, grape, olive, and citrus are very relevant for evaluating the impact of polyploidy on plant stress adaptation since they are clonally propagated crops (De Ollas et al., 2019). Extensive studies are still required to decipher the impact of polyploidy at the rootstock or scion level on agronomical phenotypic traits. Additionally, better understanding of the associated genome expression regulatory mechanisms induced by polyploidy is required as well. Overall, the production of 3x and 4x genotypes is quite straightforward nowadays and numerous grafted plants of agronomic interest could benefit from the ploidy-induced novel phenotypes.

AUTHOR CONTRIBUTIONS

MR, JO, and RM outlined and prepared the first draft of the manuscript. MR, JO, JS, and RM wrote the manuscript and produced the tables and figures. All authors revised the subsequent drafts of the manuscript and approved the final version.

FUNDING

MR was supported by the Spanish MINECO Ministry, project AGL2011-26490 and the Generalitat Valenciana grant DOCV [2010/8910]. JO and JS were supported by the "Innov'agrumes" project funded by FEDER and the "Collectivité de Corse." RM was supported by the "CAVALBIO" project funded by FEDER and the Guadeloupe Region and by the "TROPICSAFE" project funded by European Union's Horizon 2020 research and innovation programme under grant agreement No. 727459.

ACKNOWLEDGMENTS

We are grateful to Dr. Pablo Aleza and Dr. Patrick Ollitrault for their review and suggestions to improve the MS.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 December 2020
doi: 10.3389/fpls.2020.509481

Development and Cytomolecular Identification of Monosomic Alien Addition and Substitution Lines of Triticale (×*Triticosecale* Wittmack) With 2S^k Chromosome Conferring Leaf Rust Resistance Derived From *Aegilops kotschyi* Boiss

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Edited by:
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Reviewed by:
Zujun Yang, University of Electronic Science and Technology of China, China
Rudolph Fredua-Agyeman, University of Alberta, Canada

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *01 November 2019*
Accepted: *14 October 2020*
Published: *14 December 2020*

Citation:
Kwiatek MT, Ulaszewski W, Belter J, Phillips D, Skowrońska R, Noweiska A and Wiśniewska H (2020) Development and Cytomolecular Identification of Monosomic Alien Addition and Substitution Lines of Triticale (×Triticosecale Wittmack) With 2S^k Chromosome Conferring Leaf Rust Resistance Derived From Aegilops kotschyi Boiss. Front. Plant Sci. 11:509481. doi: 10.3389/fpls.2020.509481

Alien chromosome introgression has become a valuable tool to broaden the genetic variability of crop plants via chromosome engineering. This study details the procedure to obtain monosomic addition and monosomic substitution lines of the triticale carrying 2S^k chromosome from *Aegilops kotchyi* Boiss., which harbors *Lr54* + *Yr37* leaf and stripe rust-resistant gene loci, respectively. Initially, *A. kotschyi* × *Secale cereale* artificial amphiploids (2*n* = 6*x* = 42 chromosomes, UUSSRR) were crossed with triticale cv. "Sekundo" (2*n* = 6*x* = 42, AABBRR) in order to obtain fertile offspring. Cytomolecular analyses of five subsequent backcrossing generations revealed that 2S^k chromosome was preferentially transmitted. This allowed for the selection of monosomic 2S^k addition (MA2S^k) lines of triticale. Finally, the 2S^k(2R) substitution plants were obtained by crossing MA2S^k with the nullisomic (N2R) plants of triticale. The presence of 2S^k chromosome in subsequent generations of plants was evaluated using SSR markers linked to *Lr54* + *Yr37* loci. Disease evaluation of the monosomic 2S^k(2R) substitution plants for the reaction to leaf and stripe rust infection were carried out under controlled conditions in a growth chamber. The results showed significant improvement of leaf rust resistance severity of monosomic substitution plants compared with control ("Sekundo"). In contrast, the introgression of the *Lr54* + *Yr37* loci did not lead to improvement of stripe rust resistance. In summary, the creation of monosomic addition and monosomic substitution lines of triticale is the starting point for the precise and guided transfer of *Lr54* + *Yr37* loci. The results showed that the developed materials could be exploited for the development of triticale varieties with resistance to leaf rust.

Keywords: back-cross, fluorescence in situ hybridization, molecular markers, monosomic, resistance genes, triticale 2, Aegilops kotschyi

INTRODUCTION

Wild relatives and related species with homoeologous genomes are important for broadening the genetic variability of crop plants. For many crops, wild relatives provide a vast reservoir for most of agronomically important traits. A large number of the highest yielding wheat (*Triticum aestivum* L.) cultivars carry portions of alien chromatin introgression from related crops (*Secale cereale* L.) or weedy species (*Aegilops* sp., *Dasypyrum* sp., *Elymus* sp. *Haynaldia* sp.) (Gill et al., 2011). The *Aegilops* genus is the closest wild relative of bread wheat (*T. aestivum*; $2n = 6x = 42$ chromosomes; AABBDD) or triticale (× *Triticosecale* Wittmack; $2n = 6x = 42$; AABBRR). It contains 11 diploid, 10 tetraploid, and 2 hexaploid species and provides a vast reservoir of valuable genes, which were eliminated during the domestication and breeding of cultivated cereals.

Aegilops kotschyi Boiss. ($U^kU^kS^kS^k$, $2n = 4x = 28$) is valuable from genetic and breeding point of view as it carries genes associated with disease resistance, drought, heat, salt tolerance, as well as other beneficial traits (Schneider et al., 2008). Rawat et al. (2009) utilized *A. kotschyi* as a source of genes to increase the iron and zinc content in seeds of wheat. *A. kotschyi* was crossed as a male parent with bread wheat line "Chinese Spring" lacking the *Ph1* locus, which is a suppressor of homeologous pairing in wheat (Riley and Chapman, 1958). The aim of their study was to bring the genes associated with increased iron and zinc content in seeds, harbored in the $2S^k$ and $7U^k$ chromosomes of *A. kotschyi*, into the genome of wheat. They obtained only a $2S^k$ and $7U^k$ chromosome substitution line, which were not very suitable for commercial exploitation due to linkage drag. It is also reported that chromosome $2S^k$ of *A. kotschyi* possess leaf and stripe rust-resistant loci of *Lr54* and *Yr37* genes (Marais et al., 2005; Prażak and Paczos-Grzęda, 2013). Marais et al. (2005) have introduced *A. kotschyi*-derived leaf and stripe rust-resistant genes *Lr54* and *Yr37* to the wheat genome by the induction of $2DS.2S^kL$ chromosome translocation.

Polyploids, such as of cultivated forms wheat or triticale, contain multiple sets of chromosomes and have highly buffered genotypes that are more permissive to benefit from alien introgression than diploids (Qi et al., 2007). Monosomic alien addition lines (MAALs) contain only one alien chromosome in addition to the receptor background chromosomes. The production of monosomic addition lines rely on backcrosses with an acceptor crop (Faris et al., 2002; Brar and Dhaliwal, 2004; Wulff and Moscou, 2014; Kwiatek and Nawracała, 2018). In traditional breeding of intraspecific hybrids, the recurrent backcrossing is commonly employed to transfer alleles at one or more loci from a donor to an elite variety (Veatch-Blohm, 2007). The expected recurrent parent genome recovery would be 99.2% at the end of six backcrossing, which is most similar to improved variety. The proportion of the recurrent parent genome is recovered at a rate of $1 - (1/2)^{t+1}$ for each of the generations of backcrossing (Singh and Singh, 2015). When generating interspecific hybrids, backcrossing is required to generate addition, substitution, and translocation lines. In wheat and triticale, chromosome pairing between homologous chromosomes is strictly regulated by the expression of *Ph1* and *Ph2* loci (Riley and Chapman, 1958; Sears, 1977). Thus, subsequent backcrosses eliminate the alien chromatin gradually. However, the alien chromosome can be transmitted to the offspring preferentially, which contributes to the saturation of specific chromosomes (Endo, 2007).

Some *Aegilops* chromosomes were reported to be transmitted preferentially to the offspring in the process of backcrossing to produce alien chromosome addition lines of wheat (Endo, 2007) and triticale (Kwiatek et al., 2016c). The gametocidal activity of genes located on chromosome $4S^{sh}$ from *A. sharonensis* Eig is an example of when a chromosome is preferentially eliminated (Endo, 1990). Such chromosomes were reported to carry gametocidal factors (*Gc*s) (Endo, 1985). These chromosomes are known to remain in host plants in a "selfish" way. The preferential transmission of *Gc* chromosomes result from the elimination of gametes that lack them, while the gametes with the *Gc* chromosomes remain fertile (Nasuda et al., 1998). *Gc* factors cause extensive chromosome breakage, which results in the induction of non-functional gametes and exclusive transmission of the *Gc* chromosome to the offspring (Nasuda et al., 1998). *Gc*s have been reported in *A. geniculata*, *A. triuncialis*, *A. caudata*, *A. cylindrica, A. longissima*, *A. sharonensis*, and *A. speltoides*. Their activity is mostly confined to 2, 3, and 4 homeologous groups of C, S, S^1, S^{sh}, and M^g genomes. It has been reported that *Gc* genes can be constructively utilized for development of addition, deletion and translocation stocks in wheat (Endo, 2007) and triticale (Kwiatek et al., 2017b).

Monosomic alien addition and substitution lines (MAALs and MASLs) are widely used as linking bridges for the transfer of desirable genes from wild species into elite cultivars. For example, wheat-rye addition, substitution, and translocation lines are used as pre-breeding materials for the improvement of wheat (Lukaszewski, 2000, 2016) and triticale (Lukaszewski, 2006). MASLs can be used to identify favorable genes in wild species, allowing for more accurate and rapid transfer of such genes to create introgression lines. Such initiatives provide the opportunity to examine the effect of specific alien (Edet et al., 2018) and to construct the physical maps of specific chromosomes (Kynast et al., 2001). MASLs are produced by crossing monosomic or nullisomic acceptors with appropriate addition lines (Sears, 1972). The missing chromosome of acceptor plant is substituted by the donor homoeologue. The production of substitution lines for alien chromosomes is the initial step for direct introgression of specific alien chromosome segments into the acceptor genome (Lukaszewski, 2000, 2017). One of the most common procedures that reduce the amount of alien chromosome present is the induction of crop-alien Robertsonian translocations (RobTs). This approach requires a set of specific genetic stock plants, including MAALs or MASLs as a donor, and monosomic plants with a single acceptor chromosome (Kwiatek et al., 2017b).

The main aim of this study was to generate the monosomic addition and substitution line of triticale with introgression of alien $2S^k$ chromosome derived from *A. kotschyi*, which could be used for efficient study and transfer of genes responsible for leaf and stripe rust resistance.

MATERIALS AND METHODS

Plant Material

An artificial amphidiploid line of *Aegilops kotschyi* × *Secale cereale* (2*n* = 6*x* = 42; $U^kU^kS^kS^kRR$) (**Figure 1**) was obtained by Wojciechowska and Pudelska (2002) from a cross between *A. kotschyi* Boiss. (no. 14,808; kindly provided from Prof. Taihachi Kawahara, Kyoto University, Kyoto, Japan; **Figures 2a,b**), and diploid rye "Dankowskie Złote" (Danko Hodowla Roślin sp. z o.o.). Triticale "Sekundo" (Danko Hodowla Roślin Sp. z o.o.) was used as a pollinator for F_1 seed production and five subsequent backcrosses (**Figure 3**). Nullisomic (N2R) line of triticale were developed by Kwiatek et al. (2016c) and used for crossing with monosomic alien addition of $2S^k$ chromosome to produce monosomic $2S^k$(2R) substitution plants of triticale (**Figure 3**). Cross-hybridization was made in greenhouse of the Institute of Plant Genetics of the Polish Academy of Sciences. Anthers of maternal plants were emasculated and spikes isolated using paper bags, in order to avoid uncontrolled pollination. Mature stigmas were pollinated with the pollen of triticale "Sekundo." Crossing efficiency percentage was calculated by dividing of the total amount of seeds with total number of pollinated flowers.

Cytogenetic Studies

Accumulation of cells at mitotic metaphase and fixation was carried out according to Kwiatek et al. (2017b). Root meristems were digested at 37°C for 2 h and 40 min in an enzymes solution containing 0.2% (*v*/*v*) Cellulase Onozuka R-10 and Calbiochem cytohelicase (1:1 ratio) and 20% pectinase (Sigma), in 10 mM citrate buffer (pH 4.6). Digested root tips were placed on slides with a drop of ice-cold 60% acetic acid and squashed. The coverslips were removed with a razor after liquid nitrogen treatment.

Probe Preparation and Fluorescence *in situ* Hybridization

Total genomic DNA was purified using GeneMATRIX Plant & Fungi DNA Purification Kit (EURx, Gdańsk, Poland). DNA of *Aegilops sharonensis* Eig (a progenitor of the S^k-genome of *A. kotschyi*; PI 551020, U.S. National Plant Germplasm System) and *A. umbellulata* (a progenitor of the U^k-genome of *A. kotschyi*; PI 298905, U.S. National Plant Germplasm System) were labeled by nick translation with Atto-488NT and Atto-550NT kits (Jena Bioscience, Jena, Germany), respectively. Blocking DNA from triticale "Sekundo" was prepared by boiling for 30–45 min (1:50 probe:block ratio). Chromosomes were identified using fluorescence *in situ* hybridization (FISH) with the repetitive sequences from pTa-86, pTa-535, pTa-103 (centromere specific), and pTa-k374 (homologous to ribosomal DNA sequence 28S) clones described by Komuro et al. (2013). Clones were amplified from genomic DNA of "Chinese Spring" wheat (Kwiatek et al., 2016b; Goriewa-Duba et al., 2018) and labeled using Atto-488NT, Atto-550NT, and Atto-647NT nick translation kits (Jena Bioscience, Germany). FISH/genomic *in situ* hybridization (GISH) experiments were performed according to Kwiatek et al. (2017b). Slides were examined with the Olympus BX 61 automatic epifluorescence microscope equipped with Olympus XM10 CCD camera. Image processing was performed using Olympus Cell-F (version 3.1; Olympus Soft Imaging Solutions GmbH: Münster, Germany). Chromosomes of *Aegilops* and triticale were identified by comparing the signal patterns of the probes (Badaeva et al., 2004; Komuro et al., 2013; Kwiatek et al., 2013; Ruban and Badaeva, 2018).

SSR Marker Analysis

The SSR marker S14-410 (forward primer: 5′-ACCAATT CAACTTGCCAAGAG-3′; reverse primer: 5′-GAGTAACATG CAGAAAACGACA-3; Smit, 2013) closely linked to the *Lr54* + *Yr37* loci on the $2S^k$ chromosome was used to genotype genomic DNA of the plant materials. Genomic DNA of *Aegilops kotschyi*, triticale "Sekundo" and the hybrid plants were isolated using Plant DNA Purification Kit (EurX Ltd., Gdańsk, Poland). PCR reactions were performed in a LabCycler thermal cycler (SensoQuest Biomedizinische Elektronik, Goettingen, Germany). The PCR reaction consisted of 150 nM each primer (Merck KGaA, Darmstadt, Germany), 0.2 mM of each nucleotide, 1.5 mM $MgCl_2$, 0.2 units of Taq-DNA hot-start polymerase (TaqNovaHS, Blirt, Gdańsk, Poland), and 50 ng of genomic DNA as a template. The PCR conditions were as follows: 5 min at 95°C, then 35 cycles of 30 s at 94°C, 30 s at 61°C, 1 min at 72°C, and 5 min at 72°C. Midori Green Direct (Nippon Genetics Europe) was added to each amplification product and analyzed by gel electrophoresis on 2% agarose gel (LabEmpire, Poznań, Poland). Gels were visualized and photographed using EZ GelDoc System (BioRad, Hercules, CA, United States).

Evaluation of Leaf Rust Symptoms in Growth Chamber

Evaluation of the response of monosomic substitution plants on leaf rust infection was conducted in the growth chamber (at IPG PAS) using leaf and stripe rust urediniospores, which were collected separately from triticale fields in three localizations in Wielkopolska region: IPG PAS Experimental Station in Cerekwica, Poland (52° 31′ 16″ N, 16° 41′ 30″ E); Experimental Station of the Poznań University of Life Sciences (PULS), Dłoń, Poland (51°41′22″N 17°04′23″E); and Experimental Garden of the Department of Genetics and Plant Breeding (PULS) in Poznań (52° 25′ 26″ N, 16° 54′ 07″ E). The leaf and stripe rust symptoms were scored in two independent and separate experiments (**Figure 4**). Both experiments were repeated. Each experiment repeat included forty monosomic $2S^k$(2R) substitution plants and forty control plants ("Sekundo"), which were sprayed with leaf rust urediniospore solution containing 0.1% Tween 20, at three-leaf stage. Another 160 plants were sprayed with stripe rust urediniospore solution. The inoculated plants were then incubated in a humid growth chamber free from light for 10 days. After inoculation, the plants were maintained under a day/night photoperiod of 18/6 h, a temperature of 16–22°C. Winter triticale cv. "Sekundo" was taken as the susceptible control. The infection type of each individual was scored at three timepoints [10, 15, and 20 days post-inoculation (*dpi*)] using an

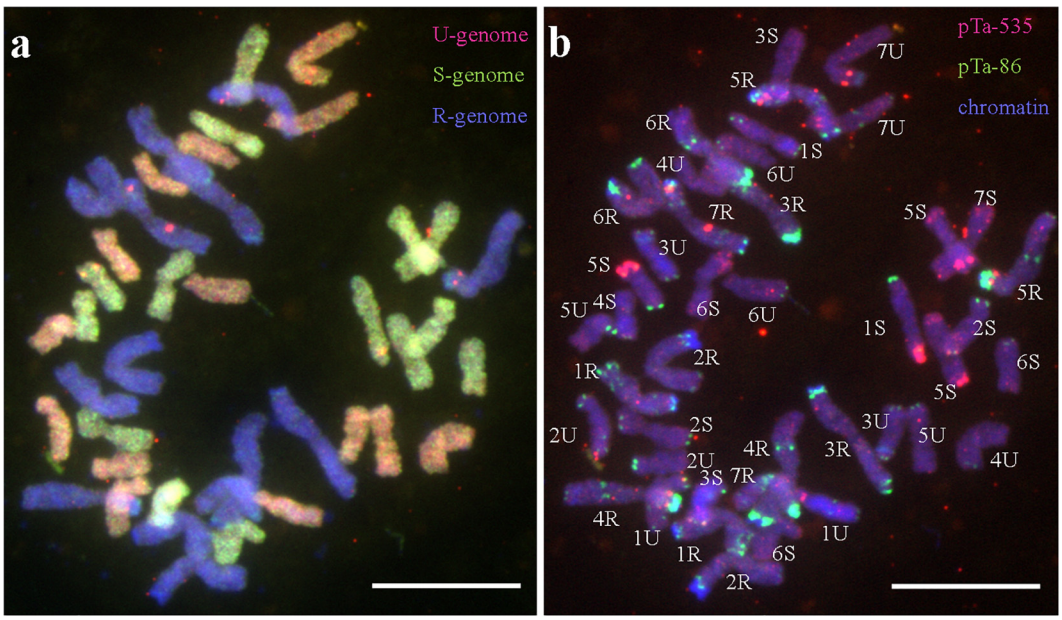

FIGURE 1 | Flourescence/genomic *in situ* hybridization performed on mitotic chromosomes of *A. kotschyi* × *S. cereale* amphiploid. **(a)** U^k- and S^k-genome chromosomes are labeled with Atto-550 (red) and Atto-488 (green), respectively. R-genome chromosomes labeled with DAPI (blue). **(b)** Probes pTa-535 and pTa-86 are labeled with Atto-550 (red) and Atto-488 (green), respectively. Chromosomes counterstained with DAPI (blue). Scale bar, 10 μm.

infection scale adapted from Roelfs (1988) and transformed into nine-grade scale (1, high resistance; 9, susceptibility, **Tables 2**, **3**; McNeal et al., 1971). The phenotypic data were analyzed using analysis of variance (ANOVA) and Tukey's highest significant difference (HSD) test.

RESULTS

Production of Monosomic $2S^k$ Addition Plants From Triticale-*A. kotschyi* Hybrids

Seven F_1 plants were produced from a cross between hexaploid triticale "Sekundo" ($2n = 6x = 42$ chromosomes; AABBRR) and hexaploid *A. kotschyi* × *S. cereale* ($2n = 6x = 42$; UUSSRR; **Figure 2c**) hybrid, as a pollen donor. All seven F_1 plants possessed 42 chromosomes including a complete haploid set of chromosomes of *A. kotschyi* (**Figure 5a** and **Table 1**). The number of R-genome chromosomes was 14. Fifteen BC_1F_1 seeds were obtained from 375 flowers of F_1 plants, which were backcrossed using triticale pollen. FISH experiments revealed that chromosomes $1U^k$, $2U^k$, and $7S^k$ were eliminated (**Figure 5b** and **Table 1**). Fiftyone BC_2F_1 seeds were obtained by subsequent backcrossing of BC_1F_1 with triticale "Sekundo." Fifty BC_2F_1 plants were karyotyped (**Table 1**), with the progressive elimination of *Aegilops* chromosomes was observed. The number of U-genome chromosomes varied between 1 and 2. The number of S^k-genome chromosomes was much more diversified and varied between 2 and 6 (**Figure 5c** and **Table 1**). However, two chromosomes ($2S^k$ and $4S^k$) were present in all 50 BC_2F_1 plants (**Table 1**). The BC_2F_1 plants were pollinated with triticale "Sekundo" pollen, and a total number of 234 seeds were obtained. FISH was carried out on 100 BC_3F_1, revealing that chromosome $7U^k$ was present in all plants. Chromosome number of S^k-genome varied between 2 and 3 (**Figure 5d**). As before, chromosomes $2S^k$ and $4S^k$ were present in all 100 BC_3F_1 plants (**Table 1**). Fifty randomly selected plants of 93 BC_4F_1 hybrids were karyotyped using FISH and GISH techniques. Chromosomes of U^k-genome were completely eliminated, but surprisingly chromosomes $2S^k$ and $4S^k$ were present in all 50 BC_4F_1 plants (**Figure 5e** and **Table 1**). The last backcross resulted in formation of 216 BC_5F_1 seeds (**Table 1**). One hundred plants were karyotyped and 99 of them carried additional $2S^k$ chromosomes ($2n = 43$) (**Figure 5f** and **Table 1**). Chromosome $2S^k$ was not present in one BC_5F_1 plant ($2n = 42$) (**Table 1**), and this plant showed centric breaks in three pairs chromosomes (**Figure 6**). Therefore, an investigation of six generations of triticale Sekundo × (*A. kotschyi* × *S. cereale*) hybrids (F_1 to BC_5F_1) by means of molecular cytogenetic methods (FISH and GISH) revealed subsequent elimination of *Aegilops* chromosomes.

Production of Monosomic $2S^k$(2R) Substitution Plants From Triticale-*A. kotschyi* Hybrids

A total number of 468 seeds were obtained from a cross between nullisomic N2R triticale plants ($2n = 40$; **Figures 7a,b**) and monosomic $2S^k$ addition line ($MA2S^kAL$) of triticale (**Figures 7c,d**), as a pollen donor. 6,486 flowers of N2R plants were emasculated and pollinated with $MA2S^k$ pollen. The

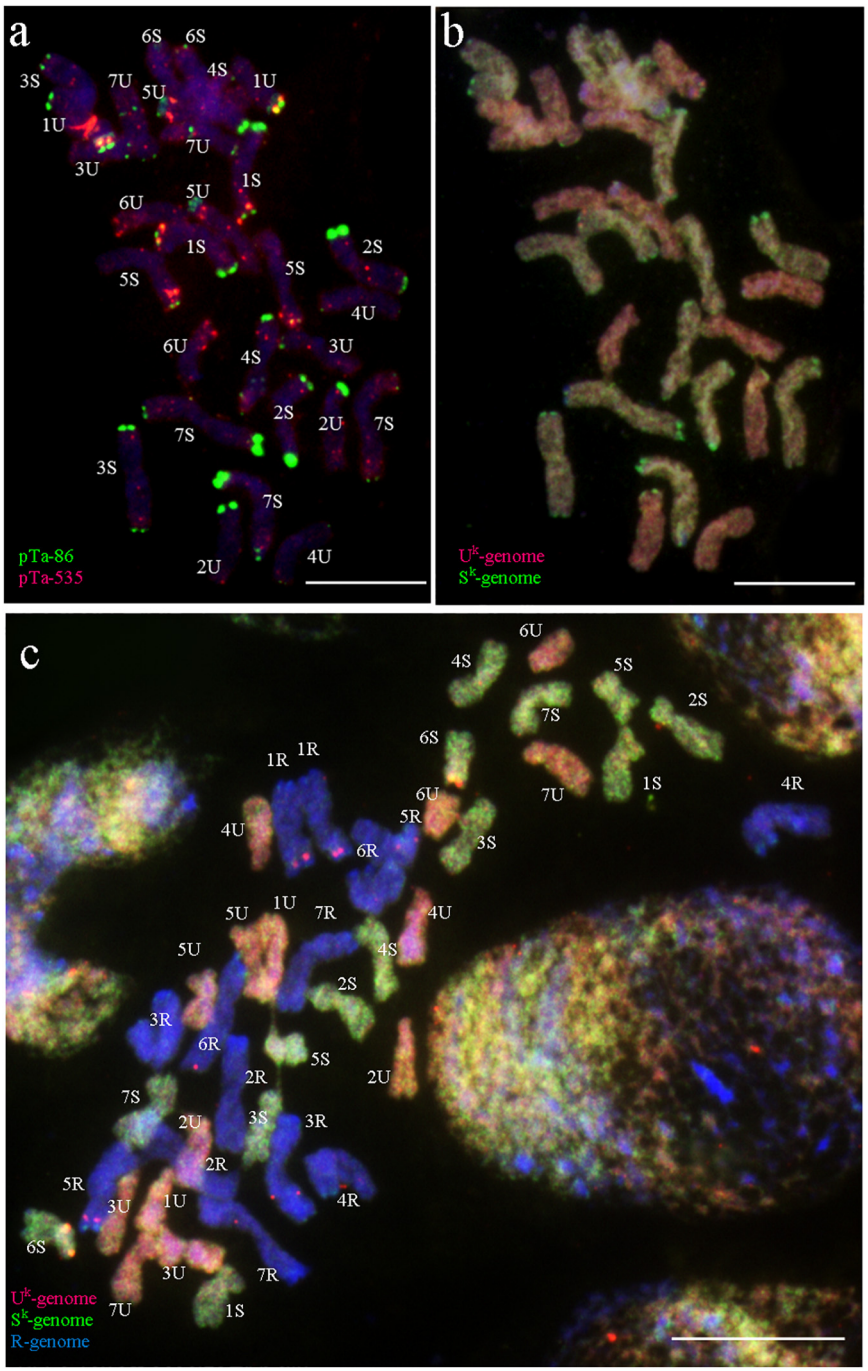

FIGURE 2 | Fluorescence/genomic *in situ* hybridization performed on mitotic chromosomes of *A. kotschyi* Boiss (2*n* = 6*x* = 42; $U^kU^kS^kS^k$; no. 14808 using **(a)** pTa-86 (Atto-488, green), pTa-535 (Atto-550, red). Genomic *in situ* hybridization using U-genome (Atto-550, red) and S-genome (Atto-488, green) probes performed on mitotic chromosomes of **(b)** *A. kotschyi* and **(c)** *Aegilops kotschyi* × *Secale cereale* (2*n* = 6*x* = 42; UUMMRR). **(a,c)** Chromosomes counterstained with DAPI (blue). Scale bar, 10 μm.

crossing efficiency was 7.22%, and 468 plants were obtained. The FISH/GISH analyses revealed that each offspring plant carried 42 chromosomes, including 2R and $2S^k$ chromosomes, which were in monosomic condition (**Figure 7e,f**). The presence of monosomic or nullisomic 2R chromosome was not observed.

Lr54 + Yr37 SSR Markers Analysis

PCR with SSR marker S14-410, linked to *Lr54* + *Yr37* loci, was conducted on *A. kotschyi*, triticale "Sekundo," the monosomic substitution $M2S^k$ (M2R) line and nullisomic N2R triticale plants (2*n* = 40). The same protocol was used to examine

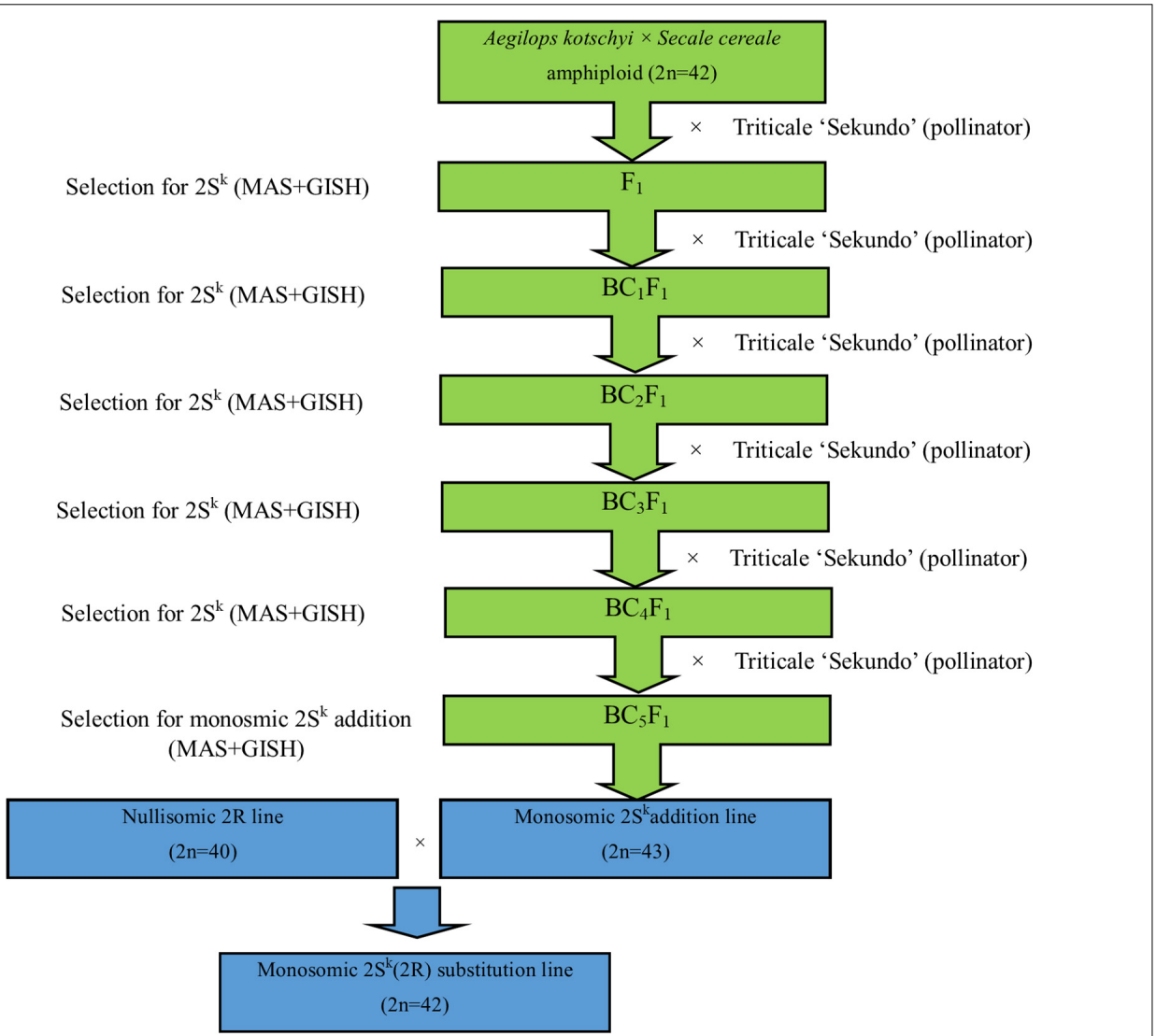

FIGURE 3 | Pedigree of monosomic 2S^k(2R) substitution plants of triticale "Sekundo."

TABLE 1 | Frequencies of individual *Aegilops kotschyi* chromosomes in five subsequent generations of backcrossing with triticale.

Generation	Pedegree	Number of pollinated flowers	Number of seeds	Number of plants (FISH)	Number of plants with												
					U-genome chromosomes							S-genome chromosomes					
					1	2	3	4	5	6	7	1	2	3	4	5	6	7
F_1	Sekundo × AkSc*	710	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
BC_1F_1	F1 × Sekundo	375	15	15	0	0	13	2	11	13	15	1	15	12	15	5	1	0
BC_2F_1	BC_1F_1 × Sekundo	488	51	50	0	0	0	42	0	0	50	5	50	41	50	23	36	0
BC_3F_1	BC_2F_1 × Sekundo	715	234	100	0	0	0	0	0	0	100	0	100	0	100	0	23	0
BC_4F_1	BC_3F_1 × Sekundo	480	94	50	0	0	0	0	0	0	0	0	50	0	50	0	0	0
BC_5F_1	BC4F_1 × Sekundo	487	216	100	0	0	0	0	0	0	0	0	99	0	0	0	0	0

**AkSc, Aegilops kotschyi* × *Secale cereale amphiploid (2n = 6x = 42 chromosomes, $U^kU^kS^kS^kRR$).*

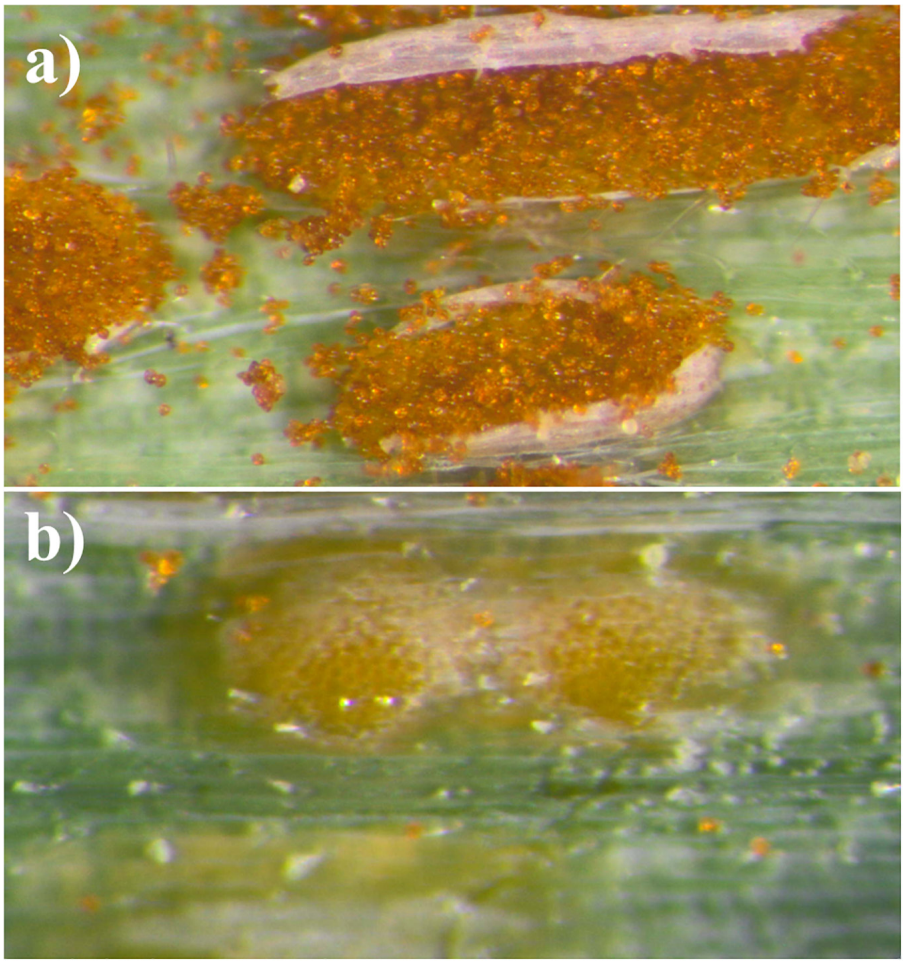

FIGURE 4 | Symptoms of **(a)** leaf rust and **(b)** stripe rust observed on leaves of triticale "Sekundo."

TABLE 2 | Means of leaf rust infection levels scored 10, 15, and 20 days post-inoculation (dpi).

Plant material	Experiment	No of plants tested	Means of infection levels			
			10 dpi	15 dpi	20 dpi	Mean ± SD
Monosomic 2S^{k}(2R) substitution plants	1	40	2.60	3.00	3.30	2.97 ± 0.48
Triticale cv. "Sekundo"	1	40	5.90	6.43	6.88	6.40 ± 0.73
Monosomic 2S^{k}(2R) substitution plants	2	40	2.70	2.98	3.20	2.96 ± 0.42
Triticale cv. "Sekundo"	2	40	5.86	6.45	6.95	6.43 ± 0.75

*F ratio = 1,267.62 (**Supplementary Table 1**).*

TABLE 3 | Means of stripe rust infection levels scored 10, 15, and 20 days post-inoculation (dpi).

Plant material	Experiment	No of plants tested	Means of infection levels			
			10 dpi	15 dpi	20 dpi	Mean ± SD
Monosomic 2S^{k}(2R) substitution plants	1	40	2.85	3.18	3.85	3.29 ± 0.77
Triticale cv. "Sekundo"	1	40	2.73	3.20	3.80	3.24 ± 0.79
Monosomic 2S^{k}(2R) substitution plants	2	40	2.70	3.15	3.78	3.21 ± 0.79
Triticale cv. "Sekundo"	2	40	2.67	3.30	4.00	3.33 ± 0.85

*F ratio = 0.5 (**Supplementary Table 2**).*

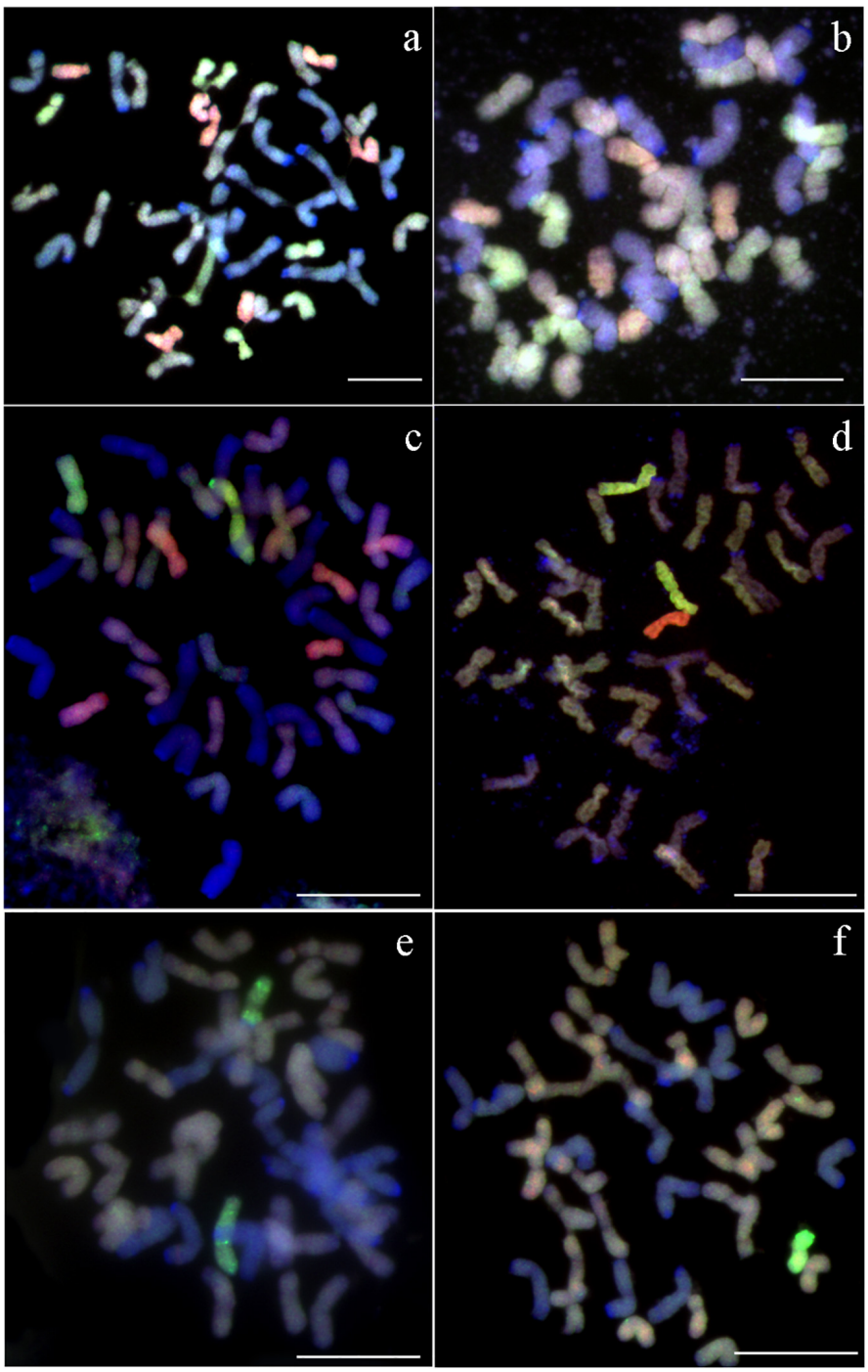

FIGURE 5 | Genomic *in situ* hybridization performed on mitotic chromosomes of **(a)** F_1, **(b)** BC_1F_1, **(c)** BC_2F_1, **(d)** BC_3F_1, **(e)** BC_4F_1, and **(f)** BC_5F_1 triticale-*Aegilops* introgression plants. A- and B-genome chromosomes are labeled with DAPI (light blue), as well as R-genome chromosomes (dark blue). U^k- and S^k-genome chromosomes are labeled with Atto-550 (red) and Atto-488 (green), respectively. Scale bar, 10 μm.

the offspring derived by subsequent backcrossing. A 410-bp amplicon for the S14-410 marker was identified in the control sample of *A. kotschyi* and in all hybrid plants of F_1 to BC_4F_1 generations (**Figure 8**). Screening for the presence of the S14-410 marker in 100 BC_5F_1 plants showed that 410 bp amplicon was present in 99 plants. Similar screening was carried out in 468 plants, created by cross-hybridization of N2R triticale plants ($2n$ = 40) and M2S^{k}AL plants ($2n$ = 43). PCR reaction with the genomic DNA of all recombinant plants yielded a 410-bp fragment.

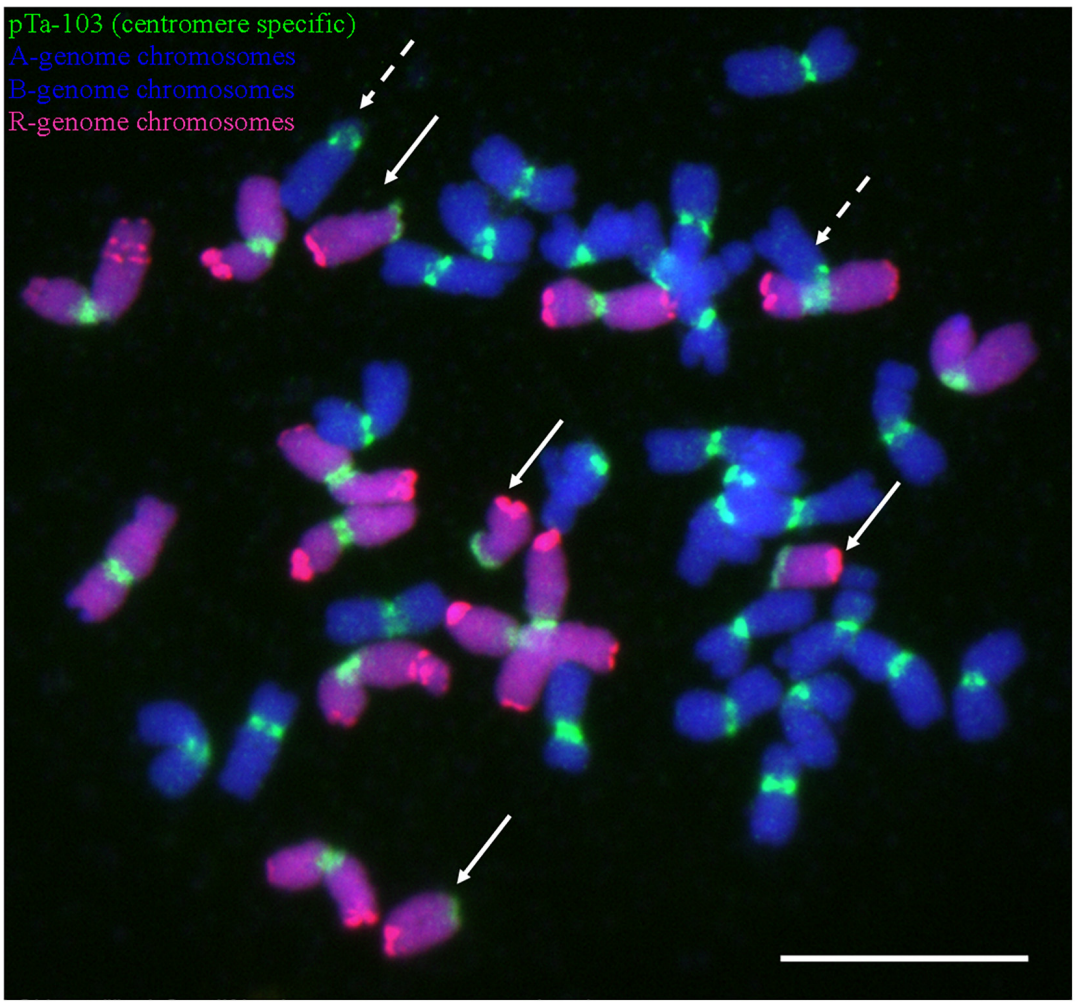

FIGURE 6 | Centric breaks of chromosomes in BC_5F_1 plant screened using multi-color GISH/FISH. Centromere regions were mapped using pTa-103 (Atto-488, green) probe. Dotted arrows, chromosome aberrations of A- or B-genome (DAPI, blue) of triticale; solid arrows, chromosome aberrations of R-genome (Atto-550, red) of triticale. Scale bar, 10 μm.

Evaluation of Leaf Rust Symptoms

The leaf and stripe rust response of two sets of 80 plants of triticale monosomic $2S^k(2R)$ substitution line was evaluated at the seedling stage in the growth chamber and compared with phenotypes of acceptor cultivar "Sekundo" as controls (**Table 2**). The mean scores for two replications of control plants (6.40 ± 0.73 and 6.43 ± 0.75) showed that the inoculation solution was effective for induction of the leaf rust infection. The mean score of three independent evaluations of infection level (10, 15, and 20 dpi) of two replications of monosomic $2S^k(2R)$ substitution plants was similar (2.96 ± 0.42 and 2.97 ± 0.48) (**Table 2**). The differences were significant at $\alpha = 0.01$ (**Supplementary Table 1**). The scores for two replications of the leaf rust response experiment did not differ significantly (**Supplementary Table 1**). In contrast, the stripe rust responses of the monosomic $2S^k(2R)$ substitution plants and the control triticale cv. "Sekundo" at the three successive points did not differ significantly ($\alpha = 0.01$) (**Table 3**). Plants of cv. "Sekundo" showed moderate stripe rust resistance (3.24 ± 0.79 and 3.33 ± 0.85), as well as monosomic substitution plants (3.21 ± 0.79 and 3.29 ± 0.77). ANOVA test for all thee timepoints showed that there is no statistically significant differences between stripe rust response of monosomic substitution plants compared with triticale "Sekundo" controls (**Supplementary Table 2**).

DISCUSSION

Alien and cultivated species are closely related and possess homeologous genomes (or sub-genomes). Hence, it is possible to introduce the desirable variation into crop plants using a backcrossing program. However, isolation barriers exist that prevent the formation of interspecific hybridization, and present a significant obstacle for the exploration of alien genetic variation in crop improvement. The generation of artificial amphidiploids can facilitate the transmission of desirable genetic

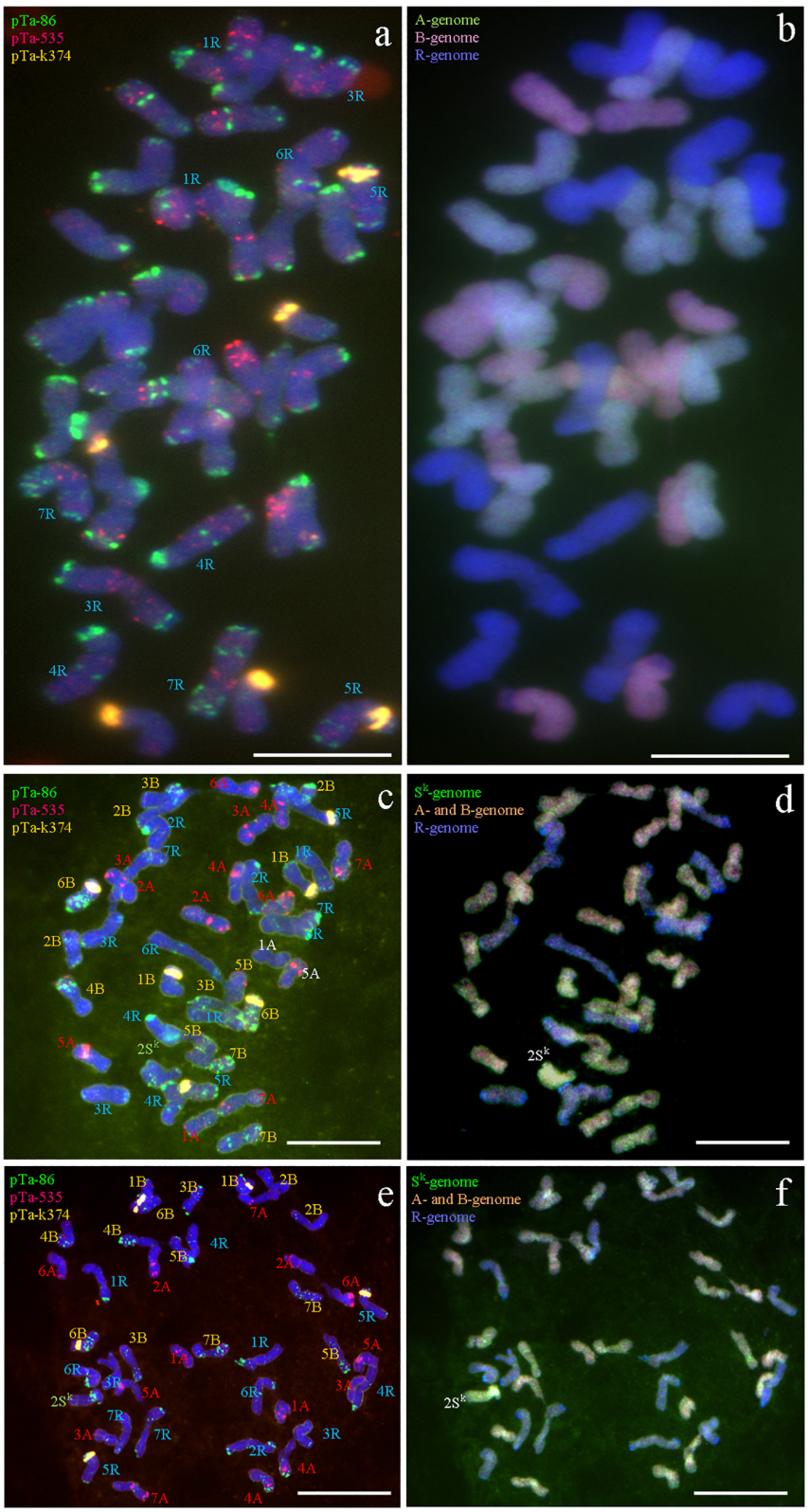

FIGURE 7 | Fluorescence and genomic *in situ* hybridization performed on mitotic chromosomes of: **(a,b)** nullisomic 2R plant of triticale (2*n* = 40); **(c,d)** monosomic $2S^k$ addition plant of triticale (2*n* = 43); **(e,f)** monosomic $2S^k$(2R) substitution plant of triticale (2*n* = 42). pTa-86 (Atto-488; green), pTa-535 (Atto-550; red), and pTa-k374 (Atto-647; yellow) probes were used for FISH. Genomic probes of A-, B-, R-, and S^k-genome were used for GISH. Scale bar, 10 μm.

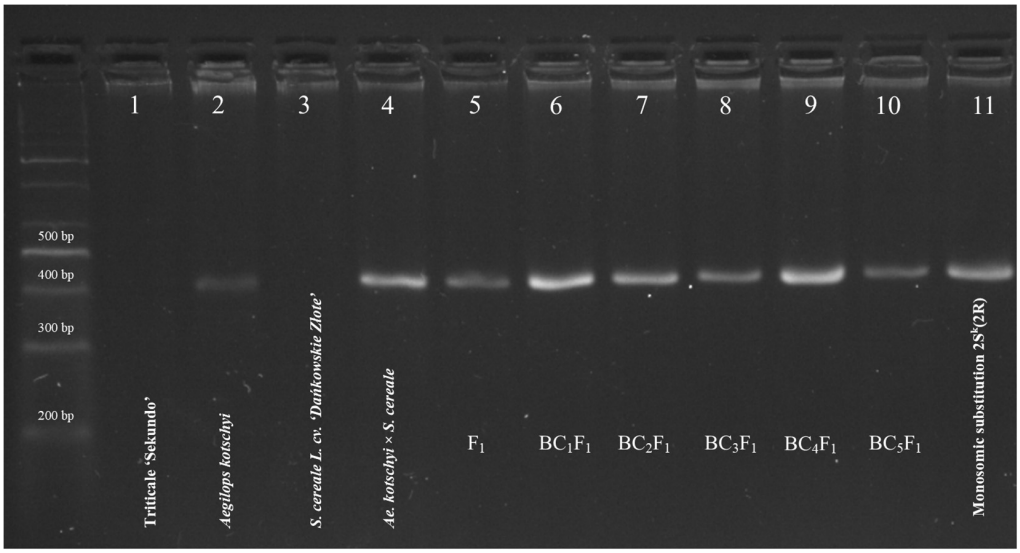

FIGURE 8 | Amplification products (410 bp) of S14-410 marker linked to loci of *Lr54* + *Lr37* leaf and stem rust resistance genes on $2S^k$ chromosome of *Ae. kotschyi*. (1) triticale "Sekundo" (AABBRR); (2) *Aegilops kotschyi* ($U^kU^kS^kS^k$); (3) *Secale cereale* L. "Dańkowskie Złote"; (4) *Aegilops kotschyi* × *Secale cereale* ($2n = 6x = 42$; UUMMRR); (5) F_1; (6) BC_1F_1; (7) BC_2F_1; (8) BC_3F_1; (9) BC_4F_1; (10) BC_5F_1; and (11) monosomic $2S^k$(2R) substitution plant of triticale ($2n = 42$). Size standard: Thermo Scientific™ GeneRuler™ 100 bp DNA Ladder.

material from wild species to crops. In this study, synthetic *A. kotschyi* × *S. cereale* ($2n = 6x = 42$, $U^kU^kS^kS^k\underline{RR}$) amdiphiploid plants were used as a donor of wild genetic material to widen the genetic variation in hexaploid triticale "Sekundo" ($2n = 6x = 42$, AABB$\underline{RR}$).

Similar artificial amphidiploid forms were used to transfer *Lr32* and *Lr39* leaf rust resistance genes from *A. tauschii* × *S. cereale* ($2n = 4x = 28$; DDRR) (Kwiatek et al., 2015) and *Pm13* powdery mildew resistance gene from *A. variabilis* × *S. cereale* ($2n = 6x = 42$, $U^vU^vS^vS^v\underline{RR}$) (Kwiatek et al., 2016a) into cultivated triticale. It was reported that the presence of an R-genome chromosome set resulted in semi-fertile F_1 plants that were capable of producing an F_1 via cross-hybridization of artificial *Aegilops biuncialis* × *S. cereale* ($2n = 6x = 42$, $U^bU^bM^bM^bRR$) amphiploid and hexaploid triticale (Kwiatek et al., 2017a). In this study, seven F_1 plants were obtained via cross-hybridization of *A. kotschyi* × *S. cereale* and triticale. The first backcross with triticale "Sekundo" resulted in rapid elimination of the *Aegilops* chromosomes. The elimination range of the U^k-genome chromosomes was far higher when compared with the rate of S^k-genome chromosome loss (**Table 1**). The elimination of alien chromosomes was also observed in the BC_2F_1 generation. Interestingly, only $7U^k$, $4U^k$, $2S^k$, and $4S^k$ chromosomes were present in all of BC_2F_1 plants. In subsequent generations of triticale hybrids, obtained via successive backcrosses, the number of *Aegilops* chromosomes was highly reduced and the plants of BC_4F_1 carried $2S^k$ and $4S^k$ chromosomes only.

This pattern could be a result of gametocidal action of the *Gc* locus on $2S^{sh}$ of *Aegilops sharonensis* (Endo, 1985). Moreover, similar "breaker" element described in *A. sharonensis* (Maan, 1975). These loci have been mapped to a region proximal to a block of sub-telomeric heterochromatin on chromosome $4S^{sh}$L (Knight et al., 2015). The diploid *A. sharonensis* ($2n = 2x = 14$; $S^{sh}S^{sh}$) is a direct ancestor and a donor of S^k-genome of *A. kotschyi* (Kihara, 1954; Badaeva et al., 2004; Ruban and Badaeva, 2018). Investigations into the origin of *A. kotschyi* showed that the U^k and S^k subgenomes are very similar to the diploid progenitors: *A. umbellulata* ($2n = 2x = 14$; UU) and *A. sharonensis*, respectively (Zohary and Feldman, 1962; Zohary, 1999; Ozkan et al., 2001; Feldman and Levy, 2012). It is a distinct possibility that there are gametocidal loci on $2S^k$ and $4S^k$ chromosomes of *A. kotschyi*, and those loci could be orthologous to *A. sharonensis* analogs.

In the presented study, 99% of BC_5F_1 monosomic alien addition plants of triticale carried an additional $2S^k$ chromosome. Endo (2007) suggested, that when the gametocidal action is intense, gametes without the alien chromosome may suffer severe chromosome damages, resulting in sterility. In this study, one BC_5F_1 plant without $2S^k$ chromosomes suffered minor chromosome aberrations (**Figure 3**). It could be hypothesized that gametes without $2S^k$ can be fertile and develop into plants with chromosome aberrations as no chromosome fragment has been lost. This genetic phenomenon is of particular interest to breeders. Linking *Gc* loci with *Lr54* + *Yr37* leaf and stripe rust-resistant genes in triticale varieties would ensure maintenance of these traits in subsequent generations without the need for selection. But, on the other hand, triticale plants with centric chromosome breaks can be selected and used for the induction of Robertsonian translocations (RobTs) (Kwiatek and Nawracała, 2018).

The molecular analyses with the S14-410 marker confirmed that the preferential transmission of the $2S^k$ chromosome

occurred in all hybrid plants, from the F_1 generation to BC_4F_1. The *Lr54* + *Yr17* marker identification reflected the results of cytogenetic analysis of BC_5F_1 plants. The key result of this study was the development of monosomic $2S^k$(2R) substitution lines, which can be used for induction of chromosome translocations. There are several reports describing the methodology used for the production of monosomic substitutions. According to Faris et al. (2002), this can be achieved by crossing an alien chromosome addition line containing the gene of interest with a line that is monosomic for a homoeologous wheat chromosome. This approach was applied for development of 1RS.1DL chromosome translocation in triticale "Rhino" (Lukaszewski and Curtis, 2006). The 1RS.1DL translocation was later used for the induction of multi-breakpoint translocation lines (Lukaszewski, 2006, 2017).

It is reported that triticale is infected by the races specific to both: wheat and rye; however, it was noticed that triticale is more easily attacked by the wheat physiological forms of the rusts than by the rye ones (Arseniuk, 1996). After inoculation, a significant improvement of the resistance level was observed in introgressed plants in comparison with triticale cv. Sekundo plants. The mean level of leaf rust resistance was high for two independent replications of the experiment (2.96 ± 0.42 and 2.97 ± 0.48). Such low infection rate can be considered as a result of *Lr54* gene expression. In a similar study, Marais et al. (2005) developed a 2DS.2S^{k}L wheat-*A. kotschyi* line (called S14 translocation). The authors reported 96% resistant plants (72 tested) which were tested for resistance to eight *Pt* pathotypes (UVPrt2, UVPrt3, UVPrt4, UVPrt5, UVPrt8, UVPrt9, UVPrt10 and UVPrt13) and two *Pst* pathotypes (6E16A- and 6E22A-) endemic to South Africa. Moreover, it was shown that the S14 translocation evidently had preferential transmission (Marais et al., 2005). Intriguingly, monosomic $2S^k$(2R) substitution triticale plants, as well as control plants revealed the same, moderate resistance against stripe rust. In general, it is reported that triticale is more resistant against stripe rust as wheat. Moreover, triticale infection by *P. stiiformis* is highly dependent on plant growth stage and pathogen race (Rodriguez-Algaba et al., 2020). Hence, it could be possible that triticale "Sekundo" possesses a partial resistance against stripe rust and *Yr37* did not increase it.

In this study, the monosomic $2S^k$(2R) substitution plants were obtained through crossing between monosomic $2S^k$ addition line of triticale (M2S^{k}AL), containing *Lr54* loci, with another triticale line that was nullisomic for 2R chromosome pair (N2R). The cross-hybridization between M2S^{k}AL and N2R plants resulted in no offspring with the 2R chromosome in a monosomic condition (M2R). Hence, it can be hypothesized that gametocidal action of $2S^k$ chromosome killed gametes lacking it. Moreover, it could be possible that the inhibiting or suppressing factor of the $2S^k$ gametocidal action in triticale is located on the 2R chromosome. It is already known that some cultivars of common wheat possess genes that suppress the function of the *Gc* factors. Tsujimoto and Tsunewaki (1985) reported that that a suppressor gene (*Igc1*), that controls the suppression of *Gc* gene action on chromosome 3C of *A. truncialis*, is located on 3B chromosome of wheat "Norin 26." Similarly, Endo (1988) postulated that gametocidal action of the 4S chromosome of *A. sharonensis* or *A. longissima* is suppressed by the presence of 4B chromosome of wheat. It could be plausible that both the *Gc* gene and the suppressor loci are located on chromosomes of the same homoeologous group; however, these hypotheses require future investigation.

The classical methods basing on backcrossing and selection are still significant in plant breeding. There are several countries, which are considered as key crop producers (for example, France, Germany, Poland, etc.), place a strict set of regulations on the cultivation of genetically modified (GM) crops. Hence, classical breeding methods, enriched by the molecular (and cyto-molecular)-assisted selection and chromosome manipulation are still worthy endeavors. In addition, the genetic mechanisms that regulate the process of segregation distortion should be seriously taken into consideration as a natural tool for chromosome manipulation and engineering.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MK: conceptualization, methodology, formal analysis, writing original draft preparation, project administration, and funding acquisition. MK, WU, JB, and HW: validation. MK, JB, WU, RS, and AN: investigation. MK and HW: resources and supervision. MK and WU: data curation. MK and DP: writing—review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This research and the APC were funded by the National Centre for Research and Development, Poland (Narodowe Centrum Badań i Rozwoju, Polska), grant number LIDER/3/0004/L-8/16/NCBR/2017.

ACKNOWLEDGMENTS

We would like to acknowledge and thank Dr. Zofia Banaszak at the Danko Breeding Station, for providing seeds of triticale cultivars. In addition, we would like to thank all of the reviewers and manuscript editor for their careful review of the manuscript and for their excellent suggestions for improving our initial work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.509481/full#supplementary-material

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