EMERGING FUNGAL PLANT PATHOGENS

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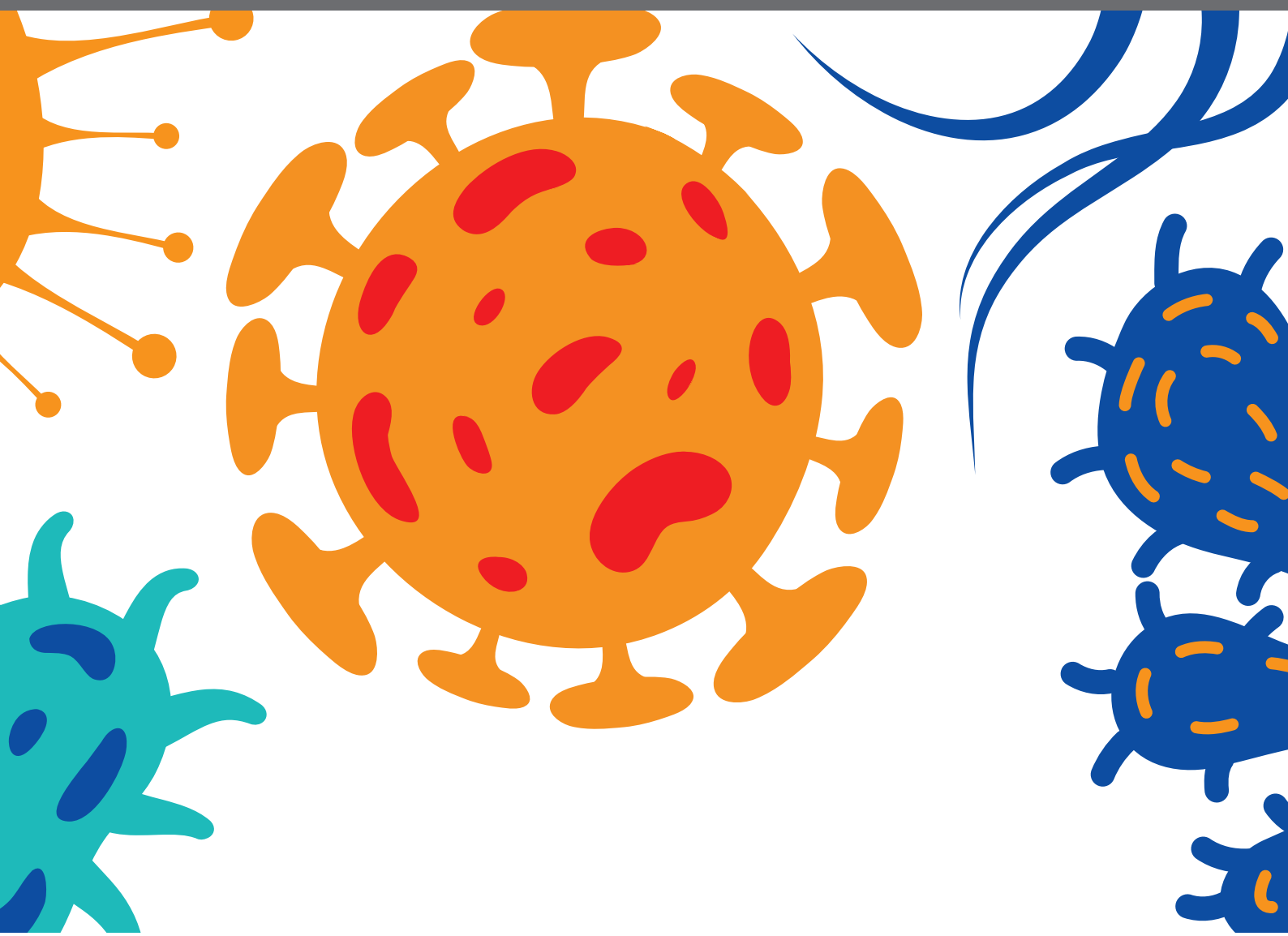

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EMERGING FUNGAL PLANT PATHOGENS

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EDITORIAL
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Editorial: Emerging Fungal Plant Pathogens

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Editorial on the Research Topic

Emerging Fungal Plant Pathogens

The occurrence of new and emerging phytopathogenic fungal pathogens is on the rise but has largely been overlooked because of inadequate detection methods (Fisher et al., 2012). Factors associated with such a phenomenon can be attributed to plant pathogens expanding beyond their normal geographic ranges due to globalization and international commerce, adaptive potential, climate and ecological changes as well as modern agricultural practices such as modified land uses and the profuse use of antifungal agents in agricultural practices (El-Sayed and Kamel, 2020). Emerging fungal pathogens are an increasing threat to ecosystems, global health, food security and global economy but remain neglected and understudied despite their potential devastating impact on economically important crops (Fones et al., 2020). These emerging pathogens can act as "true reservoirs" for future disease epidemics, but there are still numerous scientific challenges and research gaps to be resolved as to how these fungal pathogens are transmitted, evolving, adopting novel ecological strategies, switching hosts and causing infections. There is published evidence that common saprophytic fungi belonging to *Cryptococcus*, *Aspergillus* and *Penicillium* species are now emergent as potential plant pathogens. The latter can represent a major threat to staple crops such as rice, wheat, maize and potatoes either during cultural practices or during the post-harvest/storage stages (Alshannaq and Yu, 2017). If these pathogens are not detected and accurately identified in a timely matter and targeted disease-management strategies are not implemented, global food security could potentially be dramatically affected (Fones et al., 2020). To generate and promulgate better scientific insights into this new area of research, we proposed the Research Topic "Emerging Fungal Plant Pathogens". In this Research Topic, we accepted 10 articles, including 5 reviews and 5 original articles that focus on fungal characterization of emerging plant pathogenic fungi based on polyphasic approaches, their functional roles in diseases, their control methods, taxonomy, phylogeny, and evolution. It is anticipated that this Research Topic will enable plant pathologists to gain better insights into the phytopathogenic lifestyles, identification, phylogeny, host associations and evolution of emerging fungal pathogens. Several authors have contributed papers to this Research Topic and an overview of the scientific content is summarized below.

Gunasinghe et al. presented a comprehensive account of *Neopseudocercosporella capsellae*, the causative agent of white leaf spot of Brassicaceae, as an emerging pathogen. This pathogen was recently reported as causing damage to economically important Brassicaceae crops, including

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oilseed rape, vegetables, condiment, and forage brassicas. This review discusses the factors affecting the emergence of the disease and efficient disease control measures. Previously, a lack of information on critical aspects of this pathogen's life cycle limited the development of effective control measures. Gunasinghe et al. confirmed the ability of *N. capsellae* to switch between two morphologies (septate hyphae and single-celled yeast phase) on a range of artificial culture media (*in-vitro*) or *in planta* on the host surface before infection occurs. It was shown that the hyphae-to-yeast transformation occurs through the production of two morphologically distinguishable blastospore (blastoconidia) types (meso-blastospores and micro-blastospores) and arthrospores (arthroconidia).

Bipolaris sorokiniana (syn: *Cochliobolus sativus*) is the causal agent of a wide range of cereal diseases. Al-Sadi summarized the latest findings on *B. sorokiniana*, with specific emphasis on genetic, chemical, cultural, and biological control measures. Hazelnut (*Corylus heterophylla*), an important nut crop in China, is reportedly in decline due to the destructive effects of fungal branch canker and dieback. To document the main fungal pathogens from Chinese wild hazelnut, Gao et al. isolated 51 fungi. Three *Cytospora* and two *Diaporthe* species were identified based on morphological observations coupled with multi-locus phylogenetic analyses, and they also provided additional data on three new species, *viz. C. corylina*, *C. curvispora*, and *D. corylicola* while two known species, *C. leucostoma* and *D. eres* are also documented herein.

Thambugala et al. discussed how fungal antagonists play an important role in controlling plant pathogens and diseases. Since modern agricultural practices are shifting towards reducing the usage of chemically synthesized pesticides, various biocontrol methods, strategies and approaches are being used in plant disease management, and fungi are highlighted as one of the successful methods. Powdery mildew caused by *Erysiphe cichoracearum* seriously affects the yield and quality of tobacco leaves, and once it occurs, it often results in substantial economic losses. Jiao et al. investigated the biocontrol efficiency of *Bacillus amyloliquefaciens* YN201732 against *E. cichoracearum*. The results from *in vitro*, spore germination, and greenhouse-pot studies demonstrated that antimicrobial lipopeptides, especially bacillomycin D and fengycin, may contribute to the prevention and control of tobacco powdery mildew. A new fungal species, *Penicillium linzhiense* is isolated and described in Liang et al. with the potential for usage as a biocontrol agent, especially for economically important phytopathogens or emerging pathogens causing diseases on citrus or rice. Recent advancements in the improvement and application of molecular methods for diagnosing widespread and emergent plant pathogenic fungi are discussed in Hariharan and Prasannath. Grasslands are an ecologically and economically important component of the earth's vegetation, and fungal communities in grasslands play a huge role on the stability of grasslands. Karunarathna et al. addressed the taxonomy, phylogeny and ecology of grassland fungal pathogens and their interactions in grassland ecosystems. This review highlights the importance of understanding the behavior of fungal pathogens in highly diverse grasslands, providing novel insights for controlling diseases in commercial crop fields.

Climate change seriously impacts agricultural practices and food security. It has the potential to negatively affect numerous economically important crops in the future. In addition, perennial crops, such as tea, are particularly vulnerable. Climate change will not only affect crops but also likely affect crop-associated fungal pathogens. Tibpromma et al. predicted how future climatic conditions will impact tea and its associated pathogens.

In summary, this Research Topic with 10 scientific contributions consolidates and expands our knowledge regarding recent advances in plant fungal pathogens and their control measures. To facilitate the effective management of diseases, it is vital to accurately and rapidly detect and identify plant pathogenic fungi, especially ones emerging on economically important crops. In this context, the research articles published herein provide taxonomic information on fungal species supplemented with DNA sequence data to assist future plant pathologists and mycologists better identify similar species.

AUTHOR CONTRIBUTIONS

SK, SM and RJ drafted the editorial, and all authors contributed to editorial revision. All authors contributed to the article and approved the submitted version.

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REVIEW
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White Leaf Spot Caused by *Neopseudocercosporella capsellae*: A Re-emerging Disease of Brassicaceae

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White leaf spot can cause significant damage to many economically important Brassicaceae crops, including oilseed rape, vegetable, condiment, and fodder *Brassica* species, and recently has been identified as a re-emerging disease. The causal agent, *Neopseudocercosporella capsellae,* produces foliar, stem, and pod lesions under favorable weather conditions. *N. capsellae* secretes cercosporin, a non-host specific, photo-activated toxin, into the host tissue during the early infection process. The pathogen has an active parasitic stage on the living host and a sexual or asexual saprobic stage on the dead host. Where the sexual stage exists, ascospores initiate the new disease cycle, while in the absence of the sexual stage, conidia produced by the asexual stage initiate new disease cycles. Distribution of the pathogen is worldwide; however, epidemiology and disease severity differ between countries or continents, with it being more destructive in Subtropical, Mediterranean, or Temperate climate regions with cool and wet climates. The pathogen has a wide host range within Brassicaceae. *Brassica* germplasm show varied responses from highly susceptible to completely resistant to pathogen invasion and significant susceptibility differences are observed among major crop species. Cultural practices only provide effective disease control when the climate is not conducive. An increase in the susceptible host population and favorable weather conditions have together favored the recent rise in white leaf spot disease occurrence and spread. The lack of understanding of variation in pathogen virulence and associated resistant gene sources within brassicas critically limits the potential to develop efficient control measures.

Keywords: ***Neopseudocercosporella capsellae*, white leaf spot, Brassica, oilseed rape, cercosporin, disease resistance, disease management**

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INTRODUCTION

White leaf spot disease caused by *Neopseudocercosporella capsellae* (Ellis & Everhart) Videira & Crous is an important disease on many Brassicaceae including oilseed, wild, vegetable, condiment, and fodder *Brassica* species (Petrie and Vanterpool, 1978; Sumner et al., 1978; Barbetti and Sivasithamparam, 1981; Cerkauskas et al., 1998; Ocamb, 2014). Recently, there has been

a worldwide increase in pathogen activity and the disease is now identified as a re-emerging disease on oilseed rape and/or oriental *Brassica* vegetables, particularly in the UK (Inman, 1992; Anonymous, 2016), the USA (Ocamb, 2014, 2016) and in Australia (Van de Wouw et al., 2016; Murtza et al., 2019). While *N. capsellae* is commonly a leaf pathogen, it also produces pod and stem lesions resulting in "gray stem" disease (Petrie and Vanterpool, 1978; Inman et al., 1999). Optimum climatic conditions for white leaf spot disease development are 15–20°C (Koike et al., 2007) with frequent precipitation (Fitt et al., 1992).

The pathogen has a wide host range (Boerema and Verhoeven, 1977; Gudelj et al., 2004), infecting a variety of cultivated crucifers, including oilseed, and vegetable brassicas. Also, *N. capsellae* has been isolated from leaf lesions on "wild" or "weedy" crucifers such as wild radish (*Raphanus raphanistrum*) and wild turnip (*Brassica rapa* ssp *sylvestris*) (Marchionatto, 1947; Deighton, 1973; Morris and Crous, 1994; Francis and Warwick, 2003; Maxwell and Scott, 2008). It has also been recorded causing disease on false flax (*Camelina sativa*), a recently introduced oilseed crop in Europe (Föller and Paul, 2002), chinese cabbage, mustard type *Brassica* vegetables, and cauliflower (Lancaster, 2006). Moreover, *N. capsellae* can produce leaf spots interspersed with symptoms caused by other *Brassica* pathogens such as *N. brassicae* (Chevall.) Videira & Crous (syn. *Mycosphaerella brassicicola*), the cause of ringspot (Fitt et al., 2006), Pyrenopeziza *brassicae* Sulton & Raw, the cause of light leaf spot, and *Leptosphaeria maculans* Ces. & De Not., the cause of blackleg disease, resulting in severe disease epidemics (Thomas et al., 2019).

The Hosts, Brassicaceae

The family Brassicaceae contains a diverse collection of economically important weeds and cultivated varieties including major sources of cooking oil, vegetables, and condiments that contribute to 12% of the world's edible vegetable oil production and 10% of the world's vegetable production (Cheng et al., 2011). *Brassica* species have broad phenotypic plasticity as demonstrated by differences between oilseed varieties, root vegetable crops such as turnip, and leafy forms such as cabbage. Among six widely cultivated vegetable and oilseed crops, three amphidiploid species: *B. napus* (AACC, 2n = 38), *B. juncea* (AABB, 2n = 36), and *B. carinata* (BBCC, 2n = 34) were formed through interspecific hybridization between three diploid *Brassica* species: *B. rapa* (AA, 2n = 20), *B. nigra* (BB, 2n = 16), and *B. oleracea* (CC, 2n = 18) (Song et al., 1988). This broad host diversity impacts on the pathogen host range, pathogen diversity, epidemiology, and disease reaction of the host-pathogen interaction.

Economic Impacts

The impact of white leaf spot disease have become increasingly important and economically significant outbreaks have been reported on a variety of *Brassica* crops worldwide (Petrie and Vanterpool, 1978; Penaud, 1987; Inman, 1992; Ocamb, 2014). Severe losses due to white leaf spot disease can occur at the seedling stage (Ocamb, 2014) or in older plants when susceptible varieties are grown under environmental conditions favorable for disease development (Reyes, 1979; Penaud, 1987; Barbetti and Khangura, 2000). In both these situations, and particularly when the environment is conducive, white leaf spot causes significant yield losses of at least 30% in oilseed brassicas predominantly through defoliation and the development of pod lesions (Penaud, 1987; Barbetti and Khangura, 2000). Pod infections by *N. capsellae* can cause 15% yield losses in France (Penaud, 1987). A recent study in Australia demonstrated a linear decline in rapeseed yield (up to 32%) in the field with increasing white leaf spot disease incidence and severity (Murtza, You and Barbetti, unpubl). In leafy greens, the disease causes yield reductions by reducing the quality of the foliage or making them unacceptable to consumers. The disease also can cause problems in commercial operations such as mechanical harvesting (Sumner et al., 1978).

Despite white leaf spot disease being a long-standing severe disease of Brassicaceae worldwide, there are no previous reviews relating to this disease. This review evaluates research into epidemiology and disease management. Particular focus is on biological aspects to explain the basis of the recurrence of severe epidemics, the infection process, the pathogen diversity, and on the identification, deployment, and mechanisms of host resistance.

CURRENT DISTRIBUTION

Neopseudocercosporella capsellae has been recorded from all subcontinents except Antarctica, over a wide range of climatic conditions from temperate to tropical, including the principal oilseed rape or mustard producing countries of the European Union, China, India, Canada, Australia, and Japan (CMI, 1986). However, the disease severity and incidence differ geographically due to variation in pathogen populations, host species, cultivars grown, different agricultural practices adopted, and prevailing local climatic conditions (West et al., 2001), in particular, temperature, humidity, and rainfall (Siebold and Von Tiedemann, 2012).

The UK and Europe

In continental Europe, white leaf spot is widespread on oilseed rape (Perron and Souliac, 1990; Perron and Nourani, 1991; Inman, 1992; Söchting and Verreet, 2004; Krzymański, 2006), or other *Brassica* crops, including swede (*B. napobrassica*), turnip (*B. rapa* subsp. *rapa*), mustard (*B. juncea*), chinese cabbage (*B. rapa*, subspecies *pekinensis* and *chinensis*), and cabbage (*B. oleracea*) (Koike et al., 2007). The disease is most common and severe in northern Europe (Koike et al., 2007), particularly where oilseed rape is grown as a winter crop (West et al., 2001). Countries with temperate and/or mixed temperate and Mediterranean climates such as France have historically had economically damaging disease on oilseed rape (Tromas and Vincent, 1988; Perron and Nourani, 1991). Since its detection in Poland (1987), white leaf spot disease has remained a common disease on oilseed rape (Frencel et al., 1991; Starzycki et al., 2007). White leaf spot has also become increasingly important in oilseed rape crops in the UK (Anonymous, 2016). The trend toward wetter and warmer winters in parts of the UK and Europe is suggested as the cause (Inman, 1992; Rosenzweig et al., 2001).

Canada and the USA

In Canada, white leaf spot is one of the common diseases of both oriental vegetables and oilseed rape (Petrie and Vanterpool, 1978; Petrie et al., 1985; Cerkauskas et al., 1998), and severe outbreaks have been reported (Reyes, 1979; Petrie, 1986). The major oilseed and oriental vegetable fields are in Alberta and Saskatchewan, where oilseed rape is grown during wet, mild summers that favor disease initiation and spread.

In the USA, white leaf spot is a common disease of *Brassica* crops, particularly on vegetable brassicas (Sumner et al., 1978, 1991; Kahn et al., 2007; Ocamb, 2014; Ocamb et al., 2015). For example, in the Pacific Northwest, which has a Mediterranean type climate with mild and wet winters, control of the disease is a priority (Carmody, 2017). Recently, conditions have been warmer, and with higher winter rainfall (Dalton et al., 2017), which along with the presence of a susceptible host species, could be the cause of the increase in white leaf spot. Apart from vegetable crops, current interest in brassicas as cover crops, seed crops, oilseed, and biofuel crops or in broadacre crop rotations (Claassen, 2016) has contributed to increased diversity of *Brassica* crops grown in the region (Carmody, 2017) including the highly susceptible *B. juncea* (Gunasinghe et al., 2014). In California, high white leaf spot incidence on crucifers has been associated with high winter rainfall (Campbell and Greathead, 1978). Long-term climate change predictions of warmer winters and increased precipitation, will favor greater white leaf spot disease severity and spread in the USA.

Australia

White leaf spot has been reported from major oilseed *Brassica* growing states of Australia and is considered an important disease (Barbetti and Sivasithamparam, 1981; Murtza et al., 2018). Oilseed rape (predominantly spring *B. napus* cultivars but also *B. juncea*) is a major winter crop in the Mediterranean climatic regions through to the more temperate climatic regions (Charles et al., 2010; Hope et al., 2015). The pathogen was first found on a range of vegetable brassicas in 1956, 1984, and 1987 (Eshraghi et al., 2005), and only later on oilseed rape, *B. napus* in 1979 (Barbetti and Sivasithamparam, 1981), and on *B. juncea* in 2005 (Eshraghi et al., 2005). In more recent studies, white leaf spot was recorded as widespread and damaging on *B. napus* and *B. juncea* (Couchman and Hollaway, 2016; Van de Wouw et al., 2016; Murtza et al., 2018), as well as on oriental *Brassica* vegetables (Burt et al., 2006; Len, 2009).

There is an increasing white leaf spot disease incidence in all oilseed-growing regions (Van de Wouw et al., 2016; Murtza et al., 2018). Several changes may have led to this increase. There has been a recent increase in susceptible host populations. First, through sowing blackleg disease resistant *B. napus* varieties (Sprague et al., 2006) some of which are highly susceptible to white leaf spot disease (Eshraghi et al., 2007; Gunasinghe et al., 2014, 2016d). Secondly, through the recent wide deployment of drought tolerance but highly susceptible *B. juncea,* (Burton et al., 2003; Gunasinghe et al., 2014, 2016d, 2017a). The virulent Victorian isolates of *N. capsellae* strains may have also spread throughout Australia (Murtza et al., 2019). Finally, environmental factors may have led to conditions more favorable for white leaf spot disease.

Asia

White leaf spot is not reported as a damaging disease on oilseed rape or vegetable brassicas in the two major canola growing countries in Asia, China, and India, although the pathogen's occurrence has been recorded (Gangopadhyay and Kapoor, 1976; CMI, 1986). In India, the pathogen was first reported on vegetable *B. rapa* in 1973 (Gangopadhyay and Kapoor, 1976). In both countries, oilseed rape is grown as a winter crop in regions with cool but dry winters (Lal et al., 2001; Wang, 2002). In China, it is mainly grown in the south across the Yangtze River valley (Yang and Zheng, 2016) and in India in the northern states such as Rajasthan, Haryana, Punjab, and Uttar Pradesh (Chauhan et al., 2011). Dry weather during the crop season, and/or possibly differences in susceptibility of the locally grown species or varieties, could contribute to the comparatively low white leaf spot incidence and severity in China and India.

In tropical Asian regions (Taiwan, Sri Lanka) or subarctic regions (Russia), *N. capsellae* has been found on *Brassica* crops or weedy brassicas (CMI, 1986). However, there are no records of significant damage to crops. In 1966, white leaf spot was recorded as a new disease in the Soviet Far East on cruciferous vegetables (Nelen, 1966). However, Jedryczka et al. (2002) reported that white leaf spot was absent during their survey of North-Western or Central Russia in 1996–2000. The prevailing harsh climatic conditions (extremely low temperatures) may not favor the pathogen survival and disease development in these regions.

TAXONOMY, MORPHOLOGY, AND PHYSIOLOGY OF THE PATHOGEN

Taxonomy

Neopseudocercosporella capsellae belongs to the Cercospora complex (Cercosporoid), where differentiation within the complex is based on morphological characteristics of spores and reproductive structures (Braun, 2001). Re-evaluations of morphological characters of cercosporoid hyphomycetes based on molecular data are underway, and comprehensive revisions of some genera have been published (Crous et al., 2001; Videira et al., 2016). Within the complex, *N. capsellae* is in the class *Dothideomycetes,* the family Mycosphaerellaceae (Deighton, 1973), and the genus *Neopseudocercosporella* (Videira et al., 2016). Twenty-six synonyms are documented for this pathogen previous to the recent name change to *Neopseudocercosporella capsellae* (Videira et al., 2016). The other member of the genus, *N. brassicae* (Chevall.) Videira & Crous (syn. *Mycosphaerella brassicicola*), the cause of ringspot disease, is closely related to *N. capsellae* (Videira et al., 2016), however the two species are reproductively isolated morphospecies (Gudelj et al., 2004). Although, the two species can produce coexisting leaf spots on *Brassica* species (Fitt et al., 2006), leaf symptoms caused by each of the individual pathogens are distinguishable (Petrie and Vanterpool, 1978; Corlett, 1999). Despite this, based on recent molecular data, Videira et al. (2016) suggest that these pathogens could be synonymous, but this is currently unresolved.

The teleomorph: *Mycospharella capsellae* was first reported in 1991 (Inman et al., 1991). A full taxonomic description of the anamorph was proposed by Deighton (1973), and the teleomorph *M. capsellae* and its' sexual structures have been described by Inman et al. (1991).

Growth and Colony Characters on Artificial Media

On artificial media, the pathogen's growth rate is noticeably slow, taking 3 weeks for a colony to reach 1–2 cm in diameter (Crossan, 1954). Slow growth is a likely reason for under-reporting of the pathogen as the disease causal agent. On a variety of culture media, it produces dark to olivaceous-gray stromatic colonies with dentate margins (Inman, 1992). Optimum growth from hyphae occurs between 20–24°C and at pH 5.5–7.0 on potato dextrose agar (PDA) (Okullo'kwany, 1987). Young colonies produce thin and hyaline hyphae becoming thick-walled, septate, brown hyphae with stroma-like or sclerotia-like structures, which give rise to conidia (Crossan, 1954). However, *N. capsellae* does not sporulate on commonly used artificial media including PDA (Miller and McWhorter, 1948; Crossan, 1954), but produces conidia when the growing colonies on V8 or distilled water agar are exposed to near-UV light around 365–370 nm (Inman et al., 1991). Petrie and Vanterpool (1978) observed and extracted a red/purple/pink pigment produced by *N. capsellae* mycelial mats. More recently, this pigment has been confirmed as the mycotoxin cercosporin (Gunasinghe et al., 2016b).

Cercosporin—The Toxin Produced by the Pathogen

Cercosporin (4,9-dihydroxyperylene-3,10-quinon) plays a significant role in pathogenesis by *N. capsellae* (Gunasinghe et al., 2016b, 2017b). Cercosporin applied to susceptible *Brassica* cotyledons induces disease symptoms, and there is a strong positive correlation between the degree of sensitivity to cercosporin and the degree of host susceptibility to *N. capsellae* (Gunasinghe et al., 2016b). It has been proposed that the high susceptibility of the oilseed varieties of *B. juncea* (Indian mustard) from India is due to high sensitivity to cercosporin, that facilitates the early establishment of the pathogen on the host tissue (Gunasinghe et al., 2016b, 2017b). Further, across different isolates, *in vitro* cercosporin production on V8 juice agar and the level of observed virulence on *Brassica* seedlings was highly correlated (Gunasinghe et al., 2016b).

As recorded in other *Cercospora* species (Daub and Ehrenshaft, 2000), *N. capsellae* secretes cercosporin into the host tissue during the infection process (Gunasinghe et al., 2016b, 2017b), and cercosporin has been isolated from diseased lesions of 16 different hosts (Fajola, 1978) including white leaf spot lesions on oilseed rape caused by *N. capsellae* (Gunasinghe et al., 2016b). Cercosporin is a non-host specific toxin, with broad toxicity against a wide range of organisms, including bacteria, fungi, plants, and animals (Daub, 1982). Cercosporin plays a prominent role in invasion of susceptible hosts and/or assists in the interactions with other organisms on the leaf surface (Hartman et al., 1988). It belongs to a group of photosensitizing molecules that absorb light energy and convert it into a long-lived electronically excited triplet state that produces activated oxygen species (Hartman et al., 1988; Leisman and Daub, 1992; Daub and Ehrenshaft, 2000). The light-activated cercosporin damages host tissue through peroxidation of cell membranes resulting in rapid cell death and nutrient leakage. This leakage of nutrients into the intercellular spaces facilitates the pathogen's initial establishment on the plant host (Daub, 1982).

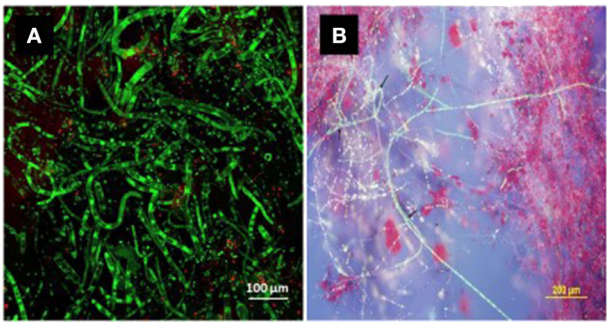

FIGURE 1 | Bright green fluorescing hyphae of *Neopseudocercosporella capsellae* (isolate UWA Wlra-7) on malt extract agar detected by confocal microscopy. The green fluorescence confirms the cercosporin inside the hyphae in a chemically reduced state protecting the pathogen from its toxicity (Daub et al., 2000; Chung et al., 2002) [**(A)** cercosporin producing hyphae and, **(B)** cercosporin producing hyphae and secreted red cercosporin crystals among green hyphae].

Temperature, light conditions, and growth medium interact significantly to regulate *in vitro* cercosporin accumulation, and patterns of regulation vary among different species or even among isolates of the same species (Jenns et al., 1989). The production of cercosporin is not completely inhibited in the dark, although light is a major factor required for the process (Jenns et al., 1989; You et al., 2008). Salts, metal ions, and buffers all influence cercosporin production, but not changes in pH (You et al., 2008). The effect of temperature (Daub and Ehrenshaft, 2000) and composition of the growth medium (Fajola, 1978; Jenns et al., 1989; Gunasinghe et al., 2016a), is highly isolate-specific. While, PDA is the best medium for toxin production by other cercosporin producers (Fajola, 1978; Jenns et al., 1989), malt extract agar or V8 agar is better for *N. capsellae* (Gunasinghe et al., 2016a,b) (**Figure 1**). However, conclusions about whether an isolate produces cercosporin cannot be made based on cultural studies alone (Daub et al., 2000) as some isolates that do not produce cercosporin on artificial culture media, do produce it *in planta* (Upchurch et al., 1991).

DISEASE EPIDEMIOLOGY

Host Plant Infection and Symptoms

Under favorable environmental conditions, *N. capsellae* conidia (Petrie and Vanterpool, 1978; Penaud, 1987; Barbetti and Khangura, 2000) or ascospores (Inman et al., 1999) produce lesions on the leaves, while only conidia are responsible for stem and pod lesions that appear later in the crop season

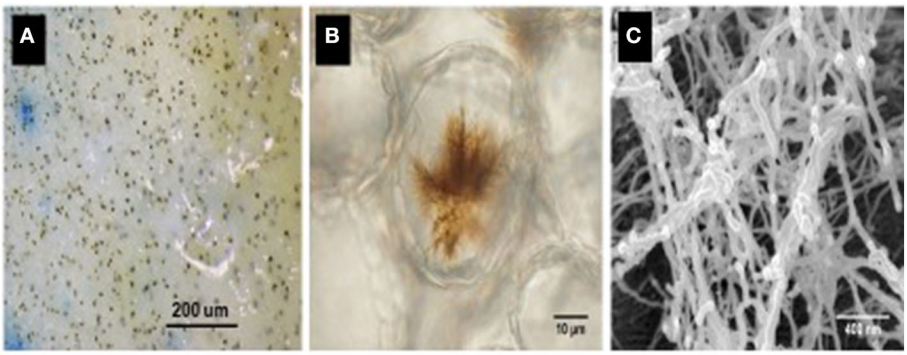

FIGURE 2 | Brown infection structures produced by *Neopseudocercosporella capsellae* on a highly susceptible *Brassica juncea* cotyledon, 24 h post-inoculation as seen under a light microscope (decolorized by immersing in acetic acid: ethanol: water (2:2:1) solution at 25°C for 3–4 days) **(A,B)**. Scanning electron micrograph illustrating fine thread-like structures observed inside the host within the cortical tissue of an infected cotyledon **(C)**.

(Inman et al., 1991). Optimal conidial germination occurs at 20–24°C and is inhibited below 8°C or above 28°C (Crossan, 1954). Germination is usually from apical cells, or less frequently from basal cells (Morris and Crous, 1994). The conidia can produce multiple germ tubes from each conidial cell (Petrie and Vanterpool, 1978; Gunasinghe et al., 2016c) and cleavage at a septum of a conidium can produce multiple conidia, each producing a germ tube to infect the host (Gunasinghe et al., 2016c). Hyphae of germinating spores invade the host tissue through natural openings such as stomata (Crossan, 1954; Gunasinghe et al., 2016c). Temperatures of 18–19°C and high humidity (100%) with at least 8 h of continuous leaf wetness are ideal for white leaf spot infections (Inman, 1992). During the early infection stage on highly susceptible host species/cultivars, *N. capsellae* produces unique brown structures that contain cercosporin. These brown structures form highly branched networks on the cotyledon surface and/or internally within the leaf cortical tissue (Gunasinghe et al., 2017b) (**Figure 2**). After successful infection, the appearance of disease symptoms on oilseed rape occurs within 6–8 days at the optimum temperature range of 15–20°C and the pathogen takes longer to produce lesions below optimum temperatures (Perron and Nourani, 1991; Inman et al., 1997).

White leaf spot lesions first appear on older lower leaves, and severe infections may induce leaf fall (Barbetti and Khangura, 2000). The symptoms on brassicas can vary depending upon the degree of leaf surface waxiness or the host species (Crossan, 1954). In particular, on oilseed rape, *N. capsellae* initiates infections on leaves observable as numerous brown, elongated spots that later become white or pale beige (**Figure 3A**). These leaf lesions can be up to 1 cm in diameter and subsequently coalesce to form large irregular shaped lesions (**Figure 3B**). The mature lesions, usually surrounded by a chestnut-brown margin with a distinct delimitation between healthy and diseased tissues, are visible on upper and lower leaf surfaces. The pathogen produces evenly distributed tufts of conidiophores on upper and lower surfaces (amphigenous) of the diseased leaf (Deighton, 1973).

Related *Brassica* pathogens can also develop similar symptoms on leaves. Ringspot disease symptoms caused by *N. brassicae* could be misleading particularly when developed under dry conditions in the absence of spermogonia and/or perithecia in typical zonate arrangement (Petrie and Vanterpool, 1978; Corlett, 1999). On oilseed rape, the early development stage of blackleg caused by *L. maculans* can resemble white leaf spot, as leaf lesions are first visible as brown discolored areas. However, white leaf spot lesions are easily distinguished from mature blackleg lesions, which have small black pycnidia within the lesions and exhibit shot-holes when they age (Brun et al., 1997). Light leaf spot disease of brassicas caused by *P. brassicae* causes irregular lesions with white spore pustules (Claassen, 2016) that can be misidentified as white leaf spot disease. However, older light leaf spot lesions are much larger than *N. capsellae* lesions and have a bleached and papery center, and the infection of the leaf can cause leaf distortion (McCartney and Lacey, 1990).

The leaf spot symptoms in *Brassica* species with waxy leaves (cabbage) or turnip, mustard, and chinese cabbage varieties, differ from each other and may not resemble the white leaf spot lesions described on other hosts (Crossan, 1954) (**Figures 3C–F**). For example, on cabbage, initial dark gray or black dendritic lesions may resemble downy mildew symptoms caused by *Hyaloperonospora parasitica* (Pers.), but mature white leaf spot lesions become more or less rectangular or rounded with well-defined margins with ashy black center (Miller and McWhorter, 1948). In contrast, typically round, semi-transparent larger lesions with brownish-gray centers with well-defined brown or tan margins are typical symptoms on turnip and wild mustard (Miller and McWhorter, 1948).

The pathogen can colonize stems and pods as the crop matures. The infection of stems or pods initially results in gray to black lesions (**Figure 4**). Geographic areas in which the sexual stage occurs, numerous tiny dark specks appear within the gray-speckled areas due to the formation of the sexual stage (Petrie and Vanterpool, 1978). These stem lesions are somewhat superficial and do not damage the pith (Inman, 1992). Pod lesions can be mistaken for *Alternaria* spp. lesions. However,

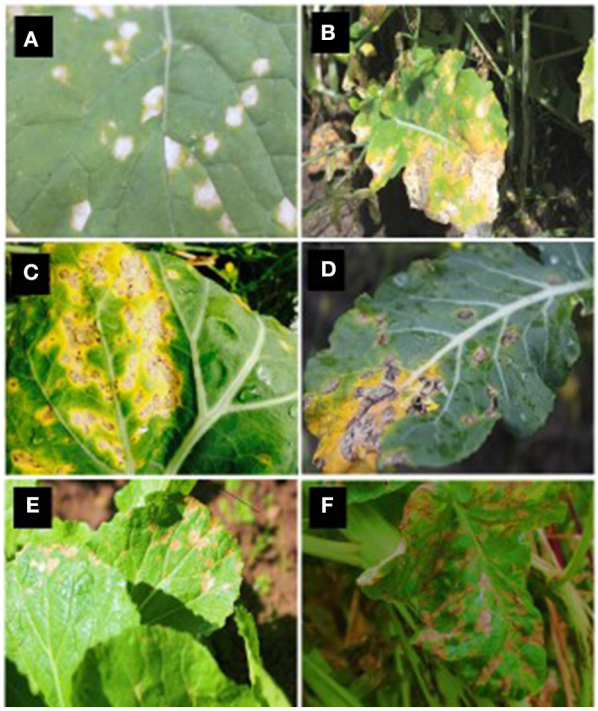

FIGURE 3 | White leaf spot symptoms caused by *Neopseudocercosporella capsellae* on different hosts. Leaf lesions on oilseed rape: *Brassica napus* **(A)** and *B. juncea* **(B)**, vegetable brassicas: *B. oleracea var. capitata* (cabbage - **C**), *B. oleracea var. italica* (broccoli - **D**), *B. campestris* var. *chinensis* (Chinese cabbage - **E**), and *Raphanus sativus* (radish - **F**).

FIGURE 4 | Stem and pod lesions caused by *Neopseudocercosporella capsellae* on *Brassica nigra* **(A)** and *B. juncea* **(B)**.

N. capsellae lesions have dark reticulation within the brown pod-spot and a less well-defined margin (Lane and Gladders, 2008) (**Figure 4**). These pod lesions can expand rapidly to cover large areas with depressed centers (Carmody, 2017). By harvest, plants can become completely discolo'red with the entire field of plants appearing purple or gray (Anonymous, 2020).

Disease Cycle and Survival Mechanisms

During a typical disease cycle, *N. capsellae* maintains the infection chain by surviving on crop debris as a saprobe (Penaud, 1987; Inman, 1992; Barbetti and Khangura, 2000). Less frequently, the pathogen moves from a crop to a susceptible weedy host (green bridge) (Petrie and Vanterpool, 1978) such as *R. raphanistrum* (wild radish) (Gunasinghe et al., 2016a) and wild mustard (*Sinapsis arvensis*) (Miller and McWhorter, 1948) and back to a crop.

The saprobic stage of *N. capsellae* could be either resting structures produced by the asexual stage (Petrie and Vanterpool, 1978; Penaud, 1987; Barbetti and Khangura, 2000) or the sexual stage (Inman et al., 1991). Simultaneous occurrence of stromatic hyphae that produce conidia (Barbetti and Sivasithamparam, 1981; Penaud, 1987) and the sexual stage that produces ascospores (Inman, 1992) has not been recorded. Nevertheless, one or the other is responsible for the pathogen survival in different localities. The pathogen, *N. capsellae* is, therefore, capable of completing its disease cycle with (Inman, 1992), or without the sexual stage (Penaud, 1987; Barbetti and Khangura, 2000).

When the sexual stage is present, separate roles for ascospores and conidia are proposed, and there is no concurrent occurrence of both spore types (Inman et al., 1999). The sexual stage is monocyclic and is only initiated at the end of the crop season on hard crop residues such as stems, racemes, or pods, but not on leaf residues or green tissue, with ascospores released early in the next crop season (Inman et al., 1991). These ascospores require both wetness and light for induction. They are usually released in response to dew or rain, and occur within a diurnal cycle that peaks between 05.00–19.00 h. These air-borne ascospores are responsible for the first appearance of the disease in newly sown crops in autumn (Inman, 1992).

Secondary disease spread is by asexual splash-dispersed (Fitt et al., 1992) conidiospores produced on mature leaf lesions. Rain splash can only deliver conidia up to 10–20 cm vertically (Walklate et al., 1989), and therefore, the vertical progress of the disease can be halted by several weeks of dry weather during the stem elongation period (Inman et al., 1993). The asexual stage is polycyclic, the pathogen completes several life cycles in one cropping season (Inman, 1992). Development of the sexual stage is the primary survival mechanism in the UK, and failure to produce adequate stem or pod lesions would be a limiting factor for the pathogen's survival and subsequent disease carryover (Inman et al., 1993).

Elsewhere, in the absence of the sexual stage, *N. capsellae* survives the intercrop season through asexual resting structures commonly found on crop debris (Petrie and Vanterpool, 1978; Penaud, 1987; Barbetti and Khangura, 2000), including leaf remains (Crossan, 1954). Dark brown, vacuolated hyphae with larger cells bearing large nuclei (Inman, 1992) present in older parts of the stem or leaf lesions are responsible for producing these resting structures known as stromatic mats (Petrie and Vanterpool, 1978) or stromatic knots (Reyes, 1979). These stromatic mats are capable of producing conidial inoculum under favorable conditions to infect autumn/winter sown crops in Australia and France (Penaud, 1987; Barbetti and Khangura, 2000) or summer-sown crops in Canada (Petrie and Vanterpool, 1978) (**Figure 5**). Crossan (1954) demonstrated that *N. capsellae* persists as thick, dense mycelium under the epidermis, for at least

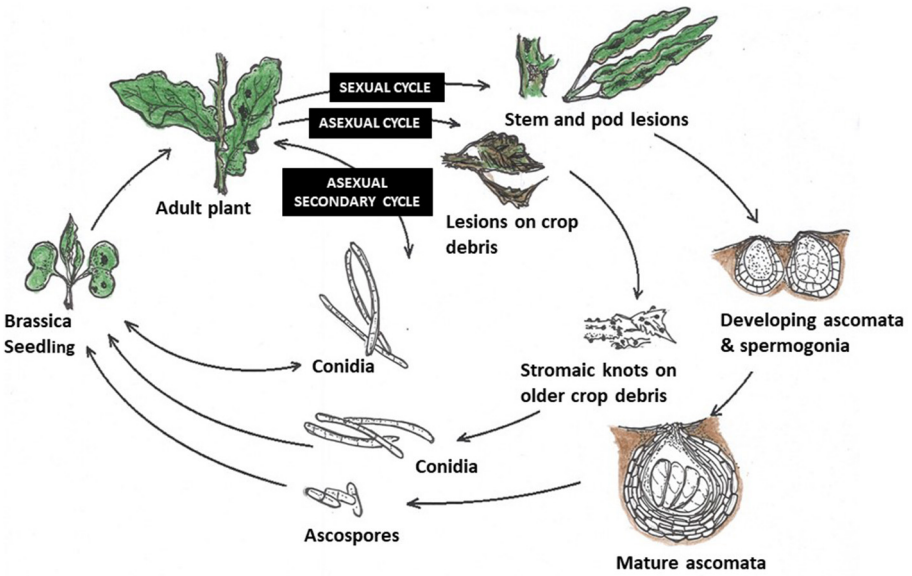

FIGURE 5 | Life cycles of *Neopseudocercosporella capsellae*: (i) Sexual cycle—as found in the UK on oilseed rape (Inman et al., 1999)—Initial infection is by ascospores. The first symptoms on leaves are by contamination from air-borne ascospores produced by mature ascomata. Mature lesions on leaves then produce conidia, which are splash-dispersed and responsible for secondary infections within the crop. Sexual structures on stem and pod lesions develop and release ascospores in the following crop season. (ii) Asexual cycle—proposed life cycle for *N. capsellae*, without the sexual stage (initial infection is from conidia) as happens in Australia (Barbetti and Khangura, 2000), Canada (Petrie and Vanterpool, 1978) and France (Penaud, 1987). In the absence of any sexual stage, carryover to initiate new epidemics is likely from splash-dispersed conidia produced by infested crop residues. (iii) Secondary cycle - secondary disease spread within and between crops is reliant upon splash-dispersed conidia produced on mature leaf lesions initiated by either ascospores or conidia.

9 months on turnip leaf residues during the hot, humid summers, and cool, wet winters in North Carolina, USA. The impact of different environmental conditions on the survival rate of these stromatic hyphae to produce conidial inoculum is unknown.

Teleomorph (Sexual) Stage Occurrence

Mycosphaerella capsellae, the sexual stage of *N. capsellae* naturally occurs in the UK (Inman, 1992). However, whether *N. capsellae* is heterothallic or homothallic is unknown. All attempts to produce ascospores with different isolate combinations from the UK only showed development up to the spermatial stage and did not form ascospores under laboratory conditions (Inman et al., 1991). The sexual stage has not been found in other European countries. This occurs with some other *Brassica* foliar pathogens. For example, the production of the sexual stage of *P. brassicae* (light leaf spot) is frequent in the UK, but absent in Germany (Siebold and Von Tiedemann, 2012) or Poland (Karolewski et al., 2004). Similarly, the sexual stage of *Alternaria* spp. (Alternaria blight) is seldom found in Germany, but is frequent in the UK (Siebold and Von Tiedemann, 2012). The widespread climatic variations in oilseed growing areas across these regions may cause these differences (Karolewski et al., 2004).

The only report of the sexual stage of *N. capsellae* outside of the UK is in Canada, where the spermatial stage on stems of *Capsella bursa-pastoris* was identified but did not progress to the next stage to complete the sexual cycle (Petrie and Vanterpool, 1978). Inman et al. (1991) reported that the stromatic knots in older lesions are the primodia for ascomata or spermogonia, which is the initial stage of the sexual cycle. These primordia can possibly switch from the asexual stage to the sexual stage under particular conditions. However, the conditions required for the progression of the spermatial stage into spermogonia or ascomata are unknown (Inman et al., 1991). Unlike in Europe, UK, and Australia, oilseed rape in Canada is grown in summer (West et al., 2001) and the sexual stage should, therefore, be able to resist harsh winter weather conditions such as subzero temperatures and snow cover, to be able to release ascospores after the sexual stage. However, climatic differences across the seasons in Canada may not meet the requirements of the different stages of the *N. capsellae* sexual cycle.

It is still possible that there is a cryptic sexual stage taking place in Australia or continental Europe as there have been no systematic surveys to determine the existence of the sexual stage in these regions. Inadequate understanding of the genetic basis of initiation of sexual reproduction, and environmental conditions required to develop sexual structures, limit prediction of the existence of the sexual stage. Therefore, the presence of the sexual stage in some continents/countries, including Australia, remains uncertain.

Pathogen Dispersal and Disease Spread

Current literature suggests, where the sexual stage does not occur, that the less efficient conidial dispersal mechanism could limit disease initiation, development, and spread, as both, initial infection and secondary disease spread depend on short distance splash-dispersed conidia (Petrie and Vanterpool, 1978; Penaud,

1987; Barbetti and Khangura, 2000). However, *N. capsellae* conidia have been readily collected in spore traps positioned 1 m or more above infested residues and even in adjacent fields, suggesting that some air-borne conidial dispersal also occurs over greater distances than predicted from rain-splash alone (MJ Barbetti unpubl.).

Primary pathogen dispersal media for *N. capsellae* are rain (Fitt et al., 1992) and wind (Inman et al., 1999). In the UK, the introduction of the disease into adjacent locations is likely to be via air-borne ascospores that can travel longer distances (Inman et al., 1999). In areas where only conidia are produced, pathogen introduction over short distances would likely be through rain splash (Fitt et al., 1992), and over moderate distances by water, soil, animals (Crossan, 1954), and, as suggested above, possible short distance air dispersal. Long-distance dispersal is likely to be by infected seed, infected crop residues, machinery, and people (Crossan, 1954; West, 2014). However, two independent studies by Petrie and Vanterpool (1978) and Carmody (2017) found no evidence for transmission of disease through infected seeds.

DISEASE RESISTANCE MECHANISMS

Limited information is available on disease resistance mechanisms associated with *N. capsellae*. Gunasinghe et al. (2016c) named at least two possible resistance mechanisms against pathogen invasion in highly resistant *B. carinata* genotype ATC94129P. The first is where conidia on the cotyledon surface rapidly disintegrated leading to poor conidial germination, and it was hypothesized that this effect was due to physical or chemical factors related to epicuticular wax crystalloids (**Figure 6A**). The second is where rapid stomatal closure occurs upon pathogen recognition (**Figures 6A,B**) and low stomatal density limits the number of entry points for the pathogen, as *N. capsellae* does not produce appressoria or special infection structures that enable direct penetration of the host plant epidermis (Crossan, 1954; Gunasinghe et al., 2016c). In *B. juncea* and *B. napus,* impeded germination, growth, and penetration in less resistant varieties resulted in reduced infection rates and consequently less disease development. Apart from low stomatal density, the degree of leaf wettability is another non-biochemical character responsible for resistance in oilseed rape against *N. capsellae* (Inman, 1992).

Although some pre-invasive host resistance mechanisms are known for *N. capsellae,* a detailed histopathological study is needed to understand possible post-invasion resistance mechanisms. For instance, hypersensitive reactions (Li et al., 2003) and lignin formation (Sharma et al., 2008) limiting the growth of the pathogen inside the host tissue (Li et al., 2008), have been reported against other *Brassica* foliar pathogens. Further, the specific details of the biochemical and molecular events occurring on host plant tissues during disease progression in *N. capsellae—Brassica* spp. pathosystem remains largely unknown with no study describing the biochemical changes in the host that accompany challenges by this pathogen. Systematic studies to understand biochemical mechanisms and molecular pathways responsible for *N. capsellae* resistance in highly resistant species such as *B. carinata* will offer new avenues for enhancing resistance in susceptible species.

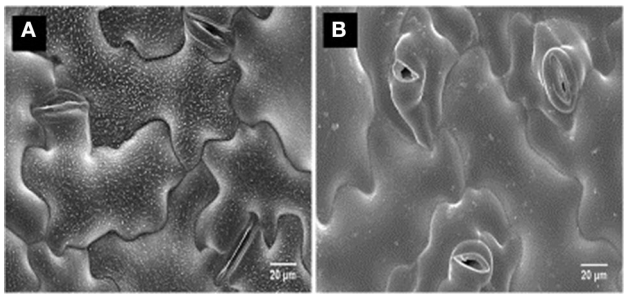

FIGURE 6 | CryoSEM of a susceptible vs. a resistant genotype of *Brassica carinata*, after a challenge inoculation of the cotyledons with *Neopseudocercosporella capsellae*: highly resistant *B. carinata* genotype ATC94129P **(A)**, highly susceptible *B. carinata* genotype UWA#012 **(B)**.

PATHOGENIC SPECIFICITY WITHIN *N. CAPSELLAE*

In a gene-for-gene pathosystem, pathogens have specific races/pathotypes that are compatible with resistance genes in the host. In these cases, knowledge of race structure is critical in developing and deploying host resistance. The pathogens most likely to overcome genetic resistance are those with greater evolutionary potential, have a mixed reproduction system, a high potential for genotype flow, large effective population sizes, and high mutation rates (McDonald and Linde, 2002).

Little is known about the existence of races in *N. capsellae* worldwide. In Australia, it is not known if the pathogen is endemic or exotic. Recent investigations of Western Australian isolates (Gunasinghe et al., 2016a) and Australia wide isolates (Murtza et al., 2019) revealed wide-ranging genetic and/or pathogenic variation present in *N. capsellae,* more than was expected in the absence of any known sexual stage. Genetic analysis of Western Australian pathogen isolates using its rDNA sequencing and phylogenetic analysis identified two genetically distinct populations: isolates recovered from *Brassica* crops and the isolates recovered from the weedy species *R. raphanistrum* (Gunasinghe et al., 2016a). A comprehensive study in 2015–2016 (Murtza et al., 2019) revealed significant variation among isolates from Western Australia, Victoria, and New South Wales based on its rDNA sequencing. There were also differences between isolates collected in 2015/2016 and those collected in 2005 (Murtza et al., 2019). The Australian isolates clustered separately from the North American, European, and Asian isolates (Gunasinghe et al., 2016a; Murtza et al., 2019).

Current data support the hypothesis that *N. capsellae* populations are rapidly evolving, both in genetic and virulence diversity and/or migration is occurring. These changes are agriculturally important as previously resistant cultivars become

susceptible in Australia (Murtza et al., 2019). Mutation, migration, and recombination through nuclear or cytoplasmic exchange and parasexuality are likely to cause novel variations in asexually reproducing pathogen populations (Burdon and Silk, 1997). Of these, spontaneous mutation has shown strong effects on the diversity of clonal populations. Mutation was responsible for all new genetic variation within clonal lineages of *Phytophthora infestans* (Goodwin et al., 1995). As *N. capsellae* is likely to be exotic to Australia, diversity of the founder population and/or possible multiple introductions in the past could have affected the diversity of the current pathogen population. It is also possible that a cryptic sexual stage could be occurring in Australia, and a systematic search of crop and non-crop hosts should be conducted to test this hypothesis. Currently, the only known country to have mixed sexual and asexual reproduction systems is the UK (Inman, 1992), and a comprehensive comparison of the UK and isolates from other regions would be useful. However, in two studies based on its rDNA sequencing, Australian isolates were distinct from isolates in other countries (Gunasinghe et al., 2016a; Murtza et al., 2019). In another study using its rDNA sequencing, Oregon isolates showed 99.0% similarity with a single UK isolate (Carmody, 2017), which may be due to a single introduction into the USA, assuming the UK isolate was representative of its population. However, there are no studies on *N. capsellae* to understand or define the genetic variability between countries or continents, nor to determine the center of the origin or long-distance pathogen dispersal patterns.

The study by Gunasinghe et al. (2016a) demonstrated significant differences in virulence of *N. capsellae* isolates within Western Australia, with substantial variation depending upon the particular isolate × host combination. Percentage disease indices caused by 52 different isolates were within the range of 49.7–37.9 for highly susceptible *B. juncea* (cv. Rohini), and 22.7–4.5 with moderately resistant *B. napus* (cv. Trilogy). Murtza et al. (2019) confirmed that variation in virulence occurs widely among *N. capsellae* isolates across Australia. A study in Oregon, USA reported significant variation in virulence among nine white leaf spot isolates (Carmody, 2017). More extensive screening studies would show if pathotype/race-specific resistance(s) were present. If they are present, the development of a standard host differential set would enable characterization of existing pathotypes/races of *N. capsellae*. Such studies are critical for breeding of cultivars with effective and durable resistance.

DISEASE MANAGEMENT

While host resistance is generally the preferred and most cost-effective option for disease management, cultural controls, such as residue removal, crop rotation, good hygiene practices, and fungicides can all be utilized to manage white leaf spot disease, providing conditions are not conducive for severe disease development.

Recommended cultural practices to prevent white leaf spot epidemics are typically aimed at reducing initial levels of inoculum or the spread of the established pathogen. These cultural disease control methods are preventive and indirect in action against the pathogen, and therefore, awareness of the biology of the pathogen facilitates the disease management practices by identifying the most vulnerable stage/s of the pathogen to attack (Ogle and Dale, 1997). Although, research to support the effect of cultural methods on white leaf spot disease are absent, destroying crop residues, and crop rotation are recommended to reduce the inoculum levels of necrotrophic pathogens with similar biology and survival mechanisms on crop residues (Bokor et al., 1975; Ogle and Dale, 1997).

Crop rotation, the most successful and widespread disease control practice, does provide pathogen control. This practice has been highly effective when the pathogen's survival period on the susceptible host or host residues is shorter than the rotation intervals between susceptible crops (Ogle and Dale, 1997). As *N. capsellae* has a survival period on crop residue of <1 year (Crossan, 1954), a 2–3-year crop rotation should be effective in controlling white leaf spot (Duff et al., 2006). However, other important oilseed rape pathogens such as *L. maculans* can survive longer, so 4 years between canola crops is recommended for Australia, Canada, and Europe (Bokor et al., 1975; West et al., 2001; Kutcher et al., 2013), and a minimum of 4–5-year rotations without crucifers in the USA (Carmody, 2017). Hence, there is a need to define *N. capsellae* survival periods across different continents and climates.

Stubble management is highly recommended for *N. capsellae* (Koike et al., 2007). Deep plowing of stubble reduces pathogen inoculum levels in the UK (West et al., 2001) and the USA (Ocamb, 2016). Intensive flailing and burial of residues are among the recent recommendations in Oregon to control foliar diseases, including white leaf spot (Ocamb, 2016). In Australia, stubble burning is also beneficial in controlling oilseed rape diseases, including white leaf spot (Bokor et al., 1975; Duff et al., 2006), however, the risk of erosion and reduced organic matter inputs are increased. Further, maintaining proper hygiene by removing alternative hosts (volunteer host plants, weeds, or wild relatives) would reduce inoculum carryover (Duff et al., 2006). Other strategies, such as manipulation of sowing and harvesting practices, could be used to protect the most susceptible growth stage of the crop from pathogen infection (Ogle and Dale, 1997). For instance, early or delayed sowing so that plants develop during a dry period will reduce the seedling infection rate by *N. capsellae*.

Greater spacing within and between rows reduces the potential of white leaf spot disease spread within-crop by modifying the microenvironment to reduce disease development and spread (Carmody, 2017). Overhead irrigation facilitates the spread of foliar pathogens by inoculum splash and increasing periods of leaf wetness, and high humidity, that all favor infections (Ogle and Dale, 1997). As *N. capsellae* depends on short distance splash-dispersed conidia for secondary disease spread, minimizing overhead irrigation is important. Avoiding susceptible varieties is a useful disease control strategy. Widespread planting of highly susceptible genotypes increases losses in the current season and provides high levels of inoculum for the following seasons. Host susceptibility to *N. capsellae* is increased under nitrogen deficiency, while a

TABLE 1 | Fungicides reported as effective in controlling white leaf spot disease on *Brassica* crops.

Fungicide	FRAC[a] group	Country	Crop	Reference
Prochloraz	3	Germany	Turnip	Amelung and Daebeler, 1988
		UK	Oilseed rape	Inman, 1992
		France	Oilseed rape	Penaud, 1987
Maneb	M03	USA	Turnip	Chandler, 1965; Sumner et al., 1978
Chlorothalonil	M05	USA	Turnip	Chandler, 1965; Sumner et al., 1978
Mancozeb	M03	Australia	Oilseed rape: *B. napus*	Barbetti, 1988
Iprodione	2	USA	*Brassica* and *Raphanus* seed crops (except Canola)	Ocamb, 2016
Flusilazole	+3	UK	Oilseed rape	Inman, 1992
Carbendazim	1	UK	Oilseed rape	Inman, 1992
Benomyl	1	UK	Oilseed rape	Carmody, 2017
		France	Oilseed rape	Penaud, 1987

[a]*FRAC group, fungicide resistance action committee fungicide group with the potential for cross-resistance.*

balanced supply of nitrogen decreases the disease potential (Duff et al., 2006).

Generally, fungicide treatment is not recommended to control white leaf spot disease in the absence of any other disease, as fungicides may not be cost-effective (Inman, 1992). However, in severe epidemics or when multiple diseases co-occur such as blackleg together with white leaf spot, application of fungicides can be beneficial and economic. If conducive weather continues after initial infections, fungicide applications are recommended every 2–3 weeks to prevent disease spread (Ocamb, 2016). If white leaf spot disease was present in the previous season, foliar fungicide application before the main rains early in the season can reduce or even prevent the disease, and monthly fungicide applications throughout the season will reduce the carryover of the pathogen in infected residues (Ocamb, 2016). In general, fungicides effective on other foliar pathogens will also reduce *N. capsellae* (Ocamb, 2016). Foliar fungicides that effective on white leaf spot are listed in **Table 1**.

With the increasing importance of *N. capsellae* (Inman, 1992; Ocamb, 2014; Ocamb et al., 2015; Murtza et al., 2018, 2019; Thomas et al., 2019), more effective and reliable control measures are needed, particularly if climate changes lead to more conducive conditions for the development of severe white leaf spot epidemics.

Breeding for disease resistance to *N. capsellae* offers the best avenue for cost-effective control of white leaf spot. However, white leaf spot has not been a priority in the past (Inman, 1992), nor is it a priority in current breeding programs, the latter due primarily because the impact and importance of white leaf spot has often been underestimated, certainly in the case of oilseed rape. Previously Brun and Tribodet (1991) optimized a method to evaluate the resistance in *B. napus,* and more recently Gunasinghe et al. (2014) developed a rapid controlled environment cotyledon screening assay to reliably identify *Brassica* germplasm resistance to *N. capsellae.* This research now allows breeders to more efficiently identify phenotype resistance in breeding programs and has prompted a resurgence of interest in developing resistant varieties.

TABLE 2 | Details of six field trials conducted in Western Australia, used to extract data for meta-analysis to identify susceptibility differences among three common *Brassica* species to *Neopseudocercosporella capsellae*.

Reference	Number of trials	Method of disease assessment
Gunasinghe et al., 2014	3	AUDPC[a] for percentage leaves diseased
Gunasinghe et al., 2016d	2	AUDPC for percentage leaves diseased
Gunasinghe et al., 2017a	1	Percentage leaf disease index

[a]*AUDPC, Area under the disease progress curve.*

Variations in Susceptibility/ Resistance and Promising Resistant Sources

A wide range of variation, from highly susceptible to complete resistance, is present against *N. capsellae* within and between Brassicaceae species. Field screening in Western Australia identified significant susceptibility/resistance variation in major Brassicaceae crops and sources of resistance to a mixture of Australian isolates of *N. capsellae* (Eshraghi et al., 2007; Gunasinghe et al., 2014, 2016d, 2017a). These results clearly demonstrate the significant potential to incorporate resistance against white leaf spot disease into commercial germplasm of oilseed, forage, and vegetable cruciferous crops through effective breeding strategies.

Meta-Analysis to Identify Susceptibility Variations Between Species

To better understand the differences in susceptibility/resistance in two common broadacre oilseed rape species (*B. juncea* and *B. napus*) and one vegetable *Brassica* species (*B. oleracea*) a meta-analysis of previously published data was conducted. Fixed and random-effects models were employed to combine data from 6 independent screening trials conducted in 2012 (Gunasinghe et al., 2014), in 2013 (Gunasinghe et al., 2016d), and in 2016 (Gunasinghe et al., 2017a) in Western Australia. Data on sample

TABLE 3 | Result of meta-analysis using random-effects model and standard REML procedure to compare disease ratings across three species, *Brassica napus*, *B. juncea* and *B. oleracea*, upon artificial inoculation of *Neopseudocercosporella capsellae*.

Genus	Estimate	Standard error	Z	p	Lower bound	Upper bound
B. juncea (intrcpt)	110.39	18.20	6.067	<0.001	74.72	146.06
B. napus	−49.28	24.16	−2.04	0.041	−96.63	−1.93
B. oleracea	−72.61	29.94	−2.42	0.015	−131.30	−13.93

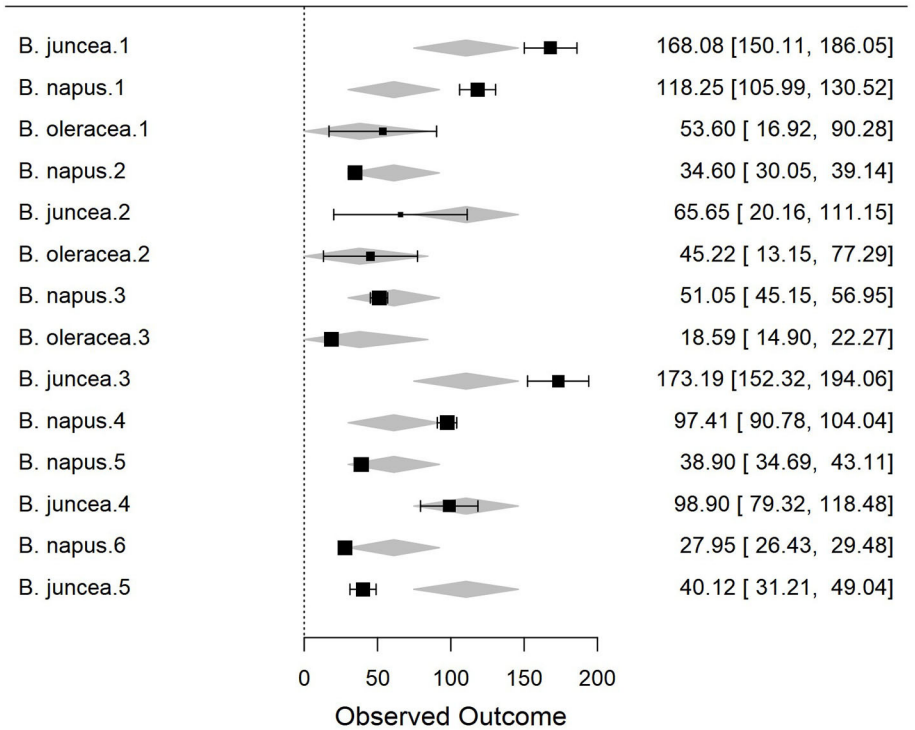

FIGURE 7 | Forest plot of the meta-analysis using the random-effects model and standard REML procedure. The effect sizes of three species compared: *Brassica juncea*, *B. napus,* and *B. oleracea*. For individual study, outcome value or effect estimates (box) along with the confidence intervals (CI) and overall effect (diamond) are shown.

size, means, and dispersion statistics were extracted from each of the three species in each of the six studies, as listed in **Table 2**. A standardized measure of effect size was then calculated using this information and the random-effects model was fit using the standard REML procedure. The model outputs include estimated study effect sizes and 95% confidence intervals (CIs) for the study effects. Results indicate significant differences in resistance/susceptibility levels across the three species tested. *B. juncea* appears to exhibit greater susceptibility than the other two included species: *B. napus* and *B. oleracea* (**Table 3** and **Figure 7**). A more comprehensive meta-analysis is required, when additional input data becomes available in the future, in order to further investigate and define this apparent effect with greater precision.

Known Resistant Sources

The majority of *B. carinata* varieties screened in the above studies showed complete resistance to white leaf spot disease with at least 20 of the 32 tested cultivars/genotypes with complete resistance (**Figure 8A**) and with the remainder highly susceptible, suggesting a single major resistance gene involved in the resistance in that species. In contrast, the presence of genotypes with high, low, and intermediate resistance in two other species, *B. juncea* and *B. napus*, suggests resistance to these is more likely to be polygenic (Gunasinghe et al., 2014). Although Indian *B. juncea* (**Figure 8B**) and *B. napus* varieties were highly susceptible to white leaf spot disease, the varieties from China were markedly less susceptible under Australian field conditions (Gunasinghe et al., 2014). Therefore, the range in genetic diversity of oilseed rape germplasm can differ between India, China, and Australia, resulting in different levels of resistance for each of the three oilseed rape species depending on the country of origin.

The resistance of forage or vegetable brassicas to *N. capsellae* is variable. *B. oleracea* species display high resistance to Australian isolates (Gunasinghe et al., 2014) or to the US Pacific Northwest isolates of *N. capsellae* (Carmody, 2017). Furthermore, one or

FIGURE 8 | *Brassica carinata* genotype ATC94129P showing complete resistance **(A)** and highly susceptible **(B)** *B. juncea* under equal disease pressure after inoculation of a mixture of *Neopseudocercosporella capsellae* isolates.

more genotypes within *B. oleracea* var. *acephala* (completely resistant), *B. oleracea* var. *capitata*, and *B. oleracea* var. *italica* (highly resistant) are excellent sources of resistance for Australia. In contrast, most Asian leafy vegetables such as *B. rapa* var. *rosularis*, *B. campestris* var. *chinensis, and B. rapa* are extremely susceptible to Australian isolates of *N. capsellae* (Gunasinghe et al., 2016d).

Availability of diverse sources of resistance provides a strong foundation for a breeding program to incorporate resistance into new commercial cultivars. However, it would be beneficial to have further information about resistance inheritance, identification of resistance gene/s, mapping resistance gene locations in the genome, and identification of tightly linked molecular markers (Michelmore et al., 1991; Roberts, 1992; Young, 1996). Within the Brassicaceae, the minimal incompatibility to hybridization will facilitate the use of host plant resistance (Song et al., 1988). Utilizing wild and weedy species (Uloth et al., 2013), and their introgressions into both *B. napus* (Barbetti et al., 2014; You et al., 2016), and *B. juncea* (Barbetti et al., 2014; Rana et al., 2017, 2019; Atri et al., 2019), and resistances introgressed from the B genome of *B. carinata* into *B. napus* (Barbetti et al., 2014; You et al., 2016) has been effective in developing resistance against *Sclerotinia sclerotiorum*. A similar strategy should be effective in developing brassicas with resistance against *N. capsellae*, for example, as there are both immunity and high levels of resistance found within *B. carinata* to *N. capsellae.* In addition, this species offers benefits, such as resistance to other oilseed diseases (Katiyar et al., 1986; Tonguc and Griffiths, 2004; Subramanian et al., 2005), high levels of drought tolerance (Katiyar et al., 1986; Cardone et al., 2003), and resistance to pod shattering (Salisbury and Barbetti, 2011). To prevent ongoing and future yield losses from this disease, incorporation of white leaf spot disease resistance into future varieties is urgently required. This will be even more urgent should the highly susceptible *B. juncea* be more widely adopted across canola growing regions.

CONCLUSION

A lack of information exists on critical aspects of the *N. capsellae–Brassica* pathosystem. These deficiencies include a lack of understanding of critical components of disease epidemiology, pathogen genetics, pathogen lifecycle, and population biology. For example, comprehensive studies on population genetics and diversity would provide critical information to link the current findings of Australian pathogen populations to the global *N. capsellae* populations, to identify long-distance pathogen dispersal methods and patterns, and most importantly, fully explain the reproductive behavior of the pathogen. An appropriately designed genetic study should identify whether the population is clonal and if there is a cryptic sexual stage, define the relative degrees of sexual vs. asexual reproduction within the population by differentiating asexually reproduced clones and sexually reproduced individuals (Wang et al., 1997). Also, appropriate genetic studies will assist in defining if mating types of *N. capsellae* occur. Together with genetic studies, systematic surveys of the sexual stage, in regions where to date there are no records of the sexual stage, will resolve the current uncertainty of occurrence or non-occurrence of the sexual stage. Together, such new information will confirm and/or re-define the pathogen's disease cycle in the absence of the sexual stage and provide the current missing knowledge about disease epidemics in different localities and seasons.

Currently, most of the pathogen populations, except in the UK, are clonal, suggesting that those populations have less diversity and lower evolutionary potential. However, this should not undermine the potential future effects of *N. capsellae*, as mutation and natural selection favor the rapid establishment of more virulent strains in clonal fungal pathogen populations (Burdon and Chilvers, 1982; Burdon and Silk, 1997). The need for future studies in this area is highlighted by Murtza et al. (2019), who showed that geographically diverse *N. capsellae* pathogen populations could result in differences in virulence and pathogenicity.

In the absence of the teleomorph stage in the disease cycle, disease initiation remains poorly understood. If conidia are the primary, and in many instances the only, inoculum, as proposed by the current literature, clearly there is a more substantial role for conidia in initiating and spreading the disease than has been considered to date. Currently, the larger conidia (compared with ascospores) have been assumed unlikely to be efficiently dispersed by wind (Fitt et al., 1992) and, as such, less successful than airborne ascospores for pathogen movement. Therefore, experiments are needed to definitively define the role of conidia in the disease cycle, where the sexual stage is absent. Windborne dispersal of conidia is possible, as suggested by unpublished field observations noted in this review. Studies to understand and define the spatial and diurnal movement patterns of conidia are, therefore, critical to fully understand the epidemiology of white leaf spot, particularly with environments where strong winds prevailing during the growing season, as occurs across much of Southern Australia.

Overall, existing knowledge gaps in disease epidemiology impede the potential for reliably predicting climate change effects

on white leaf spot epidemics. Since precipitation is a likely critical factor controlling white leaf spot disease epidemics, a better understanding of the current and predicted future variation in local patterns of precipitation, and their consequent effects on white leaf spot epidemics, is critical to explain current and forecast future disease epidemics across different countries and continents. It remains unclear from limited available data whether forecast climate changes will create a more or a less-conducive environment for future white leaf spot epidemics in different localities and countries where brassicas are grown. Strict cultural practices, identification of more cost-effective chemical control options, and selection of more resistant varieties all offer significant opportunities for greater reduction of white leaf spot incidence, severity, and overall management. While cultural controls offer greater avoidance of infested crop residues, fungicidal control could offer immediate control options in current disease-conducive environments. However, these areas of disease management overall remain poorly understood or defined. The identification of host resistance offers the best long term and most cost-effective management of white leaf spot. While a range of effective resistance sources has been identified across different brassicas, there is little understanding of the different types of resistance mechanisms. Even more critical in terms of practical management of white leaf spot disease is the current disconnect between identified host resistances and their uptake by *Brassica* breeding programs, which needs to be addressed urgently if improved long term management is to be secured through the deployment of effective host resistance.

AUTHOR CONTRIBUTIONS

NG: worked with DB to perform the meta-analysis and wrote the manuscript with input from all authors. MB and MY: added extra information and involved in critical revisions. DB: performed meta-analysis, and drafted the methodology. SN: conceived the idea, helped with reading, organization and critical revisions.

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REVIEW
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Fungi vs. Fungi in Biocontrol: An Overview of Fungal Antagonists Applied Against Fungal Plant Pathogens

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Plant pathogens cause severe losses or damage to crops worldwide and thereby significantly reduce the quality and quantity of agricultural commodities. World tendencies are shifting towards reducing the usage of chemically synthesized pesticides, while various biocontrol methods, strategies and approaches are being used in plant disease management. Fungal antagonists play a significant role in controlling plant pathogens and diseases and they are used as Biocontrol Agents (BCAs) throughout the world. This review provides a comprehensive list of fungal BCAs used against fungal plant pathogens according to modern taxonomic concepts, and clarifies their phylogenetic relationships because thewrong names are frequently used in the literature of biocontrol. Details of approximately 300 fungal antagonists belonging to 13 classes and 113 genera are listed together with the target pathogens and corresponding plant diseases. *Trichoderma* is identified as the genus with greatest potential comprising 25 biocontrol agents that have been used against a number of plant fungal diseases. In addition to *Trichoderma*, nine genera are recognized as significant comprising five or more known antagonistic species, namely, *Alternaria*, *Aspergillus*, *Candida*, *Fusarium*, *Penicillium*, *Pichia*, *Pythium*, *Talaromyces*, and *Verticillium*. A phylogenetic analysis based on partial sequences of the 28S nrRNA gene (LSU) of fungal antagonists was performed to establish their phylogenetic relationships.

Keywords: biocontrol agents, disease control, fungicides, plant diseases, plant pathogens, phylogeny, *Trichoderma*

INTRODUCTION

Plant pathogens including fungi, bacteria, viruses and nematodes cause serious losses or damage to crops worldwide and significantly reduce the quality and quantity of agricultural commodities. These losses pose a major threat to global food production annually (El Ghaouth et al., 2002; Dean et al., 2012; Singh, 2014; O'Brien, 2017). Moreover, pathogenic infection in the field or in post-harvest

storage can affect the health of humans and livestock, especially if the pathogen produces toxins in or on consumable products (Brimner and Boland, 2003; Menzler-Hokkanen, 2006).

Various methods, strategies, and approaches are used in the management of plant diseases. These encompass the development of resistant varieties through plant breeding, genetically engineered plants, use of agrochemicals and physical methods (i.e., heat treatments, UV irradiation, modified or controlled atmosphere, cold storage, and inducing resistance by applying elicitors), application of biological control agents and good agronomic and horticultural practices (Stevens et al., 1997; Wisniewski et al., 2000; Droby, 2006; Singh and Chawla, 2012; Gupta and Sharma, 2014; Singh, 2014; O'Brien, 2017). These approaches have contributed significantly to the remarkable improvements in crop productivity and quality over the past few decades (Punja, 1997; Droby, 2006; Chandrashekara et al., 2012).

Biological Control: Overview and Significance

Biological control approaches of plant diseases include any reduction in the amount or the effect of pathogens (disease-producing activity) that is achieved through the induction of biological mechanisms or the action of naturally occurring or introduced antagonists, that occurs by manipulating the microenvironment to favour the activity of antagonists (Baker, 1987; Stirling and Stirling, 1997). Microbial biocontrol agents (BCAs) for plant diseases are usually fungal or bacterial strains isolated from the phyllosphere, endosphere or rhizosphere and they play an important role in controlling plant-pathogenic organisms. Biocontrol agents or microbial antagonists prevent infection of the host plant by the pathogen, or establishment of the pathogen in the host plant. The principal mechanisms for the control have been assumed to be those that act primarily upon the pathogens. The antagonists can exhibit several direct or indirect mechanisms of action involved in biological disease control. These mechanisms include; antibiosis (where an inhibitory metabolite or antibiotic is produced by the antagonist), mycoparasitism (where the antagonist derives some or all of its nutrients from the fungal host), induced resistance (induction of plant defense response against plant pathogens) and growth enhancement (BCAs promote plant growth while the effects of the disease are being reduced and also through microbial hormones such as indoleacetic acidand gibberellic acid). Secretion of extracellular hydrolytic enzymes by the antagonist, competition for space and nutrients between organisms and detoxification of virulence factors are other actions involved in biological disease control (Wilson et al., 1991; Punja, 1997; Heydari and Pessarakli, 2010; Chandrashekara et al., 2012; Singh, 2014; Zhang et al., 2014; Deketelaere et al., 2017). Recent studies have demonstrated that effects such as induced systemic or localized resistance by microbial BCAs on plants are also crucial. These fungi or bacteria can colonize the root epidermis and outer cortical layers and release bioactive molecules that cause walling-off of the fungal thallus or bacterial colonies (Harman, 2006). Consequently, they will alter the transcriptome and the proteome machinery of plants substantially. This alteration in the plant's genetic material will provide certain additional advantages to the plant, such as increased plant growth and nutrient uptake in addition to induction of pathways for resistance in plants.

World trends are shifting towards reducing the use of agrochemicals in the management of plant diseases. Considerable research effort today is focused on seeking safe, eco-friendly and effective alternatives to synthetic, chemical fungicides to reduce the decay loss in harvested commodities and to control crop diseases in the field that lead to significant economic losses (Stirling and Stirling, 1997; Wisniewski et al., 2000; Droby, 2006; Chandrashekara et al., 2012). Due to the aforementioned mechanisms, biological control agents for plant diseases are gaining stature as viable alternatives to synthetic pesticides given their perceived increased level of safety and minimal negative environmental impacts. It is imperative to continue this line of research, since regulations on the use of new and existing fungicides are becoming more and more stringent. In particular, this has led to extensive researches on the use of microbial antagonists as protective agents and many fungal diseases can now be controlled by microbial antagonists. As a result, commercial products containing microbial BCAs have been successfully exploited in modern agriculture (e.g., *Trichoderma* based products and biopesticides based on *Bacillus thuringiensis*) (Menzler-Hokkanen, 2006).

A significant amount of harvested fruits and vegetables is lost annually due to microbial spoilage and this loss can range from 10%–50% depending on the commodity and country (El Ghaouth et al., 2002; Janisiewicz and Korsten, 2002). Developing countries experience greater losses due to inadequate storage and transportation facilities, and improper handling methods that are employed during harvesting and transit (Pathak, 1997; El Ghaouth et al., 2002; Nabi et al., 2017). The harvested yield might have been infected by one or several pathogens prior to harvest or they may become infected during transit and storage. (Punja, 1997; Janisiewicz and Korsten, 2002; Nabi et al., 2017). Several researches have been carried out to identify effective biocontrol agents for post-harvest disease management and as a result, biocontrol antagonists are now employed to control postharvest diseases worldwide. A few examples of these applications are indicated here. Mohamed and Saad (2009) found that the application of specific strains of *Pichia anomala* was a safe and effective biocontrol agent against Diplodia postharvest rot of guava fruit caused by *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. Alvindia and Natsuaki (2008) and Sangeetha et al. (2009) demonstrated the superior biocontrol potential of *Trichoderma* species for the management of the postharvest crown rot complex of banana caused by a variety of fungal pathogens including *Colletotrichum musae*, *Fusarium verticillioides*, and *Lasiodiplodia theobromae*. The search for suitable biological-control systems has largely taken place in the last fifty years and there has been considerable interest in the use of antagonistic microorganisms for the control of postharvest diseases. (Droby, 2006; Heydari and Pessarakli, 2010;

Wisniewski et al., 2016). Bacteria associated with plants are known to develop biofilms on plant surfaces and within intercellular spaces of plant tissues, which act as microniches. It has been reported that the conditions within these microniches created because of the biofilm formation are markedly different from those of the ambient environment, which will eventually lead the microbial cells to effect functions that are not possible alone. This may influence the ecology of the bacteria they harbor and the relationship of bacteria with plants, which directly influence the development of strategies for biological control of plant disease and for assuring food safety (Morris and Monier, 2003).

Fungal Antagonists

The potential for the application of fungal biological control agents against plant pathogens has largely increased because fungi have a comparatively high reproductive rate (sexually as well as asexually), a short generation time and they are target specific. Furthermore, in the absence of the host, they can survive in the environment shifting their mode of parasitism to saprotrophism thus maintaining sustainability. Many fungal species possess mechanisms that allow them to efficiently protect plants from diseases caused by plant pathogenic fungi (**Figure 1**).

History of Fungal Biological Control Applications

Since ancient times man has attempted to increase crop production and control disease severity of crop plants by altering cultivation practices, which reduce both initial inoculum as well as infection rate (Singh and Chawla, 2012; Gupta and Sharma, 2014). With the finding of microorganisms and their interactions, many methods have been employed to control pathogens through the use of fungal antagonists.

Roberts (1874) showed the antagonistic action of microorganisms in liquid cultures between *Penicillium glaucum* and bacteria, introducing the term antagonism as used in microbiology. Hartley (1921) made the first attempt at direct application of biological control of plant pathogens by inoculating soil with microorganisms that were thought to have antagonistic potential. He inoculated forest nursery soils with thirteen antagonistic fungi to control damping-off caused by *Pythium debaryanum*. (Baker, 1987; Gupta and Sharma, 2014). Weindling (1932, 1934) described the potential of *Trichoderma lignorum* (*T. viride*) to control plant-pathogenic fungi by mycoparasitism and reported the first use of a known antimycotic-producing antagonist in plant disease control (Baker, 1987). Later, Weindling (1941) noted that *Trichoderma* species excrete an antimycotic that was toxic to plant pathogens including *Rhizoctonia solani* and *Sclerotinia americana*, and named it gliotoxin. This was the first record of the use of a known antimycotic-producing antagonist in plant disease control (Baker, 1987; Howell, 2003). The discovery of penicillin by A. Fleming in 1928, and its purification and use in pharmaceutical production, significantly stimulated studies on antagonists of plant pathogens (Baker, 1987).

Development of modern biotechnological approaches lead to increase the potential usage of fungal antagonists against a wide range of plant diseases. Numerous researches and experiments have been carried out during the past few decades to identify new fungal BCAs and evaluate their effectiveness under different environmental conditions.

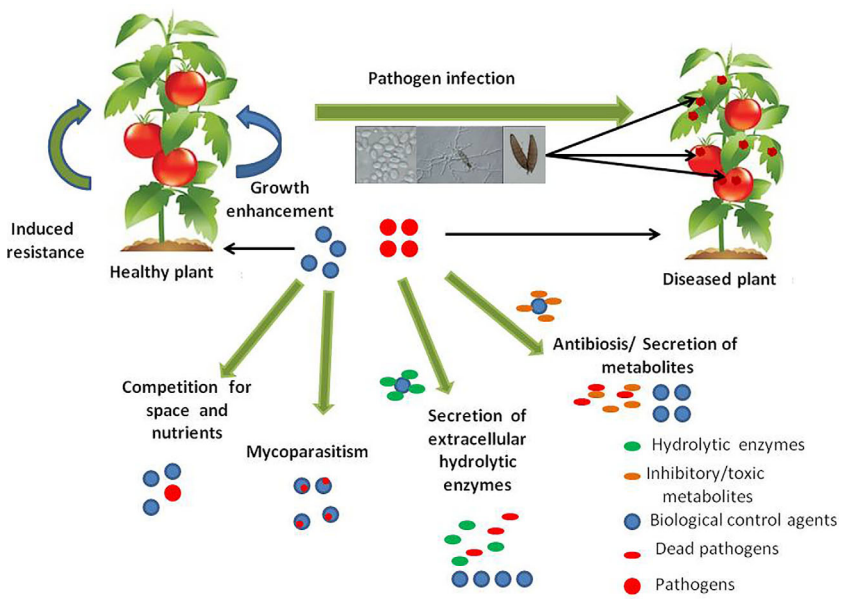

FIGURE 1 | Key mechanisms of action involved in biological control of plant fungal diseases by fungal antagonists.

Commercialization of Fungal Biological Control Agents

Commercial uses and applications of biological control of plant diseases have been slow mainly due to their variable performances under different environmental conditions in the field as well as due to their host specificity. To overcome this problem, it is essential to develop new formulations of BCAs with a higher degree of stability, efficiency and survival using new biotechnological practices (Heydari and Pessarakli, 2010). Several criteria have to be satisfied for upscaling a particular BCA to reach the stage of commercialization (Punja, 1997). Commercialization of biological control agents is expensive as it involves many steps such as isolation in pure culture or enrichment of the microorganism, identification and characterization, the development of a suitable formulation, mass production, efficacy testing of the product, inspection of storage stability, finding an industrial partner, attention to human and environmental safety matters, registration and marketing (Punja, 1997; Stirling and Stirling, 1997; Janisiewicz and Korsten, 2002; Montesinos, 2003). A number of biologically based products are being sold worldwide for the control of fungal plant pathogens and generally they are produced as granules, wettable powders, dusts, and aqueous or oil-based liquid products using different mineral and organic carriers (Ardakani et al., 2009; Nega, 2014). Several microbial antagonists have been patented and evaluated for commercial uses (Wisniewski et al., 2000; El Ghaouth et al., 2002; Schena et al., 2004; Nabi et al., 2017) and these agents are frequently recommended for plants (Albajes et al., 2000; Fravel, 2005; O'Brien, 2017). Some commercialized fungal BCAs used to control plant fungal diseases and their particulars are listed in **Table 1**.

Integrated Applications of BCAs With Synthetic Fungicides for the Control of Plant Fungal Pathogens

Synthetic fungicides consisting of inorganic or organic compounds are commonly used in developed agricultural systems to control plant diseases, including post-harvest diseases, and to safeguard crop yield and quality mainly due to their relatively low cost, ease of application, and effectiveness. Chemical agents such as Captan, dithiocarbamates, thiabendazole (TBZ) and imazalil (IMZ) are widely used in the control of plant fungal pathogens (Lucas et al., 2015; Perez et al., 2016; Gupta, 2018). However, the massive and indiscriminate use of synthetic fungicides in crop protection and post-harvest food preservation has resulted in resistance to some fungicides and also led to severe effects on humans, animals, and wildlife resulting in widespread adverse ecological effects (Gupta, 2018; Gupta, 2019). Significant biocontrol of postharvest diseases of fruits and vegetables can be achieved with both field and postharvest applications (Janisiewicz and Korsten, 2002). The combined or integrated applications of a BCA with a synthetic fungicide or physical additives, either simultaneously or in rotation, would be expected to result in an enhanced degree of disease suppression, provided that the biocontrol agent is compatible with the fungicide used (Punja, 1997; Janisiewicz and Korsten, 2002; Droby, 2006; Eshel et al., 2009).

Modern Biotechnological Approaches Used in Plant Fungal Pathogen Biocontrol

Biological control of plant diseases using fungal BCAs has developed considerably in recent years with the application of genomics, genetic engineering and recombinant DNA

TABLE 1 | Some commercialized fungal Biocontrol Agents (BCAs) for plant fungal diseases and their specifications.

Biocontrol agent	Product	Target Pathogen(s) or crop disease	Manufacturer or distributor
Ampelomyces quisqualis	AQ10® Bio Fungicide	Powdery mildew	Ecogen Inc, USA, Israel
Anthracocystis flocculosa (*Pseudozyma flocculosa*)	Sporodex L	Powdery mildew	Plant Products Co., Canada
Candida oleophila	Aspire	Post-harvest diseases	Ecogen Inc, USA, Israel
Paraphaeosphaeria minitans (*Coniothyrium minitans*)	Contans WG; KONI	*Sclerotinia sclerotiorum* and *S. minor*	Prophyta Biologischer Pflanzenschutz GmbH; Germany, Bioved Ltd, Hungary
Clonostachys rosea (*Gliocladium catenulatum*)	Primastop	Damping-off, seed rot, root and stem rot, and wilt diseases	Kemira Agro OY, Finland; RegWest Co., USA
	Prestop	Soil-Borne and foliar diseases of greenhouse vegetables, herbs and ornamentals	Danstar Ferment Ag., Switzerland; AgBio, Inc., USA
Fusarium oxysporum (non-pathogenic)	Fusaclean; Biofox C	Wilt diseases	SIAPA, Italy; Natural Plant Protection, France
Phlebiopsis gigantea	Rotstop®	Root rot diseases	Kemira Agro Oy, Finland
Trichoderma virens (*Gliocladium virens*)	Soilgard®	Soil-borne pathogens; *Rhizoctonia* and *Pythium* species	Certis USA
Trichoderma harzianum	RootShield®	Root rot diseases; *Pythium*, *Fusarium*, *Rhizoctonia*, *Thielaviopsis* and *Cylindrocladium* species	BioWorks, Inc., USA
Trichoderma harzianum	Trichodex	Grey mould (*Botrytis cinerea*); *Rhizoctonia*, *Sclerotinia* and *Colletotrichum* species	Makhteshim Agan Industries, Israel
Trichoderma harzianum and *T. polysporum*	Binab T	Root rot diseases, pruning wounds in ornamental, shade, and forest trees	BINAB Bio-Innovation AB, Sweden
Trichoderma viride	*Trichoderma Viride* Trieco	Soil-borne fungal diseases	Ecosense Lab (I) Pvt. Ltd., India

techniques. These techniques have been developed to a high degree of precision and have been applied to the improvement of fungal strains for agro-industrial processes. Development of new crop varieties or clones that are resistant to plant pathogens offers a commonly acceptable and potentially long-term control option. Furthermore, many studies have been conducted to identify genetic traits of fungal antagonists and determine their potential to enhance biocontrol activity (Janisiewicz and Korsten, 2002; Droby, 2006; O'Brien, 2017). A few examples are pointed out here; (a). the introduction of multiple lytic enzyme-encoding genes into *Trichoderma virens* genome resulted in a strain that secreted a mixture of glucanases and showed greatly enhanced inhibition of the pathogens *Pythium ultimum* (Oomycota, Chromista), *Rhizoctonia solani*, and *Rhizopus oryzae* (Djonovic et al., 2007); (b). McDougal et al. (2012) presented a method for the genetic transformation of *Cyclaneusma minus*, the causal agent of Cyclaneusma needle-cast, using protoplasts generated by incubation with Glucanex™ enzyme. *Cyclaneusma minus* was transformed with a gene encoding green fluorescent protein (GFP), which was allowed to identify several *Trichoderma* strains with potential for biocontrol of the disease. The interaction between *C. minus* and the *Trichoderma* strains, in the interaction zone where GFP expression was lost, was determined by a dual culture technique to be fungicidal; (c). Yakoby et al. (2001) generated reduced-pathogenicity mutants of the avocado fruit pathogen *Colletotrichum gloeosporioides* using insertional mutagenesis by restriction enzyme mediated integration (REMI) transformation and these isolates can be used for the biological control of anthracnose caused by *C. gloeosporioides*.

Aims of This Study

The present study aims to provide a comprehensive list of fungal BCAs that are used against fungal pathogens of crop plants, and clarify their phylogenetic relationships as these are often wrongly mentioned and interpreted in the literature of biocontrol. In this review, the main researches conducted during past the fifty years to evaluate the interactions between fungal antagonists and fungal plant pathogens were highlighted. Thus, this review is meant to serve as an updated database for applications of potential fungal antagonists against particular plant fungal diseases employed by different researchers worldwide. This can also serve as a useful tool to select and compare suitable and most applicable fungal antagonistic applications for fungal disease management in ongoing practices and researches. Many fungal antagonists and causative agents of plant diseases are presently identified and described based on the traditional classification and taxonomic systems. This review is the first comprehensive study of potential fungal antagonists applied against fungal plant pathogens based on a phylogenetic analysis and provides an extensive list of fungal BCAs used against fungal plant pathogens according to modern taxonomic concepts.Therefore, in this review the fungal antagonists and fungal pathogens are presented and listed using updated taxonomic nomenclature, which helps to avoid complications and misunderstandings. Also, the phylogenies of potential fungal BCAs are shown and discussed.

A variety of biological control methods are available nowadays, however they should be further developed before they can be effectively adopted. Therefore, this study makes a significant contribution for a greater understanding in developing such approaches with the help of the detailed information presented.

MATERIALS AND METHODS

Data Collection and Presentation

Research data on fungal antagonists used against fungal plant pathogens were collected from resources published during the past fifty years. The collected data are summarized in the **Supplementary Table 1**, which includes information on *Fungal biocontrol agent*, *Disease and host* as well as *Pathogen*. The disease or the pathogen suspension/control rate equal to or greater than 50% in each case is indicated with an asterisk (*) after the pathogen. Several potential fungal-like taxa causing plant diseases have also been taken into account to show the broad spectrum of activity of fungal BCAs and they are indicated with [@]. Different applications and treatment methods (*in vitro* and *in vivo* or both) have often been used when evaluating the effect of fungal BCAs in the research papers we considered. Thus, the disease/pathogen suppression percentage was accounted based on the maximum inhibition shown (if different conditions were applied).

Phylogenetic Analysis of Fungal Antagonists

Sequence data of the 28S nrRNA gene (LSU) from ex-type, ex-epitype, or ex-neotype strains of fungal antagonists listed in this study were downloaded from the NCBI's GenBank nucleotide database (**Supplementary Table 2**). If no ex-type strains were available, sequences from voucher, authentic or reference strains were included in the analysis. *Hyphochytrium catenoides* (EF594059) was chosen as the outgroup taxon.

Sequences were aligned with Bioedit 7.1.3.0 (Hall, 1999), and the consensus sequences were further improved with MUSCLE implemented in MEGA 5v (Tamura et al., 2011). Alignments were checked and optimized manually when necessary. The phylogenetic tree was generated by maximum likelihood (ML) criterion using RAxML-HPC2 BlackBox (8.2.10) (Stamatakis, 2006; Stamatakis et al., 2008) on the CIPRES Science gateway portal V 3.3 (Miller et al., 2010) and a Bayesian analysis was performed with MrBayes v. 3.2.6 (Ronquist and Huelsenbeck, 2003). The general time-reversible model of evolution including estimation of invariable sites and assuming a discrete gamma distribution with default parameters was used for the ML analysis. The model of evolution (GTR + I + G) was determined with MrModeltest 2.2 (Nylander, 2004) under the Akaike Information Criterion (AIC) implemented in PAUP v. 4.0b10. Bayesian inference (Rannala and Yang, 1996; Zhaxybayeva and Gogarten, 2002) was determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.0b4 (Huelsenbeck and Ronquist, 2001). Six simultaneous Markov

chains were run for 7,000,000 generations and trees were sampled every 100th generation. The first 20% of the trees (14000), representing the burn-in phase of the analysis, were discarded, while the remaining trees were used to calculate posterior probabilities (PP) in the majority rule consensus tree. The best scoring RAxML tree was selected and visualized with MEGA v. 5 (Tamura et al., 2011) and the graphical layout of the tree was created using PowerPoint 2010 version. ML Bootstrap support values (MLBS) greater than or equal to 50% and Bayesian posterior probabilities (PP) greater than or equal to 0.90 are indicated at the nodes of the branches. Alignments were deposited in TreeBASE (www.treebase.org) under the submission number 26869.

RESULTS

Phylogenetic Analysis and Taxonomy of Fungal Antagonists

After alignment the LSU dataset consisted of 1,030 characters (including alignment gaps) for 218 ingroup taxa and the outgroup taxon. The Bayesian tree had had a topology identical to the ML tree presented. (data not shown). Fungal BCAs are distributed in four phyla (Ascomycota, Basidiomycota, Glomeromycota and Mucoromycota) and thirteen classes *viz*. Sordariomycetes, Dothideomycetes, Eurotiomycetes, Leotiomycetes, Saccharomycetes (Ascomycota), Agaricomycetes, Exobasidiomycetes, Ustilaginomycetes, Microbotryomycetes, Cystobasidiomycetes, Tremellomycetes (Basidiomycota), Glomeromycetes (Glomeromycota), Mucoromycetes (Mucoromycota) in the kingdom fungi (**Figure 2**). The results show the phylogenetic placement of the various fungal BCAs within the kingdom fungi and confirm the current taxonomic placement of those BCAs. Most of the fungal BCAs belong to the class Sordariomycetes.

Fungal Antagonists and Their Potential Against Plant Pathogens

Approximately 300 species or varieties belonging to 113 fungal genera are identified as BCAs for plant fungal pathogens based on the previous studies (**Supplementary Table 1**). Nine genera are recognized as potential genera, which consist of five or more known antagonistic species (**Table 2**). They are *Alternaria*, *Aspergillus*, *Candida*, *Fusarium*, *Penicillium*, *Pichia*, *Talaromyces*, *Trichoderma*, and *Verticillium*. *Trichoderma* is the most prominent genus comprising 25 BCAs and they are widely used in controlling plant diseases caused by fungi. *Alternaria*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Lasiodiplodia*, *Penicillium*, *Phytophthora*, *Sclerotinia*, and *Verticillium* are identified as common and most abundant plant pathogenic genera, to which frequently BCAs are applied for disease control.

DISCUSSION

The present study provides a comprehensive list of fungal BCAs used against a wide range of fungal plant pathogens, and establishes their phylogenetic relationships using a phylogenetic analysis based on the available authentic 28S nrRNA gene (LSU) sequence data. This will help clarify the currently correct names for the species since they have often been wrongly quoted and interpreted in the literature of biocontrol. It will further help with searches for the species in earlier literature where the old names were used.

In the phylogenetic analysis presented in this review, fungal BCAs were distributed in thirteen classes *viz*. Sordariomycetes, Dothideomycetes, Eurotiomycetes, Leotiomycetes, Saccharomycetes (Ascomycota), Agaricomycetes, Exobasidiomycetes, Ustilaginomycetes, Microbotryomycetes, Cystobasidiomycetes, Tremellomycetes (Basidiomycota), Glomeromycetes (Glomeromycota), Mucoromycetes (Mucoromycota) in the kingdom fungi (**Figure 2**). The results confirmed the taxonomic placement of the various fungal BCAs and most of the fungal BCAs belong to the class Sordariomycetes. However, DNA sequences for some of the species are not currently available and therefore it is essential to have sequence data from authentic strains to confirm their taxonomy.

The main and common issue that we found when preparing this review was the method used by the authors to identify the fungal antagonists. Most of the publications have not used an appropriate identification method and they mainly followed the traditional identification methods and resources. However, molecular DNA sequencing and phylogenetic tools have been used in some of the recent publications to identify the antagonists and the pathogen correctly (Grondona et al., 1997; Rubini et al., 2005; Sriram and Poornadchanddra, 2013; Hung et al., 2015; Kheireddine et al., 2018). In addition, the nomenclature and classification of several fungal species have been subjected to change during the past few decades due to the modern taxonomic approaches and views. Therefore, corrections on those changes are mandatory in order to avoid misinterpretations. In this study, we have given the current names of the fungal species and the names commonly used in each particular research paper are mentioned in the brackets where applicable (**Table 2**).

Trichoderma is a species rich, asexual genus in the family *Hypocreaceae* (Hypocreales, Sordariomycetes) and currently includes 433 species epithets in Index Fungorum, but DNA sequence data for most of the species are not available in GenBank. The sexual morphs of *Trichoderma* species are linked to *Hypocrea*. Members of the genus *Trichoderma* are biotrophic, hemibiotrophic, saprobic or hypersaprobic on various plants, or other fungi (Maharachchikumbura et al., 2016) and have been identified as the antagonists with greatest potential. They have been employed in several applications in plant fungal disease control. Twenty-five known *Trichoderma* species are included in **Table 2** and these species have great potential for significantly controlling more than 100 fungal plant pathogens worldwide. Out of these species *Trichoderma harzianum* can be considered as the most common and commercially developed BCA used for a wide range of plant fungal diseases. *Trichoderma* species produce a number of metabolites and these metabolites play a major role in biological control mechanisms (Reino et al., 2008). Apart from

that, *Aspergillus* and *Penicillium* species also play a vital role as BCAs next to *Trichoderma* (**Supplementary Table 1** and **Table 2**).

Some *fungus-like* genera (*Globisporangium*, *Hyphochytrium* and *Pythium*) belonging to Oomycota, Chromista, are also important as BCAs and have been used to control plant fungal diseases (**Table 3**). Considering the activity of *fungus-like* BCAs, *Pythium oligandrum* may be considered as the one with the greatest potential as a BCA.

Application of one or more biocontrol agents combined with physical and/or chemical control treatments can be considered as

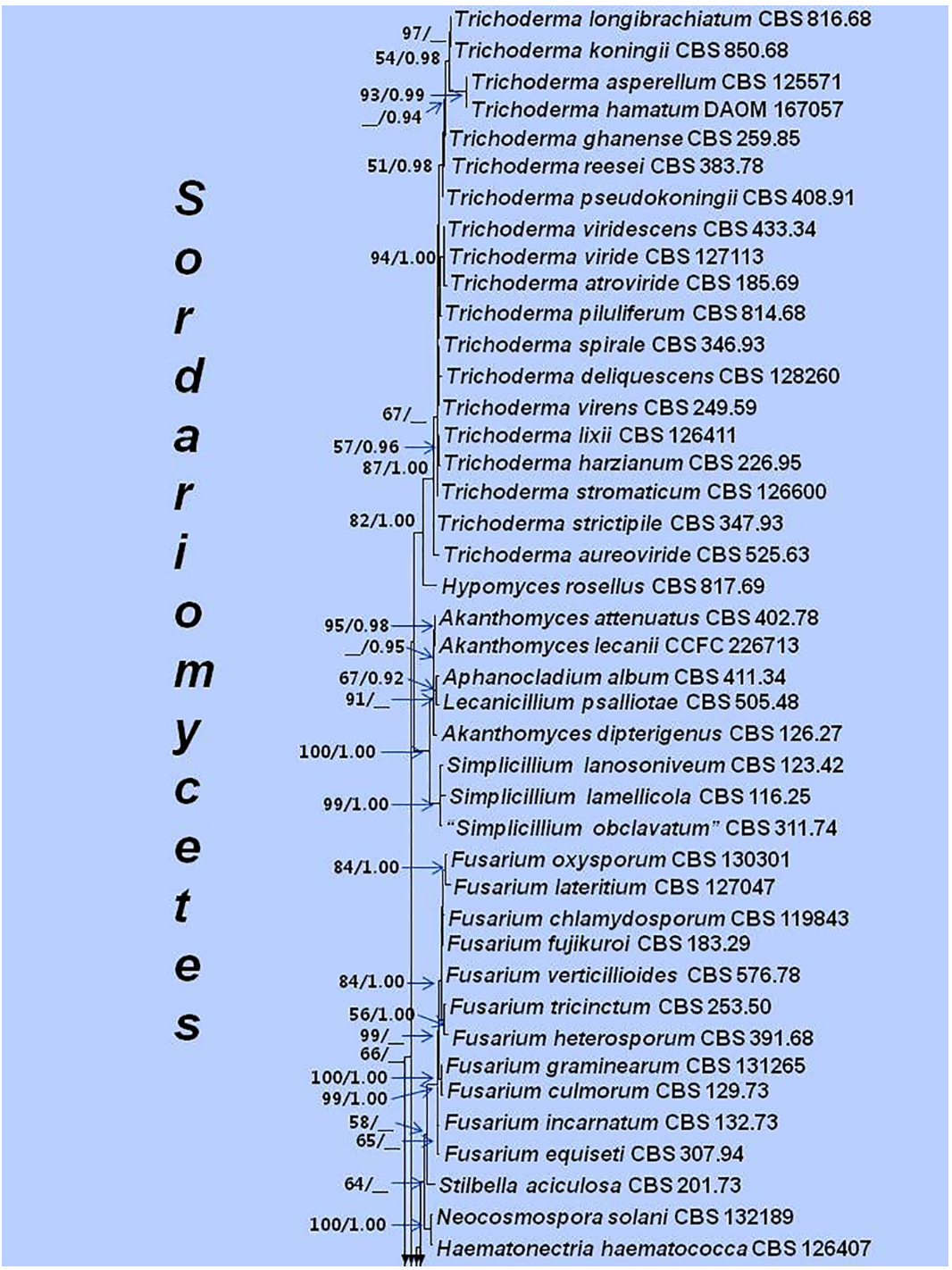

FIGURE 2 | Continued

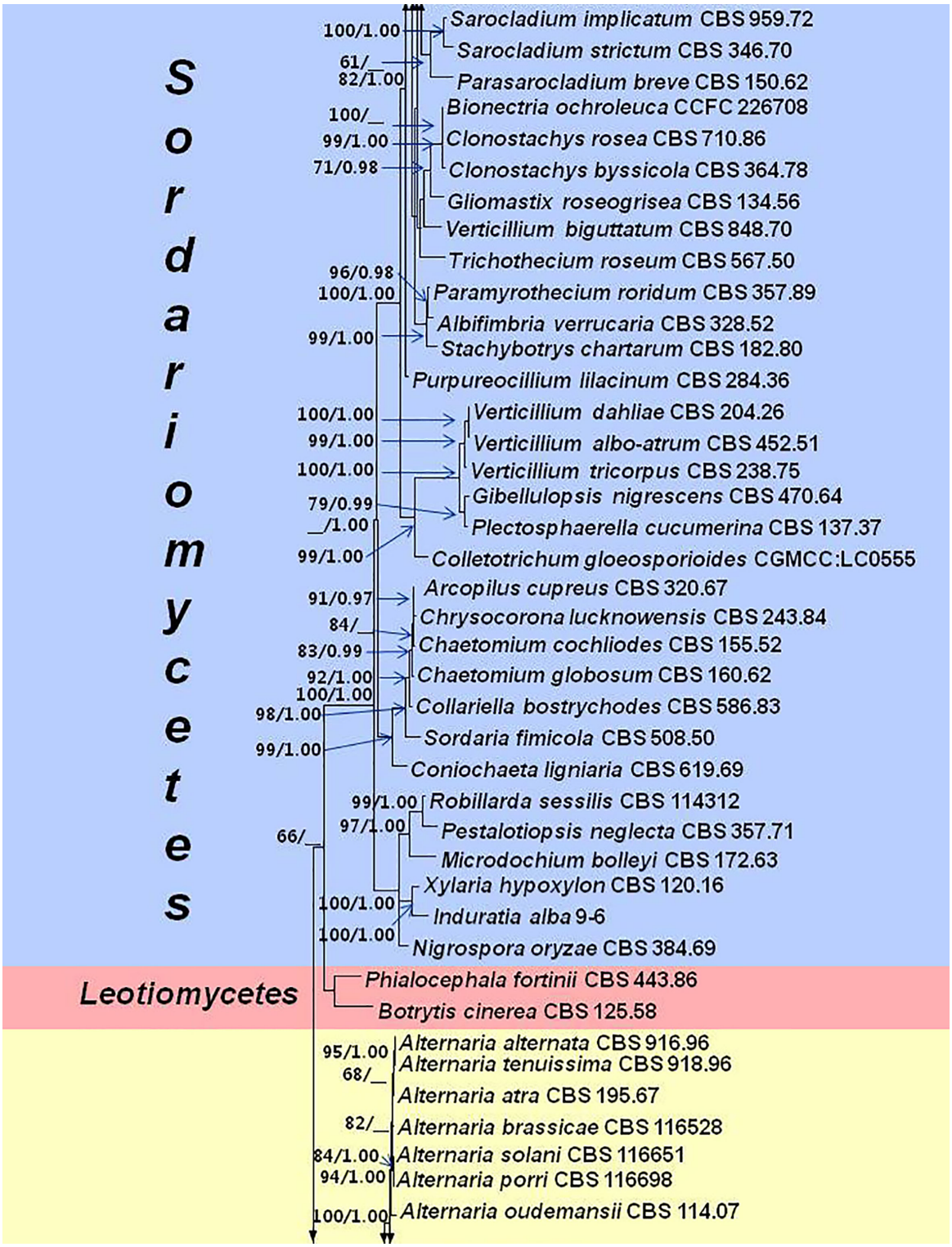

FIGURE 2 | Continued

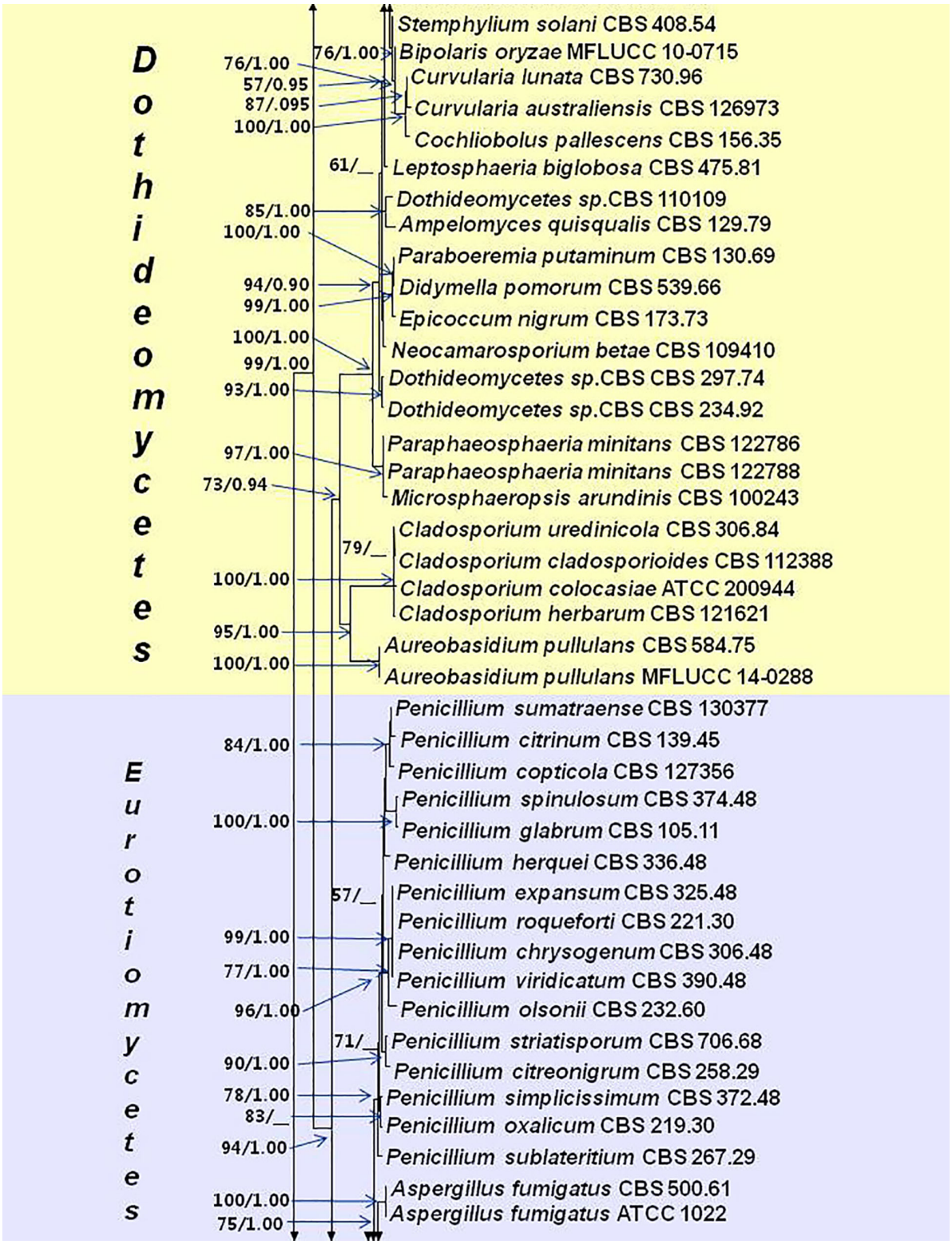

FIGURE 2 | Continued

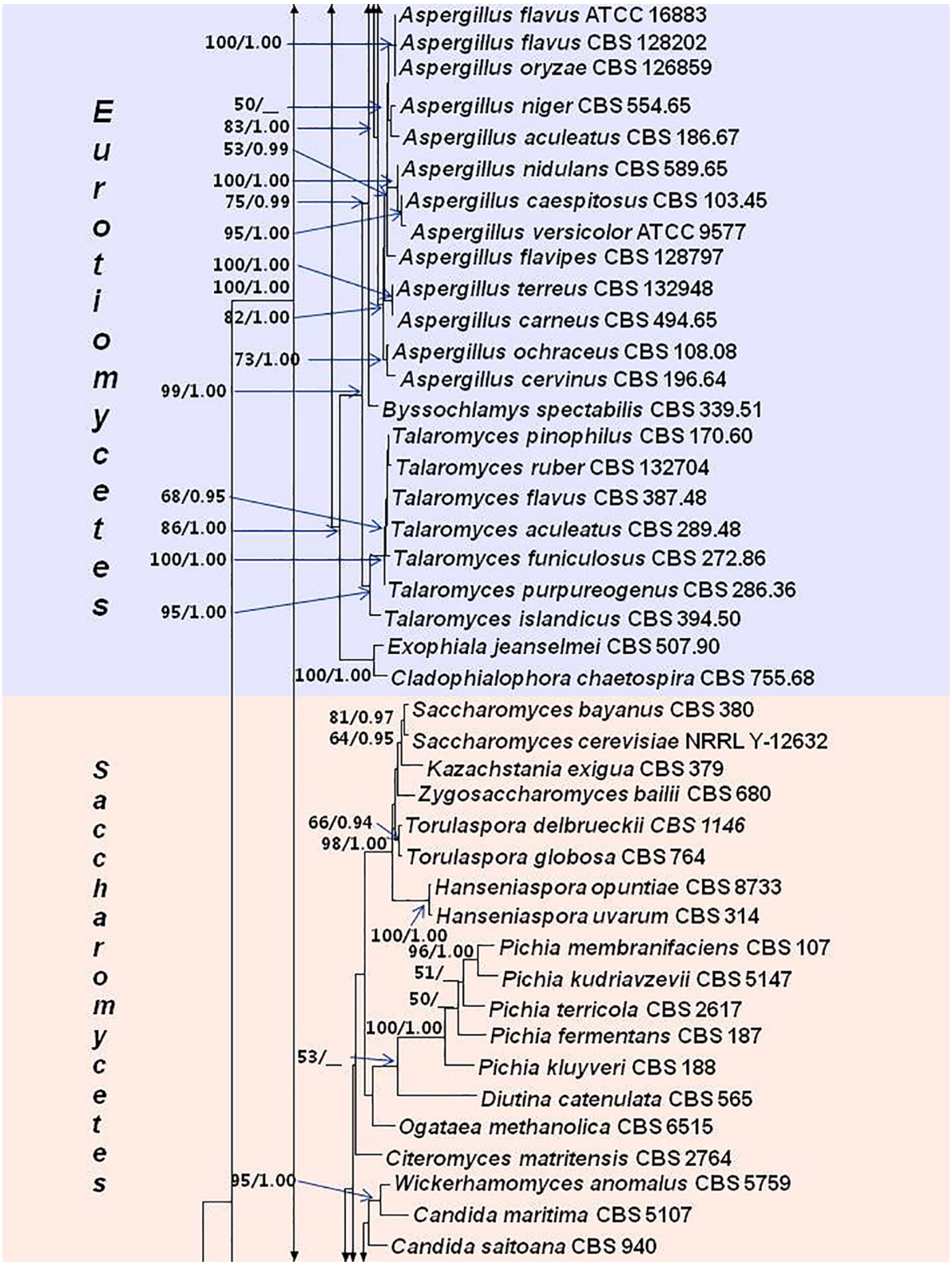

FIGURE 2 | Continued

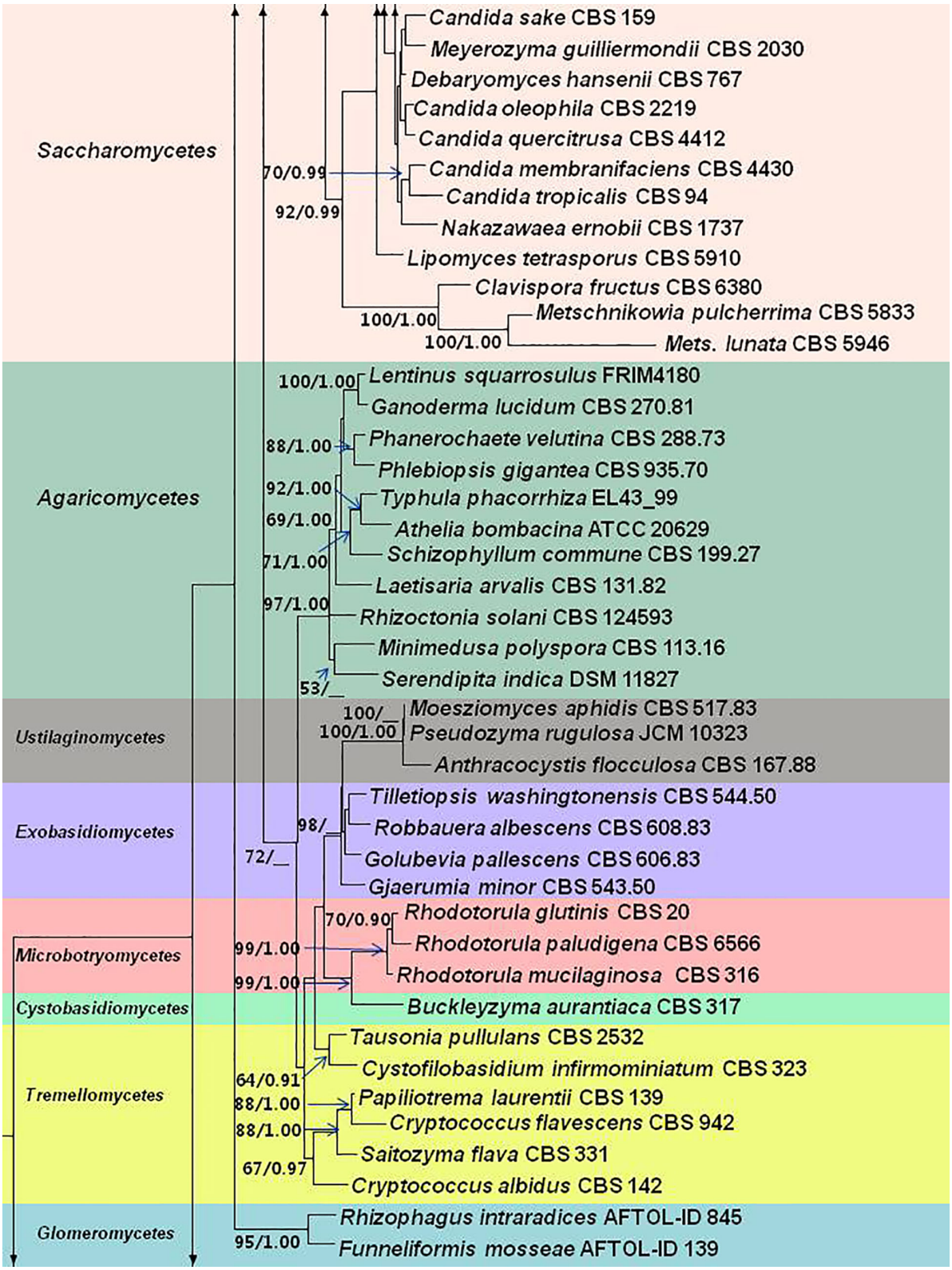

FIGURE 2 | Continued

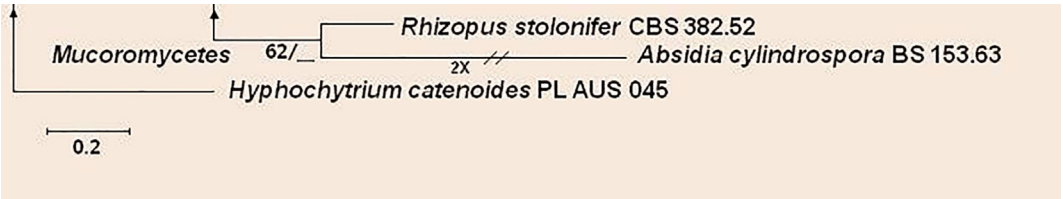

FIGURE 2 | Phylogram resulting from maximum likelihood (RAxML) analysis of sequence alignment of the 28S nrRNA gene (LSU) sequences of fungal antagonists. ML bootstrap values (MLBS) ≥ 50% and Bayesian posterior probabilities (PP) ≥ 0.90 are at each node. The tree was rooted to *Hyphochytrium catenoides* (PL AUS 045). Classes are indicated with coloured blocks to the left of the tree.

a useful strategy in achieving an enhanced performance against plant diseases. Sometimes, it is difficult to select individual strains with a broad spectrum of activity against several plant pathogens that cause severe damages and infections on plants or fruits. Mixed cultures of the microbial antagonists appear to provide better control of plant diseases over individual strains (Guetsky et al., 2001; Freeman et al., 2004; Xu et al., 2011) and these BCAs should be compatible and applied properly with a correct formulation. The application of antagonist mixtures improves the efficacy of biocontrol and are used in many agricultural fields including post-harvest disease control systems. (Datnoff et al., 1995; Nagtzaam et al., 1998; Guetsky et al., 2001; Janisiewicz and Korsten, 2002; Freeman et al., 2004; Droby, 2006; Xu et al., 2011; Doley et al., 2017; Nabi et al., 2017). In a study of the biological control of Armillaria root rot of strawberry plants by Raziq and Fox (2005), all the strawberry plants treated with *Trichoderma harzianum* isolates or *T. viride* isolate alone died by the end of the experiment, while 50% of them survived when treated with a combination of any of the antagonists with *Dactylium dendroides*. Calvo et al. (2003) improved the biocontrol efficiency of postharvest diseases of apples (*Penicillium expansum* and *Botrytis cinerea*) by using mixtures of yeasts (*Rhodotorula glutinis*, *Cryptococcus albidus* and *C. laurentii*) without increasing the amount of antagonists applied. Haggag and Nofal (2006) revealed that the multi-biocontrol agents namely *Trichoderma koningii*, *T. hamatum*, *Pseudomonas fluorescens*, *P. putida*, *Tilletiopsis minor*, and *T. washingtonensis* were more effective against *Botryodiplodia* disease (*Lasiodiplodia theobromae* = Botryodiplodia theobromae) on some Annona cultivars when applied in combination than when applied individually or even when applied in any combination of two agents. Furthermore, applications of multi-biocontrol agents resulted in a significant increase of fruit yield. Doley et al. (2017) showed that the incidence of stem-rot in groundnut caused by *Sclerotium rolfsii* was significantly lowered by combined inoculation of both *Glomus fasciculatum* along with *Trichoderma viride* as compared to when either *G. fasciculatum* or *T. viride* was applied alone.

Application of BCAs with inorganic and organic substances and chemicals such as bicarbonates and chlorides of alkali metals, minerals and other elements (Piano et al., 1997; Droby et al., 1997; Tian et al., 2002a; Gamagae et al., 2003; Tian et al., 2005; Karabulut et al., 2005; Cao et al., 2012) or low dosage of fungicides (Chand-Goyal and Spotts, 1996; Spotts et al., 1998; Qin and Tian, 2004; De Cal et al., 2009) are widely used to control plant pathogenic diseases including post-harvest diseases. A few potential examples of these applications are indicated here. Thus Tian et al. (2005) investigated the synergistic biocontrol effects of *Cryptococcus laurentii* and *Rhodotorula glutinis* combined with silicon (Si) against *Alternaria alternata* and *Penicillium expansum* moulds in jujube fruit stored at different temperatures and found that combinations of these two biocontrol agents with 2% Si is most effective in controlling the diseases on jujube fruit stored at 20°C. The studies of Karabulut et al. (2005) demonstrated that postharvest applications of sodium bicarbonate within a hydrocooler significantly controlled postharvest diseases of sweet cherries. Liu et al. (2010) revealed that tea polyphenol alone or in combination with biocontrol agents has great potential in commercial management of postharvest diseases in fruits. Larena et al. (2010) enhanced the adhesion of *Epicoccum nigrum* conidia to peach surfaces by adding 2.5% methylcellulose to the conidial formulation of *E. nigrum* and this improved the biocontrol of brown rot caused by *Monilinia laxa*. Cao et al. (2012) found that Boron improves biocontrol activity of *Cryptococcus laurentii* against *Penicillium expansum* in jujube fruit. The study by Gamagae et al. (2003) showed that the use of sodium bicarbonate at 2% with the biocontrol agent *Candida oleophila* reduces anthracnose caused by *Colletotrichum gloeosporioides* on papaya during storage. Sometimes, these integrated applications increase the efficiency of the particular BCA and sometimes indirectly improve plant productivity. Ordentlich et al. (1990) showed that integrated treatment of the fungicide captan and the biocontrol agent *Trichoderma harzianum* resulted in a reduction of *Verticillium dahlia* colonization of potato stems (Verticillium wilt), increasing marketable and total potato yield of cultivars Draga by 84% and 46% respectively, and total yield of cultivar Desiree by 80%. However, only BCA alone applications (without combining with organic or inorganic chemical substances) are considered in this review.

Fungal biological control agents act through several mechanisms (*see the introduction*) when controlling plant diseases. However, these mechanisms may cause risks to non-target species including mycorrhizal and saprophytic fungi, soil bacteria, other plants, insects, aquatic and terrestrial animals, and humans. Possible non-target effects of any BCAs or method used

TABLE 2 | Number of known fungal species in each genus with a potential Biocontrol Agent (BCA) activity against plant fungal pathogens.

Phylum	Class	Genus	Number of known species with a potential BC activity against plant fungal pathogens
Ascomycota	Dothideomycetes	***Alternaria***	**08**
		Ampelomyces	01
		Aureobasidium	01
		Bipolaris	01
		Cladosporium	04
		Curvularia	03
		Didymella	01
		Epicoccum	01
		Leptosphaeria	01
		Microsphaeropsis	02
		Neocamarosporium	01
		Paraboeremia	01
		Paraphaeosphaeria	01
		Phaeotheca	01
		Stemphylium	01
	Eurotiomycetes	***Aspergillus***	**16**
		Cladophialophora	01
		Exophiala	01
		Paecilomyces	01
		Penicillium	**17**
		Talaromyces	**08**
	Incertae sedis	*Gonatobotryum*	01
		Teratosperma	01
	Leotiomycetes	*Botrytis*	01
		Phialocephala	01
		Cadophora	01
	Saccharomycetes	***Candida***	**08**
		Citeromyces	01
		Debaryomyces	01
		Diutina	01
		Hanseniaspora	02
		Kazachstania	01
		Lipomyces	01
		Metschnikowia	03
		Meyerozyma	02
		Nakazawaea	01
		Ogataea	01
		Pichia	**05**
		Saccharomyces	02
		Torulaspora	02
		Wickerhamomyces	01
		Zygosaccharomyces	01
	Sordariomycetes	*Acremonium*	02
		Akanthomyces	03
		Albifimbria	01
		Aphanocladium	01
		Arcopilus	01
		Bionectria	01
		Coniochaeta	01
		Chaetomium	03
		Clonostachys	02
		Collariella	01
		Colletotrichum	02
		Fusarium	**12**
		Gibellulopsis	01
		Gliomastix	01
		Haematonectria	01
		Hypomyces	01
		Lecanicillium	01
		Metapochonia	01
		Metarhizium	01
		Microdochium	01
		Muscodor	03
		Neocosmospora	01
		Nigrospora	01
		Paramyrothecium	01
		Parasarocladium	01
		Pestalotiopsis	01
		Plectosphaerella	01
		Purpureocillium	01
		Robillarda	01
		Sarocladium	01
		Simplicillium	02
		Sordaria	01
		Stachybotrys	01
		Stilbella	01
		Trichoderma	**25**
		Trichothecium	01
		Verticillium	**05**
		Xylaria	01
Basidiomycota	Agaricomycetes	*Athelia*	01
		Ganoderma	01
		Laetisaria	01
		Lentinus	01
		Minimedusa	01
		Phlebiopsis	01
		Rhizoctonia	01
		Schizophyllum	01
		Serendipita	01
		Trametes	01
		Typhula	01
		Waitea	01
	Cystobasidiomycetes	*Buckleyzyma*	01
	Exobasidiomycetes	*Gjaerumia*	01
		Robbauera	01
		Tilletiopsis	02
	Microbotryomycetes	*Rhodotorula*	03
	Tremellomycetes	*Cystofilobasidium*	01
		Naganishia	01
		Papiliotrema	02
		Saitozyma	01
		Tausonia	01
	Ustilaginomycetes	*Anthracocystis*	01
		Moesziomyces	02
Glomeromycota	Glomeromycetes	*Claroideoglomus*	01
		Diversispora	01
		Funneliformis	01
		Gigaspora	01
		Rhizophagus	03
		Septoglomus	01
		Simiglomus	01
Mucoromycota	Mucoromycetes	*Absidia*	01
		Rhizopus	01

(Continued)

TABLE 3 | Fungal-like species (Oomycota, Chromista) used as Biocontrol Agents (BCAs) in the past few decades against fungal pathogens/diseases of different host plants.

Biocontrol agent	Disease and host	Pathogen	References
Hyphochytrium catenoides	Phytophthora root rot of soybean	*Phytophthora megasperma* f. sp. *glycines*	Filonow and Lockwood, 1985
Globisporangium ultimum (*Pythium ultimum*)	Barley powdery mildew	*Blumeria graminis* f. sp. *hordei*	Haugaard et al., 2001
Pythium acanthicum	Damping-off of cucumber	*Globisporangium ultimum* (*Pythium ultimum*)	Ali-Shtayeh and Saleh, 1999
	Fusarium ear blight of wheat	*Fusarium culmorum* & *Microdochium nivale*	Diamond and Cooke, 2003
Pythium nunn	Damping-off disease of cucumber	*Globisporangium ultimum* (*Pythium ultimum*)	Paulitz and Baker, 1987
Pythium oligandrum	Seedling and taproot diseases of sugar beet	*Aphanomyces cochlioides*	Takenaka and Ishikawa, 2013
	Seedling disease of sugar beet	*Aphanomyces cochlioides*	Takenaka et al., 2006
	Grey mould of grapevine	*Botrytis cinerea*	Mohamed et al., 2007
	Grey mould of tomato	*Botrytis cinerea*	Le Floch et al., 2003
	Grey mould of strawberry	*Botrytis cinerea*	Meszka and Bielenin, 2010
	Cercospora leaf spot in sugar beet	*Cercospora beticola*	Takenaka and Tamagake, 2009
	Foot rot pathogens of pea	*Didymella pinodella* (*Phoma medicaginis* var. *pinodella*)	Bradshaw-Smith et al., 1991
	Fusarium head blight	*Fusarium graminearum*	Takenaka et al., 2003
	Fusarium crown and root rot of tomato	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	Benhamou et al., 1997; Gerbore et al., 2014
	Seed rot of tomato	*Globisporangium ultimum* (*Pythium ultimum*)	He et al., 1992; Gerbore et al., 2014
	Pythium damping-off in cress and sugar-beet	*Globisporangium ultimum* (*Pythium ultimum*)	Vesely, 1977; McQuilken et al., 1990; McQuilken et al., 1992
	Damping-off disease of cress	*Globisporangium ultimum* (*Pythium ultimum*)	Al-Hamdani et al., 1983
	Damping-off of cucumber	*Globisporangium ultimum* (*Pythium ultimum*)	Ali-Shtayeh and Saleh, 1999
	Seedling diseases of cotton	*Globisporangium ultimum* (*Pythium ultimum*)	Martin and Hancock, 1986; Gerbore et al., 2014
	Pre-emergence damping-off disease of sugar beet	*Globisporangium ultimum* (*Pythium ultimum*)	Martin and Hancock, 1987
	Pythium root rot of tomato	*Pythium dissotocum*	Vallance et al., 2009; Gerbore et al., 2014
	Crown and root rot of tomato	*Phytophthora parasitica* (*Phytophthora nicotianae*)	Picard et al., 2000
	Damping-off of wheat	*Pythium ultimum* var. *ultimum*	Abdelzaher et al., 1997
	Leaf spot of strawberry	*Ramularia grevilleana* (*Mycosphaerella fragariae*)	Meszka and Bielenin, 2010
	Damping off disease of tomato	*Rhizoctonia solani*	He et al., 1992; Gerbore et al., 2014
	Black scurf of potato	*Rhizoctonia solani*	Ikeda et al., 2012
	Seedling disease of sugar beet	*Rhizoctonia solani*	Takenaka et al., 2003
	Powdery mildew of strawberry	*Sphaerotheca macularis* (*Podosphaera aphanis*)	Meszka and Bielenin, 2010
	Verticillium wilt of pepper	*Verticillium dahliae*	Al-Rawahi and Hancock, 1998; Rekanovic et al., 2007
	Verticillium wilt of olive	*Verticillium dahliae*	Varo et al., 2016
Pythium periplocum	Grey mould of grape-vine	*Botrytis cinerea*	Paul, 1999a
	Damping-off of cucumber	*Globisporangium ultimum* (*Pythium ultimum*)	Ali-Shtayeh and Saleh, 1999
Pythium radiosum	Grey mould of grape-vine	*Botrytis cinerea*	Paul, 1999b

in the field should be determined before their applications. Continuous monitoring and the use of molecular techniques to identify and follow the movement of BCAs are also necessary and negative biological impacts can then be avoided (Brimner and Boland, 2003). Cross-protection can be defined as the protection conferred on a host by infection with one strain of a microorganism that prevents infection by a closely related strain of that microorganism. This method has been widely used to control the plant diseases caused by *Fusarium* and *Verticillium* species (Matta and Garibaldi, 1977; Larkin and Fravel, 1998; Nel et al., 2006; Zhu et al., 2013).

The efficiency of a particular BCA against plant diseases can be altered by many factors such as; environmental factors, time of treatment, season of the application, nature or technique of the treatment and the frequency of the application (McLaughlin et al., 1990; Wu and Hsiang, 1998; Ali-Shtayeh and Saleh, 1999; Schisler et al., 2002; Bhagat and Pan, 2007; Lal et al., 2009; Perelló et al., 2009; Padder and Sharma, 2011). The same BCA

can show different efficiencies at *in vitro*, *in vivo*, and greenhouse/field conditions (Wokocha et al., 1986; Larena et al., 2003; Campanile et al., 2007; Padder and Sharma, 2011; Ommati et al., 2013). The dual culture method is the most widely used and simplest *in vitro* technique to determine the activity of BCAs against pathogens and sometimes the results obtained from this method may differ from results found in the *in vivo*, field or greenhouse conditions (Padder and Sharma, 2011; Zheng et al., 2011; Kheireddine et al., 2018). Therefore, it is hard to expect the same result for the same BCA in the field compared to the laboratory or greenhouse applications and it is essential to carry out tests in both *in vitro* and *in vivo* before scaling up the particular BCA. However, in this review, the maximum disease control situations are stipulated in **Table 2**, in case where different environmental conditions have been used in the respective publication.

FUTURE ASPECTS

An assay based on modern taxonomic approaches is highly recommended to identify the antagonists and the pathogens correctly. Also, verified antagonistic cultures must be obtained from reputable culture collections in a case when researchers are planning to use known strains. These practices will largely minimize the confusion related to this field in the future. Moreover, modern biotechnological and genetic engineering tools have contributed immensely towards the development of new fungal strains with high capacity and efficiency of biocontrolling. Last, but not least, fungal extracts and secondary metabolites produced by various fungal species (Mathivanan et al., 1998; Park et al., 2005; Pal and McSpadden Gardener, 2006; Koitabashi and Tsushima, 2007; Reino et al., 2008; Stoppacher et al., 2010; Lou et al., 2011; Cimmino et al., 2013) will also play a significant role in future bio-control methods of plant pathogens.

AUTHOR CONTRIBUTIONS

KT contributed to conception and design of the study. DD and KT performed the phylogenetic analysis. AP, SK, and IP wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.604923/full#supplementary-material

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Recent Advances in Molecular Diagnostics of Fungal Plant Pathogens: A Mini Review

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Phytopathogenic fungal species can cause enormous losses in quantity and quality of crop yields and this is a major economic issue in the global agricultural sector. Precise and rapid detection and identification of plant infecting fungi are essential to facilitate effective management of disease. DNA-based methods have become popular methods for accurate plant disease diagnostics. Recent developments in standard and variant polymerase chain reaction (PCR) assays including nested, multiplex, quantitative, bio and magnetic-capture hybridization PCR techniques, post and isothermal amplification methods, DNA and RNA based probe development, and next-generation sequencing provide novel tools in molecular diagnostics in fungal detection and differentiation fields. These molecular based detection techniques are effective in detecting symptomatic and asymptomatic diseases of both culturable and unculturable fungal pathogens in sole and co-infections. Even though the molecular diagnostic approaches have expanded substantially in the recent past, there is a long way to go in the development and application of molecular diagnostics in plant diseases. Molecular techniques used in plant disease diagnostics need to be more reliable, faster, and easier than conventional methods. Now the challenges are with scientists to develop practical techniques to be used for molecular diagnostics of plant diseases. Recent advancement in the improvement and application of molecular methods for diagnosing the widespread and emerging plant pathogenic fungi are discussed in this review.

Keywords: polymerase chain reaction, molecular identification, next-generation sequencing, fungal plant diseases, emerging fungal pathogens

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INTRODUCTION

Fungal plant pathogens are among the foremost biotic factors that cause devastating disease in crops (Doehlemann et al., 2017). About 8,000 species of fungi and oomycetes are linked with diseases in plants (Horst, 2008; Fisher et al., 2020). Pathogenic fungi infect plants at any phase from the seedling stage to the seed maturing stage under natural environmental conditions, either alone or in concert with other kinds of phytopathogens (Narayanasamy, 2011). The most common diseases caused by plant pathogenic fungi are anthracnose, blight, canker, damping off, dieback, gall, leaf spot, powdery mildew, rust, root rot, scab, and wilt (Iqbal et al., 2018; Hussain and Usman, 2019; Jain et al., 2019). These diseases can generate significant losses in yield (Godfray et al., 2016), quality

and quantity (Shuping and Eloff, 2017) in various agricultural systems (Rodriguez-Moreno et al., 2018) of economically important agronomical (Leonard and Szabo, 2005; Asibi et al., 2019), horticultural (Agrios, 2009; Wenneker and Thomma, 2020), floricultural and ornamental (Darras, 2016; Lecomte et al., 2016), and forest (Ritz, 2005; Marčiulynas et al., 2020) plant species worldwide (Malcolm et al., 2013).

The increasing world population necessitates well-organized plant disease management and control in agriculture to assure food security and safety (FAO et al., 2018; Sarrocco and Vannacci, 2018). An efficient and effective framework for early alert and quick response is a crucial element to combat against phytopathogenic fungi (Sankarana et al., 2010; Nagrale et al., 2016). Diagnosis of fungal plant pathogen is of significance in the area of plant protection as it contributes to improving crop vigor and health. Therefore, fungal disease management requires accurate diagnosis of diseases which is chiefly based on the identification of causative agents. Moreover, it is essential to confirm fungal plant diseases even though the diagnosis of such diseases based on the external symptoms is already made to a satisfactory level. Further, an entire list that covers a known plant disease, its typical sign and symptoms, and its known potential phytopathogen for a precise host is a requisite for disease diagnosis (Thind, 2015).

Various advances have been made in the field of phytopathogenic fungal diagnosis. Conventional fungal disease diagnostic methods have utilized visible signs after phytofungal infections including propagules of fungi *viz.* conidia, sclerotia, or mycelia on the external surfaces of flora, or fungal disease symptoms caused by fungal pathogens after infection (Nezhad, 2014; Tör and Woods-Tör, 2017). These approaches are the cornerstone of fungal disease diagnostics. Widely used conventional methods include isolation and culturing, reinoculation, microscopic techniques and biochemical tests (Tan et al., 2008; Sharma and Sharma, 2016), which have some drawbacks in that they are tedious and require knowledge and expertise in fungal plant pathology and taxonomy (McCartney et al., 2003; Pryce et al., 2003). Immunological-based diagnostic methods are built on the antigen-antibody binding principle and some issues have been noted, such as low sensitivity and affinity in assays, and potential interference from contaminants (Meng and Doyle, 2002). Furthermore, detection of fungal plant pathogens has not been effective due to the high inconsistency and phenotypic serological plasticity of fungi (Luchi et al., 2020). Thus, the implementation and development of novel and effective diagnostic methods to thwart fungal plant disease are urgent. For these reasons, plant-fungal diagnosis has moved to molecular approaches that facilitate pathogen recognition and quantification. Molecular assays can overcome drawbacks of conventional and serological methods in fungal diagnostics.

Modern developments use high throughput molecular detection strategies for plant infecting fungi. These include standard polymerase chain reaction (PCR), real-time PCR, nested PCR, loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), and nucleic acid sequence-based amplification (NASBA) (Aslam et al., 2017; Cheng et al., 2020). PCR restriction fragment length polymorphism (PCR-RFLP) and PCR denaturing-gradient gel electrophoresis (PCR-DGGE) are the methods suitable for genotyping more than for species identification (Johnston-Monje and Mejia, 2020). Further, molecular techniques cover magnetic capture-hybridization PCR (MCH-PCR), *in situ* PCR, co-operational PCR, multiplex PCR, DNA macro and micro arrays, next-generation sequencing (especially RNA-Seq based), etc. (Kumar et al., 2016). Greater confidence, accuracy, specificity, and sensitivity of DNA based molecular techniques (Capote et al., 2012; Midorikawa et al., 2018) permit the diagnosis of phytopathogens at primary stages of infection even though they are present at lower DNA concentrations (Luchi et al., 2013; Rollins et al., 2016).

Additionally, bioinformatics databases such as GenBank at the National Centre for Biotechnology Information (NCBI), Nucleotide Sequence Database Collaboration at the European Bioinformatics Institute (EBI), MycoBank, etc. offer platforms for documenting mycological nomenclatural novelties, storing and retrieving facilities of nucleotide sequences of plant infecting fungi which further accelerate the molecular tools to potentially diagnose and perform species delimitation among existing and evolving fungal species. Rapidly emerging and novel fungal plant pathogens threaten the global economy. Hence, rapid and accurate detection and identification of phytopathogenic fungi is crucial. This review aims to summarize various molecular techniques in fungal plant pathogen diagnosis along with their advantages and drawbacks. It also examines the available molecular tools used to diagnose previously present, emerging, and re-emerging plant pathogenic fungi in various agricultural crops.

MOLECULAR TOOLS FOR DETECTION OF FUNGI

Preanalytical Steps in Molecular-Based Diagnosis of Plant-Infecting Fungi

Detection and diagnosis of fungal plant pathogens using molecular techniques require preanalytical steps such as genomic DNA extraction that efficiently lyse fungal cells and recover the DNA, purification and quantification of extracted DNA. Several protocols for isolation of DNA from plant infecting fungi are available (Doyle, 1991; Cenis, 1992; Chi et al., 2009; Zhang et al., 2010; Gontia-Mishra et al., 2014; Yang et al., 2016). Recent fungal plant disease diagnostic approaches use commercial DNA extraction kits (Martinelli et al., 2015; Moffat et al., 2015). However, laboratories are dependent on standard protocols embracing lyophilization of mycelia, disruption of chitin cell wall by grinding and isolation of DNA in a buffer containing chemicals, removal of proteins by phenol-chloroform mixture, and precipitation with propanol. The isolated fungal DNA can then be purified using standard methods including pelleting, silica membrane (Mancini et al., 2016), spin filter, and silica coated magnetic particle separation (Tsui et al., 2011). Finally, the concentration of fungal DNA in the samples can be determined using UV spectrophotometer and

further it can be diluted with ultrapure PCR grade water to provide an appropriate DNA concentration (Abdullah et al., 2018).

Polymerase Chain Reaction (PCR) Based Assays

End-Point PCR

Advent of PCR revolutionized the accurate identification of various plant pathogens in disease management, including fungi (Ma and Michailides, 2007). In this *in vitro* technique, a piece of DNA template is exponentially amplified (Caetano-Anolles, 2013) through repeated cycles of denaturation, annealing, extension, final extension, and final hold reactions at various temperatures using specific primers, deoxyribonucleotide triphosphates (dNTPs), and a thermostable Taq DNA polymerase in buffer solution (Griffiths, 2014). In end-point PCR, designing either specific oligonucleotides that target certain fungal species or universal primers to amplify multiple pathogens followed by sequencing allows the accurate detection of fungal plant pathogens. For each set of nucleotide sequences of fungal isolates, the identity of each isolate can be determined by comparison against ex-type cultures available in the NCBI GenBank database using Basic Local Alignment Search Tool (BLAST) analysis. The presence of a target revealed in agarose gel electrophoresis assures the existence of targeted phytopathogenic fungi (Mirmajlessi et al., 2015).

End-point PCR systems are considered a cost-effective choice compared to other existing molecular diagnosis options for fungal plant pathogens. However, end-point PCR assays can be time-consuming and it is difficult to design primer sets to delineate closely-related fungal pathogens. Sikdar et al. (2014) dealt with diagnosing *Phacidiopycnis washingtonensis* and *Sphaeropsis pyriputrescens* (which cause speck rot and *Sphaeropsis* rot diseases in apple, respectively) using end-point PCR and real-time PCR assays. They found that the quantitative real-time PCR approach was more sensitive than the end-point PCR approach for rapid diagnosis. Different types of PCR-based molecular diagnosis and examples of each type are given in **Table 1**.

Nested PCR

Nested PCR is a modified version of end-point PCR that uses two sets of primer pairs aimed at two rounds of PCR amplification to enhance specificity and sensitivity. Nesting also aids usage of comparatively non-specific PCR primers in the initial round of PCR for amplification of numerous pathogens, followed by the use of pathogen-specific primers in the next round (Bhat and Browne, 2010). Twig blight and crown rot of pomegranate are emerging diseases in pomegranate cultivation that are caused by *Pilidiella granati*. A nested PCR assay improved both sensitivity and detection of *P. granati* and made it possible to diagnose the causative agent when the sample contained DNA as low as 10 pg of *P. granati* (Yang X. et al., 2017). Great yam disease caused by *Colletotrichum gloeosporioides* (Raj et al., 2013) and eucalyptus dieback disease caused by *Cylindrocladium scoparium* (Qiao et al., 2016) were also detected by this technique. Sensitivity of detection using nested PCR could be enhanced from 10- to 1000-fold over an end-point PCR assay (Ippolito et al., 2002; Silvar et al., 2005). However, nested PCR assays are time-consuming and have an increased risk of cross-contamination due to the manipulation of previously-amplified samples, which can create false-positive outcomes (Raj et al., 2013). Therefore, nested PCR and end-point PCR methods that may produce amplicon contamination would not be recommended to be used as reliable diagnostic methods.

Multiplex PCR

Multiplex PCR assay uses one reaction mixture with various primer pairs, and allows simultaneous amplification of several pathogens (Sint et al., 2012). The generated amplicons can then be separated and visualized using electrophoresis. Designing primers for the multiplex assay is crucial, and specific sets of primers should have similar annealing temperatures for successful amplification (Zhao X et al., 2014). A concurrent diagnostic assay to detect 12 fungi associated with fruit rot in cranberry was established using the multiplex PCR method. Fungal pathogens *Allantophomopsis cytisporea*, *A. lycopodina*, *Phyllosticta elongate*, *Coleophoma empetri*, *Colletotrichum fiorinae*, *C. fructivorum*, *Fusicoccum putrefaciens*, *Monilinia oxycocci*, *Phomopsis vaccinia*, *Phyllosticta vaccinia*, *Physalospora vaccinia*, and *Strasseria geniculate* linked with cranberry fruit rot were effectively identified with the use of *ITS-LSU* and *TEF-1*α gene regions (Conti et al., 2019). The pathogenic fungi *Fusarium oxysporum*, *Bipolaris cactivora*, *Phytophthora nicotinae*, and *Phytophthora cactorum* are threats to the cactus industry that potentially affect its export sector. This problem has been resolved using multiplex PCR assays. It was noted that the diagnosing tool was sufficient to detect and identify these quarantine fungal pathogens in grafted cacti (Cho et al., 2016). Though multiplex PCR assays are quick and reliable in nature, the assays are potentially expensive and resource-intensive, and decreased sensitivity associated with the multiplex methods (Pallás et al., 2018).

Quantitative PCR

Quantitative PCR (qPCR), permits detection and quantification of particular DNA or RNA sequences of phytopathogenic fungi in a PCR reaction mixture in real-time. The relative number of copies of target DNA and RNA sequences can be estimated by projecting a *Ct* (cycle threshold) value of the fungal samples using sequence-specific primers (Balodi et al., 2017). Fluorescent dyes such as SYBR Green I, Eva Green, Molecular Beacons, or sequence-specific fluorescence-labeled reporter probes such as TaqMan (Badali and Nabili, 2012) are used to monitor the reaction during amplification steps. The basic principle is that the fluorescent signal is proportional to the amount of amplicon produced in each cycle and can be generated by an intercalating dye or from the breakdown of a dye-labeled reporter probe during amplification (Alemu, 2014). A hypervirulent and emerging fungal plant pathogen, *Cryphonectria parasitica* causes various diseases (blight, lethal bark cankers, wilting, and

TABLE 1 | PCR based approaches to diagnose pathogenic fungi in different crops.

Assay	Diagnosed fungi	Host	Disease	Target gene[a]	Reference
End-point PCR	*Cercospora tezpurensis* sp. nov.	*Capsicum assamicum*	Leaf spot	*ACT*, *CAL*, *HIS* and *TEF-1α*	Meghvansi et al., 2013
End-point PCR	*Exobasidium maculosum*	Blueberry	Leaf and fruit spot	*LSU-rDNA*	Brewer et al., 2014
End-point PCR	*Golovinomyces cichoracearum sensu lato*	*Cannabis sativa*	Hemp powdery mildew	*ITS*	Pépin et al., 2018
End-point PCR	*Cercospora* cf. *flagellaris*	*Cannabis sativa*	Hemp leaf spot	*ITS, TEF-1α, CAL, HIS* and *ACT*	Doyle et al., 2019
End-point PCR	*Neopestalotiopsis clavispora* and *Colletotrichum siamense*	Macadamia	Leaf spot	*ITS, TUB2, TEF-1α, ACT* and *GAPDH*	Prasannath et al., 2020
Nested PCR	*Puccinia striiformis* f. sp. *tritici*	Wheat	Stripe rust	*PSR*	Wang et al., 2009
Nested PCR	*Phytophthora cactorum*	Strawberry	Crown rot	*ITS*	Bhat and Browne, 2010
Nested PCR	*Colletotrichum gloeosporioides*	*Dioscorea* spp.	Greater yam anthracnose	*ITS*	Raj et al., 2013
Nested PCR	*Pilidiella granati*	Pomegranate	Twig blight and crown rot	*SSU-rDNA*	Yang X. et al., 2017
Multiplex PCR	*Fusarium Verticillioides* and *F. subglutinans*	Maize	Stalk rot and ear rot	*gaoB*	Faria et al., 2012
Multiplex PCR	*Neofabraea alba*, *N. perennans* and *N. keinholzii*	Apple	Bull's eye rot	*TUB2*	Michalecka et al., 2016
Multiplex PCR	*Fusarium oxysporum* f. sp. *cubense* lineage VI strains	*Musa* spp.	Dessert/beer bananas	*TEF-1α* and *RPC2*	Ndayihanzamaso et al., 2020
Quantitative PCR	*Didymella bryoniae*	Cucurbits	Gummy stem blight	*RAPD*	Ling et al., 2010
Quantitative PCR	*Ramularia collo-cygni*	Barely	Ramularia leaf spot	Not mentioned	Havis et al., 2014
Quantitative PCR	*Rhizoctonia solani*	Tobacco	Target spot	*ITS*	Zhao Y. Q. et al., 2014
Quantitative PCR	*Magnaporthe oryzae*	Rice	Rice blast	*18S-28S rDNA*	Sun et al., 2015
Quantitative PCR	*Verticillium longisporum*	*Brassica napus*	Wilt and stem stripe	*TUB2*	Depotter et al., 2017
Quantitative PCR	*Pyrenophora tritici-repentis* and *Parastagonospora nodorum*	Wheat	Tan (yellow) spot and Septoria blotch	*ToxA*	Abdulla et al., 2018
Quantitative PCR	*Fusarium culmorum*	Cereals	Foot and root rot and Fusarium head blight	*COX2*	Bilska et al., 2018
Quantitative PCR	*Fusarium guttiforme*	Pineapple	Fusariosis	*TEF-1α* and *TUB2*	Carnielli-Queiroz et al., 2019
End-point PCR and quantitative PCR	*Phacidiopycnis washingtonensis* and *Sphaeropsis pyriputrescens*	Apple	Speck rot and Sphaeropsis rot	*ITS*	Sikdar et al., 2014
End-point PCR and quantitative PCR	*Guignardia citricarpa*	*Citrus* spp.	Citrus black spot	*ITS*	Faganello et al., 2017

[a]*ITS, internal transcribed spacer; LSU-rDNA, large subunit ribosomal DNA; SSU-rDNA, small subunit ribosomal DNA; TUB2, β-tubulin 2; TEF-1α, translation elongation factor 1-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACT, actin; CAL, calmodulin; HIS, histone H3; PSR, Puccinia striiformis f. sp. tritici repeat sequence; gaoB, galactose oxidase B; RPC2, DNA-directed RNA polymerase III subunit RPC2; RAPD, random amplified polymorphic DNA; ToxA, exotoxin A; COX-2, cyclooxygenase-2.*

dieback) in chestnut trees, *Castanea dentata* and *C. sativa* (Murolo et al., 2018; Jain et al., 2019). Molecular diagnosis by qPCR allowed the detection of *C. parasitica* using *rDNA ITS* sequences with a sensitivity of 2 fg of genomic DNA which was equivalent to the single spore of a pathogen (Chandelier et al., 2019).

An emerging fungal pathogen *Ramularia collo-cygni* shows typical symptoms of small, brown spots on leaves, sheaths, and awns, which made it difficult to accurately diagnose this disease using conventional techniques (Havis et al., 2015). A qPCR test was developed and submitted as the first report on molecular detection of *R. collo-cygni* in barley seed (Havis et al., 2014). An aggressive and emerging British *Verticillium longisporum* is another example of a novel fungal pathogen that was diagnosed with a qPCR approach (Depotter et al., 2017). A qPCR was able to distinguish and quantify *Diaporthe helianthi* and *D. gulyae*, the fungal pathogens of Phomopsis stem canker in sunflower. These causative agents from the same genus were successfully screened using the assay (Elverson et al., 2020). *Pyrenophora tritici-repentis* and *Parastagonospora nodorum* cause co-infections in wheat and share common physiognomies, making it a challenge for traditional disease diagnosis. Two dual-labeled probes with unique fluorogenic reporters (permitting DNA sequences from *P. triticirepentis* and *Pa. nodorum* to be amplified in parallel but independently of each other) were custom designed to perform a duplex qPCR assay, and results obtained were accurate and appropriate to simultaneous differentiation and suitable for high throughput screening of multiple pathogens (Abdullah et al., 2018). This technique is fast and very sensitive (Sikdar et al., 2014), and can provide reliable information on pathogen load (Garrido et al., 2009) and high throughput quantification of target DNA in biological areas (Schena et al., 2013). Further, the TaqMan probe offers an extra level of specificity (Shuey et al., 2014). However, qPCR needs a specialized instrument and cost of the instrument and probe can be high (Abdullah et al., 2018).

BIO-PCR

BIO-PCR assay is a modification of end-point PCR technique which involves a pre-assay incubation step in a diseased sample to increase the biomass of the causal agent. This technique is mainly used to concentrate target pathogens by growing the target pathogen in a growing media that prevent the growth of non-target microorganisms to improve detection (Schaad et al., 1995) and has been effectively used to detect seed-borne fungal pathogens (Kumar et al., 2020). A seed and airborne lupin anthracnose disease caused by *Colletotrichum lupini* was diagnosed using the BIO-PCR method. Incubation of the seeds with amended Yeast Malt Broth was done to enrich *C. lupine* biomass and a species-specific primer pair was developed based on *rDNA IGS* sequence. The established BIO-PCR protocol allowed the detection of *C. lupine* in *Lupinus* spp. (Pecchia et al., 2019). The seed-borne fungal pathogens *Alternaria alternata*, *A. radicina*, and *A. dauci* were detected using specific primers of *ITS* in rDNA with the help of a deep-freeze-blotter method during the BIO-PCR assay (Konstantinova et al., 2002). High sensitivity, elimination of PCR inhibitors and detection of living cells to avoid false positives are the advantages over end-point PCR techniques (Marcinkowska, 2002; Fatmi et al., 2005). Limitations of this technique are that it is time consuming and costs are incurred when selective media is used for the assay (Schena et al., 2004; Mancini et al., 2016).

Magnetic-Capture Hybridization PCR

Magnetic-capture hybridization PCR (MCH-PCR) uses DNA isolation with a purification phase that contains hybridization with single stranded DNA (ssDNA) probe on magnetic beads followed by the PCR amplification of target DNA sequences (Jacobsen, 1995). This PCR assay was chiefly established to deal with PCR inhibitors in plant extracts during DNA isolation steps. The magnetic beads used are coated with a biotinylated oligonucleotide that is specific to a DNA region of the fungal pathogen of interest (Walcott et al., 2004). The hybridization of double stranded DNA (dsDNA) and magnetic beads allows for separation of the complex from inhibitors (Capote et al., 2012). MCH and real-time based PCR assays were evaluated for two cucurbit seed pathogens, *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae*, that cause bacterial fruit blotch and gummy stem blight, respectively. The assay facilitated simultaneous detection of both tested pathogens in cucurbit seed samples (Ha et al., 2009). Designing of a capture probe used in MCH-PCR involves selection of oligonucleotide probe sequence from highly conserved regions of fungal pathogens (Langrell and Barbara, 2001). The selected sequence can be examined *in silico* for specificity using BLAST. The 5' end of the probe is then biotin-labeled (Chen and Griffiths, 2001) to allow attachment with streptavidin-coated magnetic beads (Johnson et al., 2013). MCH-PCR would decrease the total detection time, increase PCR sensitivity, and remove most of the inhibitors of the amplification reaction and excess of nontarget DNA (Amagliani et al., 2006).

Isothermal Amplification Based Methods

Rolling Circle Amplification

Rolling circle amplification (RCA) is an isothermal enzymatic assay that exploits DNA or RNA polymerase to generate ssDNA or RNA molecules. Requirements for an RCA assay are DNA polymerase, homologous buffer, short DNA/RNA primer, a circular template, and deoxynucleotide triphosphates (Gu et al., 2018). In this assay, DNA amplification using phi29 DNA polymerase with a strand displacement activity to extend a single or multiple primers annealing to a circular DNA template is essential (van Emmerik et al., 2020). The strand displacement process allows newly synthesized DNA template to displace the formerly synthesized DNA molecule to release ssDNA (Bhat and Rao, 2020). Long ssDNA with 100– 1,000 tandem repeats of the original targeted sequence are generated after cascade of strand displacement events (Kieser and Budowle, 2020). Four padlock probes PLP-Nm, PLP-Np, PLP-Nk, and PLP-Nv targeted on *TEF-1α* segments were designed to identify *Neofabraea malicorticis*, *N. perennans*, *N. kienholzin*, and *N. vangabunda* that cause bull's eye rot in apple. An RCA assay was used to diagnose these pathogens and provided effective and sensitive results to monitor the pathogens in the quarantine sector (Lin et al., 2018). In another study, RCA was performed to detect the Fusarium head blight-causing agent *Fusarium graminearum* and other pathovars, *F. oxysporum*, *F. incarnatum-equiseti*, and *F. tricinctum* species complex with the use of padlock probes that were designed based on polymorphisms in the elongation factor *TEF-1α* (Davari et al., 2012). Simplicity, efficiency, and no need of temperature cycling devices are the advantageous of the RCA assay (Dong et al., 2013; Goo and Kim, 2016). This method can also be used to analyze gene expression, single nucleotide polymorphism, mRNA splicing and post translational modification of protein molecules (Gao et al., 2019).

Loop Mediated Amplification

Loop mediated amplification (LAMP) technique has become a significant diagnostic tool in various plant disease diagnosis over only a decade and holds huge potential in plant disease management (Le and Vu, 2017). The LAMP reaction comprises two main stages including an initial step and cycling amplification, followed by an elongation step (Panno et al., 2020). In a LAMP assay, a set of two internal primers, forward inner primer (FIP) and backward inner primer (BIP), one backward loop primer (B-Loop), and another set of two outer primers (F3 and B3), are used to recognize six unique sequences on the targeted nucleic acid. Each FIP and BIP comprises double distinct sequences corresponding to sense and anti-sense strands of targeted DNA. Inclusion of two extra loop primers such as loop forward (LF) and loop backward (LB) and Bst DNA polymerase may accelerate the LAMP method (Nagamine et al., 2002; Francois et al., 2011). Its high exponential and isothermal amplification yields 10^9–10^{10} fold target DNA in 45–60 min at 60–65°C and this temperature rage is considered to be ideal for Bst polymerase activity (Notomi et al., 2000; Chander et al., 2014). Examples of various fungal plant disease diagnoses using LAMP are shown in **Table 2**.

Uromyces betae, sugar beet rust-causing fungi, was identified within 30 min using LAMP assay which targeted the *cytochrome b* DNA sequence (Kaczmarek et al., 2019). Conventional LAMP (cLAMP) and a quantitative LAMP (qLAMP) assays were

performed to diagnose a quarantine fungal pathogen *Fusarium circinatum* that causes pitch canker in pine and other conifers. LAMP probes targeting *TEF-1α* revealed that qLAMP tests had higher specificity than cLAMP for the detection of *F. circinatum* (Stehliková et al., 2020). A widespread pathogen, *Sclerotinia sclerotiorum*, with a broad host range including rape seed, was detected using a LAMP assay targeting *Ssos5* and a visualizing indicator hydroxynaphthol blue (HNB) for detection. The identification limit of *S. sclerotiorum* using the LAMP technique was 0.1 fg μl^{-1} of genomic DNA per reaction which was significantly lower (100 fg μl^{-1}) than the end-point PCR test (Duan Y. et al., 2014). Further, the first LAMP and qLAMP detection systems using *MGG_04322* as a target in *Magnaporthe oryzae* were developed for prompt detection and accurate identification of rice blast disease (Li et al., 2019). *Fusarium odoratissimum* tropical race 4 (TR4), the cause of panama disease in banana plants, was accurately diagnosed based on a LAMP assay using *TR4* markers that were sequenced from diversity arrays technology sequencing (DArTseq) technology (Ordóñez et al., 2019). LAMP allows analysis of crude samples as it is not affected by inhibitors (Panno et al., 2019). This tool is extremely sensitive and specific due to amplification of nucleic acids accomplished by using up to six primers (Becherer et al., 2020). Isothermal and energy efficient intensification requirements of LAMP technology makes it a prime candidate for rapid and inexpensive alternative assays (Waliullah et al., 2020). Thus, LAMP is well established in several areas including medicine, agriculture, and food industries (Mori and Notomi, 2009;

TABLE 2 | LAMP based molecular diagnosis of fungal pathogens in various crops.

Pathogen	Host	Disease	Target gene[a]	Detection system	Reference
Phytophthora sojae	Soybean	Phytophthora root rot	*A3aPro*	Real time measurement of turbidity, gel electrophoresis, and hydroxynaphthol blue (HNB) visualizing indicator	Dai et al., 2012
Verticillium dahliae	Olive	Vascular wilt	*RAPD*	HNB visualizing indicator	Moradi et al., 2014
Botrytis cinerea	Fruits and flowers	Gray mold disease	*Bcos5*	HNB visualizing indicator	Duan Y. B. et al., 2014
Fusarium oxysporum f. sp. *ciceris*	Chick pea	Fusarium wilt	*TEF-1α*	HNB visualizing indicator	Ghosh et al., 2015
Colletotrichum falcatum	Sugarcane	Red rot	*SCAR*	SYBR Green I dye	Chandra et al., 2015
Didymella bryoniae	Cucurbitaceae	Gummy stem blight	*RAPD*	Calcein indicator and gel image	Yao et al., 2016
Plasmopara viticola	Grape	Grape downy mildew	*ITS*	HNB visualizing indicator	Kong et al., 2016
Calonectria osedonaviculata and *C. henricotiae*	Box wood	Boxwood blight	*TUB2*	Capillary gel electrophoresis	Malapi-Wight et al., 2016a
Fusarium oxysporum f. sp. *lycopersici*	Tomato	Tomato wilt	*SIX3*	Melting curve analysis	Ayukawa et al., 2017
Peronospora destructor	Onion	Downey mildew	*ITS*	HNB visualizing indicator	Yang K. et al., 2017
Pyrenopeziza brassicae	*Brassica napus*	Light leaf spot	*ITS* and *TUB2*		King et al., 2018
Fusarium fujkuroi and *Magnaporthe oryzae*	Rice	Bakane and rice blast	*TEF-1α* and *CAL*	Gel electrophoresis	Ortega et al., 2018
Puccinia triticina	Wheat	Leaf rust	*PTS68*	HNB visualizing indicator	Manjunatha et al., 2018
Fusarium fujkuroi	Rice	Bakane	*NRPS31*	HNB visualizing indicator	Zhang et al., 2019
Magnaporthe oryzae	Rice	Rice blast	*MGG_04322*	HNB visualizing indicator and gel electrophoresis	Li et al., 2019
Ustilago tritici	Wheat	Loose smut	*LSU-rDNA* and *ITS*	Visual observation under natural light and gel imager	Yan et al., 2019
Fusarium oxysporum f. sp. *melonis*	Melon	Fusarium wilt	*TEF-1α*	HNB visualizing indicator	Almasi, 2019
Uromyces betae	Sugar beet	Sugar beet rust	*cyt b*	FAM dye	Kaczmarek et al., 2019
Colletotrichum gloeosporioides	Strawberry	Anthracnose	*cyt b*	HNB visualizing indicator	Wu et al., 2019
Talaromyces flavus	Strawberry	Not mentioned	*rlf*	Fluorescence data	Panek and Frąc, 2019
Alternaria spp.	Pear	Pear black spot	*Aacyt-b*	SYBR Green I dye	Yang et al., 2019
Calonectria ilicicola, Dactylonectria macrodidyma and *Dactylonectria* genus	Avocado	Black root rot	*TUB2* and *HIS*	Presence of annealing curve	Parkinson et al., 2019
Phytophthora infestans	Potato	Potato late blight	*PiSMC*	HNB visualizing indicator	Kong et al., 2020

[a]*ITS, internal transcribed spacer; LSU-rDNA, large subunit ribosomal DNA; TUB2, β -tubulin 2; TEF-1α, translation elongation factor 1-α; CAL, calmodulin; HIS, histone H3; PTS68, Puccinia triticina sequence repeat; RAPD, random amplified polymorphic DNA; Bcos5, Botrytis cinerea mitogen-activated protein kinase; SIX3, secreted in xylem 3; NRPS31, non-ribosomal peptide synthetase; MGG_04322, A1b1 superfamily hypothetical protein; cyt b, cytochrome b; rlf, DNA replication licensing factor; Aacyt-b, Alternaria alternata cytochrome b; PiSMC, Phytophthora infestans specific multiple copy DNA sequence; SCAR, sequence characterized amplified region; A3aPro, an element in the upstream of the avirulent gene Avr3 in Phytophthora sojae.*

Guan et al., 2010; Panno et al., 2020). The short size of target gene fragments, using six primers that can generate difficulties in experimental design, and the extreme amount of indicator and other reaction constituents that inhibit polymerase and carryover contaminations are considered to be the drawbacks of this diagnostic tool (Tanner et al., 2015).

Nucleic Acid Sequence Based Amplification

Nucleic acid sequence based amplification (NASBA) amplifies nucleic acids under isothermal conditions. It commonly uses RNA for amplification where a single stranded RNA (ssRNA) template is changed to complementary DNA (cDNA) by reverse transcriptase. The temperature for NASBA is around 41°C and the assay utilizes avian byeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase (Chang et al., 2012). It has the ability to generate over 10^8 copies of the desired nucleic acid sequence within 30 min (Wernecke and Mullen, 2014) and amplicons are detected *via* a probe-capture hybridization using electrochemiluminescence in molecular beacons (Heo et al., 2019). NASBA does not require a thermal cycler and the amplification power is comparable or better than real-time based PCR assays (Loens et al., 2006). Moreover, NASBA requires only short reactions, has high sensitivity and stringent control, and is not affected by inhibitors and is well suited for lab-on-a-chip devices (Honsvall and Robertson, 2017). The application is very rare in plant fungal pathogen detection. However, it has potential to be used in the detection of fungal diseases in future.

Post Amplification Techniques

DNA Microarray

A DNA microarray (DNA chip, gene chip, or biochip) (Bhatia and Dahiya, 2015) is an assemblage of microscopic DNA spots attached to a solid surface (usually glass) in defined positions. The microscopic spots consist of thousands of specific DNA sequences (probes) that are used to hybridize a cDNA (target) sample. Hybridized probe-cDNA systems can be detected and quantified using fluorophore, silver, or chemiluminescence-labeled targets to define the comparative abundance of transcripts in the sample (Guigó, 2013). Advancement of DNA microarray technology has led high throughput and multiple detection of various phytopathogens including viruses, viroids, bacteria, and fungi (Tiberini and Barba, 2012; Musser et al., 2014; Nam et al., 2014; Krawczyk et al., 2017). PCR primers and florescent probes for targeting fungal potato pathogens *Rhizoctonia solani*, *Spongospora subterranea* (*ITS* region), *Alternaria solani*, *A. alternata* (*Alt_a1* gene), *Fusarium* sp. (*TEF-1α*), and *Colletotrichum coccodes* (*TUB2*) were used with qPCR microarray technology in 48-well silicon microarrays (Nikitin et al., 2018). A novel microarray assay (ArrayTube) using marker genes *ITS*, *TEF-1α*, and *16S rDNA* with well performing probes allowed the detection of various sugar beet root rot pathogens, *Aphanomyces cochlioides*, *Botrytis cinerea*, and *Penicillium expansum* (Liebe et al., 2016). DNA microarrays can be produced batch wise on standard microscope slides in a rapid, easy, consistent, and cost-efficient way (Johannes et al., 2020). A major drawback of microarray is that it can only detect sequences that the array is designed to identify (Bumgarner, 2013).

DNA Macroarray

DNA macroarrays are built by designing species-specific probes (15–30 bases of oligonucleotides) that are arrayed into well plates and fixed on a nylon or nitrocellulose membrane. The probe hybridization with PCR amplified and labeled target DNA sequence can then be detected (Clark et al., 1999; Zhang et al., 2008). An oligo-DNA macroarray assay was established with digoxigenin-labeled RNA probes to identify the microbes in the phyllosphere of apple trees (He et al., 2012). For the identification of fungi, 40 bp of oligo-DNA sequences were selected from the fungal *rDNA-ITS* region and fungal pathogens that inflict scab (*Venturia inaequalis*), Alternaria blotch (*Alternaria mali*), and Marssonina blotch (*Diplocarpon mali*) were detected along with several non-phytopathogenic fungal and bacteria (He et al., 2012). DNA macroarray based reverse-blot hybridization was performed to target the *TUB2* gene from isolated DNA and amplified using Bt2a and Bt2b primers that were labelled with digoxygenin in PCR. This array endorsed the detection and discrimination of 15 different species belonging to the genera *Cadophora*, *Campylocarpon*, *Cylindrocarpon*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Phaeomoniella* including the black-foot pathogens *Campylocarpon fasciculare*, *C. pseudofasciculare*, *Dactylonectria macrodidyma*, *D. pauciseptata*, *Ilyonectria europaea*, *I. liriodendri*, and *I. robusta* (Agustí-Brisach and Armengol, 2013). Young vine decline (YVD) is a complex disease in grapevine that causes severe mortality in young vineyards (Úrbez-Torres et al., 2015). Sixty-one species including 34 YVD fungal pathogens were diagnosed by a DNA macroarray (Úrbez-Torres et al., 2015). Macroarray offers a reliable and effective method for pathogen diagnosis even if the sample contains multiple pathogens (Zhang et al., 2008). Finite life of filters, annotations of potential new genes showing low abundance of transcripts, and large volume of filters needed to be hybridized are major limitations of macroarray (Gammill and Lee, 2008). The other pitfalls of this array are lack of pathogen quantification and the impossibility to define whether the detected DNA is from a live organism (Úrbez-Torres et al., 2015).

DNA or RNA Probe Based Assays

In Situ Hybridization

In situ hybridization (ISH) technique functions to detect mRNAs present in the fixed sample. Designing of an antisense ssRNA probe aimed to bind target mRNA (sequence of interest) is the main step of this assay. However, synthetic oligonucleotide probes and cDNA probes can also be used (Jensen, 2014). Typically, probes are labeled with the radioactive isotopes ^{35}S, ^{125}I, and ^{32}P for probe labeling as they are very sensitive and easily quantified for detection. Nonisotopic probes can use biotin, digoxigenin, tyramide, alkaline phosphatase, or bromodeoxyuridine for probe labeling. Photography, autoradiography with X-ray film, liquid emulsion, and microscopic techniques can be used in signal detection (Corthell, 2014). The rust fungi *Puccinia horiana* isolate PA-11, *Uromyces transversalis* isolate CA-07, and *Phakopsora pachyrhizi* isolate Taiwan 72-1 infecting *Chrysanthemum* × *morifolium*, *Gladiolus* × *hortulanus*, and *Glycine max* respectively, were distinguished as rust pathogens in their respective paraffin fixed plant tissues using the ISH technique (Ellison et al., 2016). ISH

enables maximum usage of tissue that is hard to obtain, but the main limitations of ISH are cost and hazardous nature of radioactive probes, and the difficulty in identification when the target has low concentrations of DNA and RNA (Jin and Lloyd, 1997).

Fluorescent *In Situ* Hybridization

Fluorescent *in situ* hybridization (FISH) is a comparatively recent and innovative technology in plant disease diagnostics. It combines the specificity in DNA sequences with the sensitivity of detection systems based on fluorochromes (Hijri, 2009; Cui et al., 2016). FISH assays include the detection of DNA or RNA sequences in cells or tissues using DNA or RNA probes, which are labeled directly or indirectly with fluorochromes (Shakoori, 2017). In normal FISH methods, fluorescently mono-labeled oligonucleotide probes are hybridized to the ribosomal RNA (rRNA) of microbial cells, and the stained cells are then visualized by wide field epifluorescence or confocal laser scanning microscopy (Lukumbuzya et al., 2019). Upon pathogen infection, pathogen-specific rRNA sequences will be present in plants. This specific information provided by RNA can be detected by FISH (Fang and Ramasamy, 2015). Southern blight in tomato is caused by a soil-borne pathogen, *Sclerotium rolfsii*. Soil smears in a DNA isolation with 0.06 pg $\mu l^{-1}$of *S. rolfsii* was effectively detected by FISH technique that used an oligonucleotide probe labeled with cyanine dyes Cy3 and Cy5 (Milner et al., 2019). Reproducibility, sensitivity, specificity, accuracy, and speed are the best features of FISH (Bozorg-Ghalati et al., 2019). It also has potential to deliver information about resolution, morphology, and identification of main pathogens in mixed species specimens (Frickmann et al., 2017). False positive results with autofluorescence materials are a common pitfall that reduces specificity during the assay (Moter and Göbel, 2000).

Next-Generation Sequencing

Next-generation sequencing (NGS) or high throughput sequencing (HTS) is a new approach for diagnostics. The development of NGS technologies has fueled innovative ways for detection and identification of phytopathogens (Chalupowicz et al., 2019). Isolation and fragmentation of DNA, library preparation, massive parallel sequencing, bioinformatics analysis, and variant/mutation annotation and interpretation are the major steps involved in DNA based NGS (Qin, 2019). Massively parallel signature sequencing, pyrosequencing, polony sequencing, and sequencing by oligonucleotide ligation detection (SOLID) are some commonly available advanced sequencing methods in HTS (Rajesh and Jaya, 2017). RNA-Sequencing (RNA-Seq) offers advanced coverage and greater resolution of the dynamic nature of the transcriptome. The Illumina HiSeq platform is the most universally functional NGS platform for RNA-Seq and has established the standard for NGS. The platform more recently released a desktop sequencer, MiSeq (Kukurba and Montgomery, 2015).

RNA-Seq based NGS can be used in the rapid identification of fungal plant pathogens inducing novel diseases. A whole-genome sequencing approach using Illumina MiSeq was established to detect the Sarcococca blight-causing novel fungal pathogen, *Calonectria pseudonaviculata* in ornamental plants. A 51.4 Mb genome of the two host isolates showed a unique single nucleotide polymorphism for the two isolates and identified both as *C. pseudonaviculata* (Malapi-Wight et al., 2016b). Datasets from population genomics built on NGS can be exploited to recuperate variations including single nucleotide polymorphisms (SNPs), insertions and deletions (INDELS), and structural variations (Potgieter et al., 2020). *Puccinia striiformis* f. sp. *tritici* (PST) is an emerging or re-emerging plant infecting fungus that causes wheat yellow (stripe) rust in wheat and triticale. Field pathogenomics was done using RNA-Seq based NGS of PST infested wheat leaves to gain insight into emergent pathogen populations. The results revealed that there was a dramatic shift in the PST population in the UK, likely due to a current introduction of a different set of emerging and exotic PST lineages (Hubbard et al., 2015). RNA and DNA based NGS approach was conducted to develop molecular diagnostics for the cucurbit downy mildew pathogen *Pseudoperonospora cubensis*. Comparative genomics using RNA-Seq of close relative species *P. humuli* identified seven specific regions in *P. cubensis* that allowed for the development of diagnostic markers (Withers et al., 2016).

Monilinia fructicola, a brown rot disease causing fungal pathogen, causes post- and pre-harvest damages in stone and pome fruits. A hybrid and hierarchical *de novo* association strategy was used to sequence the genome of *M. fructicola* Mfrc123 strain through an amalgamation of Illumina short read NGS and Pacific Biosciences (PacBio) long read third-generation sequencing platforms (Angelini et al., 2019). In another hybrid approach, the genome of the coffee rust fungus *Hemileia vastarix* was sequenced using PacBio RS II and Illumina HiSeq platforms and a total genome of 547 Mb of *H. vastatrix* race XXXIII was generated (Porto et al., 2019). The sequenced reference genomes can be used to study the genome biology and evolution with other species. The arrival of a novel pathogen is an instance where the target cannot be well-defined. Since NGS involves no prior knowledge of pathogen sequences, the whole genome of the causal organism may be sequenced without using specific primer pairs and PCR amplification (Hadidi et al., 2016; Malapi-Wight et al., 2016b). Among NGS approaches, advances in single-molecule sequencing technologies, "third-generation sequencing," has advantages over second-generation sequencing techniques (Schadt et al., 2010). The major limitation in NGS is the time consumption incurred during assembly and analysis of large amounts of sequence data (Espindola et al., 2015). Next generation applications are often restricted by low RNA yield and/or integrity, RNA stability, and impurities with DNA, salts, or chemicals (Cortés-Maldonado et al., 2020). Also, bioinformatics and mycological expertise are necessary for NGS analysis, even if the data can be easily and quickly obtained, so that knowledge about fungal and bioinformatics analyses are mandatory to avoid any misinterpretation.

CONCLUSION

Recent advances in molecular biological techniques have increased detection and diagnosis of novel, emerging, previously reported, and re-emerging fungal plant pathogens. Traditional and variant

polymerase chain reaction (PCR) based assays, isothermal and post amplification tools, hybridization techniques, and next-generation sequencing (NGS) approaches are well-known for diagnostics in phytofungal disease detection. These molecular-based approaches have successfully identified and diagnosed symptomatic and asymptomatic diseases of culturable and unculturable fungal pathogens in sole and co-infections of agriculturally important field, horticultural, floricultural, ornamental, and forest plant species. Among various PCR centered assays, quantitative PCR has been extensively used in the quantification and differentiation of causal agents when the sample load is too insignificant to detect. Currently, loop-mediated amplification (LAMP) is showing success in the fungal disease detection arena and has facilitated identification of *Alternaria* spp., *Colletotrichum* spp., *Fusarium* spp., *Verticillium* spp., *Puccinia* spp., *Botrytis* spp., etc. that cause an array of devastating diseases in plants. NGS uses various platforms to sequence fungal genomes with no prior knowledge of the pathogen's sequence, and can be used to identify novel and emerging pathogens.

The molecular methods covered in this review are accurate, effective, lab-oriented, and require sophisticated instruments to identify fungal plant pathogens. However, expertise in mycology and bioinformatics are essential to avoid any misinterpretation of the results obtained from the molecular biological analyses. Molecular techniques should become a point of care testing (POCT) by combining with other emergent technological advancements for fungal disease diagnosis. The challenges are with scientists to develop practical approaches for molecular diagnostics of crop diseases.

AUTHOR CONTRIBUTIONS

GH gathered literature and wrote the manuscript with input from KP. KP conceived the idea and provided guidance throughout and involved in critical revisions. All authors contributed to the article and approved the submitted version.

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A Novel Species of *Penicillium* With Inhibitory Effects Against *Pyricularia oryzae* and Fungal Pathogens Inducing Citrus Diseases

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A novel species of *Penicillium*, proposed as *P. linzhiense* sp.nov was isolated from soil collected in Linzhi Town, Linzhi County, Tibet Autonomous Region, China. DNA sequence analyses from eight different gene regions indicate that the isolate represents a novel species and most closely related to *P. janczewskii*. The phylogenetic analysis based on a concatenated dataset of three genes, ITS, *CaM*, and *BenA*, also confirmed the placement of the novel species within the *Canescentia* section of the genus *Penicillium*. Differences in morphology among similar species are detailed and single gene phylogenies based on ITS, *CaM* and *BenA* genes as well as a multi-loci gene phylogeny are presented. Cultural studies were performed to study inhibitory activities on plant pathogens. The results reveal a notable antifungal activity against *Pyricularia oryzae* causing rice blast with an inhibition rate up to 77%, while for other three citrus pathogens, *Diaporthe citri*, *Phyllosticta citrichinaensis*, and *Colletotrichum gloeosporioides*, inhibition rate was 40, 50, and 55% respectively. No noticeable effects were observed for *Fusarium graminearum*, *Botryosphaeria kuwatsukai*, and *Rhizoctonia solani*. Interestingly, unlike other reported members of *Canescentia*, *P. linzhiense* showed no antagonistic effect on root rotting fungi. The new taxon isolated here has the potential to be used as a biocontrol agent especially for economically important phytopathogens or emerging pathogens on diseases occurring on citrus or rice.

Keywords: *Canescentia*, antifungal activity, *Pyricularia oryzae*, citrus diseases, taxonomy, multigene phylogeny, plant pathology

INTRODUCTION

Orange is widely planted and consumed, but its production is threatened by a variety of pathogens during production, resulting in huge economic loss every year. To reduce the loss caused by pathogens, a large amount of pesticides have been applied to protect citrus from plant pathogens. Usage of these pesticides is harmful to the environment and human health (Nicolopoulou-Stamati

et al., 2016; Bisht et al., 2019). For example, mancozeb, an effective fungicide applied widely to inhibit *D. citri* on citrus in China (Chen et al., 2010; Jiang et al., 2012; Liu et al., 2018), is carcinogenic, teratogenic, and mutagenic after degrading to ethylene thiourea (ETU). Besides, with increasing pesticide abuse, pathogens are easily subjected to resistance to pesticides, leading to failure of disease control. Therefore, it is important to screen beneficial fungi for potential biological control leading to the safe production of citrus.

Some fungi are considered as biocontrol agents for inhibiting plant pathogens, but very few are associated with members of the genus *Penicillium*. Studies have reported that conidia or culture filtrate of *P. janczewskii* can induce systemic resistance of melon and tobacco to inhibit *Rhizoctonia solani* (Madi and Katan, 1998; Nicoletti et al., 2007), and one of its main secondary metabolites, pseurotin A, reveals moderate inhibition against *Erwinia carotovora* and *Pseudomonas syringae* (Schmeda-Hirschmann et al., 2008).

Penicillium is much more ubiquitous in the environment than other species of fungi and they also exist as endophytes inside plant tissues (Rashmi et al., 2019). Due to a paucity of morphological characteristics, overlap of morphs among different species and largely similar cultural characteristics, traditional morphological classification somehow been somehow unreliable to delineate species in this genus (Houbraken et al., 2014).With the development of modern molecular based phylogeny, the concept of multi-loci sequence typing (MLST) for phylogenetic species was proposed in the classification of *Penicillium*, thus making identification of strains more precise (Rakeman et al., 2002). MLST is a typing method initially used for distinguishing bacterial mutations by directly determining the nucleotide sequences of multiple housekeeping genes that are highly conservative and usually stably expressed in all cells. It was first applied by Maiden et al. (1998) in typing of the bacterial pathogen (*Neisseria meningitidis*), gradually implemented to other bacteria and fungi predominantly in epidemiology and taxonomy. Based on multiple gene locus sequence information to identify species, Visagie et al. (2014a) came up with a short standardized gene region namely DNA barcoding which was used in the identification of *Penicillium*. Up to now, there are DNA barcodes for more than 370 species accepted for *Penicillium*. Currently, the internal transcribed spacer rDNA regions (ITS) is widely sequenced as an official DNA barcode when discriminating species for fungi (Schoch et al., 2012; Lücking et al., 2020). However, ITS is not robust enough to identify species in *Penicillium* and alternative barcodes are needed to assist proper identification (Skouboe et al., 1999; Seifert et al., 2007; Visagie et al., 2014a; Nilsson et al., 2018; Tekpinar and Kalmer, 2019). The β-tubulin gene (*BenA*), the calmodulin gene (*CaM*), and the RNA polymerase II second largest subunit gene (*RPB2*) combined with ITS have been successfully employed in species-level identification of *Penicillium* (Houbraken et al., 2014; Visagie et al., 2016a; Visagie et al., 2016b; Wang et al., 2017; Diao et al., 2019). They, however, have certain limitations sometimes, such as difficulties in amplifying specific gene regions and sequence analyses due to ambiguously aligned sites and these lead to problems in resolving species concepts (Giraud et al., 2010; Houbraken et al., 2012; Chen et al., 2013; Wang and Wang, 2013; Visagie et al., 2014a; Visagie et al., 2016b). In this study, multi gene phylogenetic analyses were performed based on ITS, *BenA*, and *CaM* following the recommendations of Visagie et al. (2016b).

During the screening of fungi for potential biocontrol agents on *Citrus*, a fungal strain, Z863, was isolated from soil by selective medium dilution plate method (Houbraken and Samson, 2011). Morphological examinations and DNA sequence analyses reveals that Z863 is a new species belonging to *Penicillium* sect. *Canescentia*. Members of section *Canescentia* are soil inhabitants and there exist several studies demonstrating their potential antifungal activity predominantly related to the inhibition of soil-borne pathogens (Madi and Katan, 1998; Nicoletti et al., 2007; Schmeda-Hirschmann et al., 2008; Houbraken and Samson, 2011; Urooj et al., 2018). The aim of this paper is to introduce this taxon collected from China as a new species based on morphology supported by phylogenetic analyses of a combined dataset from ITS, *BenA*, and *CaM* genetic data. In addition, we also report results based on its potential inhibitory activities on plant pathogens.

MATERIALS AND METHODS

Sampling and Isolations

Soil samples were collected in Linzhi Town, Linzhi County, Tibet Autonomous Region (29.60146 N, 94.41736 E), China. The fungal samples were separated based on selective medium dilution plate method, in which 10 g soil to 90 ml distilled water, were shaken for 10 min at 120 rpm and diluted twice to 10^{-1} and 10^{-2} of the original concentration. Potato dextrose agar (PDA) as an isolation medium was prepared with 200 g potato, 20 g glucose, 18 g agar, 0.3 g chloramphenicol in 1,000 ml ddH_2O and sterilized at 121°C for 30 min. Three concentrations of diluent of 0.1 ml were separately pipetted into polystyrene Petri dish with 15 ml coagulated PDA and then the sterile coater was used to homogenize the diluent with three replicates for each concentration. After cultured at 25°C for 3 d, colonies were observed and mycelia (through hyphal typing) were transferred to a new PDA plate and once colonies grow up to 3 cm, they were transferred to new plates again.

Morphological Identification

Macromorphological characters were checked from Czapek's agar (CZ), Czapek yeast autolysate agar (CYA), malt extract agar (MEA), and yeast extract sucrose agar (YES) media. The medium's preparation, strain's inoculation manner, and incubation conditions were performed following the protocols of Visagie et al. (2014b).

After incubation at 25°C for 7 d, plates were checked, observed and colony's morphology was recorded. The descriptions of color are based on NBS ISCC color name notation. Afterward, 60% lactic acid was used as a floating agent for making slides, and

mycelia and conidia were examined under the microscope. Macromorphological and micromorphological details were also examined and recorded by EOS 600D Camera (Canon, China, Beijing) and Leica Microscope DM750 (Leica, China, Shanghai) with an ICC50 Camera and arrangement of photos was done in Adobe Photoshop CC 2018.

DNA Extraction and PCR

DNA of the samples was extracted by Rapid Fungi Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China) as the manufacturer's instructions.

PCR amplifications of the ITS, *BenA*, *CaM*, *RPB2*, translation elongation factor 1-α (*TEF*) regions, the large subunit (LSU) and the small subunit (SSU) of ribosomal DNA gene and tubulin gene were performed with corresponding primers listed in **Table 1**. One amplification reaction consisted of 25 μl Green Taq Mix (Vazyme, Nanjing, China), 2 μl of each primer (10 μM), 2 μl template DNA (30 ng μl^{-1}), and 19 μl ddH_2O in reaction system according to manufacturer's instructions. PCR reactions were performed by an MG96G PCR instrument (LongGene, Hangzhou, China) with the following procedure: pre-denaturing at 94°C for 2 min; subsequent 35 cycles with denaturing at 94°C for 30 s, annealing at 55°C for 40 s, extending at 72°C for 1 min; the final extension at 72°C for 10 min. After PCR reaction, products were detected by 1% agarose gel electrophoresis. Purification of products was conducted by the DNA gel purification kit (Axygen Biotech, Hangzhou, China).

DNA Sequencing and Sequence Analyses

Purified and recovered target DNA fragments were sent to be directly sequenced in an ABI PRISMA377 automatic sequencer (Sangon Biotech, Shanghai, China). Once DNA sequences were obtained, they were verified and then aligned with homologous or similar nucleotide sequences in the GenBank database by BLAST.

Three fragments including ITS, *CaM*, and *BenA* were employed for further comprehensive phylogenetic analyses. Three sets of genetic data (ITS, *CaM*, and *BenA*) were arranged and corrected by BioEdit Sequence Alignment Editor version 7.2.3, and finally a concatenated DNA sequence dataset was analyzed with Maximum Parsimony (MP) and Maximum Likelihood (ML). Phylogenetic analysis for model selections were performed by Modeltest 3.7 based on the lowest Akaike information criterion (AIC) value. The phylogenetic tree was constructed using PAUP 4.0b10 software under different optimality criteria. The stability of tree branches was evaluated by bootstrap with 1,000 replicates. Trees were processed for publication in Adobe Illustrator CC. Taxa used in the phylogenetic analyses are shown in **Table 2**.

Cultural Studies and Inhibition Activities on Plant Pathogens

Target strain and each tested pathogen (*D. citri*, *Ph. citrichinaensis*, *C. gloeosporioides*, *Py. oryzae*, *F. graminearum*, *B. kuwatsukai*, and *R. solani*) were separately inoculated onto the same 9 cm polystyrene Petri dish containing 15 ml PDA, 4 cm between two inoculums. To set up a control group, each pathogen with the same conditions as the test group was individually inoculated in a single medium at the same position in the PDA plate. Then, both groups were cultured at 25°C for 7 d.

A week later, the growth radius was measured (recorded as *R*) of the control group and the inhibition culture group (recorded as *r*). Each replicate was measured three times and the average was calculated. The inhibition rate of pathogen radius (abbreviated as *IR*) was calculated out according to the following formula:

$$IR = \frac{R - r}{R} \times 100\,\%$$

RESULTS

Isolation and Morphology

Twenty isolates with white color colony on PDA medium plate were isolated. The morphology of these isolates was

TABLE 1 | Primers for sequence amplification used in the PCR reaction.

Locus	Primer's name	Sequence (5′→3′)	Reference
ITS	ITS1	TCCGTAGGTGAACCTGCGG	(White et al., 1990)
	ITS4	TCCTCCGCTTATTGATATGC	(White et al., 1990)
BenA	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	(Glass and Donaldson, 1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	(Glass and Donaldson, 1995)
CaM	CMD5	CCGAGTACAAGGARGCCTTC	(Hong et al., 2006)
	CMD6	CCGATRGAGGTCATRACGTGG	(Hong et al., 2006)
RPB2	5F	GAYGAYMGWGATCAYTTYGG	(Liu et al., 1999)
	7CR	CCCATRGCTTGYTTRCCCAT	(Liu et al., 1999)
TEF	CEFF2	GGCTTCAACGTGAAGAACG	(Castlebury et al., 2004)
	CEFR1	CCGTKCAARCCRGAGATGG	(Castlebury et al., 2004)
LSU	LR5	ATCCTGAGGGAAACTTC	(White et al., 1990)
	LROR	ACCCGCTGAACTTAAGC	(White et al., 1990)
SSU	NS1	GTAGTCATATGCTTGTCTC	(White et al., 1990)
	NS4	CTTCCGTCAATTCCTTTAAG	(White et al., 1990)
Tubulin	T12	TAACAACTGCTGGGCCAAGGGTCAC	(O'Donnell and Cigelnik, 1997)
	T22	TCTGGATGTTGTTGGGAATCC	(O'Donnell and Cigelnik, 1997)

TABLE 2 | Strains used for phylogenetic analysis.

Species name	Strain number	GenBank accession numbers		
		ITS	*BenA*	*CaM*
Penicillium canescens	CBS300.48^T	AF033493	JX140946	KJ867009
Penicillium yarmokense	CBS410.69^T	KC411757	KJ834502	KJ867013
Penicillium radiatolobatum	CBS340.79^T	KC411745	KP016920	KP016825
Penicillium murcianum	CBS161.81^T	KP016844	KP016924	KP016824
Penicillium jensenii	CBS327.59^T	AY443470	JX140954	AY443490
Penicillium janczewskii	CBS221.28^T	AY157487	KJ834460	KJ867001
Penicillium dunedinense	CBS138218^T	KJ775678	KJ775171	KJ775405
Penicillium echinatum	NRRL917^T	KP016840	KJ866964	KJ867021
Penicillium griseoazureum	CBS162.42^T	KC411679	KP016919	KP016823
Penicillium nigricans	CBS354.48^T	KC411755	KJ866965	KJ867012
Penicillium corvianum	DAOMC250517^T	KT887875	KT887836	KT887797
Penicillium novaezeelandiae	CBS137.41^T	JN617688	KJ834477	KJ866996
Penicillium coralligerum	CBS123.65^T	JN617667	KJ834444	KJ866994
Penicillium atrovenetum	CBS241.56^T	AF033492	JX140944	KJ867004
Penicillium antarticum	CBS100492^T	KJ834503	KJ834432	KP016826
Penicillium brevicompactum	CBS257.29^T	AY484912	AY674437	AY484813
Penicillium nucicola	DAOMC250522^T	KT887860	KT887821	KT887782
Penicillium janczewskii	CBS166.81	KC411682	KJ866967	KJ866998
Penicillium janczewskii	CBS413.68	KP016838	KJ866969	KJ867014
Penicillium janczewskii	CBS279.47	KP016837	KJ866968	KJ867008

identical when examined under microscope, suggesting that they belong to the same species. One isolate, Z863 was used as representative for further studies. Results of microscopic examination showed that it is characterized by morphologies of the genus *Penicillium*. Based on phenotypic characters, this taxon belongs to the *Canescentia* section of the genus *Penicillium*. The morphological descriptions are provided in the taxonomy section.

Taxonomy

Penicillium linzhiense H-K. Wang & R. Jeewon, sp. *nov*. – Mycobank MB#838576; **Figures 1**, **2**.

In: subgenus *Penicillium*, section *Canescentia*.

Barcodes: ITS-MT461156; *BenA*-MT461157; CaM-MT461162;

Etymology. Latin, *linzhiense*, named after Linzhi, China, location where the isolates were collected.

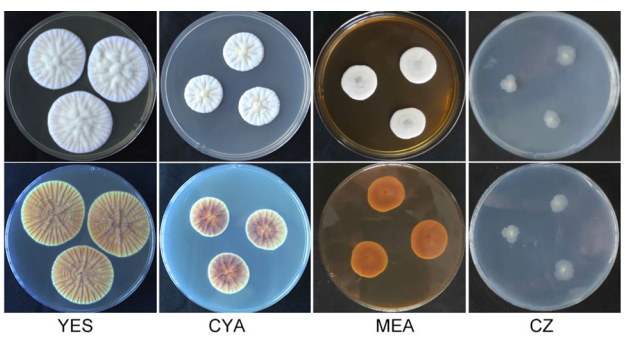

FIGURE 1 | Morphology of *Penicillium linzhiense* after incubation at 25°C on different medium for 7 days. bottom row: reverse plate.

Type strain examined: Linzhi Town, Linzhi County, Tibet Autonomous Region (29.60146 N, 94.41736 E), China, 20 Aug. 2016, collected by H-K Wang, CCTCC no: M2019870. Deposited in China typical culture preservation center located in Wuhan University, Wuhan, China.

After incubation at 25°C for 7 d on different medium plates, colonies of *P. linzhiense* displayed remarkable differences in morphology (**Figure 1**). Colonies on CYA after 7 d approached 30–32 mm, covered with many radial sulcate, thicker at the center without sulcate; margin entire to somewhat irregular; texture usually flocculent or velvet; mycelia white, moderate yellowish pink (SICC-NBS 29); center white to pinkish white (SICC-NBS 9) with exudate on the surface; soluble pigment absent. Colonies on MEA after 7 d approached 20–30 mm, uneven, navel-like bulge at the center; margin entire to somewhat irregular; mycelia white, texture velutinous; somewhat vivid pink at the center with exudate on the surface; soluble pigment absent. Colonies on CZ after 7 d approached 16–18 mm, low and plane; margin irregular; mycelia (SICC-NBS 184) very pale blue; texture fluffy; exudate and soluble pigment absent. Colonies on YES after 7 d approached 42–44 mm, covered with many radial wrinkles, thicker without radial wrinkles at the center; margin entire; mycelia white, yellowish white (SICC-NBS 92) to pale greenish yellow (SICC-NBS 104); texture mostly flocculent or velvet; exudate and soluble pigment absent.

Microscopic characters were also examined on MEA, conidiophore (**Figure 2C**), 20–100 × 2–2.5 μm, occurred in aerial or dragging hyphae with smooth walls. Broom branches (**Figure 2A**) are predominantly single-whorled, with fewer double-whorls and solitary pedicels with enlarged apices. It grew two to eight or more bottle pedicels (6–8 × 2.0–2.5 μm) per whorl, typically flask-shaped, with short and distinct necks (**Figures 2D–E**). Conidia were spherical or subspherical in shape, 2.6–4.5 μm, markedly spiny and rough (**Figure 2F**), each in a

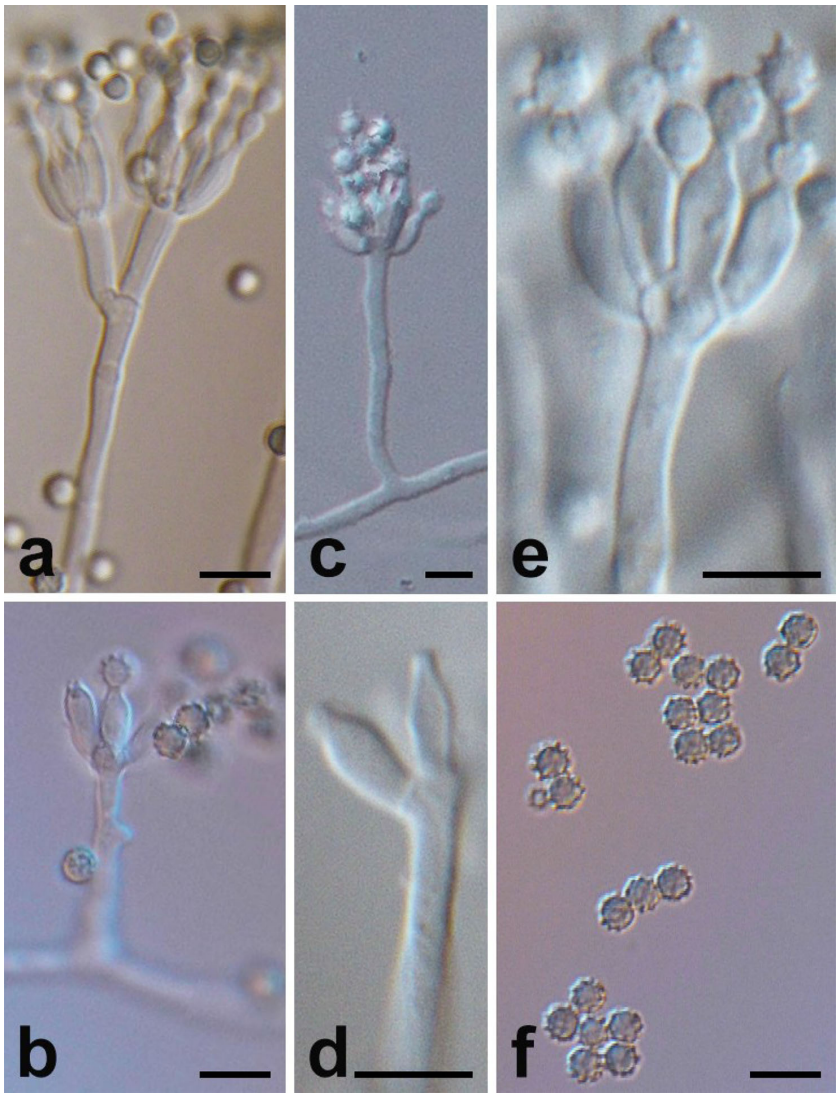

FIGURE 2 | Micromorphology of *P. linzhiense*. (Scale bars = 5 μm.) **(A)** Branching status of conidiophore. **(B)** Conidia's growth status on the bottle stem. **(C)** Conidiophore and bottle stem. **(D)** Morphology of bottle stem. **(E)** Growth manner of bottle stem on base stem. **(F)** Morphology of conidia.

bottle stem or free (**Figure 2B**). Conidial chains were loose, nearly cylindrical, or irregular.

Morphology of conidiophore and conidia of *P. linzhiense* are similar to *P. janczewskii*. The strain differs from *P. janczewskii* in that *P. linzhiense* has a light colony on CYA and is grayish-white without becoming grayish-green within 2 weeks; the conidiophore branching pattern is predominantly monoverticillate, with fewer biverticillate, and conidiophore are solitary. However, colonies of *P. janczewskii* change from grayish-green to grayish-black on CYA medium; conidiophore branching patterns of *P. janczewskii*, *P. dunedinense*, *P. echinatum*, *P. griseoazureum*, *P. nigricans* are mainly biverticillate, with terverticillate, or few monoverticillate, with two to four metula per conidiophore. This novel species can be distinguished from *P. corvianum* by the spiny conidia.

Sequencing and Phylogenetic Analyses

Eight gene fragments of strain Z863 were obtained using PCR according to the primer pairs in **Table 1**. All the sequences were uploaded to GenBank with the following accession numbers (ITS: MT461156; *BenA*: MT461157; LSU: MT461158; SSU: MT461159; *TEF*: MT461160; *RPB2*: MT461161; *CaM*: MT461162; beta-tubulin (*Tub*): MT461163).

Phylogenetic analysis (Maximum Likelihood, ML) based on a combined ITS, *CaM*, and *BenA* dataset of 21 taxa (with 1,238 characters and 130 parsimony informative characters) with *P. brevicompactum* as outgroup resulted in one tree shown in **Figure 3A** (TL = 513, CI = 0.789, RI = 0.738, RC = 0.583, HI = 0.211). Phylogeny depicts that Z863 is a new species as it constitutes a strongly supported independent lineage basal to

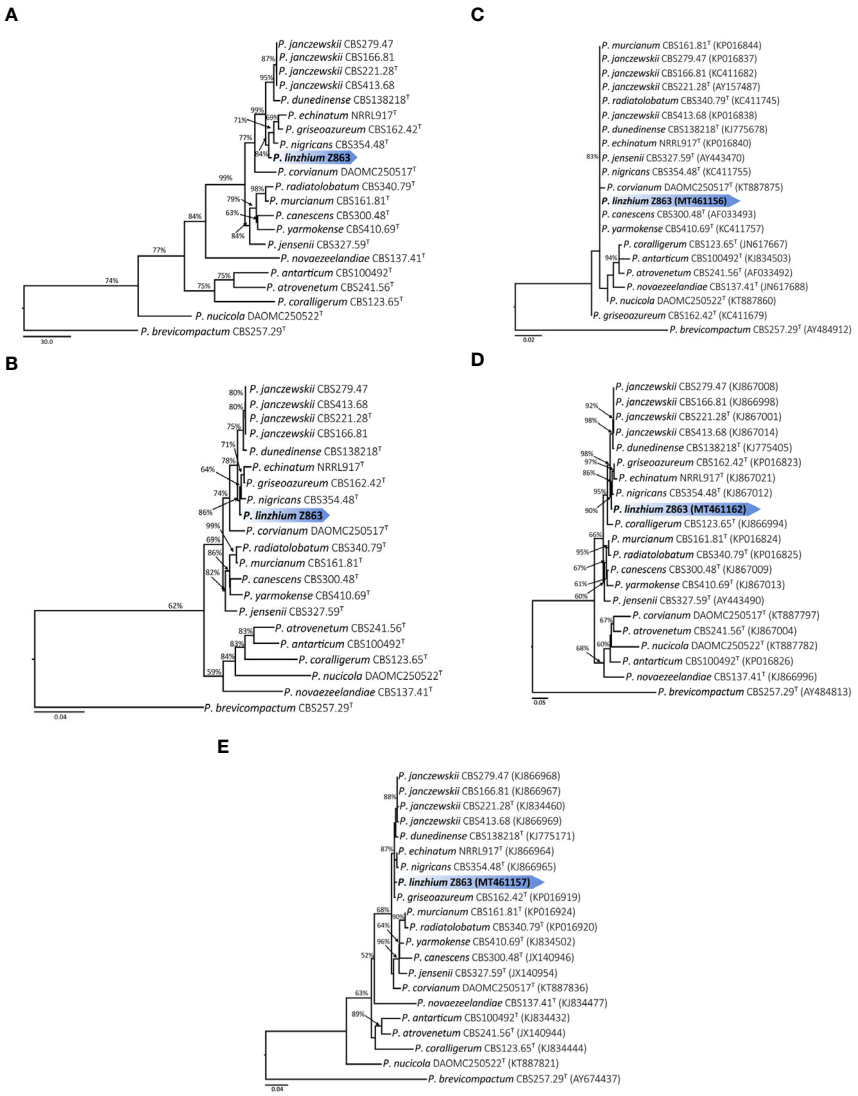

FIGURE 3 | **(A)** Maximum parsimony tree of the combined sequence of ITS, *CaM* and *BenA* of *P. linzhiense* (^{T} = ex-type; the scale bar shows the number of substitutions and the values above the nodes represent bootstrap support). The new species is marked by blue block. **(B)** Maximum likelihood tree of the combined sequence of ITS, *CaM*, and *BenA* of *P. linzhiense* (^{T} = ex-type; the scale bar shows the number of substitutions and the values above the nodes represent bootstrap support). The new species is marked by blue block. **(C)** Maximum likelihood tree derived from DNA sequence analyses of the ITS, gene region. (The scale bar shows the number of substitutions and the values above the nodes represent bootstrap support, but those support lower than 50% are not showed). **(D)** Maximum likelihood tree derived from DNA sequence analyses of the *CaM* gene region. (The scale bar shows the number of substitutions and the values above the nodes represent bootstrap support, but those support lower than 50% are not showed). **(E)** Maximum likelihood tree derived from DNA sequence analyses of the *BenA*, gene region. (The scale bar shows the number of substitutions and the values above the nodes represent bootstrap support, but those support lower than 50% are not showed).

P. janczewskii, *P. dunedinense*, *P. nigricans*, *P. griseoazureum*, and *P. echinatum* (**Figure 3A**).

The Maximum Parsimony (MP) phylogenetic tree derived from the combined dataset shown in **Figure 3B** is based on 1,238 characters with best model GTR+G+I. The phylogenetic position of Z863 is the same in the ML tree. This result also confirmed that Z863 is a new species in *Penicillium* sect. *Canescentia*. During our initial ITS sequence BLAST search in GenBank, the similarity between our strain and *P. janczewskii* (MK179261), *P. arizonense* (MH492021), *P. canescens* (KX359603), *P. murcianum* (NR_138358), *P. janczewskii* (KP016839) was 100%. This confirms that our species is undoubtedly a *Penicillium* species and belongs to *Penicillium* sect. *Canescentia*. However, one cannot rely on ITS alone for proper identification and establishing new species, especially for taxonomically complex genera (Jeewon and Hyde, 2016). Even

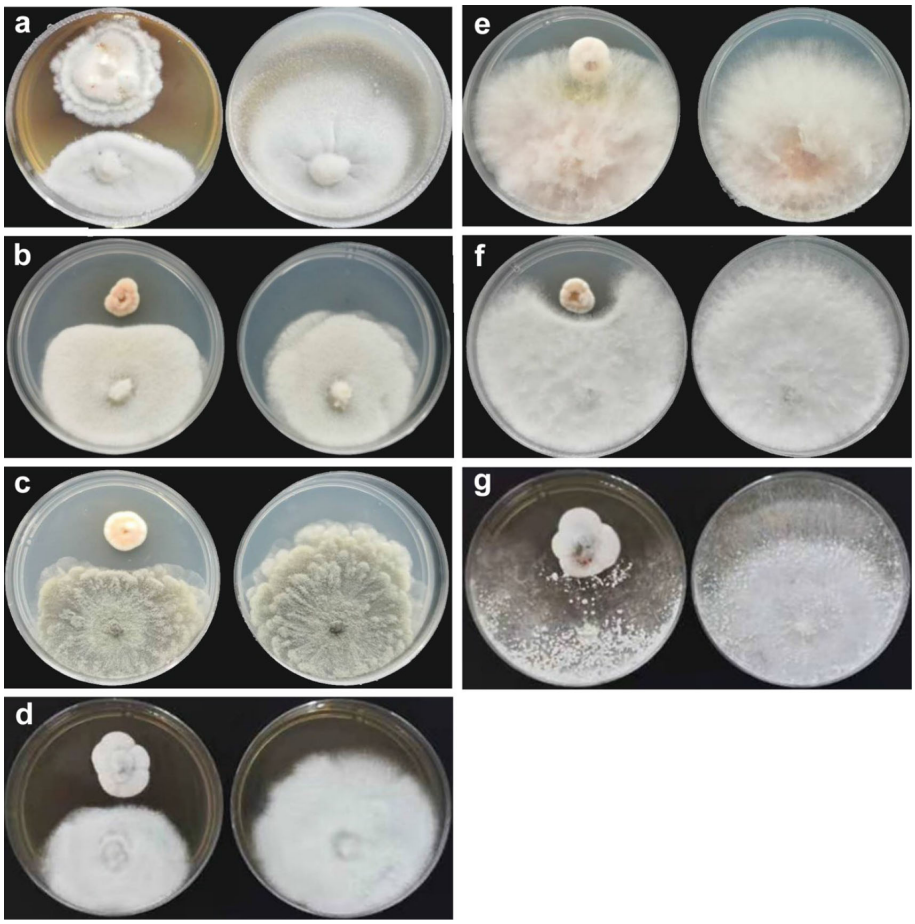

FIGURE 4 | Inhibition of *P. linzhiense* on plant pathogens. **(A)** Inhibitory effect of *P. linzhiense* on *Pyricularia oryzae* after 14 d; **(B)** Inhibitory effect of *P. linzhiense* on *Diaporthe citri* after 7 d; **(C)** Inhibitory effect of *P. linzhiense* on *Phyllosticta citrichinaensis* after 7 d; **(D)** Inhibitory effect of *P. linzhiense* on *Colletotrichum gloeosporioides* after 7 d; **(E)** Inhibitory effect of *P. linzhiense* on *Fusarium graminearum* after 7 d; **(F)** Inhibitory effect of *P. linzhiense* on *Botryosphaeria kuwatsukai* after 7 d; **(G)** Inhibitory effect of *P. linzhiense* on *Rhizoctonia solani* after 7 d. [**(A–G)** The left one was an inhibition culture group (above: *P. linzhiense*; below: the tested pathogen), and the right one was a control group].

our single gene phylogenetic analyses based on ITS alone also shows that the tree is unresolved with weak branch support and the affinities of *P. linzhiense* to *P. corvianum* (KT887875), *P. canescens* (AF033493), and P. *yarmokense* (KC411757) are not clear (**Figure 3C**). However single gene datasets based on *CaM* and *BenA* genes provided better resolution (**Figures 3D**, **E**).

Inhibition of *Penicillium linzhiense* on Plant Pathogens

Comparing inhibition culture group and control group, *P. linzhiense* showed inhibitory effects against *Py. oryzae* (**Figure 4A**), *D. citri* (**Figure 4B**), *Ph. citrichinaensis* (**Figure 4C**), and *C. gloeosporioides* (**Figure 4D**). However, the strain did not exhibit significant effect on *F. graminearum* (**Figure 4E**) and had no inhibition against *B. kuwatsukai* (**Figure 4F**) and *R. solani* (**Figure 4G**). The inhibition rate of pathogen radius (*IR*) is showed in **Figure 5**.

DISCUSSION

Penicillium is widely distributed in the environment and easily isolated from air and soil. To date many published reports have reported a ubiquitous and high frequency of *Penicillium* in soil samples from different climatic conditions and geographical regions (Grishkan and Nevo, 2004; Sharma et al., 2011; Cruz et al., 2013; Kalashnikova et al., 2016; Cecchi et al., 2019) and these species are associated with important soil function. Despite their important roles, the traditional morphological delineation of species has always been a taxonomic dilemma and currently DNA based sequence data from a combination of different genes especially ITS, *BenA*, and *CaM* should be analyzed for accurate identification. In our study, we isolated a new species, *Penicillium linzhiense* and its morphological details, ability to restrict growth of fungal pathogens and evolutionary relationships are discussed.

Our multigene phylogeny reveals that *P. linzhiense* is close to *P. janczewskii*, *P. dunedinense*, *P. echinata*, *P. griseoazureum*, and

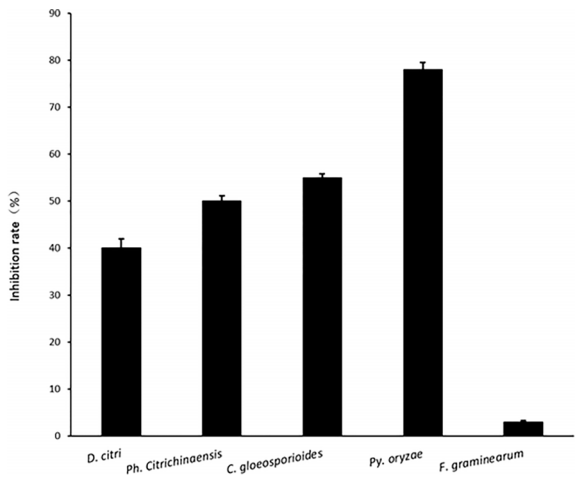

FIGURE 5 | The inhibition rate of pathogen radius (*IR*) of *P. linzhiense* against different pathogens in the confrontation culture test.

P. nigricans. However, *P. linzhiense* is distinct from species mentioned above particularly in morphs with distinctly thinner mycelium on CZ medium and with single-whorled broom branches mainly accompanied by few double-whorled one. With respect to *P. janczewskii*, *P. linzhiense* mainly differs in the color of colony after two weeks' culture and the broom branches (at the start, there is gray-white and mainly monoverticillate, with fewer biverticillate, with solitary conidiophore, and with time turns grayish green to grayish black and mainly biverticillate, with terverticillate or few monoverticillate, with two to four metula per conidiophore). When the cultural characteristics of *P. dunedinense* are compared against *P. linzhiense* at 25°C after 7 d, clear differences can be observed. On CYA medium, wrinkles of *P. linzhiense* look denser than *P. dunedinense*; on MEA, *P. dunedinense* is sulcate but *P. linzhiense* is not; on YES, *P. dunedinense* is grayish orange but *P. linzhiense* is white (Visagie et al., 2014b). As for *P. echinata*, its conidiophores have diaphragms, conidial chains that are relatively tighter than *P. linzhiense* and its broom branches are single-whorled (Matsushima, 1972), but conidiophores of *P. linzhiense* have no diaphragms and there exist few double-whorls of broom branches in *P. linzhiense*. As is shown in *Manual and Atlas of the Penicillia* (Ramirez and Martinez, 1982), both *P. griseoazureum* and *P. nigricans* on CYA and on MEA do not have any exudate but *P. linzhiense* possesses yellowish brown exudate on the colonies on CYA and MEA.

Phylogenetic analyses of a combined ITS, *CaM*, and *BenA* sequence dataset (**Figures 3A**, **B**) in the study show that *P. linzhiense* forms a distinct lineage, basal to *P. nigricans* CBS 354.48 with high bootstrap support in ML analysis (84% ML). Based on the recommendations for the establishment of new species proposed by Jeewon and Hyde (2016), we also compared % differences across all genes amplified. Comparison of ITS, *CaM*, and *BenA* nucleotides between *P. linzhiense* and *P. nigricans* CBS 354.48 reveals 0, 2, and 3 base pair differences, respectively. In the phylogram generated from maximum likelihood analysis based on ITS sequence data, *P. linzhiense* was observed to be closely related to *P. yarmokense* (CBS 410.69), *P. corvianum* (KT887875), and *P. canescens* (AF033493) (**Figure 3C**) but this relationship is unstable and unresolved. Furthermore, a comparison of DNA sequences of the ITS regions sequences between *P. linzhiense* and *P. yarmokense* shows 0 (0%) base pair differences. Although ITS barcodes play an important role in the taxonomy of *Penicillium* species, this gene region is not powerful enough to discriminate species due to their low variability (Skouboe et al., 1999; Seifert et al., 2007; Stielow et al., 2015). Upon analysis of the *BenA* sequences data, *P. linzhiense* was found to be a sister taxon to *P. echinatum* (NRRL917) (**Figure 3E**) and nucleotide comparison reveals 3 (0.8%) base pair differences between these two taxa. Thus, based on the phylogenetic analyses of the concatenated dataset and phenotypic differences and following the guidelines proposed by Jeewon and Hyde (2016), we hereby introduce *P. linzhiense* as a new species in the genus *Penicillium*.

In the inhibition studies, as we expected, *P. linzhiense* shows a distinct inhibitory effect against three important pathogenic fungi causing citrus diseases. Results also show a stronger inhibition against *Pyricularia oryzae* causing rice blast (**Figure 4**). It is recommended to perform further pathogenicity studies on *P. linzhiense*, including field experiments and the effect of metabolites to assess to what extent the latter can be used as a potential biological control agent in integrated disease management strategies. The discovery of *P. linzhiense* provides one more possibility to control citrus diseases and rice blast.

Members of *Penicillium* sect. *Canescentia* are well known as soil-borne fungi (Houbraken and Samson, 2011) and some studies pointed out they possess distinct inhibitory effect against root rotting fungi and even able to promote growth of plants (Madi and Katan, 1998; Nicoletti et al., 2007; Schmeda-Hirschmann et al., 2008; Urooj et al., 2018). Interestingly, as a member of *Penicillium* sect. *Canescentia*, *P. linzhiense* shows little inhibition against common soil-borne pathogens unlike other reported members mentioned above. Instead, *P. linzhiense* reveals its suppression on pathogens triggering plant disease aboveground. As revealed in this study, results demonstrate that *P. linzhiense* can be a potential biocontrol agent for *Py. oryzae* which causes damage to the leaves, stems, and ears of rice; *D. citri* which causes diseases at the tip of trees, new leaves, and fruits of citrus; *Ph. citrichinaensis* which is often found associated with fruits of citrus (Baayen et al., 2002); and *C. gloeosporioides* which is detrimental to leaves, branches, flowers, fruits, and fruit stalks of citrus.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.

nih.gov/genbank/, MT461156; https://www.ncbi.nlm.nih.gov/genbank/, MT461157; https://www.ncbi.nlm.nih.gov/genbank/, MT461158; https://www.ncbi.nlm.nih.gov/genbank/, MT461159; https://www.ncbi.nlm.nih.gov/genbank/, MT461160; https://www.ncbi.nlm.nih.gov/genbank/, MT461161; https://www.ncbi.nlm.nih.gov/genbank/, MT461162; https://www.ncbi.nlm.nih.gov/genbank/, MT461163.

AUTHOR CONTRIBUTIONS

RJ, HL, F-CL, and H-KW designed the study. LL did the sample collection and laboratory work. LL, RJ, PD, SSKD and H-KW are involved in phylogenetic analyses and initial writing and finalizing drafts. HL, F-CL, and H-KW contributed for the research funds. All authors contributed to the article and approved the submitted version.

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Bipolaris sorokiniana-Induced Black Point, Common Root Rot, and Spot Blotch Diseases of Wheat: A Review

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Wheat is among the ten top and most widely grown crops in the world. Several diseases cause losses in wheat production in different parts of the world. *Bipolaris sorokiniana* (teleomorph, *Cochliobolus sativus*) is one of the wheat pathogens that can attack all wheat parts, including seeds, roots, shoots, and leaves. Black point, root rot, crown rot and spot blotch are the main diseases caused by *B. sorokiniana* in wheat. Seed infection by *B. sorokiniana* can result in black point disease, reducing seed quality and seed germination and is considered a main source of inoculum for diseases such as common root rot and spot blotch. Root rot and crown rot diseases, which result from soil-borne or seed-borne inoculum, can result in yield losses in wheat. Spot blotch disease affects wheat in different parts of the world and cause significant losses in grain yield. This review paper summarizes the latest findings on *B. sorokiniana*, with a specific emphasis on management using genetic, chemical, cultural, and biological control measures.

Keywords: ***Triticum aestivum*, *Helminthosporium*, *Cochliobolus sativus*, *Drechslera*, control**

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INTRODUCTION

Wheat (*Triticum aestivum*) is among the most widely cultivated crops in the world. Wheat production exceeded 734 million tons in 2018 from 214 million ha of land (FAO, 2021). China, India, Russia, USA, and France were the largest producers of wheat in the world in 2018, accounting for more than 50% of the world's production (FAO, 2021).

Wheat production is limited by several biotic stresses, with diseases being a major limiting factor to wheat production worldwide. The total number of wheat diseases exceeds 200, but 50 diseases cause economic losses and are widely distributed (Wiese, 1987; Al-Sadi, 2016; Jarroudi et al., 2017; Lalic et al., 2017; Riaz et al., 2017; Sharma et al., 2017). Each year about 20% of wheat is lost due to diseases. Some of the major wheat diseases are rusts, spot blotch, common root rot, smut, tan spot, Septoria blotch, powdery mildew, fusarium head blight, blast and a number of viral, nematode, and bacterial diseases (Wiese, 1987; Chowdhury et al., 2013; Fetch et al., 2015; Zhu et al., 2015; Al-Sadi, 2017; Abdullah et al., 2020; Aboukhaddour et al., 2020; Gultyaeva et al., 2020). They can reduce yield or result in mortality of the infected plants. The focus of this review will be on the etiology and management of *B. sorokiniana* diseases in wheat.

The genus *Helminthosporium* is a large group of the class Hyphomycetes that includes many species pathogenic to plants and animals. This genus has been split into three genera: *Exserohilum, Bipolaris*, and *Drechslera*, on the basis of conidial ontogeny and morphology (Alcorn, 1988).

Bipolaris sorokiniana

B. sorokiniana (Sacc.) Shoemaker, (syn. *Helminthosporium sativum* Pammel, King & Bakke, *H. sorokinianum* Sacc. in Sorokin, and *Drechslera sorokiniana* (Sacc.) Subramanian & Jain, causes diseases on a number of cereals, including wheat (Tunali et al., 2008; Devi et al., 2018; Gultyaeva et al., 2018; Gupta et al., 2018b; Jamil et al., 2018; Singh et al., 2019; Villa-Rodríguez et al., 2019; Li et al., 2020). The teleomorph for this fungus is *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur, which is the sexual (perfect) state. *C. sativus* was not reported in nature, except in Zambia (Raemaekers, 1991). However, sexual reproduction of *C. sativus* has been rarely reported (Sultana et al., 2018). On the other hand, most of the reproduction of *B. sorokiniana* occurs through the production of asexual conidia (Gupta et al., 2018a).

The genus *Bipolaris* has brown conidiophores, mostly simple, producing conidia through the apical pore. The conidia are brown, several-celled (phragmosporous), elliptical, straight, or curved, germinating by one germ tube at each end (Barnett and Hunter, 1998; Navathe et al., 2020) (**Figure 1**). *B. sorokiniana* has olive-brown, ovate conidia, with tapered ends and a prominent basal scar. The conidia are 15-28 X 40-120 µm and have 3- to 10-septa (Wiese, 1987) (**Figure 1**).

DISEASES CAUSED BY *B. SOROKINIANA*

Bipolaris sorokiniana attacks different cereals, including wheat, and causes common root rot, spot blotch, and black point diseases. Root rot is one of the most widespread diseases of wheat and it occurs in all areas where wheat is grown. Losses in wheat due to common root rot and seedling blight vary. Canada lost approx. 5.7% of wheat during 1969–1971 due to common root rot, which is equivalent to $42 million (Ledingham et al., 1973). Smiley et al. (2005) estimated 35% loss in wheat yield due to crown rot in the Pacific Northwest. Spot blotch is found wherever wheat is grown, and it can cause significant losses (15-25%) in warm areas (Gupta et al., 2018a). Seed infection by *B. sorokiniana* can result in black point disease, which may result in root rot and seedling blight (Wiese, 1987; Al-Sadi and Deadman, 2010; Li et al., 2019b).

B. sorokiniana attacks several host plants from different genera and families (Wiese, 1987; Farr et al., 1989). The major plant hosts (listed by the genera name) that attacked by *B. sorokiniana* are *Agrohordeum, Agropyron, Agrostis, Ammophila, Andropogon, Arthraxon, Avena, Bouteloua, Bromus, Buchloe, Calamagrostis, Calamovilfa, Cenchrus, Chloris, Cynodon, Dactylis, Dendrobium, Dichanthelium, Digitaria, Echinochloa, Elymus, Eragrostis, Eremopyrum, Festuca, Hordeum, Hystrix, Koeleria, Linum, Lolium, Medicago, Muhlenbergia, Oryzopsis, Panium, Phalaris, Phleum, Poa, Secale, Setaria, Sorghum, Stipa, Trifolium, Triticum, Vulpia, Zea*, and *Zizania* species (Farr et al., 1989). Disease symptoms and yield losses in these hosts are variable. *B. sorokiniana* do not have host specialization (forma speciales). However, isolates have been found to differ in their aggressiveness on wheat and barley (Al-Sadi, 2016).

BLACK POINT

Importance and Etiology of Black Point

Black point is a disease of cereal seeds, exhibiting a brown to black tip at the embryo end of the grain. The affected kernels usually become heavier than normal. The disease can result in lowering quality and market value of grains, production of fungal toxins in the seeds that may become harmful to livestock, and causing seedling blight, root rot and different diseases. In addition, it can reduce seed germination, seedling emergence,

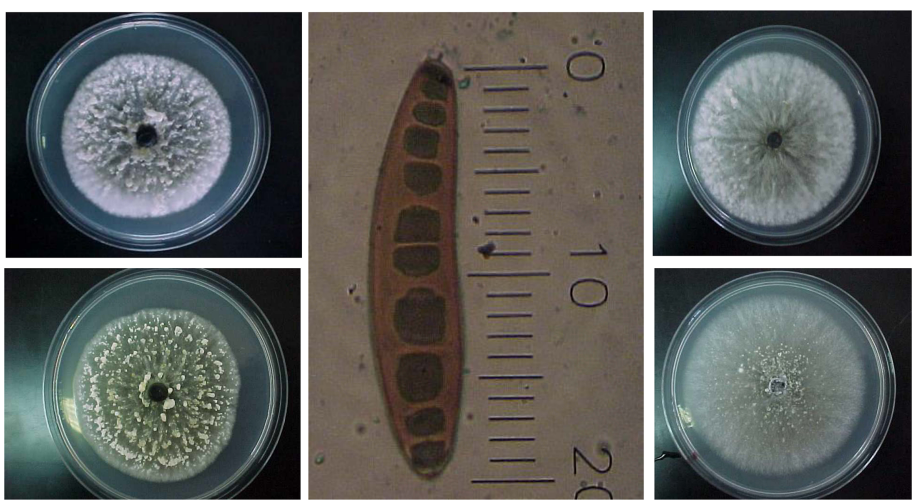

FIGURE 1 | Morphology of *Bipolaris sorokiniana* culture and spores (1 scale is equivalent to 5 µm) grown on potato dextrose agar. The mycelial growth of the four isolates shows mixed color (white and black) as explanied by Navathe et al. (2020), with varying intensities of the black color among the isolates.

total photosynthetic area, and normal growth of plants (Al-Sadi and Deadman, 2010; Neupane et al., 2010; Ghosh et al., 2018; Li et al., 2019b; Somani et al., 2019).

The disease is caused by *B. sorokiniana* (Sisterna and Sarandon, 1996; Xu et al., 2018b; Li et al., 2019b; Somani et al., 2019; Li et al., 2020). In addition, some reports indicated the association of *Alternaria alternata*, *Fusarium* spp., and *Penicillium* spp. with wheat seeds developing black point symptoms (Al-Sadi and Deadman, 2010; Gannibal, 2018; Ghosh et al., 2018; Xu et al., 2018b; Li et al., 2019b; Li et al., 2020). Black point has been reported in different parts of the world, including China, Argentina, Oman, Australia, India, and Bangladesh (Rashid et al., 1992; Sisterna and Sarandon, 2005; Al-Sadi and Deadman, 2010; Poole et al., 2015; Xu et al., 2018b).

Bipolaris sorokiniana has been reported in the embryo (Weniger, 1923) and in the endosperm of wheat seeds (Rashid et al., 1997). Seed infection in wheat increases after flowering (Andersen, 1952). It can also be affected by the genotype, location (especially in warm and humid climates) and the management practices (Zhang et al., 1990). Penetration into the seed is achieved through the ovary wall and seed coat (Han et al., 2010; Ansari et al., 2017). *B. sorokiniana* was reported to remain viable for 10 years in wheat seeds (Machacek and Wallace, 1952). The fungus can also survive as a resting mycelium for 5 years (Mead, 1942). *B. sorokiniana* is very frequently isolated from the seeds of wheat, reaching as high as 80%–90.5%, and with a common level of infection of about 9%–22%, depending on the cultivar and the prevailing conditions (Rashid et al., 1992; Rashid et al., 1997; Rashid, 1998; Li et al., 2019b).

The incidence of black point disease is affected by many factors, most importantly temperature and humidity. Higher humidity (especially above 90%), rain and relatively lower temperatures (<30 ^{o}C) after heading usually increase the disease incidence (Cromey and Mulholland, 1988; Li et al., 2019a; Li et al., 2019b).

Management of Black Point

Control of *B. sorokiniana* in the seeds of wheat could be achieved through the use of resistant cultivars, fungicides, seed treatment, or biocontrol agents. Cultivars can react differently to seed infection due to several factors such as non-compatibility to infection, restricted pathogen invasion of the seed parts due to inhibitors, or reduced testa permeability (Gannibal, 2018; Singh et al., 2019). In a study by Li et al. (2014) on 403 wheat genotypes in the North China Plain, 62.5% of the genotypes were classified as susceptible, while 37.5% were resistant to black point disease. In another study, considerable variation was found among wheat cultivars in their resistance to black point disease, with no relationship between the earliness of ripening and resistance (Cromey and Mulholland, 1988). Connert and Davidson (1988) showed that wheat cultivar resistance to black point disease can be affected by the causal agent, with some cultivars having more resistance to *B. sorokiniana* than to *A. alternata*.

A study in Pakistan revealed that tebuconazole + imidacloprid and difenoconazole + cyproconazole were the most effective chemicals for the management of black point disease of wheat (Shahbaz et al., 2018). Triazole fungicides (e.g., propiconazole and tebuconazole) inhibit the synthesis of sterols, which are building blocks of the membranes of fungal cells. This makes them ideal chemicals for the management of *Bipolaris* and other fungal pathogens (Ansari et al., 2017; Somani et al., 2019). Treating seeds with fungicides helps protect wheat seeds from infection. In addition, it helps manage diseases associated with seed infection, including root and crown rot. Some of the common fungicides used in seed treatment include fludioxonil and difenoconazole (Wei et al., 2021) and Vitavax-200 (Carboxin 37.5% + Thiram 37.5%) and Homai-80WP (Thiophanate methyl 50% + Thiram 30%) (Malaker and Mian, 2009).

The use of biocontrol agents has been effective in reducing black point disease. *Bacillus amyloliquefacien*s, *B. megaterium, Trichoderma harzianum*, and *Epicoccum* sp. were found antagonistic against the causal agents of black point disease (El-Gremi et al., 2017). The isolates also improved germination and seedling growth of wheat, with *B. amyloliquefacien*s being the most efficacious, as it was as effective as the fungicide diniconazole in increasing the weight of kennels. In another study, the antifungal compounds produced by *B. vallismortis* were effective in inhibiting black point fungi (Kaur et al., 2015; Kaur et al., 2017). A study by Mónaco et al. (2004) showed that *T. harzianum* and *T. koningii* significantly inhibited the growth and caused mycelial abnormalities in *Bipolaris sorokiniana* and *A. alternata*.

SPOT BLOTCH

Importance and Etiology of Spot Blotch

Spot blotch is a common disease on wheat in all continents (Duveiller et al., 1998; Al-Saadi et al., 2002; Neupane et al., 2010; Al-Sadi, 2016; Devi et al., 2018; Gultyaeva et al., 2018; Gupta et al., 2018a; Gupta et al., 2018b). Losses due to spot blotch are high, especially in warmer areas of the world. They have been reported to reach 16%–43% (Sharma and Dubin, 1996; Duveiller and Sharma, 2009; Ayana et al., 2018; Devi et al., 2018). In addition, the hotspot for spot blotch disease is in South Asia (Van Ginkel and Rajaram, 1998; Joshi et al., 2007; Sharma et al., 2018; Sultana et al., 2018).

Spot blotch symptoms appear as brown lesions with yellow halos, which enlarge with time to cover larger areas of the leaf. Lesions can turn olive brown in color, especially under humid conditions that promote sporulation of the fungus (Al-Sadi, 2016; Gupta et al., 2018a; Gupta et al., 2018b). *Bipolaris sorokiniana* is the pathogen responsible for spot blotch disease in wheat (Devi et al., 2018; Gupta et al., 2018a; Gupta et al., 2018b; Tembo et al., 2018; Aggarwal et al., 2019). The symptoms of *Pyrenophora tritici-repentis*-induced tan spot, and Alternaria leaf blight resemble those of spot blotch. One difference is that tan spot is characterized by the appearance of dark fruiting structures, called pseudothecia, on wheat straw, which is not the case for spot blotch (Carmona et al., 2006). Spot blotch differs from Alternaria blight by the development of dark spot areas, which represent masses of conidia that are produced at later

infection stages (Neupane et al., 2010; Viani et al., 2017). The spot blotch symptoms elongate and coalesce (Chand et al., 2010).

Leaf infection by *B. sorokiniana* could come from seeds, root or air. If the pathogen is in the soil, then infection could occur through stomata on the hypocotyl, from where the fungus progresses to the root, shoot and coleoptile (Sprague, 1950). Spore germination can occur within 4–6 h and penetration by *B. sorokiniana* occurs through stomata and epidermis (Raguchander et al., 1988).

Studies in India and Brazil have shown that spot blotch is usually favored by warm weather (Chaurasia et al., 2000; Kumar et al., 2002; Acharya et al., 2011; Singh, 2017). Also, high humidity is an important factor in enhancing symptom development (Viani et al., 2017). Infection usually starts on the older leaves (Gupta et al., 2018a). In addition, water stress and terminal heat stress have negative effects on the resistance of wheat to *B. sorokiniana* (Duveiller and Sharma, 2009).

Management of Spot Blotch

No complete resistance to spot blotch has been reported in wheat, but wheat cultivars have been reported to differ in resistance to the disease (**Table 1**) (Ahirwar et al., 2018; Ayana et al., 2018; Gurjar et al., 2018; Jamil et al., 2018; Singh et al., 2018; Tembo et al., 2018). Therefore, breeding and selecting resistant cultivars is the best option for managing spot blotch in the long term (Gupta et al., 2018a). Among 150 wheat genotypes screened in Zambia, the genotypes 19HRWSN6, 19HRWSN7, and 19HRWSN15 were found resistant (Tembo et al., 2018). In addition, a study on 60 wheat genotypes in Nepal indicated that the genotype NL750 had a high level of resistance to spot blotch, while the tolerant genotype BL1473 is able to produce good yields despite the high disease levels (Sharma et al., 2004; Rosyara et al., 2007).

Resistance can be induced using some microorganisms and compounds. The combined application of *Trichoderma harzianum* and methyl jasmonate was found to enhance the activities of defense related enzymes, including catalase, ascorbate peroxidase, phenylalanine lyase, and peroxidase (Singh et al., 2019). In addition, methyl jasmonate is known to inhibit spore germination in *B. sorokiniana*. In another study, wheat was found to strongly elicit salicylic acid signaling, followed by an enhanced expression of phenylpropanoid pathway genes, which leads to the accumulation of phenolics that play a role in the resistance against spot blotch (Sahu et al., 2016). Also (Sharma et al., 2018) showed that salicylic acid and syringic acid negatively correlated with spot blotch severity, indicating their role in disease defense.

In a study on the efficacy of 195 bacterial strains in suppressing *B. sorokiniana*, *Bacillus subtilis* TE3 strain proved to be the most efficacious in suppressing the disease (Villa-Rodríguez et al., 2019). The mechanisms of actions of the antagonistic bacterial strain were through colonizing the wheat phyllosphere and the antimicrobial compounds produced by the bacterium. Additionally, *B. safensis* and *Ochrobactrum pseudogrignonense* have been reported to promote resistance to spot blotch in wheat (Sarkar et al., 2018). However, the efficacy of biocontrol agents is usually limited by environmental factors and growing conditions.

Several fungicides have been developed and used for the management of spot botch. The yield increase in fungicide treated plots suffering from leaf diseases compared to untreated plots was 10% in Sweden (Djurle et al., 2018) and 30% in Argentina (Castro et al., 2018). The fungicides carbendazim (Yadav et al., 2013), difenoconazole (Ishikawa et al., 2012), propiconazole (Singh and Singh, 2007; Gupta et al., 2017), and Azoxistrobin (Navathe et al., 2019) were efficacious in managing spot blotch. In addition, Mishra et al. (2014) showed that silver nanoparticles act as a fungicide against spot blotch. The use of silicon was also found to improve resistance of wheat leaves to *B. sorokiniana* infection (Domiciano et al., 2010). In addition to these management strategies, balanced nutrition and crop rotation should form a part of the integrated management strategies in managing spot blotch in wheat (Sharma et al., 2005; Sharma et al., 2006; Yadav et al., 2013; Mazzilli et al., 2016; Bankina et al., 2018; Sˇvarta and Bimsˇteine, 2019). The application of nitrogen alone without phosphorus and potassium is known to increase the severity of spot blotch (Singh et al., 2012).

TABLE 1 | Examples of wheat genotypes having less susceptibility to spot blotch.

Country	Wheat genotypes/cultivars	References
Afganistan	PAMIR-94	(Bainsla et al., 2020)
Brazil	BH 1146	(Singh et al., 2016; Singh et al., 2018)
China	Ning 9415, Ning 8201	(Schlegel, 1997; Bainsla et al., 2020)
India	Chirya 7, Chirya 3, Ning 8139, Suzhou, Milan-3, HD 2888, HD 2967, WR 95, IC529962 and IC443652	(Gurjar et al., 2018; Kumari et al., 2018; Choudhary et al., 2019)
Mexico	BARTAI, WUYA	(Singh et al., 2018)
Nepal	NL750	(Sharma et al., 2004; Rosyara et al., 2007)
Zambia	19HRWSN6, 19HRWSN7 and 19HRWSN15	(Tembo et al., 2018)

COMMON ROOT ROT AND CROWN ROT DISEASES

Importance and Symptoms

Common root rot and crown rot of wheat are important diseases in most wheat-growing countries, including China, Australia, Middle East, and Europe (Fedel-Moen and Harris, 1987; Tunali et al., 2008; Al-Sadi and Deadman, 2010; Poole et al., 2015; Gupta et al., 2018b; Xu et al., 2018a). They are characterized by the development of necrotic lesions on the roots, subcrown, and crown. The lesions are dark brown to black in color. Development of symptoms on the root is usually followed by symptoms on wheat crowns (Al-Sadi and Deadman, 2010; Qostal et al., 2019).

The disease is caused by *B. sorokiniana* (Tunali et al., 2008; Xu et al., 2018a; Yue et al., 2018), which is also associated with other fungi including *Fusarium pseudograminearum, F. culmorum, Microdochium nivale*, *Pythium* spp., and *Rhizoctonia cerealis* (Moya-Elizondo et al., 2011; Saremi and Saremi, 2013; Kazan and Gardiner, 2018; Xu et al., 2018a; White et al., 2019).

Yield and quality of wheat could be reduced by common root rot and crown rot. Common root rot was reported to result in yield losses of 6%–24% (Wildermuth et al., 1992). Yield reduction due to crown rot has been estimated to range from 0 to 89% in New South Wales, Australia (Klein et al., 1991). In Queensland (Australia), crown rot caused up to 26% yield loss in some fields, with an overall reduction by 5% for the whole state (Burgess et al., 1981), while a reduction by up to 35% was reported in the Pacific Northwest, North America (Smiley et al., 2005). Reduction in yield is usually because of the effect of common root rot and crown rot diseases on the number of tillers and on the number and size of kernels (Duczek and Jones-Flory, 1993).

Common root rot is a disease of dry and warm areas. Disease severity and incidence is affected by soil moisture, soil temperature, cultural practices, pathogen population in the soil, and time of infection. Disease severity increases when the plant is under stress or grown in warm soil and less moisture (Mathieson et al., 1990; Acharya et al., 2011). In addition, the incidence of common root rot was found to be affected by the soil populations of *B. sorokiniana* at the time of planting (Boer et al., 1991). Propagules of *B. sorokiniana* can go to a depth of 40 cm in the soil, but the population of the fungus is highest in the top 10 cm (Mathieson et al., 1990).

Management of Common Root Rot and Crown Rot

Different methods have been used in the control of common root rot and crown rot of wheat. The use of the endophytic bacterium *Pseudomonas mediterranea* resulted in a significant reduction in root and crown rot of wheat in Pakistan (Ullah et al., 2020). Disease severity index of wheat common root rot decreased from 90.8% to 27.7% following the use of the actinobacterium *Nocardiopsis dassonvillei* as a biocontrol agent, which was attributed to the ability of this isolate to produce siderophores and hydrogen cyanide (Allali et al., 2019). The actinobacterium was also found to enhance growth of wheat through the production of indole-3-acetic acid. In another study, the bacterial strain *Lysobacter enzymogenes* C3 and the fungal strain *Rhizoctonia* BNR-8-2 were found to result in a significant reduction in the common root rot of wheat, which was attributed to the production of chitinases, β-1,3-glucanases and antibiotics, especially by *L. enzymogenes* C3 (Eken and Yuen, 2014). Yue et al. (2018) showed that the biocontrol fungus *Chaetomium globosum* is effective in inhibiting *B. sorokiniana* associated with wheat common root rot, which is attributed to the production of secondary metabolites by *C. globosum*.

Cultural practices are important for the management of plant diseases. Crop rotation of wheat with *Brassica carinata* was found to result in a significant reduction in common root rot and crown rot diseases (Campanella et al., 2020). In Iran, soil solarization was found effective in reducing wheat root rot (Saremi and Saremi, 2013). The use of organic agriculture helped reduce populations of *Fusarium* populations associated with crown rot of wheat in Canada (Fernandez et al., 2011), while zero tillage was found to increase wheat yields and reduce the incidence of wheat root rot in Mexico (Govaerts et al., 2006).

Different cultivars of wheat were reported to differ in resistance to common root rot (Al-Sadi and Deadman, 2010; Manghwar et al., 2018). In addition, the production of GmPGIP3 transgenic wheat plants enhanced the resistance of wheat to *Bipolaris sorokiniana*-induced common root rot as well as *Gaeumannomyces graminis* var. *tritici*-induced take-all diseases in wheat (Wang et al., 2015). Fungicides are not a good choice for the management of wheat root and crown diseases (Fernandez et al., 2010).

CONCLUSION

Bipolaris sorokiniana is a serious pathogen, not only because it results in significant yield losses, but also because it can attack most wheat organs, including roots, crown area, stems, leaves and kernels. This means that management strategies should not only focus on limiting the presence of the fungus in the aerial parts of the plants, but attention should be given to *B. sorokiniana* inoculum present in soil. In addition, it is important to develop an integrated disease management program for managing *B. sorokiniana* using cultural practices, biological control and chemical fungicides. Since the search for biocontrol agents has been given more attention during recent years, it is important to find antagonistic strains that can complement cultural and chemical practices in the field. The search for new sources for resistance should consider finding less susceptible cultivars to all diseases caused by *B. sorokiniana*, instead of focusing on one disease.

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The author confirms being the sole contributor of this work and has approved it for publication.

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Climate-Fungal Pathogen Modeling Predicts Loss of Up to One-Third of Tea Growing Areas

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Climate change will affect numerous crops in the future; however, perennial crops, such as tea, are particularly vulnerable. Climate change will also strongly influence fungal pathogens. Here, we predict how future climatic conditions will impact tea and its associated pathogens. We collected data on the three most important fungal pathogens of tea (*Colletotrichum acutatum*, *Co. camelliae*, and *Exobasidium vexans*) and then modeled distributions of tea and these fungal pathogens using current and projected climates. The models show that baseline tea-growing areas will become unsuitable for *Camellia sinensis* var. *sinensis* (15 to 32% loss) and *C. sinensis* var. *assamica* (32 to 34% loss) by 2050. Although new areas will become more suitable for tea cultivation, existing and potentially new fungal pathogens will present challenges in these areas, and they are already under other land-use regimes. In addition, future climatic scenarios suitable range of fungal species and tea suitable cultivation (respectively in CSS and CSA) growing areas are *Co. acutatum* (44.30%; 31.05%), *Co. camelliae* (13.10%; 10.70%), and *E. vexans* (10.20%; 11.90%). Protecting global tea cultivation requires innovative approaches that consider fungal genomics as part and parcel of plant pathology.

Keywords: *Camellia sinensis*, climate change, crop loss, fungal diseases, perennial crops

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INTRODUCTION

Climatic changes can alter fungal survivability and infectivity as well as host susceptibility, leading to new disease outbreaks (Elad and Pertot, 2014; Cheng et al., 2019). They may also facilitate the emergence of new virulent strains, a cause of significant concern for future epidemiological research (Garrett et al., 2006; Chen et al., 2019). Several studies on fungal diseases and climate change have revealed that the number of fungal diseases has increased alongside climate change, and it is increasingly recognized as a worldwide threat to important crops (Fisher et al., 2012). Bebber et al. (2013) found that fungal pathogens are moving poleward at 7.61 ± 2.41 km/y in the Northern Hemisphere, while climates move at about 2.7 km/y. Trade and transport are likely to be accelerating the spread of fungal pathogens. With land-use change, drought, and climate variability, fungi need adaptation or shifts in community for survival, while some fungi with higher thermal optima have higher fitness for climate change (McLean et al., 2005; Bajpai and Johri, 2019). Consequently, tea as perennial plant might not escape fungal threats unless it is relocated.

Several studies have been conducted to gain insight into the responses of perennial crops such as almonds, avocados, grapes, and walnuts to climate projections (Lobell et al., 2006). Diseases in perennial crops have been well researched, but the impact of climate change on the distribution of these fungal diseases on tea has not yet been studied (Karakaya and Dikilitas, 2018; Nowogrodzki, 2019). Tea comprises almost one-quarter of the global perennial crop market, including coffee, tree fruits, and tree nuts (**Supplementary Table S1**). There are two main varieties of cultivated tea: *Camellia sinensis* var. *sinensis* (CSS) and *Camellia sinensis* var. *assamica* (CSA) (Ahmed and Stepp, 2013). The difference of the two tea varieties can be distinguished: var. *assamica*, quick-growing with a large leaf, tall and well suited to very warm tropical climates, and with high sensitivity to cold weather, while var. *sinensis*, lower-growing shrubs, with small leaves, and able to withstand colder climates (Kaundun and Matsumoto, 2003; Ming and Bartholomew, 2007). Globally, tea is usually grown in monocultural plantations in humid climates, where genetic uniformity and low environmental variability facilitate the spread of fungal pathogens. In Yunnan (China), tea is grown in between forest, agroforest, and mixed-crop (Lehmann-Danzinger, 2000; Ahmed et al., 2010; Diez et al., 2013).

Fungal pathogens that affect tea plant leaves can lead to a significant reduction in their quantity and quality, resulting in a loss of revenue (Gulati et al., 1993; Baby, 2002; Wang L. et al., 2016; Wang Y. et al., 2016; Cheng et al., 2019; Sun et al., 2019). Approximately 507 fungal pathogenic species are associated with tea plants (Chen and Chen, 1990), and among them *Exobasidium vexans* mainly attacks tea leaves which make ~40% yield loss (Sun et al., 2019; Chaliha and Kalita, 2020). Some studies investigated the effects of future climates on the distribution of other perennial crops, including coffee in Ethiopia (Moat et al., 2017), grapevines in the United Kingdom (Mosedale et al., 2015), and cultivated grasses in Sichuan, China (Guo et al., 2014). Juroszek et al. (2020) reviewed the potential climate change effects on plant pathogens and crop disease risks during the past 30 years, but tea was not included. Modeling land with highly, moderately, and non-suitable regions for the cultivation of tea crops in Zhejiang, China under baseline climates was done by Li et al. (2012). However, this study did not include global land suitability and the impacts of fungal diseases on tea crops. Several regional studies examined climate change effects on tea yield and/or quality and also used regression models to predict tea crop yields (Wijeratne et al., 2007; Patra et al., 2013; Karunaratne et al., 2015; Boehm et al., 2016; Duncan et al., 2016; Gunathilaka et al., 2016; CIAT, 2017; Sitienei et al., 2017; Ahmed et al., 2018; Gunathilaka et al., 2018; Han et al., 2018; Zakir, 2018; Ahmed et al., 2019). Karakaya and Dikilitas (2018) dealt particularly with the biochemical, physiological, and molecular defense mechanisms of tea plants against pathogenic fungi under changing climate conditions.

None of these studies considered climate change in the context of the effects of fungal pathogens on perennial crops like tea. Ecological niche modeling (ENM) based prediction of suitable habitat for host and pathogen could provide baseline infromation on how pathogens would spread in potential tea plantation regions in the future. Therefore, we aim to generate distribution models of a host crop and its fungal pathogens to enhance understanding of how these pathogens might spread and impact potential tea plantation regions under future climatic scenarios. Also gaps in this research include changes across global plantations in the two most common commercial varieties of tea.

We reviewed the literature on where cultivated tea is globally affected by fungal pathogens and global climate projections to anticipate future tea climates as well as future tea-fungal pathogen climates (Fick and Hijmans, 2017; Větrovský et al., 2019). Baseline (baseline scenario) and future climate scenarios (2050) were used for generating suitability mapping for suitability for pathogens under current conditions with regional and global climate projections. This tea-pathogen scenario is only a case study which can be applied to many different host–pathogen distributions in different disciplines. In addition, we give pattern of spore and appressorium germination of *Co. acutatum* at growth rate peaks 25 to 27.5°C.

METHODS

We obtained data from published literature, online databases, and personnal communication. Most fungal pathogenic reports in tea originated from China and India. Bioclimatic correlations were the main factors that we used to model probability of occurrence among these pathogens. Distributions of fungal pathogens were linked to bioclimatic factors, particularly their unique temperature and precipitation requirements. We collected fungal pathogen data from "Google Scholar", "Research Gate", and "Web of Science". The main keywords used in the online literature search information regarding distribution of tea and tea pathogens were: "*Camellia sinensis*", "fungal pathogen", and "tea plantation". Those following

discovery of tea pathogens were most importantly fungal tea disease and distribution of tea. Based on the review of relevant literature, a list of tea fungal pathogens was prepared. Distribution of tea occurrence records was compiled in several locations, and final data were used for the analyses of current and projected climates. Tea leaf sample data (280 from Africa and 654 samples from Asian countries) were obtained from Global Biodiversity Information Facility (GBIF), while tea samples from America and Australia were obtained from online website (www.killgreen.io/main/us-grown-tea; www.youtube.com/c/CuppaChaTEA). Sampling includes collections from tea plantations as well as wild populations especially in China. Tea growers in Asian countries were contacted afterwards to find out from where tea leaves were collected and to get information of pathogens in tea plantations. Therefore, for comparative purposes in the case of diseases on tea, the global geographic distribution of fungal tea pathogens was summarized from existing literature and combined with information on major fungal diseases as well as the evolution of this research field. In addition, species identity of the selected fungal diseases was confirmed by published literature that was based on morphology, genomics, and pathogenicity (**Supplementary Table S2**). Three major fungal diseases on tea were selected, and these caused diseases on more than one host species. In addition, we obtained tea and the fungal pathogen distribution records from GBIF. This database includes information taken from the CABI database. In addition, we also have ground truthing data for tea that were recorded in 2013/14. The leaf sample collection records used as ground-based data come from Bangladesh, Cameroon, China, India, Kenya, Madagascar, Malawi, Nepal, Nigeria, Pakistan, Rwanda, South Africa, Sri Lanka, and Tanzania.

Based on information available on disease incidence at various geographical locations, we selected three important fungal pathogens on tea leaves that are briefly described here. The genus *Colletotrichum* represents high diversity, and several species are plant pathogens that cause diseases in many economically important crops. In this study, we selected two species of *Colletotrichum* (*Co. acutatum* and *Co. camelliae*) that are either already known to cause disease or are considered as emergent diseases on the tea plant. *Colletotrichum acutatum* is a new causal agent for brown blight on tea, which is generally considered as an endophytic fungus but is also considered pathogenic or potentially pathogenic on tea. *Colletotrichum camelliae* is the earliest known cause of tea anthracnose, and it is notable that this pathogen has been reported only from tea, and knowledge of it is limited. *Exobasidium vexans* is an obligate pathogen capable of attacking young leaves and is recognized as the most serious disease in cultivated tea. Considering the data on tea leaf-affecting pathogens, we selected *Co. acutatum*, *Co. camelliae*, and *E. vexans* for our modeling. The three pathogens used in our modeling process were selected by occurring wide range of crops in tropical and subtropical areas, also latent infection on tea (*Co. acutatum*, *Co. camelliae*) with mainly pathogen only in tea (*E. vexans*). We includeed *Co. acutatum* because this species was treated as a regulated quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO) for many years until 2011 (EPPO, 2011). This species in a broad sense is a suitable candidate pathogen for modeling disease development on tea in the context of climate change because there are economically important pathogens in tea that infect tea leaves which is the most economically important part of tea (Peres et al., 2005; Wharton and Schilder, 2008; Chen et al., 2016; Chen et al., 2017). In addition, several fungal pathogens on tea are shown in **Supplementary Table S2**. These three taxa are obligate parasites, highly pathogenic, and responsible for crop losses (Dean et al., 2012; Wang Y. et al., 2016; Wang L. et al., 2016; Li et al., 2017).

Bioclimatic Data

We obtained 19 biologically significant climate variables (bioclim) from Wordclim version 2 (www.worldclim.org/version2) (Fick and Hijmans, 2017) to generate distribution models. For future projections of both tea and fungal models, bioclimatic variables (monthly temperature and precipitation), tea and fungal models were derived from the results of 19 Earth System Models (ESM) provided by the Coupled Model Intercomparison Project-Phase 5 (CMIP5) (Taylor et al., 2012). Relative humidity data were used for tea and pathogenic distribution, as relative humidity and temperature were main factors affecting pathogens (Jones and Wint, 2015). There are four representative concentration pathways (RCP) (Van Vuuren et al., 2011) within each of the 19 ESM, ranging from RCP 2.6 (aggressive mitigation/lowest emissions) to RCP 8.5 (highest emissions scenario). All models available within each RCP were combined into a majority ensemble result (E26, E45, E60 and E85) (Zomer et al., 2015; Ranjitkar et al., 2016a). Grid cell of all the climatic layers used was adjusted to 0.0833 (~10 km spatial resolution). Tea location data was prepared with approximately 20 km spatial resolution of presence data where each 20 km^2 grid has a single point of present data. The rest of the data points, if available within 20 km^2 grid were discarded to minimize spatial autocorrelation and possible overfitting of the model output maps (Ranjitkar et al., 2014). Tea and pathogen data were extracted from tea plantation areas and pathogen incidence recordings. Information obtained from temporal distribution and climatic range was used to select appropriate bioclimate thresholds (temperature and precipitation) to project bioclimatic suitability for the pathogens. Out of these, temperature is the most vital factor affecting fungal growth that could cease at non-permissive temperatures.

In addition, data of optimum temperatures for conidia germination, appressoria formation, and anthracnose development of pathogens (*Co. acutatum* and *Co. gloeosporioides*) were obtained from available literature (Kenny et al., 2012). Temperature induced fungal pathogen growth with percent germination, appressorium, and anthracnose disease was used to genetate graph with fungal growth, in that range (**Supplementary Figure S1**).

Habitat Suitability Modeling

MaxEnt 3.4.1 (Phillips et al., 2004; Phillips et al., 2006; Phillips and Dudík, 2008) was used for generating suitability maps for tea

and pathogens in the baseline (baseline scenario) and future (2050) climate scenarios. Presence data and pseudoabsence were used, and prediction model was created using MaxEnt software. To make the model robust to current tea cultivation area, we selected pseudoabsence points using a 100 km radius of available presence data (Senay et al., 2013; Ranjitkar et al., 2014; Warren et al., 2020). Altogether, 20,000 pseudoabsence points were selected. Bioclimatic factors for both species occurrence and pseudoabsence data were extracted and used for running the model in Maxent. Altogether 20 replicates of the model with 19 bioclimatic variables (obtained from the worldclim database) were run setting MaxEnt to select 75% of occurrence localities randomly for calibration and the remaining 25% for evaluation after each run. The bootstrap method was used with maximum iteration = 5,000, convergence threshold = 0.0001, maximum background points = 20,000 and default features. Initial modeling results were thoroughly checked and high contributions and important variables were selected and retained while other highly correlated variables were removed using variance inflation factor (VIF) statistics. Highly correlated variables were indicated by VIF <10 (Ranjitkar et al., 2016a; Ranjitkar et al., 2016b). Removing variables VIF >10 eliminated multicollinearity issues that might affect modeling. The least-correlated set of variables were used to re-run model using the above mentioned modeling parameters for host and pathogen. The calibrated model was then projected onto future (2050) climatic scenarios.

Model Validation and Detect Change in Suitable Area of Tea Growing Areas

Model prediction performance was calculated using TSS (True Skill Statistics), MaxKappa, and AUC (Area Under Curve-Receiver Operating characteristics) statistics (Araujo et al., 2005). The calculated TSS is independent of prevalence—the ratio of presence to pseudo-absence data in the presence–absence predictions (Allouche et al., 2006). Both TSS and MaxKappa deal with sensitivity and specificity values of model output. Both range from −1 to +1, where +1 indicates perfect agreement and TSS >0.7 indicates a good model fit (Allouche et al., 2006). Both TSS and MaxKappa deal with sensitivity and specificity values of model output. Both range from −1 to +1, where +1 indicates perfect agreement and TSS >0.7 indicates good model fit (Allouche et al., 2006). The average model was converted to a binary model (presence/absence) applying equal training sensitivity and specificity thresholds that suit the present distribution of the focal species. Reclassified binary layers were used to estimate suitability change in the future for hosts and pathogens. We compared the model predictions in different time frames: baseline and projection in the future (2050) across four RCPs. The modeled occurrence probabilities for both current and future climatic conditions were summarized as presence–absence of tea distribution, and the overall distribution change was calculated as the difference between two distribution models for current and future conditions (Ranjitkar et al., 2016a; Ranjitkar et al., 2016b). A fuzzy logic model was employed for identifying areas where fungal pathogens overlap with tea-growing areas.

RESULTS

Tea Distribution

Modeled suitable habitats for both varieties of commercial tea under baseline and projected future climatic conditions are shown in **Figures 1**–**3**. Intentionally we did not include novel areas for tea plantations as those areas already had other land-use types, and converting to tea plantation will not be economically beneficial. This also refers to Ranjitkar et al. (2016a) who showed the evidence of the distribution of suitable habitats and impacts of climate change for the selected tree species. Tea species occurrence records of collection sites across the world are shown separately. Almost all tea species occurrence records are in the modeled suitable range of tea. Modeled area is largely represented where sample collection was larger and where sample size was small or just one to two projected grids are smaller. Sample size and representation from each geographical region affect final model output (**Figure 1**). The AUC values ranged from 0.97 to 0.99, TSS between 0.65 and 0.95, and maxKappa between 0.31 and 0.48 for all models. CSS has larger and more widely distributed habitats than CSA. The main areas suitable for CSS are forecasted across different countries in Asia, Africa, Europe, and North and South America (**Figure 1**). Commercial cultivation is just beginning in Europe and North American countries (Zhang et al., 2020). Suitable areas for tea growing under future climate scenarios will decrease in land coverage, especially in South and Southeast Asia, Central and South America, and Africa. Models revealed CSS might lose 15 to 32% of suitable area by 2050 compared to baseline. Predicted suitability loss was higher in RPC 8.5 scenario compared to RPC4.5 and least in RPC2.6 (**Figure 2**). Our modeling results revealed climatic suitability of CSA is distributed mainly across the countries in Asia, while suitable area is also predicted across African and South American countries (**Figure 1**). These suitable areas are forecast to decrease 32 to 34% by 2050. All scenarios, the minimum (RCP 2.6), the median (RCP4.5), and the maximum (RCP 8.5) climate change projections in 2050, suggested very similar trends in terms of the projected changes (**Figure 3**). Areas where major loss is detected in the model are highlighted in the inset in **Figures 2** and **3**. The contribution of each replicate to the final average model was proportional to their goodness-of-fit statistics.

Climatic Factors *vs.* Tea Disease Development and Fungal Pathogen Modeling

Suitable area of each fungal pathogens on two varieties of tea show substantial overlap in **Figures 5**–**7**, while modeled tea and its fungal pathogens with overlapping suitability, unsuitability, new habitat, and pathogen free tea suitability are shown in **Supplementary Table S3**. The model analysis indicates in

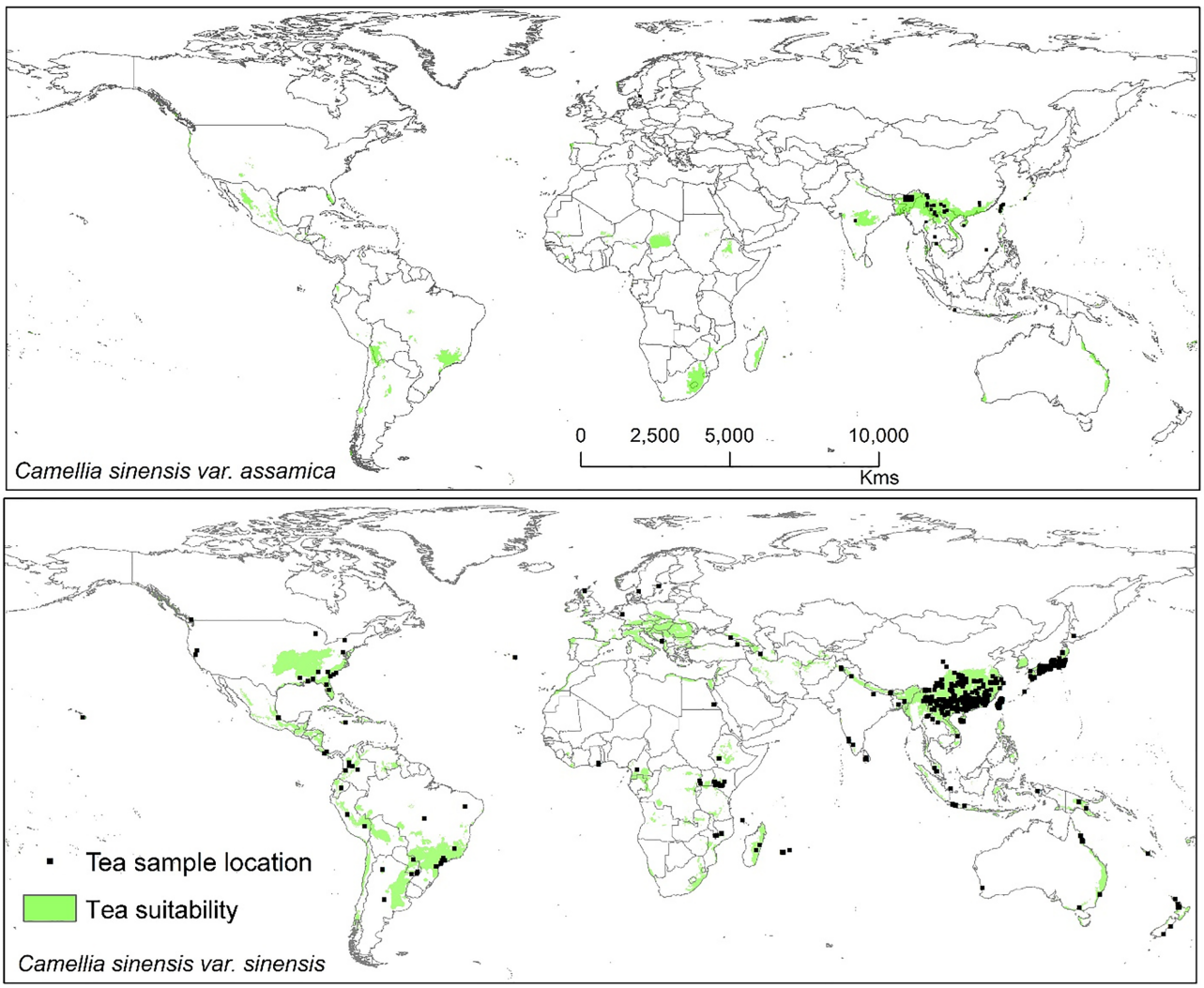

FIGURE 1 | Suitable area for *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* growing in baseline climatic conditions (green represents the suitable areas). Tea species occurrence records by different plant collectors and deposited in various herbaria across the world. Here locations of species occurrence records are plotted, and in the background tea-suitable area in baseline climatic scenario is shown.

future climatic scenarios a suitable range of fungal species that overlap with suitable tea (respectively in CSS and CSA) growing areas are *Co. acutatum* (44.30%; 31.05%), *Co. camelliae* (13.10%; 10.70%), and *E. vexans* (10.20%; 11.90%). Not only tea plantations will suffer from those pathogens but other land-use types in Ranjitkar et al. (2016a) might also be affected. Modeling habitat suitability for tea fungal pathogens reveals that their distributions differ across spatial extent (**Figure 4**). However, these pathogens are widely co-distributed with tea (**Figures 5**–**7**).

Colletotrichum acutatum

Colletotrichum acutatum is an important anthracnose pathogen with a wide host plant range worldwide (Damm et al., 2012). On tea plants, this pathogen is considered a new causal agent which can reduce quality of tea leaves when compared with *Co. gloeosporioides*, as this species has high phenotypic and genotypic diversity (Chen et al., 2017). Brown blight on tea leaves caused by *Co. acutatum* was reported by Chen et al. (2016; 2017), showing *Co. acutatum* is a suitable candidate pathogen to study climatic change and disease development on tea. The *Co. acutatum* species complex is known today as a destructive pathogen on fruits (including perennial crops), such as citrus (Peres et al., 2008), apple (Lee et al., 2007), olive (Talhinhas et al., 2011), and blueberry (Wharton and Schilder, 2008). According to our prediction, it shares a high percentage of bioclimatic suitability with tea at 44.30% (CSS) and 31.05% (CSA). *Colletotrichum acutatum* is thus a suitable candidate pathogen on tea plants that is capable of shifting to novel areas or hosts under climate change scenarios. Suitable modeled areas for *Co. acutatum* on CSA include southern China, North India, and Nepal, while suitable areas for CSS include Asia (Southern China, Japan, North India, and Nepal), Africa (Madagascar), Europe

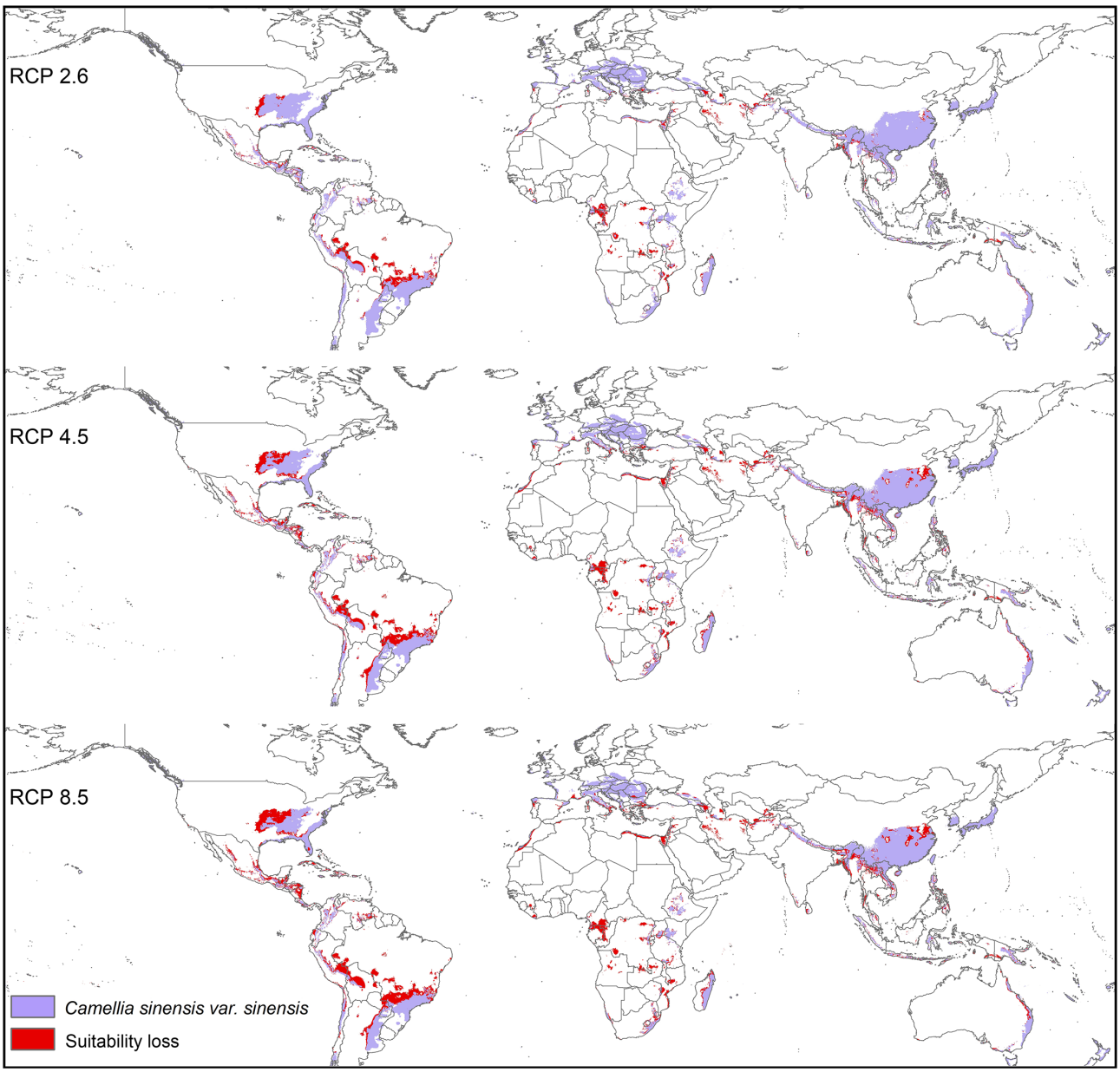

FIGURE 2 | Suitable areas for *C. sinensis* var. *sinensis* growing under future climate scenarios with baseline suitable areas and losses by 2050 (purple represents the suitable areas, and red represents lost suitable areas).

(Central and Wester), North America (United States), and South America (Southern Brazil, Uruguay, Argentina, Colombia, Peru, and Chile) (**Figure 5**).

Colletotrichum camelliae

Colletotrichum camelliae has been reported among the most prevalent tea pathogens causing damage and several diseases known as tea leaf blight, tea brown blight, or tea anthracnose (De Silva et al., 1992; Wang L. et al., 2016; Wang Y. et al., 2016; Ponmurugan et al., 2019). Orrock et al. (2020) reported that anthracnose in US tea is caused by this fungus. *Colletotrichum camelliae* is the most dominant and frequently identified pathogen in the main tea growing areas of China (Liu et al., 2015; Wang L. et al., 2016). This species is likely still a dominant pathogen on tea, especially in Asia where it affects CSS.Tea in China is likely to be further affected by this pathogen under future climates because in China it was shown that the frequency of extreme weather and climate events will be increasing (Baby et al., 1998). Suitable modeled areas for *Co. camelliae* on tea plants are found in Asia with a bioclimatic suitability percentage of 13.10% (CSS) and 10.70% (CSA) (**Figure 6**). *Colletotrichum* spp. pathogens show the way in

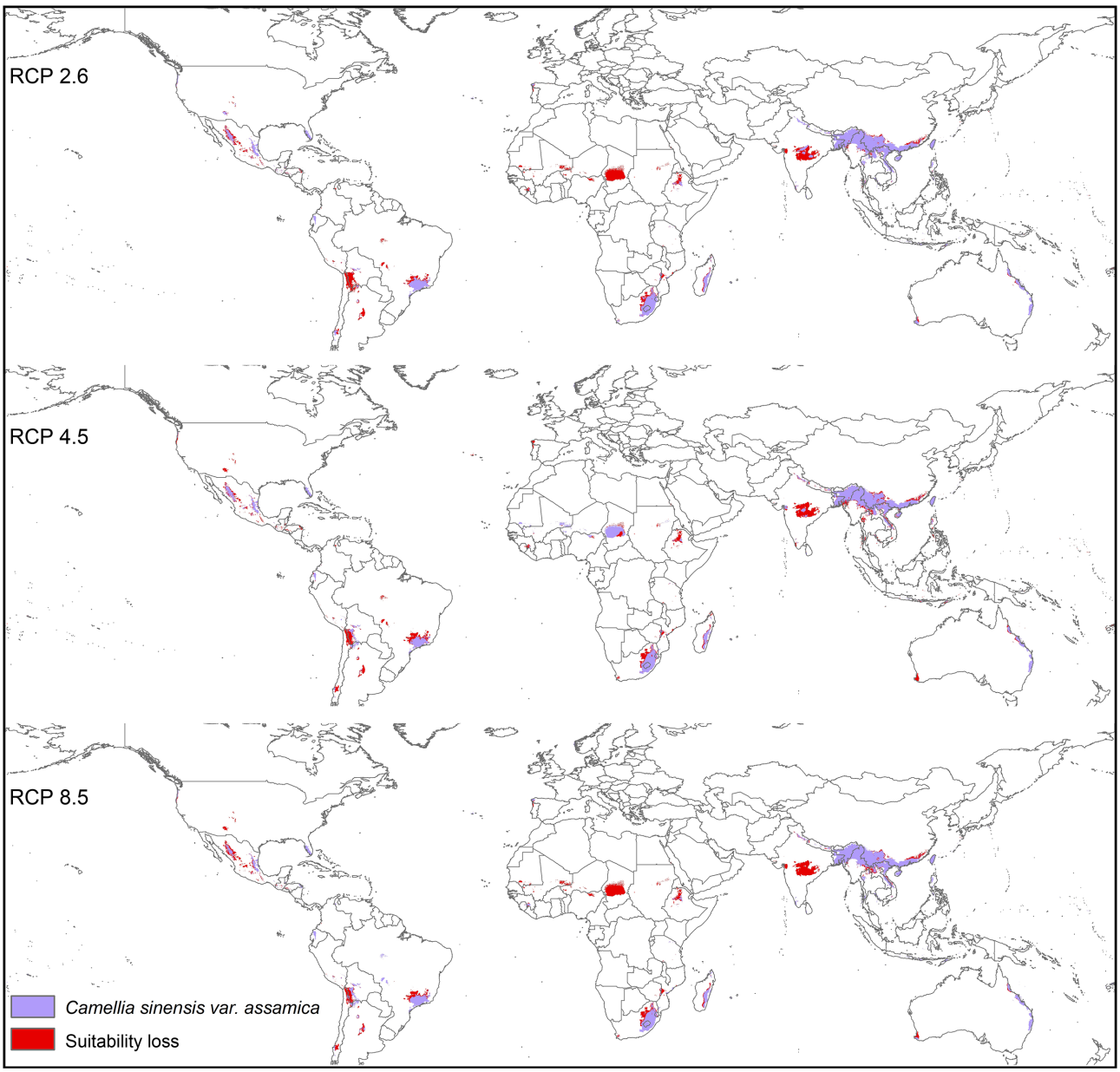

FIGURE 3 | Suitable areas for *C. sinensis* var. *assamica* growing under future climate scenarios with baseline suitable areas and losses by 2050 (purple represents the suitable areas, and red represents lost suitable areas).

which the growth rate of spore germination and onset of diseases vary in accordance to temperature change, with the temperatures >25 and <35°C representing ideal conditions for fungal growth. Tea-growing areas in most places might become favorable climatic zones for pathogens, and in these areas fungal infections in tea might become widespread. T1 = 25°C and T2 = 27.5°C are temperatures at which germination and appressorium were at their peak (**Supplementary Figure S1**). This information could foster increased human preparedness in the emergence of new latent pathogen with climate change. The results show that temperatures between 25 and 30°C foster ideal conditions for this kind of fungal growth (**Supplementary Figure S1**). Our results are in accordance with Piao et al. (2010) who commented that when rainfall or high humidity accompanies a temperature range between 23 and 35°C, the disease may become most widespread and serious. According to available literature, 25 to 28°C is the optimum range of temperatures and is justified for the selected temperature range (Pasanen et al., 1991; Pietikäinen et al., 2005; Piao et al., 2010). The germination time and growth rate of a fungus depend on temperature, humidity (50–70%), and surface nutrients (Pasanen et al., 1991; Piao et al., 2010).

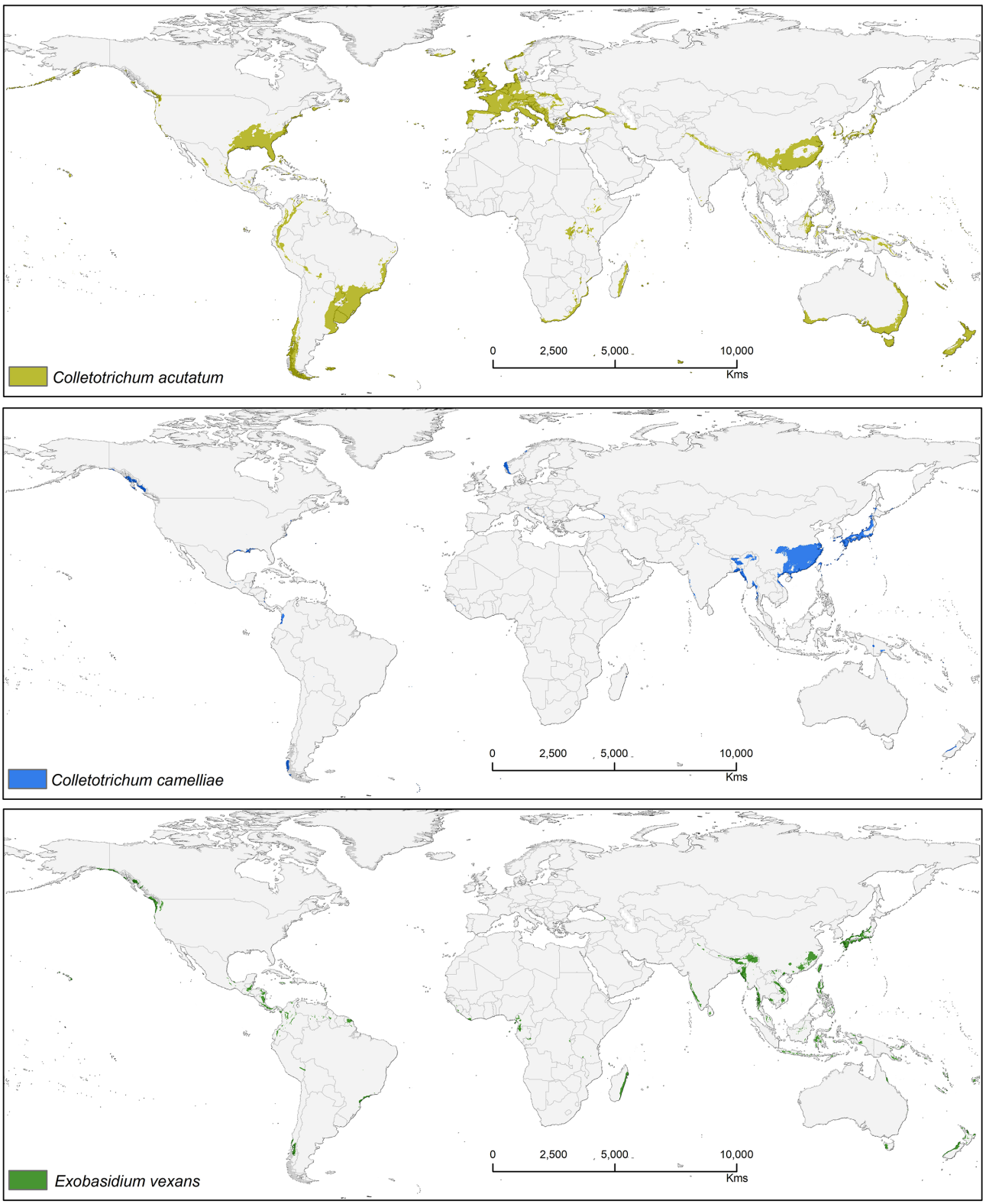

FIGURE 4 | Bioclimatic suitability for three major fungal pathogens: *Colletotrichum acutatum, Colletotrichum camelliae*, and *Exobasidium vexans*.

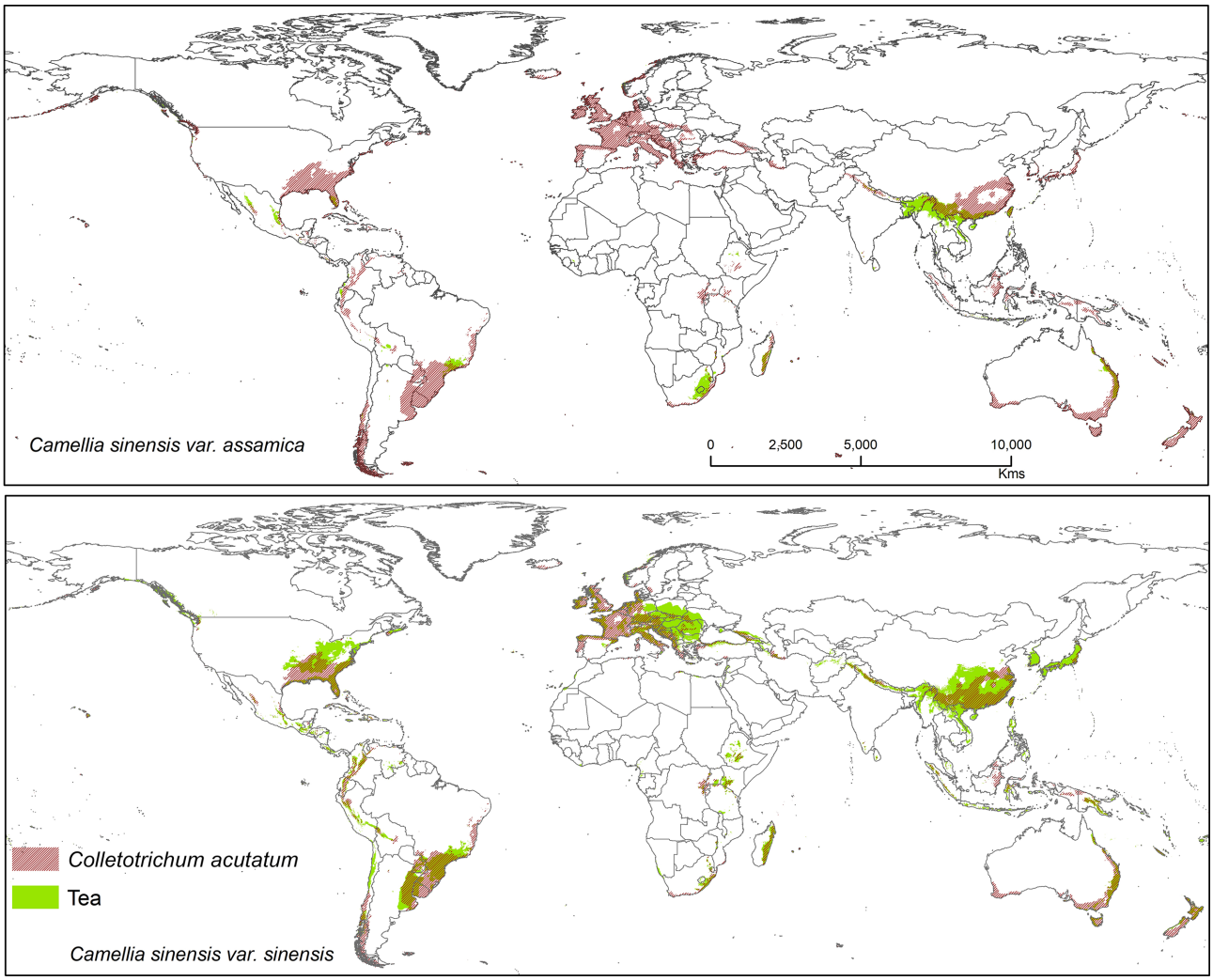

FIGURE 5 | Shows the suitable area of *Colletotrichum acutatum* on tea with suitable area of two varieties of tea as projected in the average of all future projections.

Exobasidium vexans

Exobasidium vexans is an obligate parasite or endemic pathogen which occurs only in tea plant and causes blister blight disease on tea leaf and is the most devastating disease that affects tea, especially in Asia (south and east asia) (Punyasiri et al., 2005; EPPO, 2006; Sinniah et al., 2016; Ponmurugan et al., 2019; Chalkley, 2021). This pathogen has caused severe crop/yield losses in Sri Lanka (33%), India (35–50%), and Indonesia (20–25%) (De Silva et al., 1992; Baby et al., 1998; Radhakrishnan and Baby, 2004; Basu-Majumder et al., 2010; Diez et al., 2013). This fungus is known to remain dormant during periods of unfavorable conditions, only becoming pathogenic under optimal climatic conditions. If not controlled by fungicides, tea losses due to this pathogen may range from 25 to 43% (Mur et al., 2015). Suitable modeled areas for *E. vexans* on tea are in Australia, South America, Africa, and Asia. In future, CSS maps have more overlab with pathogen maps than CSA (**Figure 7**). It has a bioclimatic suitability percentage with suitable tea of 10.20% (CSS) and 11.90% (CSA) and areas with increased future risk cluster in Asia, including China, Japan, Mynmar, Vietnam, India, Bhutan, Sri Lanka, Philippines, and Indonesia (**Figure 7**).

Correspondence analysis among the three tea pathogens used in our study with global annual temperatures, annual precipitation, and humdity indicates that these pathogens might have the ability to survive low humidity and temperatures under 15°C (**Supplementary Figures S2**–**S5**). These diseases are thus able to preceed tea into novel areas as emergent pathogens, potentially impacting new tea production initiatives in the future.

DISCUSSION

The effects of crop losses associated with pathogens and pests in major agricultural crops are better understood than those of

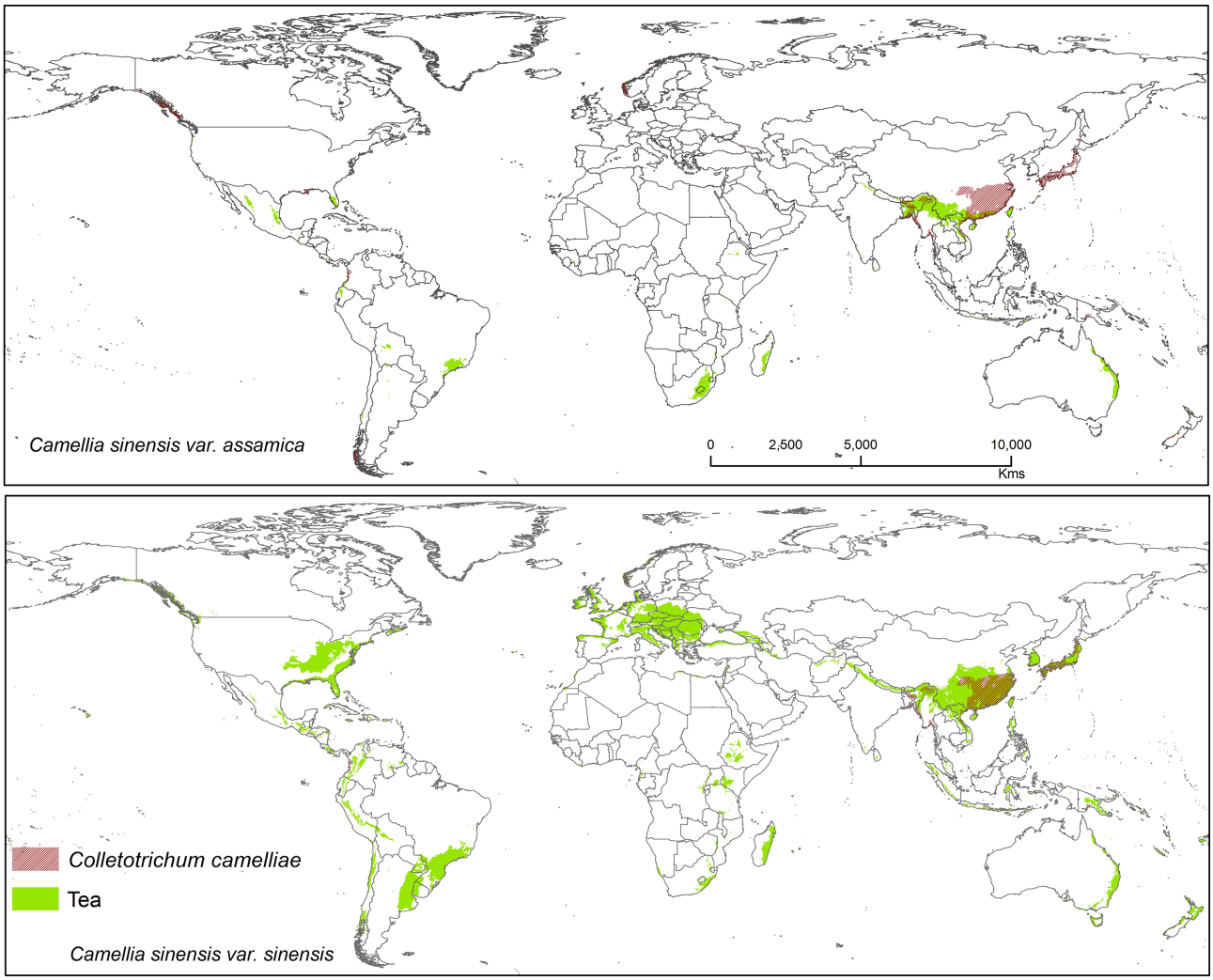

FIGURE 6 | Shows the suitable area of *Colletotrichum camelliae* on tea with suitable area of two varieties of tea as projected in the average of all future projections.

commodity crops like tea (Chen et al., 2019; Savary et al., 2019), and among perennial crops tea is unique as its leaves are the product. We modeled tea growth under future climate scenarios and found that suitable tea-growing areas could decrease 34% by 2050. In addition, fungal pathogenic mapping revealed an overlap with baseline tea-growing areas. We anticipate that climate change will render large areas unsuitable for the two dominant tea varieties, CSS and CSA. Future pathogen damage will likely be greater in growing areas for the *sinensis* variety as *C. sinensis* grows in relatively cooler areas compared to *C. assamica*, and most of the pathogens are active in these regions compared to warmer areas where *C. assamica* grows. In addition to change in suitability, temperature rise in baseline tea-plantation areas favors rapid growth and spread of fungal pathogens. We observed that future temperatures will reach optimum levels for these selected pathogens in baseline tea-plantation areas. Suitable areas of overlap for both hosts and pathogens were estimated to show potentially threatened areas (**Figures 5–7**). Furthermore, potential changes in the distribution were assessed by comparing the suitability map produced by SDM under future climates.

Tea growing areas comprise regions with diverse climates. Although climate change affects each region differently, it influences tea yields by altering precipitation levels, increasing temperatures, and encouraging pests. Bunn et al. (2015) reported that climate change will reduce the global area suitable for coffee by about 50% across emission scenarios. Compared to coffee (Bunn et al., 2015), our model for tea revealed reduction of global area suitable for tea will be less. Still, climate change will exert an enormous influence on future tea production. This future projection information identifies overlapping regions of fungal species with suitable tea while, overlapping regions potential for other plantations as well. Similarly, the impact of climate change is also reported on several agroforestry tree species in Yunnan Province, China,

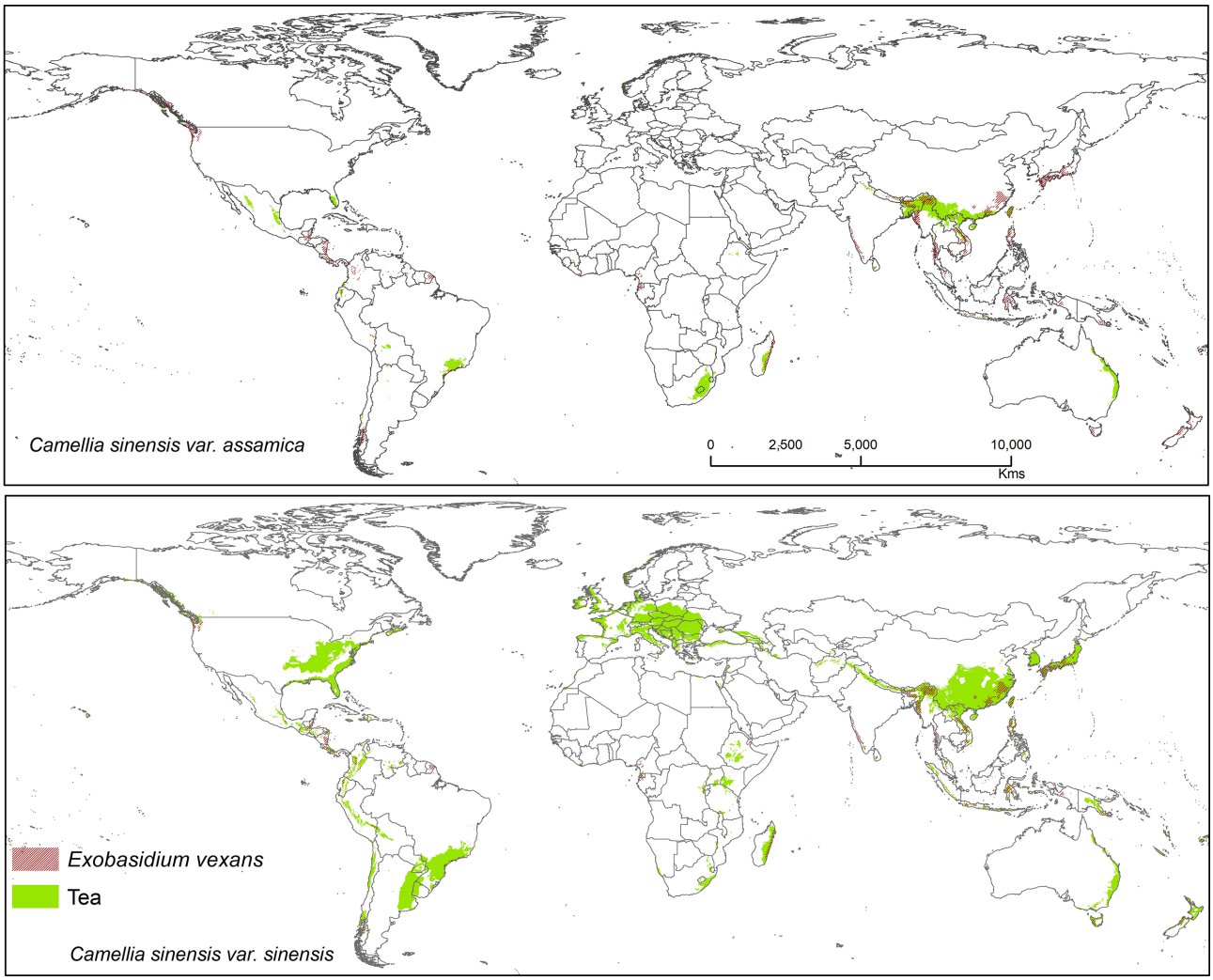

FIGURE 7 | Shows the suitable area of *Exobasidium vexans* on tea with suitable area of two varieties of tea as projected in the average of all future projections.

and it showed a decline from 7 to 18% (Ranjitkar et al., 2016a), also vegetation shifts, expansion of alpine vegetation zones (Brandt et al., 2013), and a decrease of *Abies georgei* forests under different emission scenarios between 4.6 and 25.9% in the elevational range can be seen (Wong et al., 2010). According to Orrock et al. (2020), recommendations to focus on tea research for development, such as test accessions, vary responses to the disease, applying the methods developed here to identify disease tolerant and future breeding efforts towards developing cultivars for tea production. Species distribution or niche models, such as Maxent, use the tolerance limit to critical factors based in the current species distribution and the climatic or other factors we input to the model. The predicted species distribution is a theoretical or the fundamental niche based on the input climatic factors, and there are other factors that might affect the actual distribution of species (Elith and Franklin, 2013; Seybold et al., 2020). In the current study, we use two approaches, the tea plant distribution based on climatic factors and the distribution of known pathogens in the same climatic scenario, which we believe is an advancement in the estimate of species distribution. However, there are other factors that affect the actual niche; for example, with the change of climatic conditions, the susceptibility of tea for the mentioned pathogen might also change (Toyoda et al., 2002). Modeling other factors, especially complex biotic factors, such as susceptibility to pathogens, chemical or mechanical herbivore defense, should be the concerns of future research, and the current result should be interpreted with caution.

In addition to change in suitability, temperature increases in baseline tea-plantation areas will favor rapid growth and the spread of fungal pathogens. We found that future temperatures will reach optimum levels for these selected pathogens in baseline tea-plantation areas. This also refers to plant natural microbiota interaction with new pathogens which underscores the need to

reduce the lag time between the appearance of new diseases and development of protective measures effective on a broad range of pathogens and host plants.

We are just considering 30 to 50 years of change, and within this time period genetic mutation in higher plants such as tea is not possible. However, plasticity can be considered. Due to phenotypic plasticity plants can adapt to a wide range of climates and survive in adverse conditions.

One major limiting factor of this study is the limited knowledge of tea–pathogen distributions; however, this does not imply that pathogens are absent from non-reporting regions. Climate factors other than temperature and humidity (extreme events, CO_2 fertilization, ozone, nitrogen deposition, and drought conditions) will also change, affecting tea and its interactions with pathogens. Stress-induced changes in tea from climate change have been explored by Han et al. (2018) *via* physiology, biochemistry, metabolism, and the economic and societal aspects of tea under changing climatic conditions. In addition, the identified fungi pathogens based on blast searches or only ITS sequences are unacceptable for species level because of the intraspecific variations when only one gene is used for phylogenetic analysis specially the genus *Colletotrichum* (Shi et al., 2018; He et al., 2020).

The effects of climate and pathogens on tea can be addressed in several ways. Adding shade tree species to tea plantations may mitigate damages from epidemic pests and diseases (Mukhopadhyay and Mondal, 2017). Increased knowledge of fungal pathogen genomes will facilitate the production of new fungicides (Cools and Hammond-Kosack, 2013). Understanding fungal communities or fungal pathogen–plant interactions by using next-generation sequencing, genomics, and metabolomics will help to uncover fungal infection mechanisms and plant defense mechanisms, which could be helpful in finding new targets for fungicide development and finding useful resistant genes for crop breeding (Pasiecznik et al., 2005; James et al., 2006; Peay et al., 2016; Chen et al., 2019b). Even though a number of studies have been conducted on identification of fungal pathogens on tea, only a few detailed studies have been done on fungal pathogen genomics on tea. Metagenomics have many advantages on pathogen evolution, host–pathogen interactions, determination of trait-specific genes and plant–host adaptation mechanisms (Li et al., 2016; Ren et al., 2019). Ultimately, understanding the roles of pathogens in crop production systems might even enhance food security (Gregory et al., 2009). Currently, tea plants are mostly protected from fungal diseases by fungicides, but these come with attendant harmful side effects on the tea plant, human health, the environment, and biodiversity. Fungi are distributed widely and are able to colonize and infect all plant tissues, as they have adapted many different evolutionary strategies, and several of them are able to switch modes from non-pathogenic to pathogenic (James et al., 2006; Möller and Stukenbrock, 2017). Finally, more attention is needed to understand the antagonism of endophytic fungi, such as that found in the genus *Diaporthe*, which is known as the most abundant genus of endophytes in tea plants and is associated with the symptomatic and asymptomatic tissues of *Camellia* spp. from several provinces in China (Chen, 2007; Osono and Hirose, 2009; Gao et al., 2016). Rising temperatures in tea plantations will increase their suitability for pathogens, which increase probability of pathogen dispersal and development, thus increasing the probability of future crop damage in 2050. For example, *Diaporthe theae* causes a common disease (canker disease), which creates serious problems in all tea-growing regions of the world (Ponmurugan et al., 2006). Under climate change, this species is able to affect tea leaves, leading to severe economic losses (Guarnaccia and Crous, 2017; Wang et al., 2017). Moreover, the genus *Pestalotiopsis* (including *Pestalotiopsis*-like species) needs to be considered as they also can cause several diseases on the foliage, stems, and roots of tea plant; of the *Pestalotiopsis*/*Pestalotiopsis*-like species, *Pestalotiopsis camelliae*, *Pseudopestalotiopsis theae*, and *Pseudopestalotiopsis camelliae-sinensis* are considered global tea pathogens (Joshi et al., 2009; Maharachchikumbura et al., 2011; Zhang et al., 2012; Maharachchikumbura et al., 2013; Liu et al., 2017; Chen et al., 2018) capable of causing substantial tea production losses. If there is greater variation in temperature (lower as well as higher), this disease is likely to become more serious.

This study provides an understanding of pathogens on tea, and these results will facilitate further research on the potential risks of fungal pathogens and how to prepare for risks in advance. Other perennial crops will also be challenged by new climates and pathogen interactions, also requiring additional study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

PM, SR, JX, YD, and JS made significant contributions to design the study. ST, SK, SR, and DS wrote the manuscript. SR provided the models and maps. KH, RJ, IM, and DB verified the methodology and modeling as well as providing valuable comments and suggestions. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.610567/full#supplementary-material

Supplementary Table 1 | Global perennial crop markets ($USD Billions per year).

Supplementary Table 2 | Fungal diseases reported from Camellia sinensis, confirmed by morphology, genomics and pathogenicity (the bold type shows pathogens used in this study).

Supplementary Table 3 | Modeled tea and its fungal pathogens overlapping suitability and pathogen free tea suitability

Supplementary Figure 1 | Temperature induced fungal pathogen growth with percent germination, appressorium and anthracnose disease. Temperature 25°C or above but below 30°C are better conditions for fungal growth, in that range, tea might be severely infected causing major economic loss (Kenny et al., 2012).

Supplementary Figure 2 | Temperature ranges for tea and its pathogens based on occurrence points.

Supplementary Figure 3 | Average annual temperature map of the world. Baseline tea plantations and selected pathogen occurrence areas.

Supplementary Figure 4 | Precipitation ranges for tea and its pathogens.

Supplementary Figure 5 | Annual relative humidity map of the world. Baseline tea plantations and selected pathogen occurrence areas.

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Dimorphism in *Neopseudocercosporella capsellae*, an Emerging Pathogen Causing White Leaf Spot Disease of Brassicas

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White leaf spot pathogen: *Neopseudocercosporella capsellae* causes significant damage to many economically important Brassicaceae crops, including oilseed rape through foliar, stem, and pod lesions under cool and wet conditions. A lack of information on critical aspects of the pathogen's life cycle limits the development of effective control measures. The presence of single-celled spores along with multi-celled conidia on cotyledons inoculated with multi-celled conidia suggested that the multi-celled conidia were able to form single-celled spores on the host surface. This study was designed to demonstrate *N. capsellae* morphological plasticity, which allows the shift between a yeast-like single-celled phase and the multi-celled hyphal phase. Separate experiments were designed to illustrate the pathogen's morphological transformation to single-celled yeast phase from multi-celled hyphae or multi-celled macroconidia *in-vitro* and *in-planta*. Results confirmed the ability of *N. capsellae* to switch between two morphologies (septate hyphae and single-celled yeast phase) on a range of artificial culture media (*in-vitro*) or *in-planta* on the host surface before infection occurs. The hyphae-to-yeast transformation occurred through the production of two morphologically distinguishable blastospore (blastoconidia) types (meso-blastospores and micro-blastospores), and arthrospores (arthroconidia).

Keywords: Arthroconidia, Blastoconidia, *Brassica*, dimorphism, morphological transformation, *N. capsellae*, white leaf spot

INTRODUCTION

Dimorphic fungi are capable of assuming two distinct morphologies during their life cycle, switching between a single-celled yeast form and a multi-celled hyphal form (Gauthier, 2015). Dimorphism is a highly synchronized, reversible response to an external stimulus posed by the environment (Lin et al., 2015). The majority of dimorphic fungi belong to the class Ascomycete or their 'Imperfect' relatives (Gauthier, 2015), and morphogenesis is widespread among pathogenic fungi of either mammals, plants, or insects (Gauthier, 2015). Well-known dimorphic

phytopathogenic fungi such *as Taphrina deformans*, the cause of peach leaf curl disease, and *Ophiostoma novo-ulmi*, the cause of Dutch elm disease, have had major adverse impacts on agriculture or the urban landscape, respectively (Gauthier, 2015).

In dimorphic fungi, yeast phase development occurs usually in response to a change in growth condition and/or nutritional status of the growth medium (Odds and Kerridge, 1985; Lin et al., 2015). Although diverse stimuli, including pheromones, plant lipids, plant hydrophobicity, pH, nitrogen, and quorum sensing can induce the transition of yeast-to-mycelium or *vice versa* in fungal morphogenesis in plant pathogenic fungi (Orlowski, 1994), several human fungal pathogens are solely thermally dimorphic, where temperature alone induces the morphological transformation (Orlowski, 1994; Gauthier, 2015). The search for internal factors that regulate morphogenesis or external factors that stimulate this dimorphic switch is a rapidly growing area of research, due in part to the availability of novel microscopy and molecular tools in the past decade (Gauthier, 2015). Such studies have revealed that pathogenicity, virulence, and lifecycle of dimorphic fungal pathogens are closely interrelated, and that pathogenicity may be limited to one of the alternative morphologies (Gauthier, 2015; Lin et al., 2015).

Neopseudocercosporella capsellae, the cause of white leaf spot disease, causes significant damage to many economically important Brassicaceae crops, including oilseed rape/canola, vegetable, condiment, and fodder species (Barbetti and Sivasithamparam, 1981; Petrie, 1985; Cerkauskas et al., 1998; Gunasinghe et al., 2020). The pathogen produces foliar, stem, and pod lesions under favourable weather conditions (Petrie and Vanterpool, 1978) causing yield losses in the range of 24-30% in oilseed brassicas (Penaud, 1987; Barbetti and Khangura, 2000; Murtza et al., 2021). While the pathogen distribution is worldwide, it is most destructive in regions with cool and wet climates, such as occur in France (Perron and Nourani, 1990; Penaud and Walker, 2015), the United Kingdom (Inman et al., 1993), Canada (Petrie and Vanterpool, 1978), the United States of America (Ocamb et al., 2015) and Australia (Gunasinghe et al., 2016d; Van de Wouw et al., 2016; Gunasinghe et al., 2017; Murtza et al., 2018). Over recent decades, there has been an increase in white leaf spot disease in some countries, such as the United Kingdom following wetter/warmer winters in the 1990s (Inman et al., 1993).

Despite being a common *Brassica* pathogen, important knowledge gaps exist in the *N. capsellae-Brassica* pathosystem (Gunasinghe et al., 2020), particularly on critical aspects of its epidemiology. This is in part from its very slow growth rate on artificial media (Crossan, 1954) and its secretion of cercosporin, a non-host specific, photo-activated toxin into both artificial media and during the early infection process in host tissues (Gunasinghe et al., 2016b). Air-dispersed ascospores produced by the teleomorph are responsible for the disease initiation and long-distance pathogen dispersal in the United Kingdom (Inman et al., 1992). However, elsewhere, most *N. capsellae* populations are anamorphic and thought to be clonal, where aspects of *N. capsellae* pathogenicity, disease initiation, spread, and epidemic development are not fully described and/or understood (Gunasinghe et al., 2020). For example, on rapeseed cotyledons, Australian isolates of *N. capsellae* can be observed as hyphae, as large multi-celled conidia, and what appeared to be single-celled spores that are able to reproduce by budding (Gunasinghe unpub). This suggests that a morphologic shift between the yeast phase and the hyphal form likely occurs for *N. capsellae*. Its relevance is that morphologic shift between the yeast phase and the hyphal form in other pathogenic fungi is essential for maintaining pathogenicity (Orlowski, 1994). Further, Lin et al. (2015) noted, a thorough understanding of the life cycle of dimorphic pathogens is required if they are to be effectively controlled. Hence, studies were undertaken to determine if *N. capsellae* is dimorphic and able to switch between a single-celled yeast form and a multi-celled hyphal form *in-vitro* or *in-planta* on host leaves.

METHODS

Pathogen Isolates

Three single-spored isolates of *N. capsellae* (UWA Wln-10, UWA Wlj-3, and UWA Wlra-7), stored as lyophilized ampoules containing the living pathogen preserved on vacuum dried agar cultures (Bazzigher and Kanzler, 1985) at room temperature, were obtained from the University of Western Australia (UWA) culture collection. All three isolates were collected within Western Australia. UWA Wln-10 was from infected leaves of *B. napus* at Bindoon North, UWA Wlj-3 was from diseased *B. juncea* leaves at Shenton Park, and UWA Wlra-7 was isolated from infected *Raphanus raphanistrum* leaves at West Calingiri (Gunasinghe et al., 2016d). Pathogen isolates were revived on Potato Dextrose Agar (PDA - Difco) or Malt Yeast Extract (MEA) plates and then working cultures were maintained as MEA media slants at 4°C. For *in-vitro* studies, two isolates of genetically different groups (UWA Wlj-3, and UWA Wlra-7) (Gunasinghe et al., 2016a) were used. When multi-celled conidia were needed, susceptible host plants grown in the field were spray inoculated with a mixture of hyphal fragments of all three isolates, and conidia produced on host plants lesions were collected (Gunasinghe et al., 2014).

Preparation of Mycelial Suspension and Multi-Celled Conidial Suspension for *In-Vitro* and In-Planta Studies

To prepare a mycelial suspension, each of the three *N. capsellae* isolates (UWA Wln-10, UWA Wlj-3, and UWA Wlra-7) was sub-cultured onto MEA in Petri Plates and grown for three weeks at 20°C. Erlenmeyer flasks (250 mL) containing 150 mL of Malt Extract Broth (MEB) were inoculated with agar plugs from the growing edges of each *N. capsellae* culture separately and incubated on a rotary platform shaker (Innova™ 2100, New Brunswick Scientific) at 150 rpm and 20°C. After 3-4 weeks, cultures of all three isolates with abundant mycelial growth were mixed in equal volumes and blended for 5 min (Kambrook Mega Blender, Breville Group Ltd). Mycelial mats in the mixture after

blending were removed by filtering through sterile gauze. Concentration of the filtered suspension of mycelial fragments containing 90 µm (± 10 µm) length was adjusted to 4 x 10^6 mL^{-1} using a hemocytometer and sterile distilled water (Gunasinghe et al., 2014).

To produce multi-celled conidia, field-grown plants were inoculated with mycelial inoculum. Seeds of highly susceptible, *B. juncea* genotypes Rohini, Vardan, or Prakash (Gunasinghe et al., 2014) were sown (6 seeds per pot) in sequential batches of 9 pots each, seedlings were grown in a controlled environment room (15°C, 12h photoperiod and Photosynthetic Photon Flux Density of 580µmolm^{-2} s^{-1}) for 15 to 20 days before transferring into an experimental field plot (1 x 0.5m) at the University of Western Australia, Crawley, Western Australia, where they were fertilized weekly with Thrive® (DuluxGroup (Australia) Pty Ltd), a complete nutrient solution dissolved in water. Beginning at approximately four weeks of age, field plants were spray-inoculated weekly with a mixture of mycelial fragments (4 x 10^6 fragments mL^{-1}) prepared as described above for three consecutive weeks using a handheld and operated aerosol sprayer in the late afternoon to maximize the period of high humidity to favour infections.

After 20 to 25 days post-inoculation, when disease symptoms became apparent, leaves with typical white leaf spot symptoms were collected. Leaves with lesions were gently washed with sterile deionized water (DI) before, lesions were excised and placed into glass vials containing 20-30mL of sterile DI. Multi-celled conidia were released by vigorous handshaking for four min. The pieces of the leaf lesions were removed from the glass vials with a sterile pair of forceps and the mixture was centrifuged to pellet the conidia. The supernatant was discarded and the conidial pellet was washed twice in sterile DI water and re-suspended in 2 to 3mL of sterile DI water. Multi-celled conidial concentration (4 x $10^6$$mL^{-1}$) was achieved by making adjustments based on hemocytometer counts (Gunasinghe et al., 2016c).

Dimorphism *In-Vitro*

In-*Vitro* Blastospore Formation From *N. capsellae* Hyphae and Observations of Colony Morphology During Morphological Transformation

Morphological transformation of *N. capsellae* was examined by inoculating UWA Wlj-3, and UWA Wlra-7 on 5 different media: PDA (Difco), MEA (20g, peptone: 6g, dextrose: 20g, agar: 15g, de-ionized water 1L), V-8 juice agar (clarified V-8 juice: 200mL, $CaC0_3$: 3g, agar 15g, de-ionized water 1L), Neutral Dox Yeast Agar (NDYA) ($NaNO_3$: 2g, KH_2PO_4; 1g, $MgSO_4$: 0.5g, KCL: 0.5g, $FeSO_4$ 0.1% sol: 10mL, yeast extract: 0.5g, sucrose: 30g, agar: 14g, de-ionised water: 1L), Yeast Extract Peptone Dextrose Agar (YEDPA) (Yeast extract: 10g, peptone: 10g, dextrose: 20g, agar 15g, de-ionised water 1L adjusted to pH=6) or Water Agar (WA) (agar:15g, de-ionised water 1L) using two inoculation methods (streak plate method and agar plug method). A four week old pure mycelial colony of each isolate on NDY was used for all inoculations.

For the agar plug method, a mycelial plug (5mm diameter) from the growing edge of the mycelial colony was placed in the middle of a Petri Plate containing PDA, MEA, V-8 juice agar, NDYA, YEDPA, or WA. For the streak plate method, a loop full of mycelial fragments was taken from the growing edge of the colony and dragged over the surface of the plate in a 'zig-zag' formation. There were six single plate replicates for each medium for each inoculation method. Three plates from each media inoculated with each individual inoculation method were incubated at 23°C, and the other three at 37°C, in the dark for a maximum of 4 weeks.

Stereo microscopy, light microscopy (LM), and scanning electron microscopy (SEM) techniques were used to document colony and cell morphologies. Colony morphologies of *N. capsellae* on different media incubated at the two temperatures were recorded every other day by examining individual plates. Colonies on plates were further prepared to examine with a Nikon- SMZ1500 stereomicroscope, a Nikon Eclipse Ni-U light microscope, or SEM where necessary.

LM: Cells/hyphae collected by scraping the surface of colonies were mounted on microscope slides and stained with 1% cotton blue in lactophenol. Slides were studied and photographed (1000x magnification) with a Nikon Eclipse Ni-U light microscope. SEM: Selected colonies were prepared for SEM for further analysis. Thin agar blocks (2-3 cm) with colonies were cut carefully from the culture plate with a sterile scalpel and placed on a pin mount with a carbon tab (ProSci Tech) on it or in cryovials (2mL). The samples on pin mounts were air-dried in a laminar flow cabinet for five days. Cryotubes with samples were frozen at -80°C (Revco ULT390-5-D, Thermo Scientific) for 24h and then freeze-dried (FD-8 Witeg Germany) for 24h. Dried samples were carefully placed on standard pin mounts with a carbon tab on top. Finally, all samples were sputter-coated with Mini Plasma Sputter Coater (gold: 0.1mm) and visualized and imaged using a Jeol JCM-6000 Versatile Benchtop SEM.

In-Vitro Blastospore Formation From *N. capsellae* Multi-Celled Conidia

Two drops (20µl) from multi-celled conidial suspension prepared as described before in the previous section on preparation of multi-celled conidial suspension, were placed on 10 standard glass microscope slides and incubated in a moist chamber. After a 24h incubation period, 1% cotton blue in lactophenol was added to each drop, before examining under an Olympus BX5 microscope. From 5 random slides, a total of 50 fields (10 from each) were imaged with an Olympus DP71 digital photographic system.

Morphological Analysis of *In-Vitro* Formed Blastospores (Single Spore/Cell) Types

Two measurements (length and width) were taken from more than 400 spores using the software ImageJ 1.53a (Schneider et al., 2012) from digital images taken of two different single-celled blastospore types (micro-blastospores and meso-blastospores) produced from *N. capsellae* hyphae in pure cultures or multi-celled macroconidia suspended in sterile distilled water. All the measurements for smaller bacteria-like spores (micro-blastospores) were taken from SEM images, and the larger

yeast-like spore (meso-blastospore) measurements were taken from a combination of both SEM and LM images.

Dimorphism *In-Planta*

Host Genotypes

Two genotypes from each of three major oilseed species were selected for *in-planta* studies. The six susceptible or resistant genotypes used were *B. carinata* (ATC94129P, highly resistant and UWA#012, very susceptible), *B. napus* (Hyola 42, resistant and Trilogy, susceptible), and *B. juncea* (Dune, moderately resistant and Vardan, susceptible) (Gunasinghe et al., 2014).

In-Planta Blastospore Formation From *N. capsellae* Hyphae

Seeds of each genotype were sown in 48 cell seedling trays (30 x 34 cm), four seeds per cell and four replicate cells per genotype. Ten days after sowing, plants were thinned to two per cell. Seedlings were grown in a controlled environment room (15°C, 12h photoperiod, and Photosynthetic Photon Flux Density of 580 µmol m^{-2} s^{-1}). Ten-day-old cotyledons of each seedling were inoculated by depositing a single drop (10µl) of the mycelial suspension (4 x 10^6 mL^{-1}), prepared as described earlier in the previous section on preparing of mycelial suspension, on each cotyledon lobe. The control was an identical tray of seedlings under the same conditions but inoculated with sterile distilled water. All plants were placed in clear polyethylene bags for 48h to maintain high humidity to promote infection (Brun and Tribodet, 1991). Five cotyledons for each genotype, (4 inoculated and 1 control from each of the 5 cells) were randomly sampled for LM or SEM 48h post-inoculation.

In-Planta Blastospore Formation From *N. capsellae* Multi-Celled Conidia

Collection of multi-celled macroconidia from diseased leaves and preparation of macroconidial suspension in sterile distilled water (4 x 10^6 mL^{-1}) was done as described in the previous section on preparation of muiti-celled conidia. An experiment identical to mycelial inoculation studies was conducted, but by inoculating cotyledons with a 10µm multi-celled conidial suspension rather than mycelial suspension. After 48h post-incubation, two sets of five cotyledons (four inoculated and one control) were collected separately for LM and SEM.

Sample Preparation for Microscopy and Morphological Analysis of Blastospores (Single Spore/Cell Types) Produced *In-Planta*

For LM studies, collected cotyledons were decolourized with acetic acid: ethanol: water (2:2:1) solution and stained with 1% cotton blue in lactophenol for 40 sec to prepare whole wet mounts (Gunasinghe et al., 2016c). Four inoculated and one uninoculated cotyledons were examined using an Olympus (BX51) microscope, where 25 random images from each inoculated site (50 from a cotyledon and a total of 50 x 4 = 200 for each genotype) were captured using an Olympus DP71 digital photographic system.

For SEM studies, cotyledon samples were fixed in 2.5% glutaraldehyde and cut into 4 x 4 mm pieces and transferred into glass vials with fresh 2.5% glutaraldehyde. Samples were then prepared for SEM using a BioWave microwave processor fitted with a PELCO coldspot (Gunasinghe et al., 2016c). Prepared samples were mounted on standard aluminium pin mounts with carbon tabs (ProSciTech), sputter-coated with 5 nm carbon, 3 nm platinum, and surface imaged with a field emission SEM (Zeiss Supra 55 VP). Twenty-five random images were taken from each of the inoculated areas (50 from a cotyledon and a total of 50x4 = 200 for each genotype).

Different blastospore types formed from *N. capsellae* multi-celled macroconidia or hyphal fragments were measured using the software ImageJ 1.53a. Two measurements from more than 400 micro-blastospores (bacteria-like single-celled spores) from SEM images and meso-blastospores (Yeast-like single-celled spores) from a combination of both SEM and LM images were taken.

Molecular Identification

Confirmation of the identity of the two observed morphologically different phases (Hyphae and single-celled blastospores) was undertaken by sequencing the internal transcribed spacer region (ITS1, 5.8S, and ITS4) of the rDNA operon, and comparing sequence data with available sequence information data for *N. capsellae* isolates in GenBank. Areal hyphae obtained from a pure mycelial colony and single-celled blastospores collected from a young bacterial-like colony consisted only of blastospores have been used to extract DNA. To obtain the hyphal phase, five Petri Plates containing 15mL NDY were inoculated with a mycelial plug taken from a three-week-old culture of the isolate UWA Wlj-3 on MEA plates at the mycelial phase. After 20 to 30 days of incubation at 23°C, plates were examined to select a plate with mycelial growth and no blastospores. Aerial mycelium (hyphae) from selected NDY cultures was harvested and placed in a sterile Eppendorf tube. Streak plates of YEPD (in duplicate) from the same isolate: UWA Wlj-3 were prepared from the same mycelial colony on MEA to obtain yeast phase (single cells). YEDP was used to induce the unicellular phase of the pathogen (Zhu et al., 2017). After two days of incubation at 23°C in dark, cells from the growing yeast-like colonies were aseptically transferred into a sterile Eppendorf tube.

Extraction of Genomic DNA, PCR, and Sequencing

Genomic DNA was extracted using the ISOLATE II Genomic DNA Kit (Bioline (Aust) Pty Ltd) with some modifications to the standard protocol. All samples with mycelia or single cells (yeast phase) were washed with 400µl of TE buffer in an Eppendorf tube, pelleted by centrifugation (13,000rpm, 5min) in a microfuge® 18 (Beckman Coulter ™) and the supernatant was discarded. Lysis buffer (200µl) and two metal beads were added to each of the sample and homogenized with the tissue homogenizer (FastPrep-24TM 5G Homogenizer – MP Biomedicals) for 6 seconds. Genomic DNA extraction in the homogenized samples was carried out using the ISOLATE II Genomic DNA Kit according to manufacturer instructions. All DNA samples were stored at 4°C after evaluating the quality and

concentration of DNA extracted using a DeNovix: DS-11 FX Spectrophotometer/Fluorometer.

Part of the nuclear rRNA operon was amplified with primers ITS1 (5'TTT CCG TAG GTG AAC CTG C3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC3') using the polymerase chain reaction (PCR) (Gunasinghe et al., 2016a). Spacer I, between the 18S and 5.8S rRNA gene, was amplified using the 5' primer ITS1, and spacer II, between 5.8S and 28S of the rRNA gene, was amplified with the 3' primer ITS4. PCR reactions were carried out with 50ng genomic DNA in MyTaq Red mix (Bioline (Aust) Pty Ltd). PCR conditions were: initial denaturation step at 94°C for 2 minutes, followed by 35 cycles of 1 minute at 94°C, annealing at 55°C and extension for 1.5 minutes at 72°C and a final elongation step of 72°C for 2 min (Gunasinghe et al., 2016a). PCR products were separated and visualized by gel electrophoresis at 84V for 45 minutes on a 1% (w/v) agarose gel and products of 500bp (20µl of each) were sequenced by Macrogen Inc. (Korea).

RESULTS AND DISCUSSION

Observations confirmed the morphogenic plasticity of *N. capsellae*. The species changed its morphology from septate hyphal growth to budding yeast form or *vice-versa* depending on external conditions. The morphological transformation (hyphae-to-yeast) of *N. capsellae* resulted in two common single-celled blastospore (blastoconidia) types distinguished based on gross appearance: The first, predominantly elliptical and comparatively large single-celled meso-blastospores that resembled yeast cells where average dimensions were 4.8 x 2.7µm (**Table 1** and **Figure 1A**) and the second, single-celled micro-blastospores (average dimensions of 1.9 X 0.9 µm) that resembled rod-shaped bacterial cells (**Table 1** and **Figure 1B**) and comparatively large, spherical to cylindrical arthrospores (arthroconidia) (**Figure 1C**). The formation of blastospores (Odds and Kerridge, 1985; Gauthier, 2015; Lin et al., 2015) and arthrospores (Barrera and Szaniszlo, 1985) are important aspects of fungal morphogenesis in many dimorphic or polymorphic fungal species, including Ascomycetes. Blastospores are asexually produced single-celled spores that can increase in number rapidly through repeated budding (Odds and; Kerridge, 1985; Mehrabi et al., 2006; Lin et al., 2015), while arthrospores are formed by segmentation and subsequent fragmentation of existing hyphae (Barrera and Szaniszlo, 1985).

This morphological transition in *N. capsellae* has not been previously recognized. To eliminate the potential for contamination being the source of the newly described structures, the identity of the structures was confirmed by PCR amplification and sequencing of the ITS1 and ITS4 regions of nuclear ribosomal DNA of blastospores taken from a yeast-like colony (GenBank Acc. No. MZ149251) and hyphae from a pure mycelial colony (GenBank Acc. No. MW898135). The BLAST results for both samples were 99% match to *N. capsellae*.

In-Vitro Morphogenesis

Pure Cultures on Growth Media

The formation of single-celled spores (blastospores and arthrospores) produced prominent changes in colony morphology (**Figures 2** and **3**). Morphological transformation from hyphae-to-yeast or yeast-to-hyphae was observed on all 5 tested media, although some different media favoured the formation of yeast and the other the formation of mycelial phase. Transferring hyphae to a fresh medium (subculturing) initiate yeast-like colonies on some media demonstrating hyphae-to-yeast transformation. Generally, on commonly used media like PDA or NDY, and WA, the mycelial phase dominated with an infrequent, short, initial yeast-like phase immediately after subculturing. When yeast-like growth was observed initially (**Figures 2A, B**), yeast-to-hyphae transition was initiated in colonies anytime within two-three weeks (**Figures 2C, D**). The hyphae-to-yeast transformation was also observed in mycelial colonies older than one month (**Figure 3**). At the initial growth stage on some media such as NDY, PDA, and WA, the yeast phase was present but visible only under the microscope and the proportion of yeast to mycelia and the time taken for the yeast-to-hyphae transformation differed across the media tested.

Subculturing and the method of inoculation influenced the morphological transformation of the fungus. Placing *N. capsellae* hyphal fragments onto a new growth medium promoted the initial formation of creamy white-to-tan-coloured colonies dominated by single-celled blastospores (**Figures 2A, B**). Similar growth behaviour on agar media by dimorphic pathogens has been observed. Initially formed yeast-like colonies of *Candida citri* switched to mycelial growth after prolonged incubation (Sipiczki, 2011). Odds and Kerridge (1985) claim that the *C. albicans* blastospore-dominating yeast phase was easy to obtain and maintain on artificial media, while the pure mycelial phase was difficult to attain as it is only a transient form.

Of the two inoculation methods tested, streaking favoured the formation of the yeast phase. Hyphal growth with or without initial yeast-like growth was observable on PDA, NDY, and V8 juice agar when inoculated with an agar plug (**Figures 4A, B, F**).

TABLE 1 | Spore dimensions (µm) of two types of blastospores: meso-blastospores and micro-blastospores.

	Meso blastospore *in-planta*	Meso blastospore *in-vitro*	Micro blastospore *in-planta*	Micro blastospore *in-vitro*
Average Width	2.9 ± 0.82	2.7 ± 0.64	0.65 ± 0.15	0.9 ± 0.34
Range	0.9-6.6	1.0-4.9	0.2-1.0	0.3-2.0
Average Length	3.4 ± 0.95	4.8 ± 1.24	1.1 ± 0.13	1.9 ± 0.44
Range	0.9-7.1	2.3-9.2	0.3-2.5	0.7-3.4

Values are the mean of more than 400 cells from each morphotype measured in-vitro *and in-planta.*

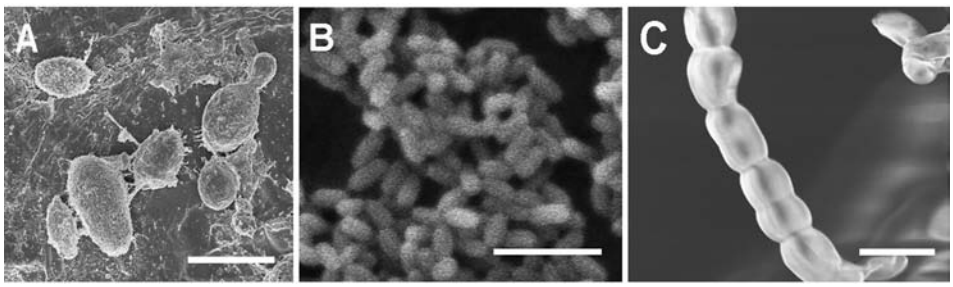

FIGURE 1 | Scanning electron micrographs of single-celled spore types with different morphologies produced by *Neopseudocercosporella capsellae* during morphological transformation from hyphae-to-yeast. **(A)** Unicellular meso-blastospores. **(B)** Unicellular micro-blastospores. **(C)** Arthrospore chain. Scale bars = 5μm.

Except for WA, yeast-like colonies appeared on streak plates of all media within 24h of inoculation (**Figures 4C, D**). On WA, yeast-like colonies, hyphal growth, or both were observed during the initial growth stage depending on the inoculation method used (**Figure 4E**). The appearance of initial yeast-like colonies immediately after subculturing was fast, within overnight incubation (**Figures 4C, D**), while it took at least 10-14 days for hyphal growth to occur when the initial yeast growth was absent (**Figures 4E, F**).

The co-occurrence of cells with different morphologies varying from single-celled blastospore types to true hyphae in a single colony was observed in *N. capsellae* colonies (**Figure 5**). However, the dominating morphotype and the architecture of the colonies seem to depend on several factors, such as isolate,

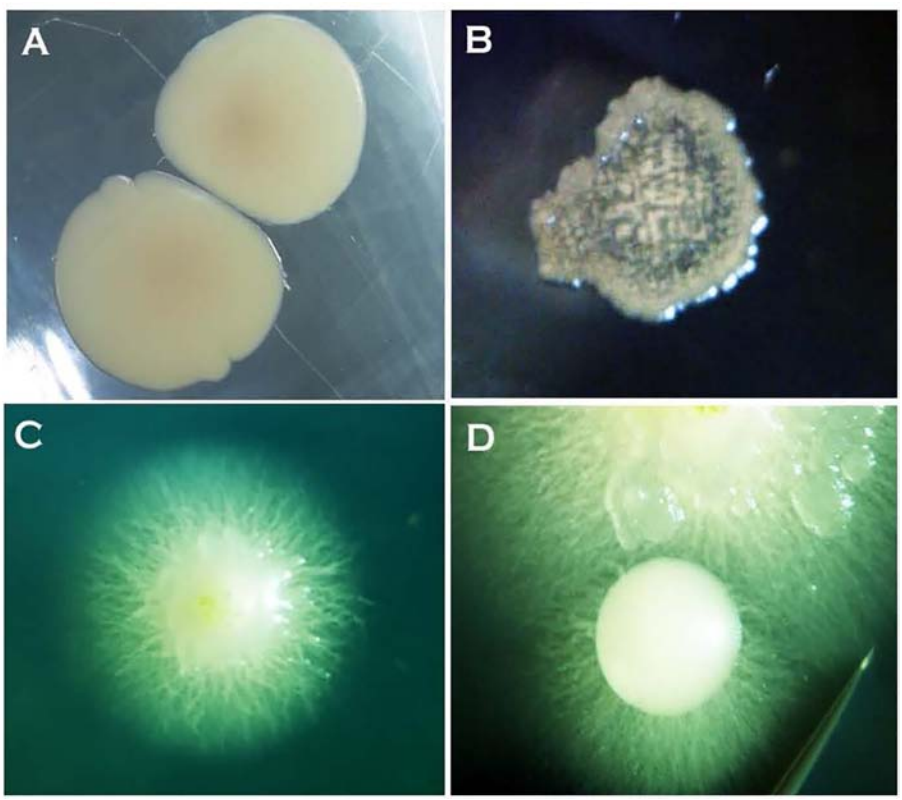

FIGURE 2 | Two-day-old bacteria-like colonies of *Neopseudocercosporella capsellae* consisting of single-celled blastopores appeared after streak-inoculation using hyphal fragments from a pure hyphal colony. **(A)** Isolate UWA Wlj-3 on NDY and **(B)** isolate UWA Wlra-7 on PDA. **(C)** and **(D)** Ten-day-old colonies of isolate UWA Wlj-3 on PDA where hyphae initiation is evident at the periphery at the transition stage from a bacterial-like initial stage.

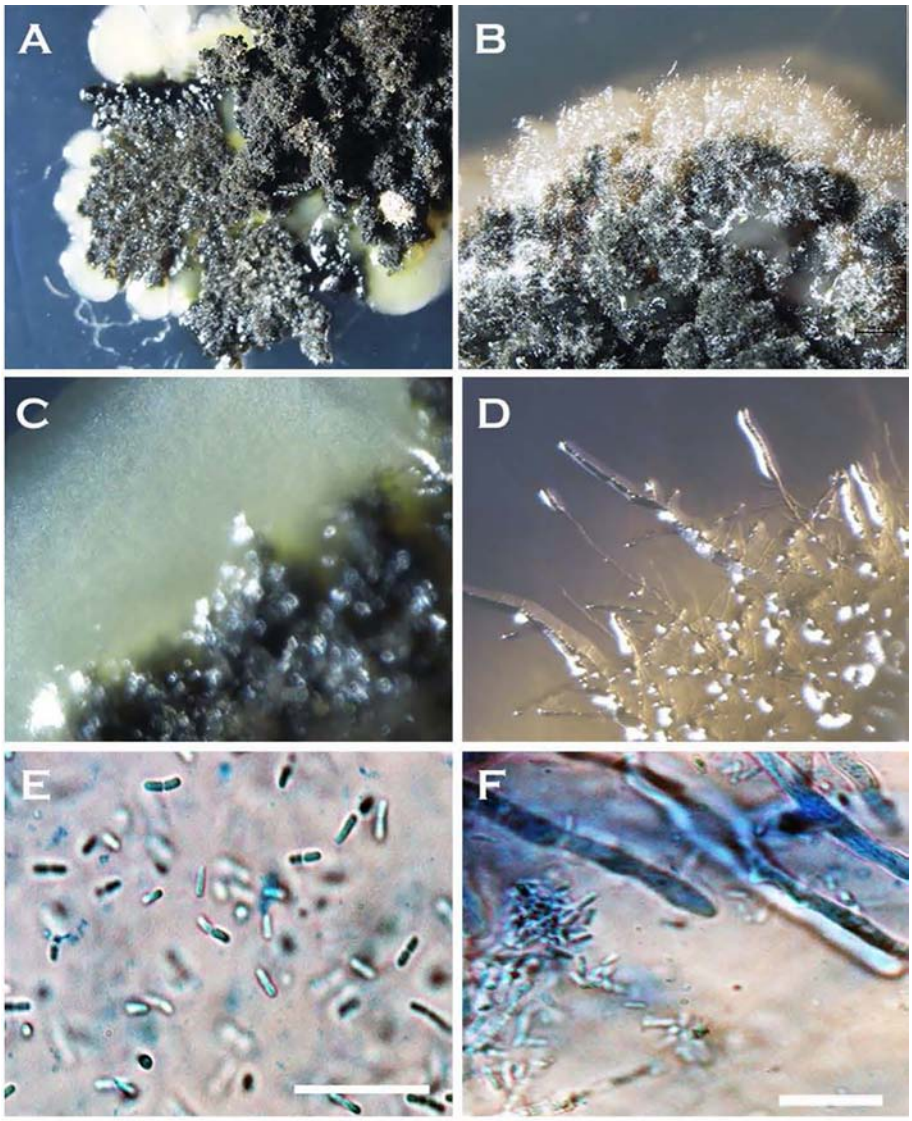

FIGURE 3 | Morphological transformation (hyphae-to-yeast) of *Neopseudocercosporella capsellae* cultures on growth media where blastospores are seen as creamy white to tan masses at the edges of colonies **(A–D)**. Colonies at the time of hyphae to yeast transformation, **(A)** seven week old colony of isolate UWA Wlj-3 on NDY, and **(B)** five week old colony of isolate UWA Wlra-7 on PDA. **(C)** Edge of the UWA Wlj-3 colony, and **(D)** edge of UWA Wlra-7 colony, where hyphae-to-yeast transformation occurred. **(E)** Blastospores from UWA Wlj-3 colony and **(F)** hyphae from UWA Wlra-7 colony, both stained with 1% cotton blue in lactophenol and as seen under the light microscope. Scale bar = 5μm.

method of inoculation, and type of the growth medium and changed over time with the age of the colony (**Figures 2** and **3**). The formation of mixed colonies with different cell morphologies has been reported in other dimorphic pathogens such as *Cryptococcus* (Levy et al., 2012; Lin et al., 2015) and *Candida* sp. (Odds and Kerridge, 1985; Sipiczki, 2011). *Cryptococcus* produces colonies with different morphologies, where yeast cells (blastospores) predominate in the colony centre and hyphae dominate at the periphery (Lin et al., 2015). Obtaining a colony consist of single-cell morphology was a difficult task for *C. albicans* on artificial culture media because of the co-existence of blastospores, hyphae, and pseudohyphae (Odds and Kerridge, 1985). Such colonies, the architecture of the colony, degree of morphological heterogeneity, and the proportion of each cell morphology of otherwise genetically identical cells, are a result of differential gene expression among cells (Levy et al., 2012) as influenced by one or more of several factors, such as gradient of signalling molecules created by the varying microenvironments across the growth medium and/or factors like the age or cell-cycle stage (Lin et al., 2015).

Apart from much larger colonies at 37°C, the same growth patterns were observed at both temperatures (23°C and 37°C).

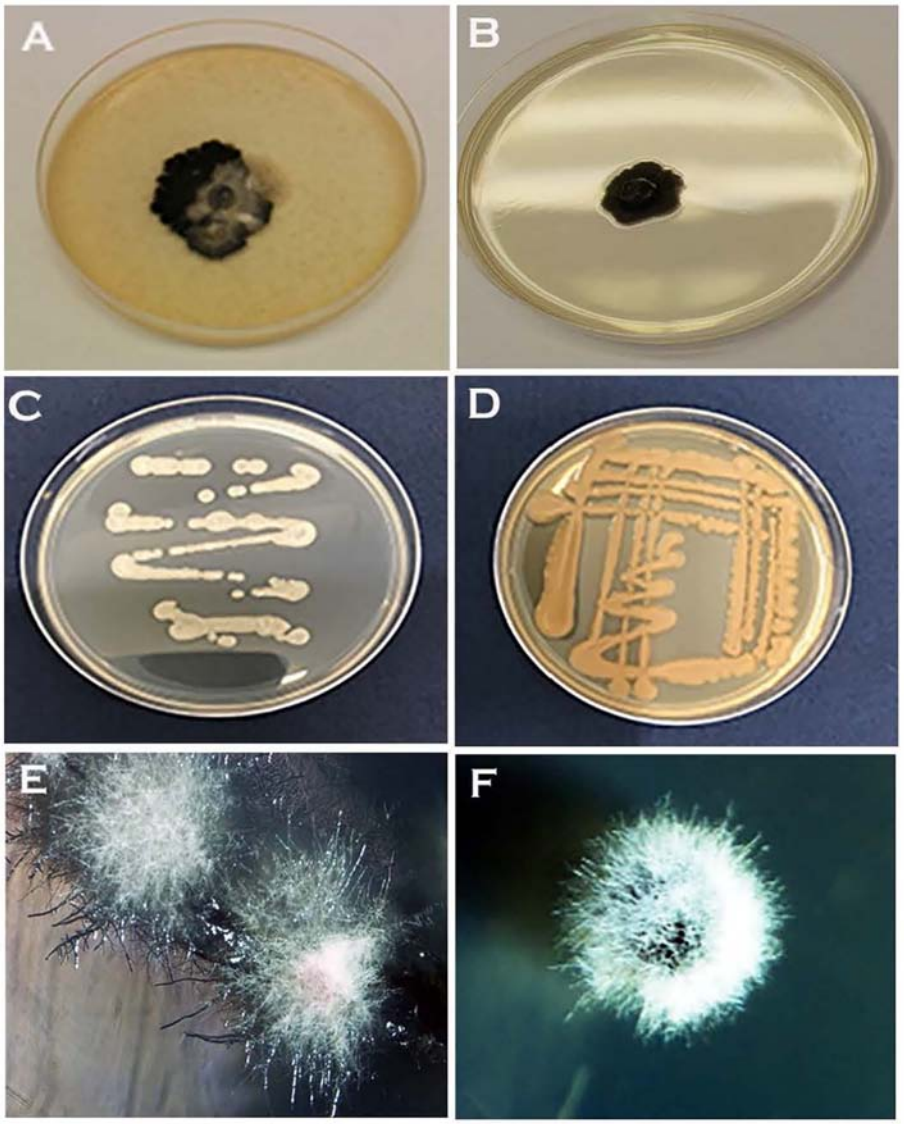

FIGURE 4 | Different colony morphologies of *Neopseudocercosporella capsellae* isolate UWA Wlj-3 grown on three different growth media, and with two different subculturing methods using a hyphal colony on a single growth medium. **(A)** Seven week old colonies on V8 juice agar and **(B)** PDA, inoculated with an agar plug. Four-day-old streak plate on **(C)** PDA and **(D)** V8 juice agar. **(E)** Four-week-old hyphal colonies on WA streak plate. **(F)** Three-week-old hyphal colony on NDY inoculated with an agar plug.

Therefore, these *in-vitro* studies at two temperatures suggest that *N. capsellae* morphogenesis is not induced solely by temperature as seen in thermally dimorphic human fungal pathogens (Dukik et al., 2017), but by more complex external and internal factors, including the growth medium.

Multi-Celled Macroconidia in Sterile Distilled Water

Multi-celled macroconidia produced both micro-blastospores and meso-blastospores or arthrospores *in-vitro* within a 24h incubation period. All three spore types were stained with cotton blue and observable under LM among the multi-celled macroconidia when suspended in sterile distilled water (**Figure 6**). However, not all multi-celled conidia produced single-celled blastospores, as some germinated by producing single or multiple germ tubes.

In-Planta Morphogenesis

Multi-celled macroconidia or hyphal fragments newly introduced onto host tissue (cotyledons) formed single-celled blastospores or arthrospores displaying the same trend of morphological transition that occurred *in-vitro*. All single-celled spore types similar to the spore types produced *in-vitro* (meso and micro-blastospores and arthrospores) were evident on cotyledons inoculated with multi-celled macroconidia

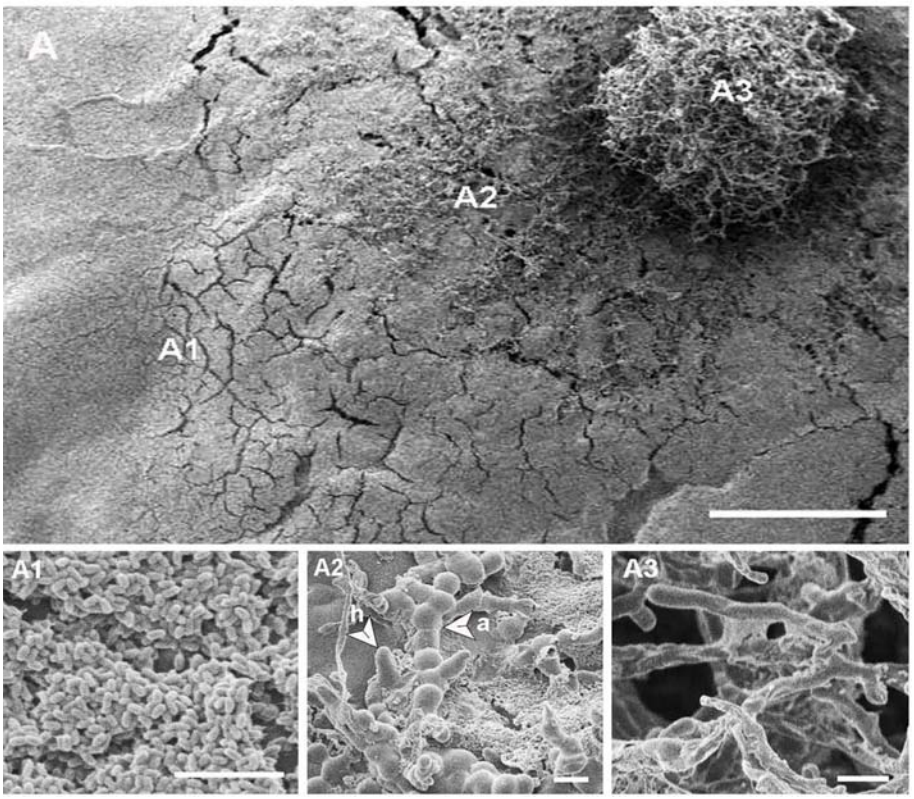

FIGURE 5 | **(A)** Scanning electron micrograph of a three-week-old heterogeneous *Neopseudocercosporella capsellae* isolate UWA Wlj-3 colony on PDA, showing cell types with different morphologies at different places in the culture, scale bar = 500µm. **(A1)** Blastospores (yeast cells) dominating at the periphery of the colony. **(A2)** Middle area of colony showing a mixture of blastospores, arthrospores (a) and hyphae (h). **(A3)** Centre of the colony dominated by true hyphae. Scale bars = 10µm.

suspensions (**Table 1** and **Figures 7A, B, E, F**) or mycelial fragments (**Figures 7C, D**) within 48h post-inoculation and this was consistent across all six test genotypes belonging to three different host species. However, germinated multi-celled macroconidia with single or multiple germ tubes were also common. Initial observations were that the amount of germinating multi-celled macroconidia or each morphologically different single-celled blastospore type could vary depending on the host, such as susceptible and resistant host species. After landing on the host tissue, the formation of a single-celled blastospore (yeast phase) on the host surface just before invasion has been observed for several dimorphic plant pathogens (Ingold, 1995; Ruiz-Herrera et al., 1995; Vollmeister et al., 2012; Cissé et al., 2013). Asexually produced diploid, multi-celled pycnidiospores of *Zymoseptoria tritici*, a dimorphic wheat pathogen causing Septoria tritici blotch (Palmer and Skinner, 2002; Mehrabi et al., 2006) or teliospores of *Ustilago maydis*, a maize pathogen causing smut disease (Ruiz-Herrera et al., 1995; Vollmeister et al., 2012), establish a short yeast-like phase on the leaf surface by producing blastospores before host invasion in the filamentous form. In *Taphrina deformans*, the cause of leaf curl disease of peach and nectarine, ascospores discharged from curled leaves produce single-celled blastospores, that overwinter and then switch back to the infectious filamentous form to invade the host (Rodrigues and Fonseca, 2003; Tavares et al., 2004; Cissé et al., 2013). This morphological transition from multi-celled conidia or hyphae-to-yeast and yeast-to-hyphae plays unique species-specific roles in virulence (Mehrabi et al., 2006; Gauthier, 2015; Lin et al., 2015). For instance, yeast cells of *T. deformans* function as an overwintering dormant spore (Cissé et al., 2013), while dissemination is the key role of yeast cells produced by *Ophiostoma ulmi* (Lin et al., 2015).

Blastospore (Blastoconidia) Formation

In *N. capsellae*, blastospores are produced during hyphae-to-yeast transition from multi septate hyphae (**Figure 3**) or by asexually produced multi-celled macroconidia (**Figures 6** and **7**). Although there can be a variety of different morphologies observed, the formation of blastospores involves three steps of bud emergence, bud growth, and spore/conidia separation. The sites at which blastospores are produced during hyphae-to-yeast transformation could be limited and for *C. albicans* these hyphal sites are adjacent and posterior to septa (Odds and Kerridge, 1985). Similarly, in *N. capsellae* yeast-like meso-blastospore formation occurred at the septa of hyphae (**Figures 8A, B**) or

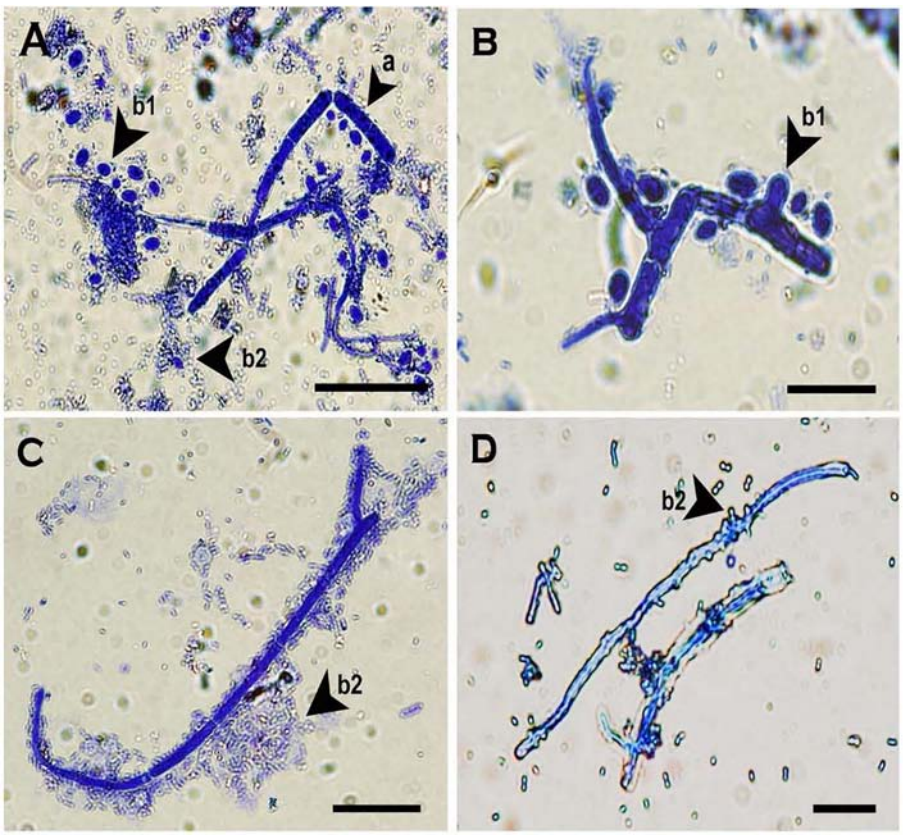

FIGURE 6 | Two morphologies of blastospores and arthrospores formed from *Neopseudocercosporella capsellae* multi-celled macroconidia suspended in sterile distilled water 24h post-inoculation. **(A)** Multi-celled macroconidia with meso-blastopores (b1), micro-blastospores (b2) and an arthrospore (a). **(B)** Multi-celled macroconidium producing meso-blastospores (b1) (arrow shows a meso-blastospore bud raised adjacent to the septa). **(C)** Multi-celled macroconidium with micro- blastospores (b2). **(D)** Micro-blastospore (b2) formed from multi-celled macroconidia (arrow shows buds of micro-blastospores). Scale bars **(A, C)** 20μm; **(B, D)** 10μm.

the tip (**Figure 8B**). However, micro-blastospore formation from hyphae was random and occurred in comparatively large numbers (**Figures 8C, D**).

Arthrospore (Arthroconidia) Formation

The formation of single-celled arthrospores from existing hyphae has long been observed during fungal morphogenesis in certain fungal species (Miyazi and Nishimura, 1971; Bibel et al., 1977; Emyanitoff and Hashimoto, 1979; Cartwright et al., 2014). These single-celled spores are produced in extensive numbers to enhance the pathogen's reproductive potential and, therefore, the primary function of arthrospore is reproduction rather than survival between seasons. However, arthrospores can also be the principal means of dissemination in certain pathogenic fungi (Barrera and Szaniszlo, 1985). Arthroconidia formation and morphogenesis in dimorphic human fungal pathogens can be stimulated by several types of environmental stress conditions related to growth medium composition, carbon dioxide tension, and increased temperatures (Rippon and Scherr, 1959; King and Jong, 1976; Bibel et al., 1977; Emyanitoff and Hashimoto, 1979). Initiation of arthro sporogenisis, the age of conidia formation, and the number of conidia formed are all highly species-specific (Barrera and Szaniszlo, 1985).

Arthro sporogenesis of *N. capsellae* was observed *in-vitro* from multi-celled hyphae on different artificial media (**Figure 9**) or multi-celled macroconidia (**Figure 10**). This is the first report showing arthrospores from *N. capsellae*, although the cleavage of multi-celled macroconidia on cotyledons to produce single cylindrical entities by this pathogen has been reported earlier by Gunasinghe et al. (2016d). Two different morphologies commonly observed for *N. capsellae* arthrospores were cylindrical (**Figures 9A, B**) or spherical (**Figures 9C, E**). Initial observations are that the size of the arthrospores and the proportion of each morphology *in-vitro* vary depending on the growth conditions. This is not unusual as other dimorphic fungal species can produce a range of arthroconidia that differ in size and/or shape and the dominating type is determined by the substrate (King and Jong, 1976; Allermann et al., 1978; Kier et al., 1980; Barrera and Szaniszlo, 1985).

The development and release of arthroconidia appear to vary depending on the fungal species (Barrera and Szaniszlo, 1985). In both: *Mucor rouxii* and *Tricophyton mentagrophytes*, arthroconidia develop inside the hyphae as cylindrical conidial

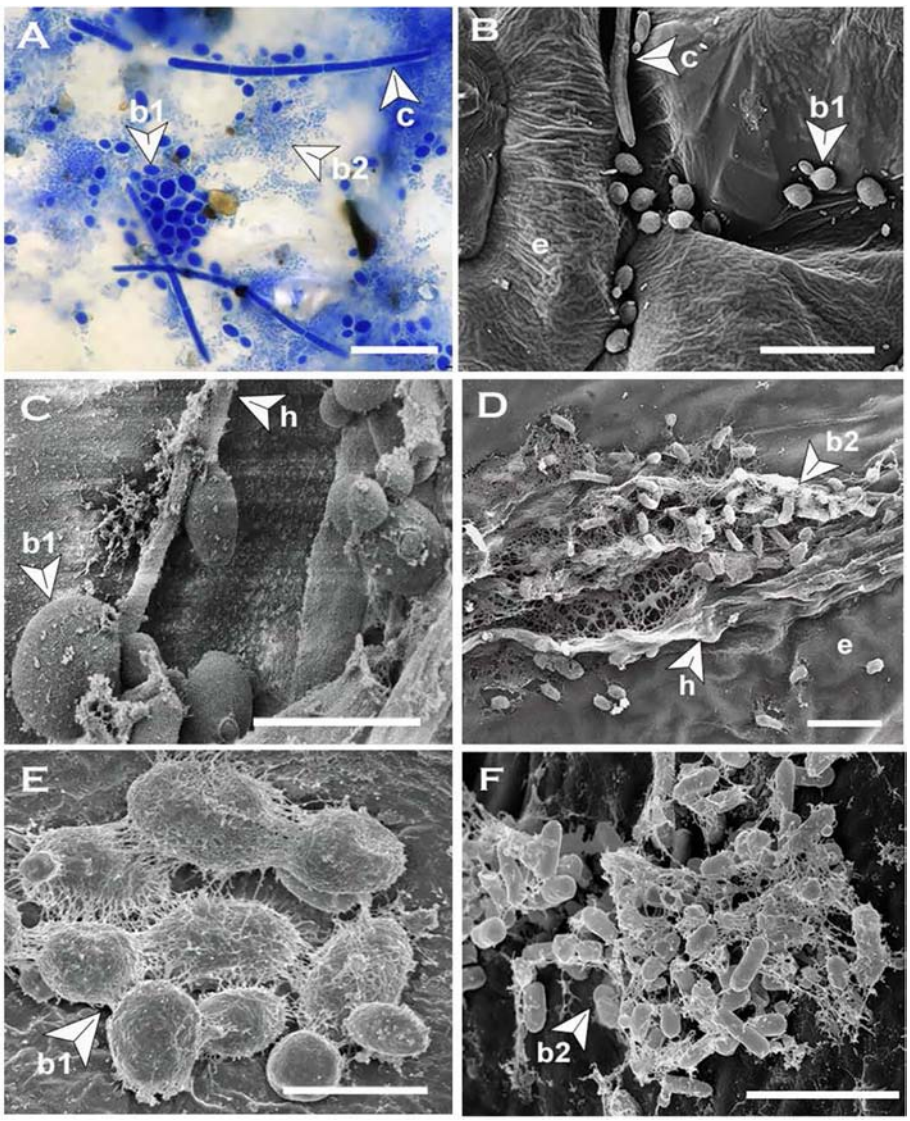

FIGURE 7 | Blastospores produced by *Neopseudocercosporella capsellae* on cotyledons of the host Brassica juncea or *B. napus*. **(A)** Multi-celled macroconidia (c), meso-blastospores (b1) and micro-blastospores (b2) on a cotyledon surface 48h post-inoculation with a multi-celled macroconidial suspension (cotyledons were decolorized by immersing in acetic acid:ethanol:water (2:2:1) solution at 25°C for 3-4 days before visualization under the light microscope (scale bar= 20µm). **(B)** Cotyledon surface inoculated with a multi-celled macroconidial suspension showing multi-celled macroconidia (c), meso-blastospores (b1), and epidermal cells (e) as seen under Scanning Electron Microscope (SEM) (Scale bar=10µm). **(C, D)** Cotyledon surface inoculated with hyphal fragments showing hyphae (h) producing meso-blastospores **(C)** (Scale bar=5µm) and micro-blastospores **(D)** (Scale bar=1µm) as seen under SEM. **(E, F)** Morphology of meso-blastospores **(E)** (Scale bar=5µm) and micro-blastospores **(F)** (Scale bar=1µm) produced in planta as seen under SEM.

chains enveloped by the original hyphal cell wall but then change their morphology to spherical just before dehiscence (Takeo and Nishiura, 1974; King and Jong, 1976). Separation of the conidia could occur by autolysis of inter-septal material in the original hyphal wall that helps the adhesion of neighbouring septal walls of conidial chains or by dehiscence of the original hyphal wall (Barrera and Szaniszlo, 1985). The arthroconidia of *Antromycopsis brousonnetiae* developed a secondary cell wall inside the hyphae and the separation of conidia is by centripetal dehiscence of the original hyphal wall (Moore, 1977).

The frequently observed release mechanism for *N. capsellae* arthrospores produced *in-vitro* was dehiscence of original hyphae. Transverse dehiscence at the septa was more common (**Figures 9C–E**) than centripetal dehiscence (**Figure 9F**). However, the release of cylindrical arthrospores by autolysis of the original hyphal wall also occurred when arthrospores were produced from multi-celled macroconidia (**Figure 10**). There is evidence that arthrospores are a superior asexual spore type providing enhanced dissemination and better establishment on the host tissue by protecting cells from host lytic enzymes

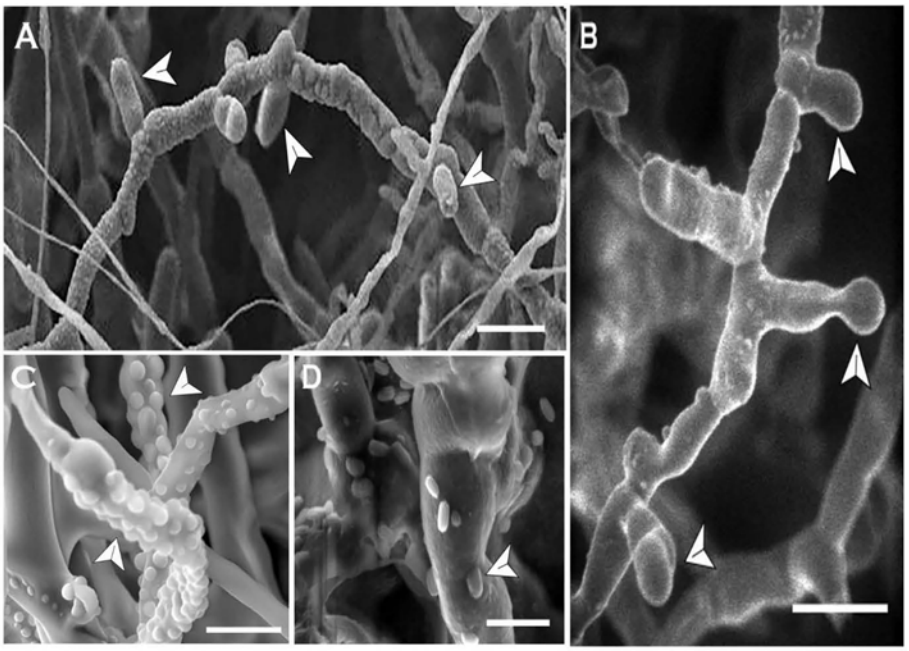

FIGURE 8 | *In-vitro* blastospore formation by *Neopseudocercosporella capsellae* hyphae as seen under the scanning electron microscope. Old septate hyphae producing meso-blastospores (arrows) at the septa **(A)** and the tip **(B)** at the transition stage (Scale bars =10μm). **(C)** Young hyphae showing buds for micro-blastospores (Scale bar = 5μm). **(D)** Old hyphae with micro-blastospore buds and single-celled micro-blastospores (arrows) separated from the original hyphae (Scale bar = 2μm).

(Duran et al., 1973), increasing antibiotic production (Nash and Huber, 1971; Queener and Ellis, 1975), and by promoting surface adherence (Kurakado et al., 2020).

Morphological Plasticity, Pathogen Biology, and Disease Epidemiology

Although white leaf spot has been a damaging disease on Brassicaceae, there is relatively little information on the pathogen's biology and disease epidemiology (Gunasinghe et al., 2020). It has been suggested that the pathogen is under-reported as the causative agent, because of the difficulties in isolation related to the slow growth on artificial media (Gunasinghe et al., 2020). As demonstrated by this study the initial growth of *N. capsellae* on artificial media can initially mimic bacterial/yeast colonies (**Figures 2A, B** and **Figures 4C, D**). This ability to form strikingly different colony morphology following *in-vitro* morphological transition creates difficulties in identification based on colony morphology which also potentially leads to under-reporting.

Morphological transformation in fungi plays critical roles in survival between seasons, dissemination, and host invasion and therefore, in virulence (Lin et al., 2015). Dimorphic or polymorphic fungi achieve this through the morphological transformation into single-celled spore types with different morphologies (blastospore or arthrospores) and/or varied physiologies (Odds and Kerridge, 1985) having altered tolerance to certain host physiological conditions making them more robust on hosts (Lin et al., 2015). In the absence of the teleomorph (sexual stage) *N. capsellae* infection cycle is not fully understood (Gunasinghe et al., 2020), as splash-dispersed multi-celled macroconidia are unlikely to be dispersed by wind (Fitt et al., 1992). The morphological transition on host leaves may provide a superior spore that can be efficiently disseminated through wind facilitating disease initiation and spread in the localities like North America, Asia, and Australia where the sexual stage and, therefore, ascospores are thought to be absent.

In the literature, polymorphism is defined as the ability of a fungus to take more than two different morphological forms (Mayer et al., 2013). *Candida albicans* provides a classic example for polymorphic pathogenic fungi, where it can form biofilms, different morphotypes for the single-celled blastospores (yeast phase), hyphae, and pseudo-hyphae (Odds and Kerridge, 1985; Kurakado et al., 2020). Formation of biofilm at the interface of yeast-to-hyphae transition was also observed for *N. capsellae* in this study. It is known that biofilm development and morphogenesis are interconnected phenomena controlled by the same set of genes for a given species (Lin et al., 2015; Kurakado et al., 2020). Further studies are recommended not only to answer the question of whether *N. capsellae* is dimorphic or polymorphic but also to define the decisive role/s of each morphotype produced understanding its morphogenesis.

CONCLUSION

N. capsellae has the morphological plasticity to produce different morphologies in response to various environmental/external

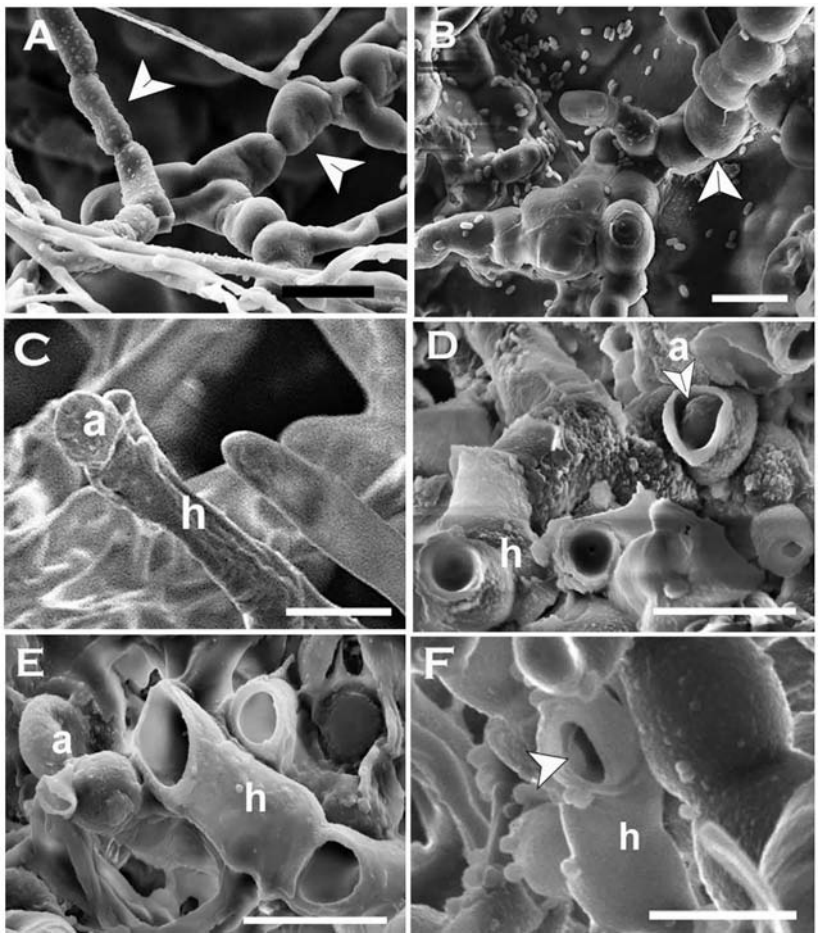

FIGURE 9 | Formation and release of arthrospores (arthroconidia) produced *in-vitro* from *Neopseudocercosporella capsellae* hyphae as seen under the scanning electron microscope. **(A, B)** Series of delicately attached arthrospores (arrows) produced by 7 week old colonies of isolate UWA Wlj-3 on NDY **(A)** and PDA **(B)**. The arthrospores break off and disseminate when disturbed (Scale bars = 5μm). **(C, D)** Releasing spherical arthrospore (a) after transverse dehiscence of the hyphae (h) at the septa. **(D, F)** Hyphal cell walls after releasing arthrospores by dehiscence along the septa **(C)** or centripetal dehiscence **(D)** (Scale bars = 5μm).

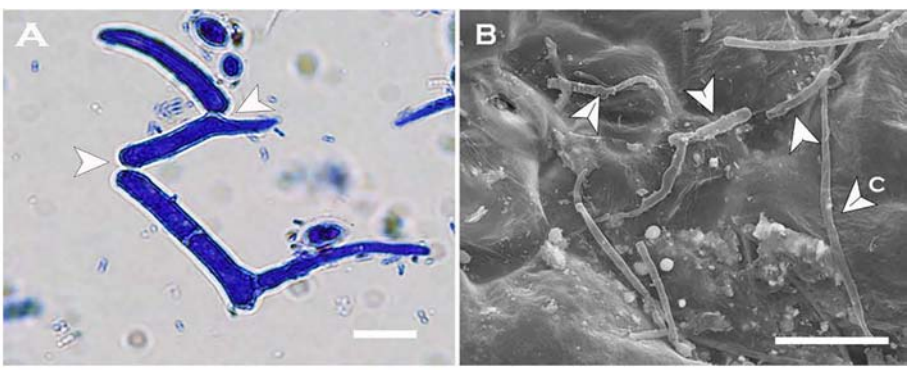

FIGURE 10 | Arthrospores produced by *Neopseudocercosporella capsellae* multi-celled macroconidia on the host. **(A)** Cylindrical arthrospore formation by disintegration of original macroconidia cell walls at septa (arrows) on the cotyledon surface as seen under the light microscope. **(B)** Scanning electron micrograph of multi-celled macroconidia (c) and germinating three arthrospores as single-celled spores by producing a single germ tube each (arrows) on the cotyledon. Scale bars = 10μm.

stimuli. During its morphogenesis, dikaryotic mycelium or asexually produced multi-celled macroconidia (2n) on leaf lesions in their parasitic phase initiate a single-celled yeast phase through the production of at least three types of morphologically distinguishable single-celled spore types. Multi-celled macroconidia on the host surface develop in three possible ways 1) they separate from the septa to produce multiple arthrospores that germinate as single entities, 2) they produce blastospores that increase in number rapidly by budding, and 3) they germinate as typical conidia by producing single or multiple germ tubes. This morphological plasticity of *N. capsellae* can be considered a cost-efficient strategy to provide a more resilient form of spores on the host enhancing inoculum potential and dissemination, both contributing to the pathogen's virulence. A systematic study is recommended to fully understand *N. capsellae* multifaceted life cycle and to define the role of morphogenesis in relation to virulence, which is largely unknown.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

NG: Designed and conducted the work and drafted the manuscript. PD: Worked with NG to conduct the study and prepared the figures. SN, MB, and MY: Gave significant contribution to the conception and the design and reviewed the manuscript for critical and important intellectual content. All authors contributed to the article and approved the submitted version.

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Bacillus amyloliquefaciens YN201732 Produces Lipopeptides With Promising Biocontrol Activity Against Fungal Pathogen *Erysiphe cichoracearum*

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Bacillus amyloliquefaciens YN201732 is an endophytic bacteria with high biocontrol efficiency and broad-spectrum antimicrobial activities. In order to clarify the main active ingredients and their antifungal mechanisms against powdery mildew of tobacco, this study is focused on lipopeptide obtained through acid precipitation and organic solvent extraction. HPLC and LCMS-IT-TOF were used to separate and identify antimicrobial lipopeptides. Findings revealed that bacillomycin D plays an important role against surrogate fungal pathogen *Fusarium solani*. Synthetic pathways of sfp, bacillomycin D, and fengycin were separately disrupted. The *sfp* gene knockout mutant *B. amyloliquefaciens* YN201732M1 only showed minor antagonistic activity against *F. solani*. While *Erysiphe cichoracearum* spore germination was inhibited and pot experiments displayed a significant decrease in tobacco powdery mildew. The spore inhibition rate of YN201732M1 was only 30.29%, and the pot experiment control effect was less than 37.39%, which was significantly lower than that of the wild type. The inhibitory effect of mutant YN201732M2 (deficient in the production of bacillomycin D) and mutant YN201732M3 (deficient in the production of fengycin) on the spore germination of *E. cichoracearum* were 50.22% and 53.06%, respectively, suggesting that both fengycin and bacillomycin D had potential effects on spore germination of powdery mildew. Interestingly, in a greenhouse assay, both *B. amyloliquefaciens* YN201732M2 and YN201732M3 mutants displayed less of a control effect on tobacco powdery mildew than wild type. The results from *in vitro*, spore germination, and greenhouse-pot studies demonstrated that antimicrobial lipopeptides especially bacillomycin D and fengycin may contribute to the prevention and control of tobacco powdery mildew. In addition, gene mutation related to lipopeptide synthesis can also affect the biofilm formation of strains.

Keywords: antifungal activity, bacillomycin, biofilm, tobacco powdery mildew, pathogen

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INTRODUCTION

Erysiphe cichoracearum is the primary cause of powdery mildew in tobacco plants, and it results in substantial reduction of yields and quality of tobacco in China (Xing et al., 2015). This pathogen is a biotrophic parasite, which is harmful to mature leaves and spreads from the bottom to the top of leaves (Dietz and Winter, 2019). Disease symptoms begin with the development of white powdery disease spots on both sides of leaves, and the spots expand followed by foliar chlorosis with pathogen spread all over the leaves, eventually leading to plant death (Glawe, 2008). The application of chemical fungicides is the main strategy for controlling tobacco powdery mildew. However, with increasing concern over fungicide resistance, environment pollution, and food safety (Koch et al., 2018; Patle et al., 2018), biological control has attracted considerable attention.

Bacillus as an ideal biocontrol bacterium has been used for biological control of crop diseases and insect pests, especially endophytic *B. amyloliquefaciens*, which are ubiquitously found to be safe microorganisms with proven excellent *in planta* colonization aptitudes (Cao et al., 2011; Liu et al., 2017) and versatility in effectively protecting plants from pathogens (Ongena and Jacques, 2008). They can compete with pathogens for nutrients, secretes antimicrobial substances, and induce the plant's defense system to resist the invasion of pathogens (Wang et al., 2017). The most direct way through which *B. amyloliquefaciens* suppresses pathogens is the production of secondary metabolites such as antimicrobial proteins, lipopeptides, polyenes, phospholipids, amino acids, nucleic acids, and polyketides (Ongena and Jacques, 2008; Chen et al., 2015; Yan et al., 2018). Low-molecular-weight lipopeptides are one of the most common types of antimicrobial substances (Koumoutsi et al., 2004). Iturin, fengycin, and surfactin are the three most studied families of cyclic peptides (LPs). With a unique chemical composition and amphiphilic ring structure, they can still maintain high activity under high temperature, ultraviolet rays, and different pH values and can also resist the hydrolysis action of peptidases and proteases (Santoyo et al., 2012; Caulier et al., 2019). The iturin and fengycin families are associated with strong antifungal activity against various fungi *in vitro*, while the surfactant family is responsible for less antifungal activity, but displays the properties of a surfactant (Romero et al., 2007). Guo et al. (2019) reported that *B. subtilis* NCD-2 inhibits pathogenic fungi by producing fengycin-type cyclic peptides, which may play a role in the suppression of clubroot pathogen on Chinese cabbage. Since the discovery of secondary metabolites secreted by *Bacillus* to inhibit or even kill plant pathogens (Stein, 2005), scientists have been working on the application of microbial antagonists to manage plant diseases (Li et al., 2018; Munir et al., 2020). Microbial communities also play a significant role to manage the bacterial wilt of tobacco (Cai et al., 2021). Studies have found that lipopeptides produced by *B. amyloliquefaciens* FZB42 are necessary for inducing resistance, biofilm formation, root colonization, and successful control of plant pathogens (Koumoutsi et al., 2004; Chowdhury et al., 2015). In addition, antimicrobial peptides are powerful weapons of biocontrol strains against other pathogenic microorganisms, and their various structural types can help *Bacillus* effectively avoid the development of pathogen resistance and thus maintain the antagonistic advantage of biocontrol agents (Raaijmakers et al., 2010).

Bacillus amyloliquefaciens YN201732 is a beneficial endophytic strain isolated from tobacco seeds that can suppress the growth of *E. cichoracearum* in tobacco and protect the host from pathogen invasion through efficient root colonization (Jiao et al., 2020). *In vitro* testing showed that strain YN201732 can effectively antagonize a variety of pathogenic fungi, and the supernatant of *B. amyloliquefaciens* YN201732 completely inhibited the conidial germination of *E. cichoracearum*, implying that it may secrete antifungal compounds (Jiao et al., 2020). In this study, the antifungal LPs were investigated using both biochemical and genetic approaches. The crude extract of lipopeptides in *in vitro* tests and gene disruption experiments confirmed that bacillomycin D and fengycin were the major compounds among the detected LPs, responsible for the inhibition of *E. cichoracearum* in tobacco. Further in-depth research on the main antimicrobial substance of *B. amyloliquefaciens* YN201732 against phytopathogen is of great significance for the development of endophyte YN201732 as biological pesticides and bio-organic fertilizers.

MATERIALS AND METHODS

Microorganisms and Plasmids

The strains and plasmids used in this study are described in **Table 1**. *Escherichia coli* TG1 competent cells were used as a host for all plasmids. *B. amyloliquefaciens* strain YN201732 was identified following the method described in Jiao et al. (2018) and stored in 50% glycerol at -80°C (in Laboratory of Biocontrol and Plant Pathology, Yunnan Agricultural University, Kunming, China). Since *E. cichoracearum* cannot be cultured purely, the pathogen was collected from the surface of naturally infected leaves. Pathogen *F. solani* preserved by the Laboratory of Biocontrol and Plant Pathology, Yunnan Agricultural University, Kunming, China, and maintained on potato dextrose agar (PDA) medium, was used as an indicator fungus to overcome the problem of *in vitro* culturing of powdery mildew pathogen.

Preparation of Crude Extracts of Lipopeptide Compounds and Separation and Purification of Organic Solvents

For lipopeptide production, liquid chromatography and mass spectrometry characterization were used. *Bacillus amyloliquefaciens* YN201732 was streaked on Luria-bertani (LB) plates and cultured at 37°C for 24 hours. After growing, a single colony was picked and inoculated into Landy liquid medium at 37°C, 160 rpm, cultured for 48 hours with shaking to prepare 2 L of fermentation broth (Branda et al., 2001). The fermented suspension was centrifuged to remove bacterial cells (50 mL centrifuge tube, 4°C, 10000 rpm, 20 minutes), and the supernatant was adjusted to pH 2.0 with 6 mol/L HCl and left at

TABLE 1 | Microorganism and plasmid used in this study.

Plasmid/Strain	Characteristics	Source or reference
Fungus		
Fusarium solani	Pathogen of *Panax notoginseng* root rot	Laboratory stock
Erysiphe cichoracearum	Pathogen of tobacco powdery mildew	Natural infected
Bacteria		
Escherichia coli TG1	*supE hsdΔ5 thi Δ(lac-proAB)*/F' [*traD36proAB*$^+$ *lacI*^{q}*lacZ ΔM15*]	Laboratory stock
B. amyloliquefaciens YN201732	Wild type	
B. amyloliquefaciens YN201732M1	*B. amyloliquefaciens* YN201732Δsfp:: Kan	This study
B. amyloliquefaciens YN201732M2	*B. amyloliquefaciens* YN201732ΔbmyA:: KanR	This study
B. amyloliquefaciens YN201732M3	*B. amyloliquefaciens* YN201732ΔfenB:: KanR	This study
Plasmid		
pUC18	Amp^{r+}, 2.7kb, ori from pBR322	TaKaRa (Dalian)
pMD18-T	Amp^{r+}, 2.7kb, linear pUC18 with T overhangs	TaKaRa (Dalian)
pBluscript KS minus	Amp^{r+}, 3.0kb, ori from pBR322	Laboratory stock
pMD18-kanR	Amp^{r+}, Kan^{r+}, pMD18 insert with KanR, 3.7kb	Laboratory stock
pBEST502	Amp^{r+}, Kan^{r+}, ori from pBR322	(Itaya and Tanaka,1990)

4°C overnight. The precipitate was collected by centrifugation at 10,000 rpm for 10 minutes. The precipitate was further washed three times with sterile deionized water, 5 mL of deionized water was also added to adjust pH to 7.0 with 1 mol/L NaOH. After freeze-drying, the powder was extracted with three to four times the amount of 95% methanol under the assistance of ultrasonic for 3 hours. After centrifugation, the precipitate was collected and extracted twice, and the extracted solution was mixed together. The pooled extraction solution was completely evaporated under pressure using a rotary vacuum evaporator to obtain a crude extract of the lipopeptide. Then the lipopeptide crude extract was sequentially extracted with petroleum ether, ethyl acetate, n-butanol, and water. After the crude extract was dissolved in 100 mL H_2O, it was extracted with 1: 1 (V: V) petroleum ether for 3 hours. During this period, it was shaken every 10 minutes and extracted 3 times. Organic layer combined extracts were concentrated and weighed with a rotary evaporator. Aqueous phase was sequentially extracted with ethyl acetate and n-butanol in the same way, and the remaining aqueous phase was directly concentrated and weighed to obtain secondary extracts of four different polar solvents. The four separated components were concentrated and dried and then dissolved in a certain amount of methanol. The solvents were volatilized and secondary extraction product was dissolved with equal amounts of methanol respectively, and four extracted fractions were collected. Four fractions were filtered through a 0.22-μM-pore-size hydrophilic membrane, which was used for the determination of antifungal activity.

Determination of Antifungal Activity of Various Components of Crude Lipopeptides Against Pathogenic Fungi

The phytopathogenic fungus *F. solani* was cultured on the PDA for 3 days, and the hyphal pieces were picked from the edge of the colony, and inoculated into a 250 mL flask containing 100 mL of a sterilized PDA broth at 25°C, with 160 rpm for 3 to 5 days. Large mycelium was removed by filtering through sterile gauze to obtain a conidial suspension, counted by using a blood cell counter, and the concentration was adjusted to 10^8 cfu/mL with sterile water. One hundred microliters (100 μL) of the conidia suspension of *F. solani* was spread on PDA plates. A volume of 100 μL of the collected fraction was dropped into the hole (5mm) 2.5 cm from the center of the Petri plate and allowed to diffuse into the agar. Plates were then incubated at 25°C, and the distance between the edges of the Petri dish and the fungal mycelium was measured after 5 days. The solvent methanol was set as the negative control, and the antimicrobial lipopeptide methanol crude extraction was the positive control at the same time. The experiment was repeated three times.

LCMS-IT-TOF Analysis of Lipopeptide Active Components

Liquid chromatography conditions: qualitative analysis of active LPs were performed using high-performance liquid chromatography (HPLC) (Agilent 1200, U.S.). A sample of the active secondary extract methanol solution was taken, filtered through a 0.22 μM microporous filter, and then loaded. The injection was 5 μL, and the column was LH-20 (MeOH) (Welch Ultimate AQ-C18, 5 μM, φ 4.6 × 300 mm). The column temperature, detection wavelength, and flow rate were 35°C, 254 nm, and 1 mL/min, respectively. The active part of the mobile phase was acetonitrile: the water (containing 0.05% trifluoroacetic acid) (v/v) was 40:60.

Mass spectrometry (MS) conditions: the high-performance liquid ion trap time-of-flight mass spectrometer was LCMS-IT-TOF (Shimadzu Corporation, Japan). LCMS-IT-TOF is an emerging spectrometry technique that is a combination of both QIT (ion trap) and TOF (time-of-flight). An accurate mass-to-charge ratio of lipopeptides can be obtained without complicated extraction, separation, and purification steps, and the types of lipopeptides can be qualitatively judged by comparing with the molecular weight of known compounds. In the ESI ion source, the detection method was positive and negative ions, and the atomizing gas flow rate was 1.50 L/min. While the temperature of the curve desolvent tube (CDL) and the heating module was 200°C, the PR vacuum degrees of the IT vacuum and TOF vacuum were 97 Pa, 1.1×10^{-2} Pa, and 1.2×10^{-4} Pa, respectively. Collision-induced dissociation technology (CID) was used to

obtain the typical fragment ions of the compound. The detector voltage was 1.50 kv. High-purity argon was used as collision gas in the collision cell with a collision energy of 20–50%. Accurate mass analysis was performed on the main chromatographic peaks, and chemical composition was confirmed by comparison with related literature.

Inhibitory Effect of Lipopeptide Gene Mutation on *Fusarium solani*

In order to further verify the role of lipopeptide antibiotics produced by *B. amyloliquefaciens* YN201732 to control disease, a double-exchange homologous recombination method (Liu et al., 2018) was used to knock out the 4'-phosphopantetheinyl transferase gene (*sfp*), non-ribosomal pathway peptide antibiotics bacillomycin D (*bmyA*) and fengycin synthetic genes (*fenB*). A 100 μL cell-free culture filtrate of *B. amyloliquefaciens* YN201732 and its lipopeptide mutants (incubated at 37° C for 48 hours in Landy medium), was loaded into 5 mm small wells from which agar pieces were taken out, and a plug (about 5 mm in diameter) of *F. solani* from a 5-day-old (25°C) PDA plate was placed in the center. Antagonistic activity of YN201732 and its mutants against *F. solani* was studied in the way of co-culture on the plate (Xu et al., 2013).

Qualitative Biofilm Determination of *Bacillus amyloliquefaciens* YN201732 and Its Mutants

A biofilm assay was carried out using a modified version of the microtiter plate as described by Liu (Liu et al., 2018). The overnight-grown YN201732 and mutant were inoculated into fresh LB culture medium, and cultured at 37°C, 160 rpm to a bacterial solution concentration of OD600 of 1.0, making them in the logarithmic growth phase. The bacterial solution was centrifuged at 4°C and 10,000 rpm for 5 minutes, and bacterial cells were collected, washed three times with 0.9% NaCl solution, and resuspended in an equal volume of biofilm growth medium (MSgg medium, 5 mM potassium phosphate(pH 7), 100 mM morpholino propanesulfonic acid (pH 7), 0.5% glutamate, 50 μg/mL tryptophan, 50 μg/mL phenylalanine, 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $MnCl_2$, 50 μM $FeCl_3$, 1 μM $ZnCl_2$, 2 μM thiamine, 0.5% glycerol) (Branda et al., 2001). Qualitative biofilm determination was performed in 24-well polyvinylchloride (PVC) microtiter plates. In total, 2 mL of Msgg culture medium was added to the culture wells, and then 10 μL each of YN201732 wild-type and YN201732M1, YN201732M2, and YN201732M3 were set up in four replicates. The microtiter plates were incubated under stationary conditions at 37°C, and the film formation of YN201732 wild-type and mutant strains was observed after 12h and 24h, respectively.

Inhibition Test of *Bacillus amyloliquefaciens* YN201732 and Its Mutants Against Spore Germination of Tobacco Powdery Mildew

The cell-free culture filtrates of *B. amyloliquefaciens* YN201732 and its mutants were mixed with 5% (w/v) water agar medium (Agar,5 g; ddH_2O, 100 mL) in proportion to make a fermentation filtrate content of 20% (v/v) culture medium, invert a plate with a thickness of about 2 mm, and use a water agar plate containing 20% (v/v) sterile LB medium as a control. Naturally-occurring powdery mildew diseased leaves were shaken to evenly spread the spores on the plate and incubated at 25°C for 24 h. Moreover, after germination, 50 mm of the sterile puncher was used to create holes from the water agar medium which was mixed with the cell-free culture filtrates of YN201732 and its mutants, an agar plug was put on the center of a glass slide. The conidia were examined using a light microscope, and the germination standard was based on the length of the germ tube exceeding half the diameter (short diameter) of the spores. The experiment was repeated three times with each treatment repeated three times with five replicates. Microspores (150) were randomly examined through a microscope. The ratio of germinated spores to total observed spores was germination rate, and the inhibition rate of strain fermentation filtrate (%) = [Control spore germination rate – Treatment spore germination rate)/Control spore germination rate] × 100.

Control Effect of *Bacillus amyloliquefaciens* YN201732 and Its Mutants on Powdery Mildew in Greenhouse

The experiment was carried out in the greenhouse of the College of Plant Protection, Yunnan Agricultural University, Kunming, China. Tobacco seedlings of Yunyan 87 (4–5-leaf-stage) were transplanted into a pot (10 cm × 12 cm) containing 200 g of autoclaved soil, 1 plant/pot. YN201732 wild type and its mutant strains were incubated in Landy medium at 37°C for 48 hours, and the cell concentration of YN201732 and its mutants was adjusted to 1×10^7 cfu/mL. The disease base was investigated with the appearance of symptoms; sprayed the leaves with YN201732 wild type and its mutants suspension once, 10 mL/plant. Sterile LB medium and azoxystrobin 50% WG 10,000 times were used as negative and positive controls. Tween 20 (0.1%) was mixed with each agent prior to spraying. Initially, the disease symptoms were recorded after 3rd day and finally, with the interval of 7 days after inoculation of YN201732 wild type and its mutants. Disease severity was evaluated based on the percentage of leaves covered by mycelia and the percentage of powdery mildew caused by *E. cichoracearum* (tobacco pest index and survey standard, 2008), and the disease index and relative control effect were calculated. This experiment was performed in 3 replicates per treatment with 20 seedlings per replicate. The whole experiment was set according to a completely randomized block design.

Statistical Analysis

The data were subjected to analysis of variance using IBM SPSS Statistics 22.0. The mean values among the treatment groups were compared using Duncan's multiple range test at the 5% level of significance ($P < 0.05$).

RESULTS

Antifungal Activity of Various Components of Crude Lipopeptides Against Pathogenic Fungi

The measurement of 4 different lipopeptides crude extract antagonistic activity is shown in **Figure 1**. The results show that the n-butanol extract had obvious antifungal activity against *F. solani*, and the average zone of inhibition diameter was 2.63 ± 0.17 cm.

LCMS-IT-TOF Analysis of Active Substances in Lipopeptide Extracts

The active components of the n-butanol extract were first optimized by HPLC and then detected by LCMS-IT-TOF mass spectrometer. Eight molecular ion peaks were detected for the corresponding seven retention time components (**Figure 2**) (m/z 1031.4, 1053.4, 1069.4, 1045.4, 1067.4, 1083.4, 1059.5, and 1081.4). By consulting relevant literature, comparing the molecular weights of lipopeptides of *Bacillus* (Yan et al., 2015; Caulier et al., 2019), it was found (see **Table 2**) that the mass-to-charge ratios m/z 1031.4, 1053.4, and 1069.4 correspond to the hydrogen ion peaks $[M + H]^+$, the sodium ion peak $[M + Na]^+$ and the potassium ion peak $[M + K]^+$, whose corresponding molecular weights are both 1030.4. This compound belongs to the *Bacillus* antimycin D of the subtilisin family. The corresponding compound is 14-carbon C14-Bacillomycin D; mass-to-charge ratios m/z 1045.4, 1067.4, and 1083.4 correspond to the hydrogen ion peaks $[M + H]^+$, the sodium ion peak $[M + Na]^+$ and the potassium ion peak $[M + K]^+$. The corresponding molecular weight is 1044.4, and the corresponding compound is C15-Bacillomycin D of 15 carbons; the mass-to-charge ratios m/z 1059.5 and 1081.4 correspond to the hydrogen ion peak $[M + H]^+$ and the sodium ion peak $[M + Na]^+$, its corresponding molecular weight is 1058.4 and the corresponding compound is C16-Bacillomycin D of 16 carbons. The same compound has different ionic forms, corresponding to three kinds of compounds, respectively. According to the above analysis, it can be found that the molecular weights of the three compounds differ in order by 14 Da, that is, one methylene group ($-CH_2$), so these three compounds should be a group of homologs.

As shown in **Table 3**, m/z 1031.4 was detected in addition to the retention time RT 18.303, and was detected once in other time periods RT 18.525, indicating that m/z 1031.4 corresponds to two isomers. This phenomenon also exists in the other seven molecular ion peaks, which are m/z 1053.4 and 1069.4, which can also be detected at two different retention times of RT 18.303

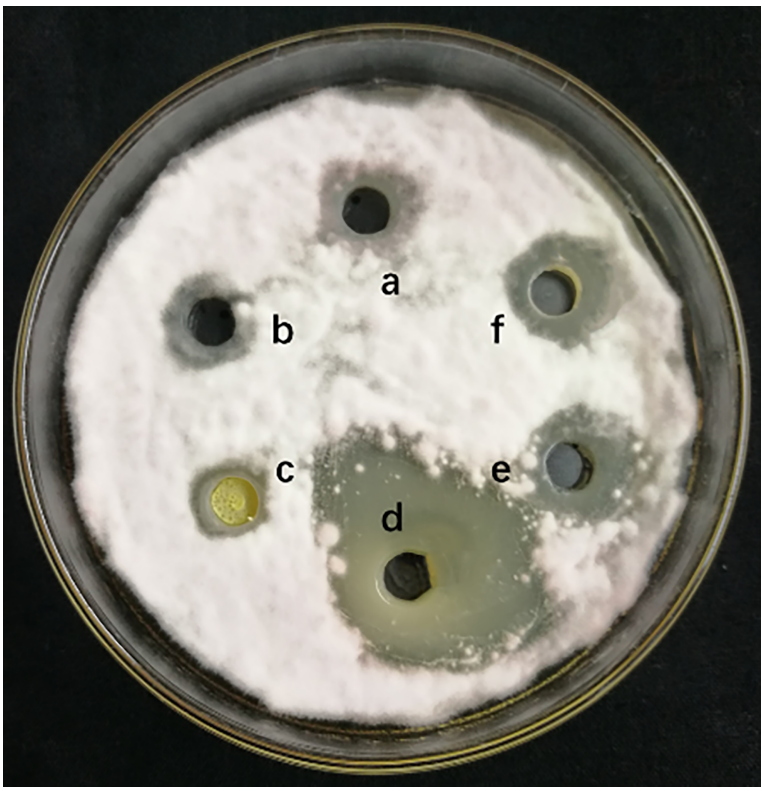

FIGURE 1 | Inhibitory effect of different lipopeptides crude extract on *F. solani.* a-methanol (negative control), b-Petroleum ether extraction component, c- Ethyl acetate extraction component, d-N-butanol extraction component, e-Water extraction component, f- Methanol crude extract(positively control). A volume of 100 μL of the collected fraction was dropped into the hole on a PDA agar plate with actively growing *F. solani*. The plates were incubated for 5 days at 25°C.

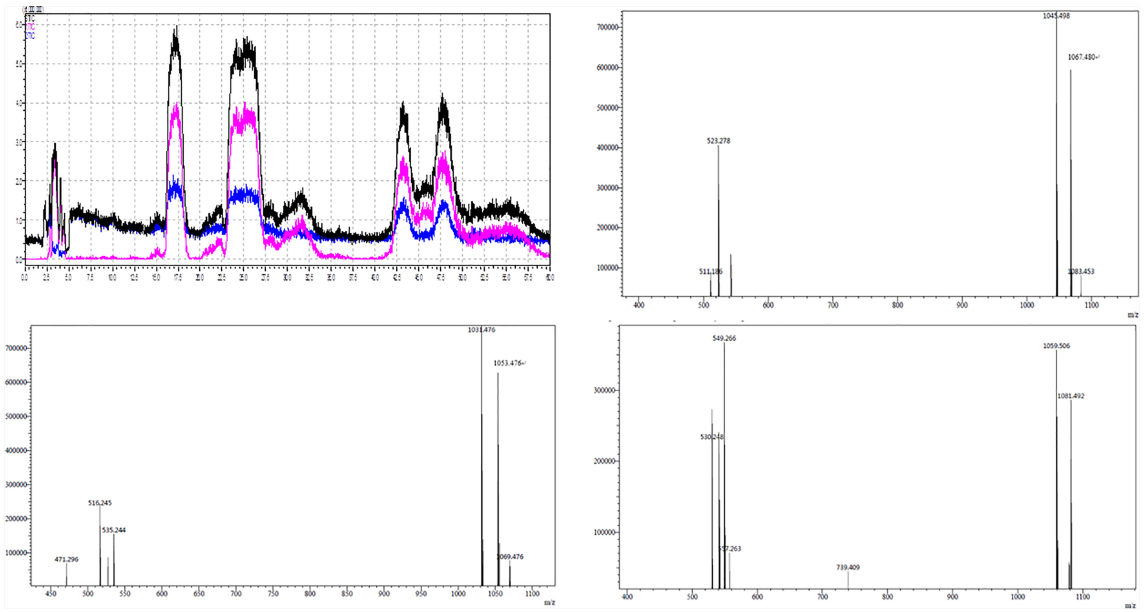

FIGURE 2 | LCMS-IT-TOF of secondary metabolite produced by *B. amyloliquefaciens* YN201732.

TABLE 2 | Mass peaks of secondary metabolite compounds in n-butanol extract from *Bacillus amyloliquefaciens* YN201732.

No.	Retention time	Mass-to-charge ratio	Molecular weight	Positive ions	Compound
1	18.303	1031.4	1030.4	$[M+H]^+$	C14-Bacillomycin D
2	18.303	1053.4	1030.4	$[M+Na]^+$	C14-Bacillomycin D
3	18.303	1069.4	1030.4	$[M+K]^+$	C14-Bacillomycin D
4	18.525	1031.4	1030.4	$[M+H]^+$	C14-Bacillomycin D
5	18.525	1053.4	1030.4	$[M+Na]^+$	C14-Bacillomycin D
6	18.525	1069.4	1030.4	$[M+K]^+$	C14-Bacillomycin D
7	25.555	1045.4	1044.4	$[M+H]^+$	C15-Bacillomycin D
8	25.555	1067.4	1044.4	$[M+Na]^+$	C15-Bacillomycin D
9	25.555	1083.4	1044.4	$[M+K]^+$	C15-Bacillomycin D
10	25.998	1045.4	1044.4	$[M+H]^+$	C15-Bacillomycin D
11	25.998	1067.4	1044.4	$[M+Na]^+$	C15-Bacillomycin D
12	25.998	1083.4	1044.4	$[M+K]^+$	C15-Bacillomycin D
13	27.328	1045.4	1044.4	$[M+H]^+$	C15-Bacillomycin D
14	27.328	1067.4	1044.4	$[M+Na]^+$	C15-Bacillomycin D
15	27.328	1083.4	1044.4	$[M+K]^+$	C15-Bacillomycin D
16	45.182	1059.4	1058.4	$[M+H]^+$	C16-Bacillomycin D
17	45.182	1081.4	1058.4	$[M+Na]^+$	C16-Bacillomycin D
18	49.837	1059.5	1058.5	$[M+H]^+$	C16-Bacillomycin D
19	49.837	1081.4	1058.4	$[M+Na]^+$	C16-Bacillomycin D

and 18.525 min; m/z 1045.4, 1067.4, and 1083.4 at Rt 25.555, 25.998, 27.329 min were detected at three different retention times; m/z 1059.4, 1081.4 were detected at RT 45.182, 49.837 min. Since m/z 1031.4, 1053.4, and 1069.4 correspond to the same compound C14-Bacillomycin D, it is speculated that C14-Bacillomycin D has two isomers; m/z 1045.4, 1067.4, and 1083.4 correspond to the same compound C15-Bacillomycin D, and it is speculated that C15 -Bacillomycin D has three isomers; m/z 1059.4 and 1081.4 correspond to the same compound C16-Bacillomycin D. It can be considered that C16-Bacillomycin D has two isomers. However, the specific structural assignment of each substance cannot be judged based on the results of LCMS-IT-TOF, and further research is needed.

Inhibitory Effect of Microbial Lipopeptide Gene Mutants on *Fusarium solani*

The *sfp*, *bmyA*, and *fenB* genes in endophyte YN201732 were knocked out, and mutant strains YN201732M1, YN201732M2, and YN201732M3 were obtained, respectively. In order to study the effect of mutant strains on the antagonistic activity of

TABLE 3 | Inhibitory effect of *Bacillus amyloliquefaciens* YN201732 and the mutants fermentation filtrate on the conidia germination of *Erysiphe cichoracearum*.

Treatment	Conidia germination rate/%	Inhibition rate/%
CK	25.54 ± 9.01^{d}	
YN201732M1	17.80 ± 7.64^{c}	30.29 ± 7.95
YN201732M2	12.71 ± 4.38^{b}	50.22 ± 5.26
YN201732M3	11.99 ± 10.19^{ab}	53.06 ± 9.04
YN201732	7.06 ± 4.75^{a}	72.37 ± 3.15

Different letters indicate that the differences are significant ($P < 0.05$) using Duncan's multiple range test.

E. cichoracearum, *F. solani* was used as indicator organisms, the supernatants of YN201732 wild-type and its mutant strains were used to perform a plate-to-pan test of *F. solani*. The results in **Figure 3** show that the supernatant of wild-type strain YN201732 inhibited the mycelial growth of *F. solani*, and the average distance between the edges of the petri dish and the fungal mycelium was 5.37 ± 0.46 mm, while the disruption of *sfp* had almost no effect on the antifungal activity against *F. solani*. Under the same condition, the supernatant of YN201732M2 showed minor antifungal activity against *F. solani*, with the average distance between the edges of the Petri dish and the mycelium being about 1.48 ± 0.46 mm. The inhibition zone of mutant YN201732M3 was 3.97 ± 0.06 mm, and the antifungal effect was slightly lower than that of the wild-type strain. These results show that antimicrobial lipopeptides except for fengycin could inhibit the pathogen of *F. solani*. These data confirmed the hypothesis that the LP, especially the fraction of bacillomycin D, plays a major role in *F. solani* inhibition.

Comparison of *Bacillus amyloliquefaciens* YN201732 and Its Mutant Biofilm Formation Capabilities

The biofilms of wild-type YN201732 and mutants YN201732M1, YN201732M2, and YN201732M3 were grown in MSgg medium for 12h and 24h, as shown in **Figure 4**. Biofilm assay with microtiter plates at both 12 hours and 24 hours' time points showed that YN201732M1 formed a thin and fragile biofilm compared to wild-type strain YN201732, which had no difference with the biofilm of strain YN201732M2 and YN201732M3. The mutants YN201732M1, YN201732M2, and YN201732M3 had an obvious growth lag in the 12h before culture. The biofilm was basically not visible to the naked eye, and the wild-type biofilm was formed. It was found that the biofilm formed by the three mutants, especially YN201732M1, after 24 h of culture was very thin and did not form a spatial three-dimensional structure, and the biomass of the biofilm was very small. However, the biofilm formed by wild-type YN201732 after 24 h of culture had a

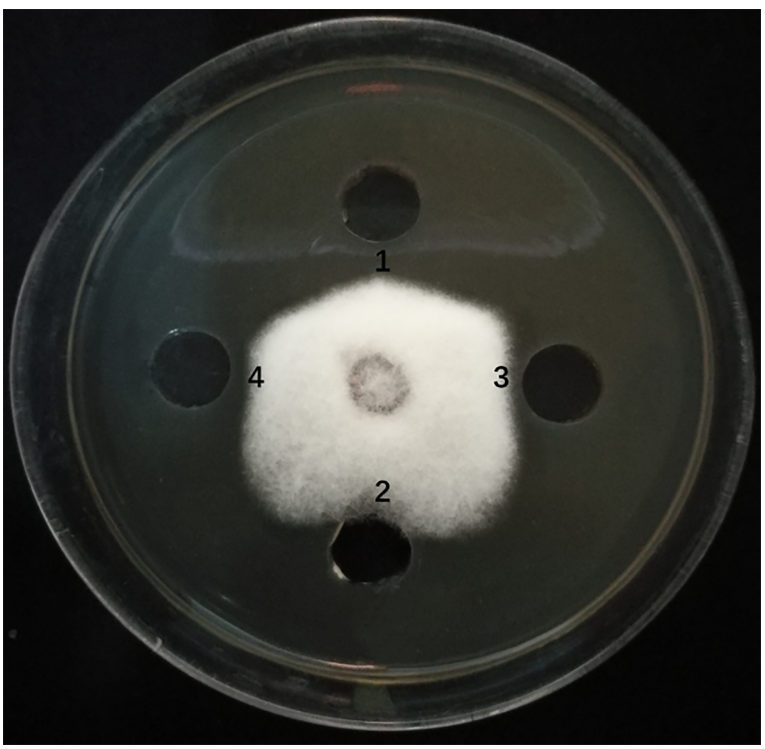

FIGURE 3 | Antagonistic effects of supernatant drawn from *B. amyloliquefaciens* wild type strain YN201732 (spot 1), the sfp-disrupted mutant strain YN201732M1 (spot 2), the bmyA-disrupted mutant strain YN201732M2 (spot 3), and the fenB-disrupted mutant strain YN201732M3 (spot 4) against the indicator organisms (*F. solani*). A volume of 100 μL of a 48h culture grown in Landy medium was dropped into the hole (5mm) on PDA agar plates with actively growing *F. solani*. The plates were incubated at 25°C for 5 days.

FIGURE 4 | Microtiter plate assay of biofilm formation by wild type and the mutant strains. Wild-type YN201732, YN201732M1, YN201732M2, and YN201732M3 cells were grown in MSgg medium at 37°C for the indicated times.

complex spatial three-dimensional structure, and wrinkles were also observed. It is speculated that lipopeptides may also be a key factor affecting the formation of biofilms.

Inhibition Effects of *Bacillus amyloliquefaciens* YN201732 and Its Mutants on the Germination of *Erysiphe cichoracearum* Conidia

Comparing the inhibitory effects of wild type and the mutants on the germination of powdery mildew spores (**Table 3**), fermentation filtrates of mutants with different antimicrobial lipopeptides were used to study the effect on the conidia germination. The inhibitory rate of *sfp* gene mutant YN201732M1 on conidial germination was only 30.29%. Antimicrobial lipopeptides may be the main factor that affects spore germination. The spore inhibition rates of *bmyA* gene mutant YN201732M2 and *fenB* gene mutant YN201732M3 were similar (50.22% and 53.06%, respectively), indicating that fengycin and bacillomycin D have significant effects on the spore germination, but YN201732M2 spore germination rate was significantly different from wild type. The difference indicated that bacillomycin D has a major effect on the spore germination of powdery mildew.

Biocontrol of *Bacillus amyloliquefaciens* YN201732 and Its Mutant Inoculation on Tobacco Powdery Mildew Under Greenhouse Conditions

From the results of YN201732 and its mutants on the management of tobacco powdery mildew (**Table 4**), it can be seen that the mutants have significantly different control effects than the wild type. From days 3 to 30, the control effect of the *sfp* gene mutant YN201732M1 was significantly different from that of wild-type YN201732 and the positive control 50% WG treatment, and there was no significant difference in the negative control. At 7 days, the mutant YN201732M2, YN201732M3 had no significant difference in disease index

TABLE 4 | Control effect of *Bacillus amyloliquefaciens* YN201732 and the mutants on tobacco powdery mildew in the greenhouse.

Treatment	Disease base	3d		7d		14d		21d		30d	
		Disease index	Control effect	Disease index	Control effect	Disease index	Control effect	Disease index	Control effect	Disease index	Control effect
CK	$16.84^{ab} \pm 2.03$	$29.02^{d} \pm 2.54$	–	$33.81^{c} \pm 2.74$	–	$42.11^{d} \pm 1.14$	–	$54.14^{d} \pm 1.93$	–	$63.72^{d} \pm 2.47$	–
YN201732M1	$15.41^{a} \pm 1.94$	$18.17^{c} \pm 2.22$	37.39	$25.02^{b} \pm 2.09$	24.59	$35.19^{c} \pm 1.87$	16.43	$46.79^{c} \pm 2.33$	13.58	$59.21^{cd} \pm 1.75$	7.08
YN201732M2	$15.99^{a} \pm 0.87$	$11.14^{ab} \pm 1.44$	61.61	$10.73^{a} \pm 0.99$	67.66	$20.64^{b} \pm 1.97$	50.99	$34.34^{b} \pm 2.74$	36.57	$54.99^{bc} \pm 3.08$	13.7
YN201732M3	$17.41^{ab} \pm 2.79$	$13.10^{b} \pm 1.68$	54.86	$11.46^{a} \pm 0.64$	65.46	$21.32^{b} \pm 2.49$	49.37	$32.59^{b} \pm 3.31$	39.8	$56.07^{bc} \pm 3.32$	12.01
YN201732	$18.81^{b} \pm 1.26$	$9.08^{a} \pm 1.10$	68.71	$9.34^{a} \pm 2.75$	71.85	$14.60^{a} \pm 0.98$	65.33	$29.24^{b} \pm 1.59$	45.99	$52.84^{b} \pm 2.59$	17.07
Azoxystrobin 50% WG	$19.02^{b} \pm 3.03$	$12.76^{b} \pm 1.33$	56.03	$12.58^{a} \pm 1.05$	62.09	$10.68^{a} \pm 1.43$	74.64	$9.17^{a} \pm 0.79$	83.06	$11.85^{a} \pm 2.68$	81.4

Different letters indicate that the differences are significant ($P < 0.05$) using Duncan's multiple range test.

compared to wild type YN201732 and the positive control, and the control effect was more than 65.46%. Starting from 14 days, compared to the positive control 50% WG, the control effect of other treatments began to decrease. The disease indexes of YN201732M2 and YN201732M3 were significantly lower than the wild-type YN201732 treatment. During days 21 to 30, mutants YN201732M2, YN201732M3, and wild-type YN201732 had no significant difference in disease index, but they were still significantly different from the negative control.

DISCUSSION

The lipopeptide compounds surfactin, iturin, and fengycin by *B. amyloliquefaciens* are considered to be the main antifungals against plant pathogens (Ongena et al., 2007; Ongena and Jacques, 2008; Li et al., 2019). Grady et al. (2019) purified two antimicrobial lipopeptides (surfactin B and surfactin C) from the fermentation broth of *B. velezensis* strain 9D-6, which inhibited *in vitro* growth of prokaryotic and eukaryotic pathogens. In our study, the plate antagonism test showed that only n-butanol extraction component had potential antagonistic activity against *F. solani*. This component was identified as the lipopeptide bacillomycin D by LCMS-IT-TOF analysis. Lipopeptide bacillomycin D produced by *B. amyloliquefaciens* FZB42 has strong antifungal activity against *F. graminearum*, can cause morphological changes in the plasma membrane and cell wall of *F. graminearum*, and induce reactive oxygen species, further accumulation eventually leads to cell death (Gu et al., 2017). In addition, the main active component of the ethyl acetate extract was detected by mass spectrometry (results not shown), but the effect of surfactin against pathogenic fungi was poor, consistent with previous report (Peypoux et al., 1999). As a biosurfactant, it is related to the limping movement of bacteria and biofilm formation. It has obvious antiviral, antitumor, and antimycobacterial activity, and its antifungal activity is poor. No fengycin compounds were detected in the mass spectrometry results of different solvent-extracted components in this test, but fengycin synthesis-related genes were cloned from the genome. It is speculated that this may be caused by the loss of fengycin during the isolation and purification process. Further research is needed to find underlying reasons.

The results of liquid phase and liquid chromatography-mass spectrometry showed that the compound bacilllomycin D may be the main active substance of bacterial strain YN201732 against *F. solani*. However, due to the complex composition of the fermentation liquid of *Bacillus* and the inclusion of various inorganic and organic compounds, a single isolation and purification method cannot completely separate these large molecular proteins and small molecular compounds. Therefore, the mechanism by which *B. amyloliquefaciens* YN201732 inhibits pathogenic fungi was needed to be further verified by gene knockout. We successfully knocked out three genes related to the synthesis of antimicrobial lipopeptides in endophyte YN201732, the 4'-phosphopantetheinyl transferase gene *sfp*, and the compound bacillomycin D and fengycin synthesis genes *bmyA* and *fenB*. Antagonistic results of the mutants and the indicator fungus *F. solani* showed that the *sfp* gene mutant YN201732M1 lost its antagonism ability, and the pathogenic fungi and strains had almost no inhibitory zones, while the bacillomycin D mutant YN201732M2 antagonism capacity decreased by about 72.44%. Fengycin antagonism was slightly reduced, but it was not significantly different from the wild type. The above results indicate that lipopeptides are the main antagonists that inhibit pathogenic fungi, and bacillomycin D produced by *B. amyloliquefaciens* YN201732 is the main active substance that inhibits the growth of *F. solani*. Previously, both fengycin and bacillomycin D were detected in the culture extract of strain *B. subtilis* 49, with antifungal activities demonstrated against *F. graminearum* and *Sclerotinia sclerotiorum* (Ramarathnam et al., 2007).

Under laboratory conditions, when *B. amyloliquefaciens* grows statically in the culture medium, a biofilm is formed at the interface between the liquid surface and the air (Donlan and Costerton, 2002; Flemming and Wingender, 2010). Pellicle biofilm greatly explains the bacteria's ability to resist adverse external conditions (Douarche et al., 2015). Three mutants in this study had a certain delay in biofilm formation compared to wild-type strains. The delay in YN201732M1 biofilm formation was the highest, indicating that the 4'-phosphopantetheinyl transferase (*sfp*) not only regulates the synthesis of lipopeptides, it also affects the formation of biofilms. It is known that bacterial biofilm formation ability is closely related to its colonization ability (Xu, 2014), so these active compounds not only kill pathogens by direct antagonizing but also affect their colonization ability through biofilm formation of biocontrol bacteria.

Powdery mildew caused by *E. cichoracearum* seriously affects the yield and quality of tobacco leaves, and once it occurs, it often results in huge economic losses (Glawe, 2008; Meng, 2014). Recent research found that *B. amyloliquefaciens* YN201732 has a good therapeutic and protective effect on tobacco powdery mildew, and the fermentation broth of the strain has a certain inhibitory effect on spore germination (Jiao et al., 2020), but the specific mechanism is still unclear. In this study, bacillomycin D, a lipopeptide compound that inhibits the activity of pathogenic fungi, was isolated and proved to play a major role in antagonizing the indicator pathogen *F. solani*. At the same time, the mutants were used to analyze the effect of the fermentation filtrate on the spore germination. The *sfp* gene mutant YN201732M1 had a poor inhibitory effect on the conidia germination of *E. cichoracearum*. Further analysis found that the spore germination inhibition rate of mutants YN201732M2 and mutant YN201732M3 was reduced, while the germination rate of YN201732M2 spores was significantly different from the wild type, and it was speculated that fengycin and bacillomycin D has an effect on the spore germination, but bacillomycin D has a profound effect. Romero et al. (2007) reported that the iturin and fengycin families of lipopeptides are the key factors for *B. subtilis* to antagonize *Podosphaera fusca* of powdery mildew of Cucurbitaceae, the lipopeptide components had a strong inhibition effect on the conidial germination of *P. fusca*. Greenhouse experiments for mutants also showed that the *sfp* gene YN201732M1 mutant had no control effect, indicating that secretion of lipopeptides is one of the main mechanisms of disease control. The effect of mutant YN201732M2 and mutant YN201732M3 is lower than the

wild type and the control effect is equivalent, which means that fengycin and bacillomycin D are the main substances to display inhibition. Bacillomycin D and fengycin antibiotic gene clusters were found by He et al. (2013) in the genome of *B. amyloliquefaciens* B9601-Y2, and MALDI-TOF-MS mass spectrometry detected the cyclic peptide compound bacillomycin D (m (z/z peak at 1031.5-1097.2) and fengycin (m/z peak at 1433.6-1543.2). It was found that bacillomycin D and fengycin are the main antifungal substances, and the two can enhance the antifungal activity of B9601-Y2 through coordination. The results of this study are consistent with previous studies; bacillomycin D and fengycin play an important role in inhibiting the activity of pathogenic fungi. Xu et al. (2013) found for the first time that bacillomycin D can affect the biofilm formation and rhizosphere colonization of *B. amyloliquefaciens* SQR9 and play a major role in antagonizing *F. oxysporum*.

CONCLUSIONS

In this study, crude lipopeptides were obtained from fermentation broth of *B. amyloliquefaciens* YN201732 using acid precipitation and organic solvent extraction methods. Only the n-butanol extracted components had antagonistic activity against indicator pathogen *F. solani*. Through LCMS-IT-TOF analysis, we confirmed that bacillomycin D is the main active substance of endophyte YN201732 against *F. solani*. Combining with the gene knockout method, it was found that the *sfp* gene knockout mutant YN201732M1 inhibited the spore germination and significantly reduced the ability to prevent and control powdery mildew, while the control effects of the *bmyA* gene knockout mutant YN201732M2 and the *fenB* gene knockout mutant YN201732M3 were both lower than the wild type indicating production of antimicrobial lipopeptides by bacteria YN201732 is one of the main mechanism responsible for the management of tobacco powdery mildew. In addition, we also confirmed that mutants of genes related to compounds synthesis also affect biofilm formation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

RJ carried out the conceptualization, data curation, formal analysis, investigation, methodology, funding acquisition, project administration, resource management, supervision, validation, software curation, visualization, and writing (original draft, review, and editing). PFH carried out the conceptualization, data curation, formal analysis, investigation, funding acquisition, project administration, resource management, supervision, validation, software curation, visualization, and writing (original draft, review, and editing). SM carried out the conceptualization, data curation, formal analysis, investigation, methodology, validation, software curation, visualization, and writing (original draft, review, and editing). YW carried out the funding acquisition, project administration, resource management, and supervision. JW carried out the funding acquisition, project administration, resource management, and supervision. MX carried out the conceptualization, data curation, formal analysis, investigation, and methodology. PBH carried out the conceptualization, data curation, formal analysis, investigation, and methodology. HY carried out the conceptualization, resource management, formal analysis, supervision, and methodology. GW carried out the conceptualization, project administration, formal analysis, supervision, and methodology. SK carried out the writing (original draft, review, and editing). YX carried out the funding acquisition, project administration, resource management, supervision, investigation, and methodology. YC carried out the conceptualization, data curation, formal analysis, funding acquisition, investigation, and methodology. YH carried out the conceptualization, data curation, formal analysis, investigation, methodology, funding acquisition, project administration, resource management, supervision, validation, software, visualization, and writing (original draft, review, and editing). All authors contributed to the article and approved the submitted version.

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Cytospora and *Diaporthe* Species Associated With Hazelnut Canker and Dieback in Beijing, China

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Hazelnut (*Corylus heterophylla* Fisch.) is an important nut crop in China but has been declining owing to the destructive effects of fungal branch canker and dieback. The identification and management of these pathogens are difficult because of the lack of attention to branch canker, insufficient understanding of phylogenetic, and overlapping morphological characteristics of the pathogens. In total, 51 strains were isolated from Chinese wild hazelnut in this study, and three species of *Cytospora* and two of *Diaporthe* were identified through morphological observation and multi-locus phylogenetic analyses (ITS, *act*, *rpb2*, *tef1-α*, and *tub2* for *Cytospora*; ITS, *cal*, *his3*, *tef1-α*, and *tub2* for *Diaporthe*). Three new species, *Cytospora corylina*, *C. curvispora*, and *Diaporthe corylicola*, and two known species, *Cytospora leucostoma* and *Diaporthe eres*, grew at 5–30°C and a pH of 3.0–11.0, with optimum growth at approximately 25°C and pH 4.0–7.0. Additionally, the effects of six carbon sources on mycelial growth were investigated. This study explored the main pathogenic fungi species of *Corylus heterophylla*, completed the corresponding database of pathogenic fungi information, and clarified their biological characteristics. Moreover, the results of this study provided a theoretical basis for *Corylus heterophylla* disease management and prevention in China.

Keywords: Ascomycota, biological characterization, Diaporthales, mycelial growth, phylogeny, taxonomy

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INTRODUCTION

Branch canker and dieback are important forest diseases caused by fungal pathogens in the phylum Ascomycota, especially those in the genera *Cytospora* (Cytosporaceae, Diaporthales) and *Diaporthe* (Diaporthecae, Diaporthales) (Adams et al., 2005; Rossman et al., 2007; Gomes et al., 2013; Udayanga et al., 2015; Fan et al., 2020). In total, 672 species epithets of *Cytospora* have been recorded in the Index Fungorum (April 2021; www.indexfungorum.org/). Recent studies reported that approximately 150 species of *Cytospora* caused branch canker and dieback on more than 130 woody host species (Spielman, 1985; Adams et al., 2002; Adams et al., 2005; Kirk et al., 2008; Fan et al., 2014a; Fan et al., 2014b; Fan et al., 2020; Pan et al., 2020; Pan et al., 2021). More than 1,137 species epithets of *Diaporthe* have been enumerated in the Index Fungorum (April 2021; www.indexfungorum.org/), and over 200 species have been accepted recently (Udayanga et al., 2011; Dissanayake et al., 2017; Yang et al., 2020). These species are pathogenic, endophytic, or saprobic to a wide range of plant hosts (Adams et al., 2002; Adams et al., 2005; Udayanga et al., 2011; Fan et al., 2020). Some of these infect the stems, branches, twigs, and even roots of many commercial plants,

and cause necrotic damage to young tissues, canker and dieback on branches, and, ultimately, the death of the host, resulting in serious economic losses (Fotouhifar et al., 2010; Udayanga et al., 2011; Udayanga et al., 2012a; Udayanga et al., 2012b; Guerrero and Pérez, 2013b). Symptoms of infected branches include fruiting bodies that immersed in the bark and erupted through the bark surface when mature; however, symptoms are not always the same (Santos et al., 2010; Jiang et al., 2020; Pan et al., 2020). Previous studies on species identification were conducted based on host affiliation and morphology, but different species can infect one host, and single species can infect multiple hosts (Adams et al., 2005; Yang et al., 2020; Zhu et al., 2020). Thus, accurate species identification requires polyphasic approaches based on ecology, additional morphological observations, and multi-locus phylogeny analyses (Harrington and Rizzo, 1999; Mostert et al., 2001; Adams et al., 2002; Adams et al., 2005; Udayanga et al., 2014a; Udayanga et al., 2014b; Du et al., 2016).

Hazelnut (*Corylus* spp.), a common tree that is extensively distributed in Asia, Europe, and North America, has important economic and nutritional value (Özdemir et al., 2001). *Corylus heterophylla* has been widely cultivated in China for centuries. More than four million acres of natural hazel grow in northern China with an annual yield of over 23,000 tons (Hu, 2016a). However, recently, global hazelnut production has declined because of the destructive effects of branch canker (Linaldeddu et al., 2016). Eastern filbert blight, one of the most destructive diseases of *Corylus americana*, was caused by *Anisogramma anomala*, which became commercially important in the 1970s (Gottwald and Cameron, 1980; Pinkerton et al., 1992; Chen et al., 2007). *Cytospora corylicola* has been recognized as a rot agent in European hazelnuts in Italy (Servazzi, 1950; Graniti, 1957; Salerno, 1961; Guerrero and Pérez, 2013a; Guerrero and Pérez, 2013b). Several fungal pathogens have been reported to cause canker and dieback of *Corylus avellana*, including *Anthostoma* (Diatrypaceae), *Diaporthe (Diaporthaceae)*, *Diaporthella* (Gnomoniaceae), *Diplodia*, *Dothiorella* (Botryosphaeriaceae), and *Gnomoniopsis* (Gnomoniaceae) (Guerrero and Pérez, 2013a; Guerrero and Pérez, 2013b; Linaldeddu et al., 2016; Wiman et al., 2019). However, these studies mainly focused on canker and dieback of European hazelnuts, and only the genera *Erysiphe* and *Trichothecium* have been recorded in China (Sun, 2013; Hu, 2016b). *Cytospora coryli* and *Diaporthe coryli*, pathogens of *Corylus mandshurica*, have been collected from Beijing and Shaanxi Province, and are of great significance for research on wild hazelnuts in China (Yang et al., 2020; Zhu et al., 2020). As *Corylus heterophylla* is the main source of hazel products in the Chinese market (Liu et al., 2014a; Liu et al., 2014b), and given the importance of *Corylus* species in the Chinese economy, the fungal pathogens associated with canker and dieback of *C. heterophylla* need to be investigated.

During an investigation of cognitive practices in Beijing, China, 51 strains were isolated from the symptomatic stems and branches of *Corylus heterophylla*. The purpose of this study was to identify these strains using polyphasic approaches and supplement a multi-locus DNA dataset of the pathogens of Chinese wild hazelnut. Additionally, the influences of temperature, pH, and six carbon sources on mycelial growth were determined to evaluate the possible role of these conditions in fungal growth.

MATERIALS AND METHODS

Sampling and Isolation

During the investigation of cognitive practices (June to August, 2019), more than 70 specimens were collected from stems and branches of *Corylus heterophylla* in Huairou District of Beijing, China. These infected stems and branches were collected from three nurseries, expressed typical canker and dieback symptoms with fruiting bodies immersed and erupted through the bark surface when mature (**Figure 1**). Twenty-five specimens were selected and taken to laboratory, observed using a stereo microscope (M205 FA) (Leica microsystem, Wetzlar, Genmany). A total of 51 strains were established by transferring the ascospores or conidial masses from the fruiting bodies on to the surface of PDA (1.8% potato dextrose agar, potato 20 g, dextrose 20 g, agar 17 g, distilled water to complete 1,000 ml) plates with diameter 90 mm. The strains were incubated in darkness at 25°C for 24 h until spores germinated. Single germinating spores were moved to new PDA plates. Specimens have been maintained at the Museum of the Beijing Forestry University (BJFC) and the working Collection of X.L. Fan (CF), housed at the Beijing Forestry University. Living cultures are deposited in the China Forestry Culture Collection Centre (CFCC).

Morphological Observations

Species identification was based on morphological features of the fruiting bodies, which was produced on the infected plant tissues, including stromata (arrangement and size), conceptacle (presence or absence), conidiomata (color, size, and shape), ostioles (number and diameter), locules (number and arrangement type), conidiophores, and conidia (size and shape), supplemented by cultural characteristics such as colony color, texture, and the presence or absence of airborne hyphae (Mostert et al., 2001; Zhang et al., 2007; Zhu et al., 2020). The morphological features were observed under a Leica stereomicroscope (M205 FA) (Leica microsystem, Wetzlar, Genmany). Micro-morphological observations determined by a Nikon Eclipse 80i compound microscope. Measuring 30 conidiomata/ascomata and 50 conidia/ascospores, determined by length, width, and length/width ratio (L/W ratio). Recording the colony diameters and describing the color was based on the color charts of Rayner (1970) after 1–2 weeks on PDA in darkness. The results were edited manually by Adobe Bridge CS v. 6 and Adobe Photoshop CS v. 5.

DNA Extraction and PCR Amplification

Scrapping the mycelium from the cellophane for DNA extraction after three days dark-incubation at 25°C. Using the modified CTAB method to extract genomic DNA (Doyle and Doyle, 1990). Using a 20 μl system of 10 μl Mix (Promega), 7 μl

FIGURE 1 | Disease symptoms of *Corylus heterophylla*. **(A–C)** Death of the hazel trees caused by *Cytospora* and *Diaporthe* in the orchards. **(D–F)** Conidiomata on a naturally infected stem in the field.

ddH_2O, 1 μl upstream primer, 1 μl downstream primer, and 1 μl template DNA to conduct polymerase chain reaction (PCR) in order to amplify gene fragments. The primers and PCR conditions are set in **Table S1**. Electrophoretic separation was conducted for the PCR amplification products in 2% agarose gels with a DNA maker 2,000 bp (Takara Biotech). Using an ABI PRISM® 3730XL DNA Analyzer with BigDye® Terminater Kit v. 3.1 (Invitrogen) at the Shanghai Invitrogen Biological Technology Company (Beijing, China) to conduct the DNA sequences. In order to acquire a consensus sequence of sequences obtained from forward and reverse primer pairs, Seqman v. 9.0.4 (DNASTAR Inc., Madison, WI, United States) was used.

Phylogenetic Analyses

Using MAFFT v. 6 (Katoh and Standley, 2013) to align ITS sequence data and editing it manually with MEGA v. 6.0 (Tamura et al., 2013), the current strains were preliminarily identified as *Cytospora* and *Diaporthe* species. To clarify their further phylogenetic position, five genes (ITS, *act*, *rpb2*, *tef1-α*, and *tub2* for *Cytospora*; ITS, *cal*, *his3*, *tef1-α*, and *tub2* for *Diaporthe*) for each genus were combined and aligned to compare with other strains in GenBank secondly. Generating subsequent alignments for each gene and adjusting them manually. Excluding the ambiguously aligned sequences from analyses. Reference sequences were retrieved from recent publications (Fan et al., 2014a; Fan et al., 2014b; Fan et al., 2015a; Fan et al., 2015b; Lawrence et al., 2017; Fan et al., 2020; Pan et al., 2020; Zhu et al., 2020; Pan et al., 2021). *Diaporthe vaccinii* (CBS 160.32) was included as the outgroup in *Cytospora* analysis (**Table S2**) and *Diaporthella corylina* (CBS 121124) was included in *Diaporthe* analysis (**Table S3**). All the datasets were performed using PAUP v. 4.0b10 for the maximum parsimony (MP) method (Swofford, 2003), RAxML for the maximum likelihood (ML) method (Stamatakis, 2006), and MrBayes v. 3.1.2 for the Bayesian Inference (BI) method (Ronquist and Huelsenbeck, 2003) for the phylogenetic analyses. All novel sequences derived from this study were deposited in MycoBank (www.mycobank.org) (Crous et al., 2004). All sequences from this study were submitted in GenBank, as shown in **Tables S2** and **S3**. The multi-gene sequence alignment files were deposited in TreeBASE (www.treebase.org; accession number: S276989).

Testing the Influence of Temperature, pH, and Carbon on Mycelium Growth

All 51 strains we collected were identified as three species of *Cytospora* and two of *Diaporthe* through morphological and phylogenetic analyses. We selected representative strains of each species to assess the influences of temperature, pH, and carbon sources on mycelium growth incubated in the dark. A mycelial plug with 5-mm-diameter was transferred on to the center of a 90 mm PDA plate and incubated in an environment of 0–40°C with a 5°C gradient (i.e., 0, 5, 10, 15, 20, 25, 30, 35, and 40°C), three repeats were set for each treatment (Fang, 1979; Zhou et al., 2020). The pH values of the PDA were regulated to a range of 2.0–12.0 using 1 mol/L NaOH and 1 mol/L HCl, to obtain pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0. Taking a mycelial plug to PDA plate with the same methods as temperature tests and incubating in darkness at 25°C, three replicates were also set. The strains of the five species were incubated in darkness at 25°C on PDA (the 20 g dextrose were replaced by 20 g fructose, galactose, maltose, sucrose, or xylose) plates to assess the utilization of these compounds as carbon sources (Zhao et al., 2019; Zhou et al., 2020). The colony diameter was measured in millimeters every 24 h for four days of incubation, and converting the data to radial growth to assess the effects of temperature, pH, and carbon sources on mycelial growth (Zhao et al., 2019). All data were analyzed with one-way ANOVA and Post Hoc of LSD and Tukey using IBM SPSS Statistics v. 22.0 (IBM Inc., Armonk, NY, USA). A p-value < 0.05 was considered significant. Graph with SE-bar were conducted in order to explain the difference in mycelium growth under different conditions.

RESULTS

Isolation

During the investigation of cognitive practices, we isolated a total of 51 strains from infected stems and branches of hazelnut trees (**Table S4**). Among the 51 strains, 14 strains were identified as *Cytospora* species (37%) and 37 strains were *Diaporthe* (73%). *Diaporthe corylicola* is the main species observed on *Corylus heterophylla* (64.71%), followed by *Cytospora curvispora* (11.76%). The rest were identified as *Cytospora corylina*, *Cytospora leucostoma*, and *Diaporthe eres*, four strains of each species, accounting 7.84% of total, respectively.

Phylogeny

Totally 14 strains of *Cytospora* obtained from hazelnut and other 224 strains retrieved from recent publications were used in the phylogenetic analyses (Spielman, 1985; Adams and Taylor, 1993; Adams et al., 2002; Adams et al., 2005; Lawrence et al., 2017; Fan et al., 2018; Fan et al., 2020; Jiang et al., 2020; Pan et al., 2020; Zhou et al., 2020; Zhu et al., 2020; Pan et al., 2021). The sequence datasets for the five genes (ITS, *act*, *rpb2*, *tef1-α*, and *tub2*) were performed in individual and combined analyses. The single gene region analysis is very similar to the tree topologies of the combined analyses. The phylogram generated here indicated 237 ingroup taxa including 3,686 characters (622 for ITS, 457 for *act*, 1,076 for *rpb2*, 775 for *tef1-α*, and 756 for *tub2*) in the multi-locus analyses, of which 2,010 characters were constant, 191 variable characters were parsimony uninformative and 1,485 characters were parsimony informative. MP analysis generated 200 parsimonious trees (TL = 9,878, CI = 0.307, RI = 0.812, RC = 0.249) and the first one is selected and shown in **Figure 2**. All trees of ML and BI analyses were in agreement and no significant difference with MP tree. MP/ML-BS (MP/ML bootstrap support) ≥50% were shown, and branches with BPP (BI posterior probability) ≥0.95 were thickened in the phylogram.

Thirty-seven strains of *Diaporthe* from hazelnut were aligned with 253 strains supplement from recent publications. The five genes (ITS, *cal*, *his3*, *tef1-α*, and *tub2*) in individual and combined were used to clarify the phylogenetic position of these *Diaporthe* species. The tree topologies generated by the MP, ML, and BI analyses were similar. All strains counted 2,983 characters including gaps (613 for ITS, 587 for *cal*, 578 for *his3*, 645 for *tef1-α*, and 560 for *tub2*), of which 1,233 characters are constant, 360 variable characters are parsimony uninformative 1,390 characters are parsimony informative. MP analysis generated 100 parsimonious trees (TL = 1,826, CI = 0.267, RI = 0.788, RC = 0.210), the first one is selected and presented in **Figure 3**. The support values were shown and branches were thickened in the phylogram at the same standard as **Figure 2**.

The current 14 strains of *Cytospora* clustered in three clades representing three species, of which one was known as *C. leucostoma* and two were distinguished from all other known taxa with high support values (MP/ML/BI = 100/100/1) as two clades. Thus, we described the two clades as *C. corylina*, *C. curvispora* here. Four strains were assigned to *Diaporthe eres*, and the remaining 33 were classified as one new species, *D. corylicola*, representing a monophyletic clade with high support values (MP/ML/BI = 100/100/1).

Taxonomy

Cytospora corylina H. Gao & X.L. Fan, sp. nov. **Figure 4**.

MycoBank MB 838643.

Holotype: CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′16.66″E, 40° 52′52.18″N, from branches of *Corylus heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (**holotype** BJFC CF20210114), ex-type living culture CFCC 54684.

Etymology: Named after the host genus on which it was collected, *Corylus*.

Description: Necrotic tissues on branches of *Corylus heterophylla*. Sexual morph: not observed. Asexual morph: Stromata immersed in bark. Pycnidial stromata ostiolated, scattered, erupted slightly through the bark surface. Multiple locules, conceptacle absent. 850–1,280 (av. = 913, n = 30) μm in diameter. Ectostromatic disc gray with one ostiole in center, discoid, circular to ovoid, 180–270 (av. = 214, n = 30) μm in diameter. Ostiole slight-brown, inconspicuous, at the same level as the disc surface. 110–185 (av. = 135, n = 30) μm in diameter. Locules numerous, circular to irregular with common walls generally invaginated. Conidiophores branched, occasionally unbranched, hyaline, approximately cylindrical,

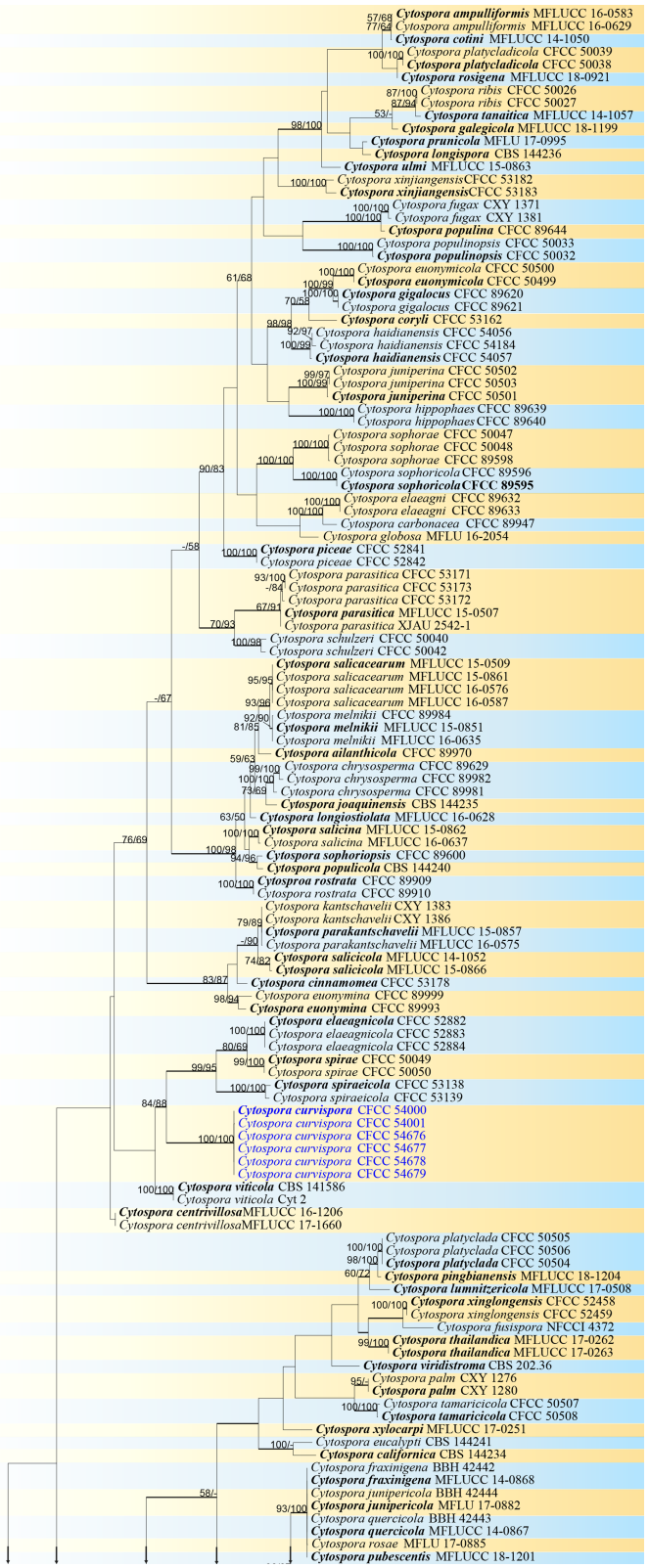

FIGURE 2 | Continued

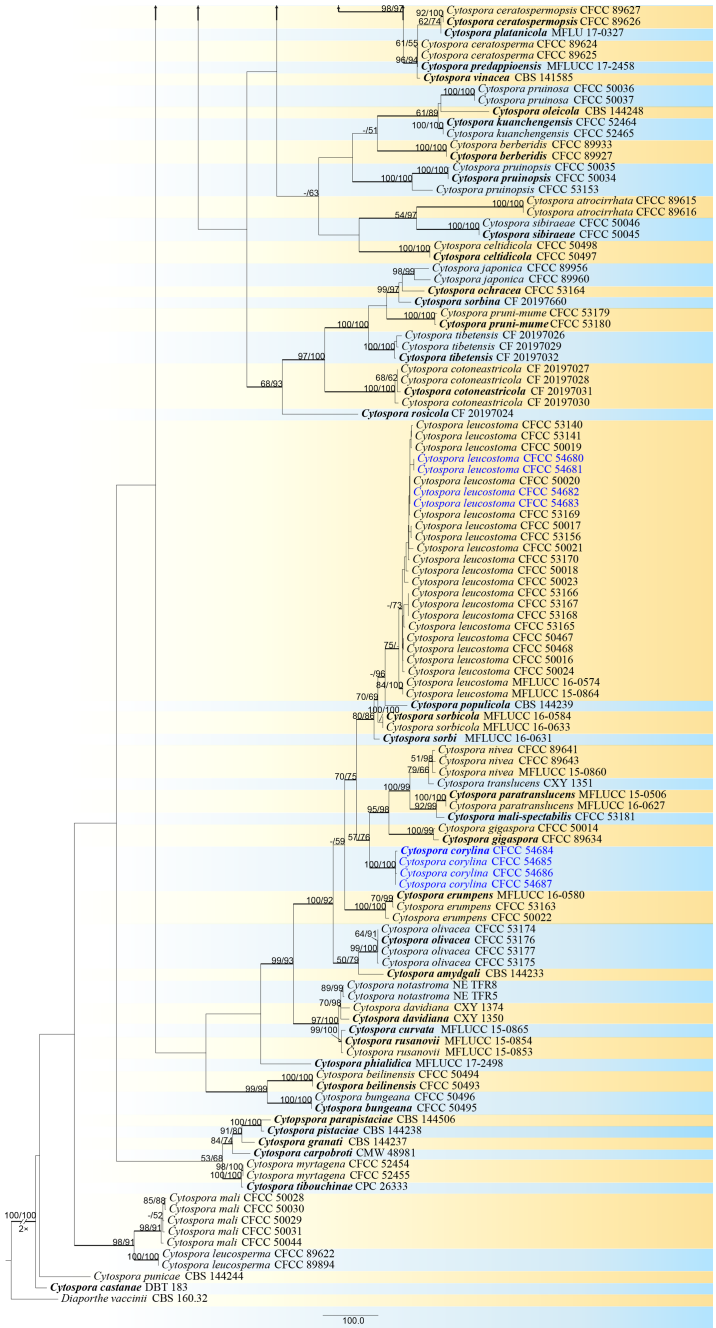

FIGURE 2 | Phylogram of *Cytospora* based on combined five genes (ITS, *act*, *rpb2*, *tef1-α*, and *tub2*). MP/ML-BS ≥50% are shown at first/second positions. Branches with BPP ≥0.95 are thickened. Strains in this study are in blue. Ex-type strains are in bold.

8.0–13.5 × 1.0–2.5 (av. = 10.8 ± 1.6 × 1.6 ± 0.4, n = 30) μm, sometimes reduced to conidiogenous cells. Conidiogenous cells enteroblastic, phialidic, subcylindrical to cylindrical. Conidia hyaline, allantoid, smooth, aseptate, thin-walled, 3.5–7.5 × 1.0–1.5 (av. = 5.8 ± 0.8 × 1.3 ± 0.2, n = 50) μm, L/W ratio 3.61–4.52 (av. = 4.27 ± 0.17).

Culture characteristics: Colonies are initially white, reaching 90 mm after two days, slightly fawn in center and becoming nearly fucous black after 30 days. Colonies concentric circles, with a thick texture, and aerial mycelium lacked. Conidiomata distributed on PDA surface irregularly surrounded by dark mycelium (**Figure 9A**).

Other specimens examined: CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′21.62″E, 40°52′47.58″N, from branches of *Corylus heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC

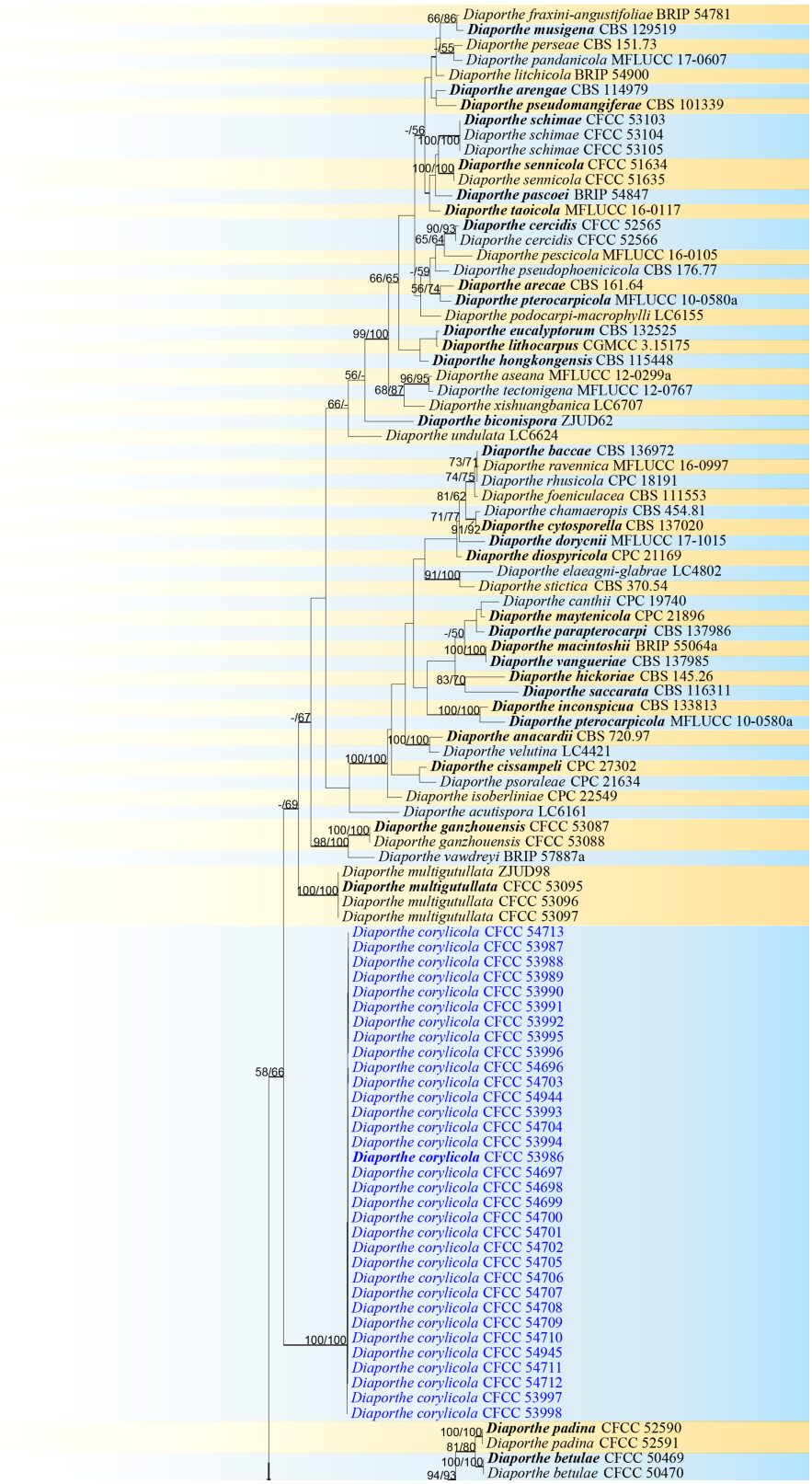

FIGURE 3 | Continued

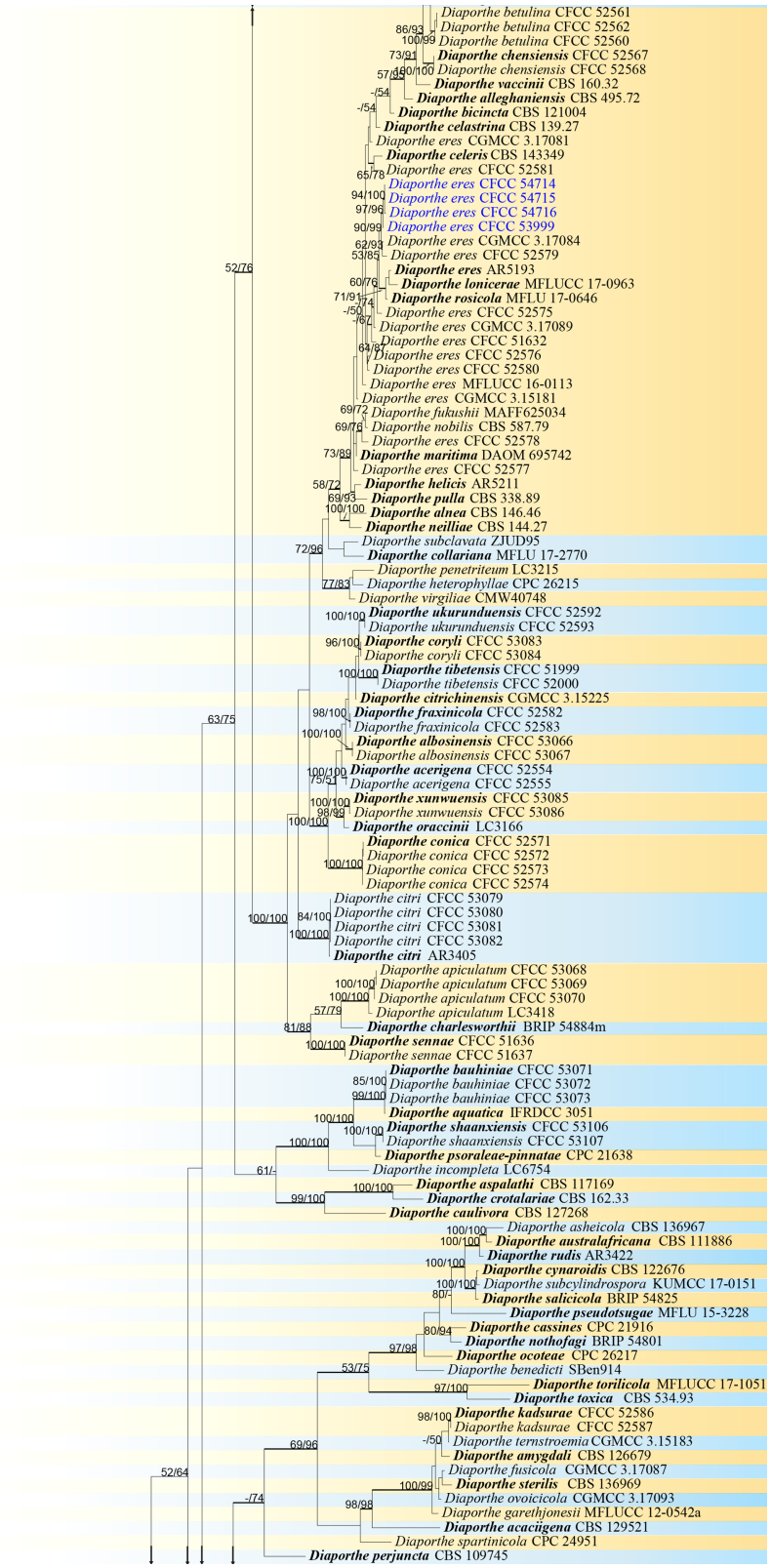

FIGURE 3 | Continued

FIGURE 3 | Phylogram of *Diaporthe* based on combined five genes (ITS, *cal*, *his3*, *tef1-α*, and *tub2*). MP/ML-BS ≥50% are shown at first/second positions. Branches with BPP ≥0.95 are thickened. Strains in this study are in blue. Ex-type strains are in bold.

C20210115), living culture CFCC 54685 and CFCC 54686; *ibid.* BJFC CF20210116, living culture CFCC 54687.

Notes: *Cytospora corylina* was collected from *Corylus heterophylla* in Beijing, China. It is significantly different from *Cytospora coryli* isolated by Zhu et al. (2020) in morphology. *Cytospora coryli* has a flat conidiomata and inconspicuous ostiole. In terms of culture morphology, The colonies of *C. coryli* are brown, conidiomata distributed radially on colony surface. The four strains representing *Cytospora corylina* clustered as a single lineage and appeared to be the most

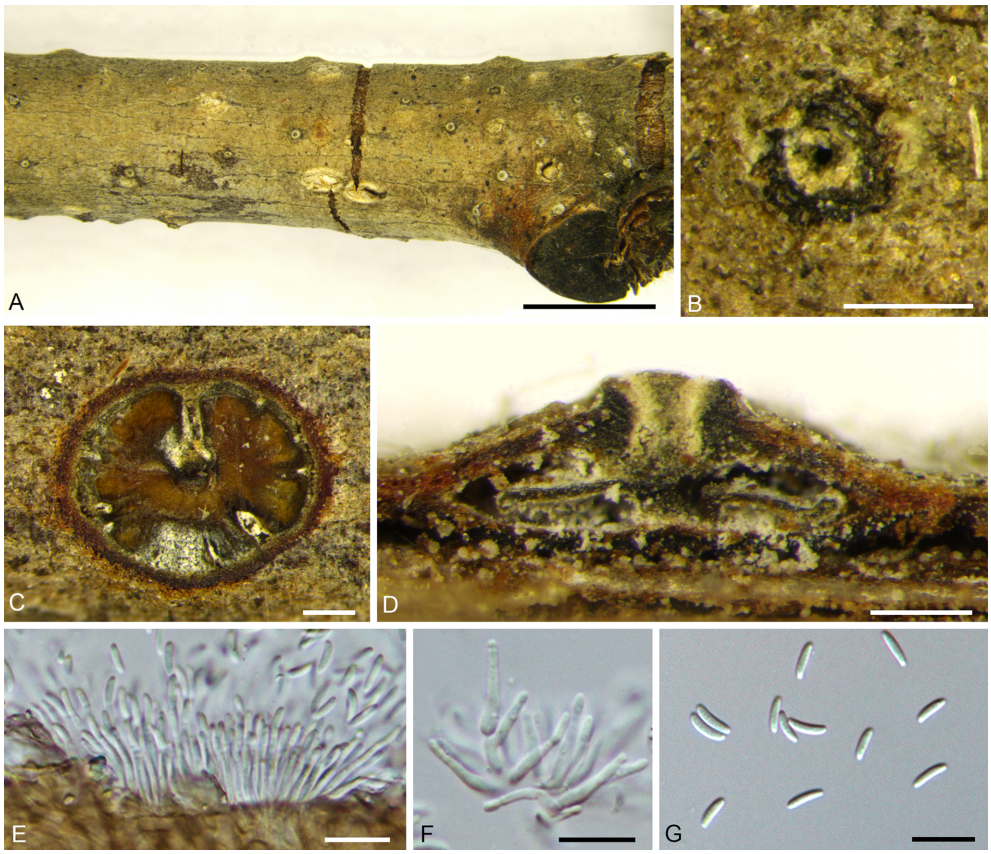

FIGURE 4 | *Cytospora corylina* from *Corylus heterophylla*. **(A, B)** Habit of conidiomata on twig. **(C)** Transverse section of conidioma. **(D)** Longitudinal section through conidioma. **(E, F)** Conidiophores and conidiogenous cells. **(G)** Conidia. Scale bars: 3 mm **(A)**; 200 μm **(B–D)**; 10 μm **(E–G)**.

closely related to *C. gigaspora*, *C. mali-spectabilis*, *C. nivea*, *C. paratranslucens*, and *C. translucens*, with support values of MP/ML/BI = 57/76/0.99. However, this strain could be distinguished from the five most related species by the conidiomata (*C. gigaspora* with a flat locule, *C. mali-spectabilis* with a column lenticular tissue in the center, and *C. nivea* with a dark conceptacle) (Fan et al., 2015b; Pan et al., 2020). In addition, *C. corylina* has a smaller conidia size (3.5–7.5 × 1–1.7 μm) than *C. gigaspora* (8.9–12.1 × 1.9–2.9 μm), *C. mali-spectabilis* (9.0–10.0 × 1.5–2 μm), *C. nivea* (6.2–9.2 × 1.7–2.4 μm), and *C. paratranslucens* (6.5–7.3 × 1.3–1.5 μm) (Adams et al., 2006; Fan et al., 2015b; Norphanphoun et al., 2017; Pan et al., 2020). Therefore, we describe this species as novel based on DNA sequence data and morphology.

Cytospora curvispora H. Gao & X.L. Fan, sp. nov. **Figure 5**. MycoBank MB 838641.

Holotype: CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26'23.64"E, 40° 52'48.37"N, from branches of *Corylus heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (**holotype** BJFC CF20210110), ex-type living culture CFCC 54000.

Etymology: Named after the character of its curved conidia.

Description: Necrotic tissues on stems and branches of *Corylus heterophylla*. Sexual morph: not observed. Asexual morph: Stromata immersed in bark. Conidiomata pycnidial, scattered, conical, erupted through the bark surface when mature, locules multiple. Conceptacle absent, diameter 1,080–1,700 (av. = 1,423, n = 30) μm. Ectostromatic disc black-brown, discoid, circular to ovoid, 480–660 (av. = 526, n = 30) μm in diameter, with one ostiole. Ostiole dark-gray to black, inconspicuous, slightly curved, at the same level as the disc surface, 40–70 (av. = 58, n = 30) μm in diameter. Locules numerous, circular to irregular with common wall generally invaginated. Conidiophores unbranched, barely branched, hyaline, approximately cylindrical, 9.5–14.0 × 1.0–1.5 (av. = 12.5 ± 1.3 × 1.1 ± 0.3, n = 30) μm, sometimes reduced to conidiogenous cells. Conidiogenous cells enteroblastic, phialidic, subcylindrical to cylindrical. Conidia hyaline, elongateallantoid to falcate, smooth, aseptate, thin-walled, 4.5–8.5 × 1.0–1.5 (av. = 6.7 ± 1.0 × 1.3 ± 0.2, n = 50) μm, L/W ratio 4.88–6.04 (av. = 5.19 ± 0.22).

Culture characteristics: Cultures are initially white, thin, reaching 70 mm after three days, turning slightly honey after 30 days and deepened continuously. Colonies are uniform, aerial mycelium lacked. Conidiomata distributed irregularly on PDA surface (**Figure 9B**).

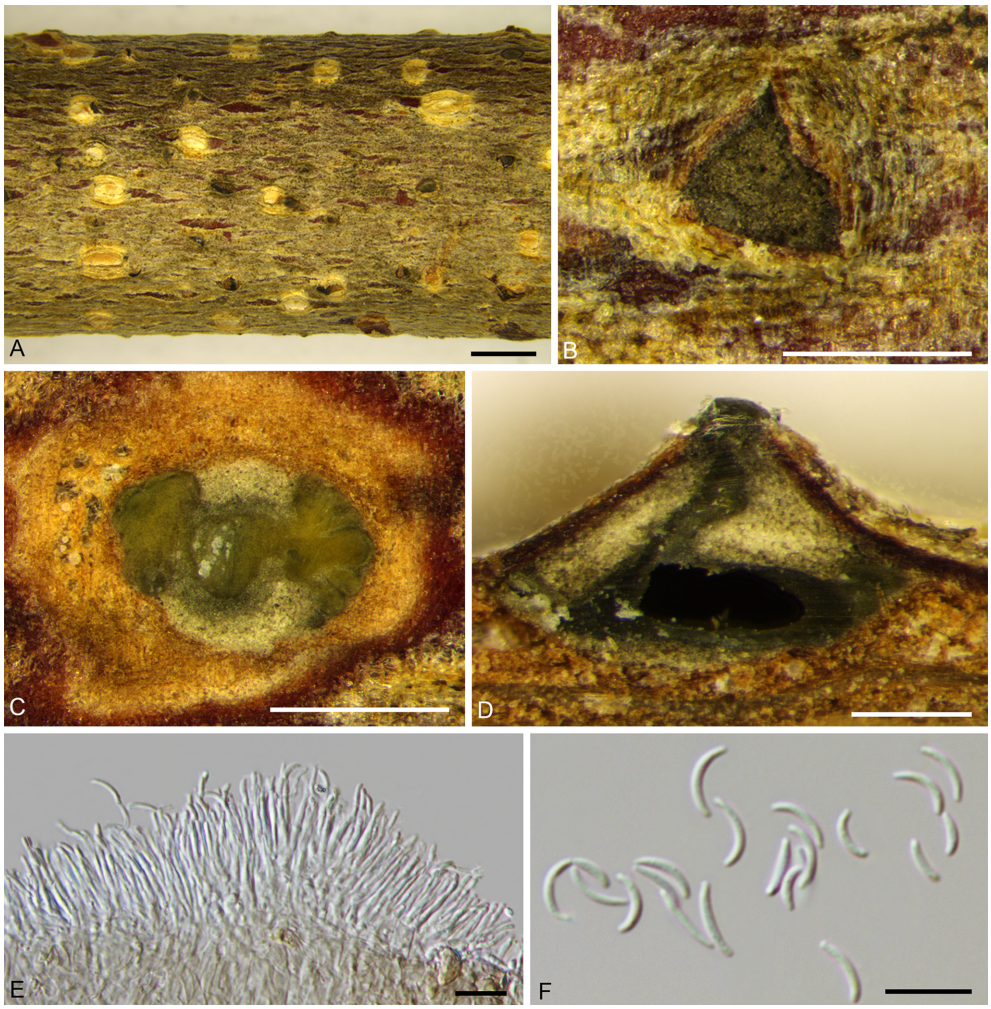

FIGURE 5 | *Cytospora curvispora* from *Corylus heterophylla*. **(A, B)** Habit of conidiomata on twig. **(C)** Transverse section of conidioma. **(D)** Longitudinal section through conidioma. **(E)** Conidiophores and conidiogenous cells. **(F)** Conidia. Scale bars: 2 mm **(A)**; 500 μm **(B–D)**; 10 μm **(E, F)**.

Other specimens examined: CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26'28.24"E, 40°52'48.73"N, from branches of *Corylus heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210111), living culture CFCC 54001; CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26'22.83"E, 40°52'50.57"N, from branches of *C. heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210118), living culture CFCC 54676 and CFCC 54677; CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26'20.22"E, 40°52'41.15"N, from stems of *C. heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210113), living culture CFCC 54678 and CFCC 54679.

Notes: *Cytospora curvispora* was isolated from *Corylus heterophylla* in Beijing, China. We can distinguish it from *C. coryli* by its curved conidia and conical conidiomata. In the phylogeny analysis, *Cytospora curvispora* clustered with *C. elaeagnicola*, *C. spiraeae*, and *C. spiraeicola* with support values of MP/ML/BI = 84/88/0.99. However, differences in their distribution and morphology were identified. *Cytospora spiraeae* and *C. spiraeicola* were isolated from *Spiraea salicifolia*, collected from Gansu Province and Beijing, respectively. *Cytospora elaeagnicola* was collected from branches of *Elaeagnus angustifolia* in the Xinjiang Uygur Autonomous Region. Morphologically, the conidia of *C. curvispora* are more curved and with a smaller width (1.0–1.5 μm for *C. curvispora*, 1.5–2.0 μm for *C. elaeagnicola*, 2.0–2.5 μm for *C. spiraeae*, 2.5–3.5 μm for *C. spiraeicola*). In addition, *C. elaeagnicola* colonies are white and have a thick texture at the center, becoming thinner at the edges; *C. spiraeae* is fawn and felt-like and *C. spiraeicola* is buff to hazel with a heterogeneous texture (Zhu et al., 2018; Zhang et al., 2019; Zhu et al., 2020). Thus, we described this finding as novel based on morphological and phylogenetic analyses.

Cytospora leucostoma (Pers.) Sacc., Michelia, 2: 264, 1881. **Figure 6**.

Description: see Fan et al. (2020).

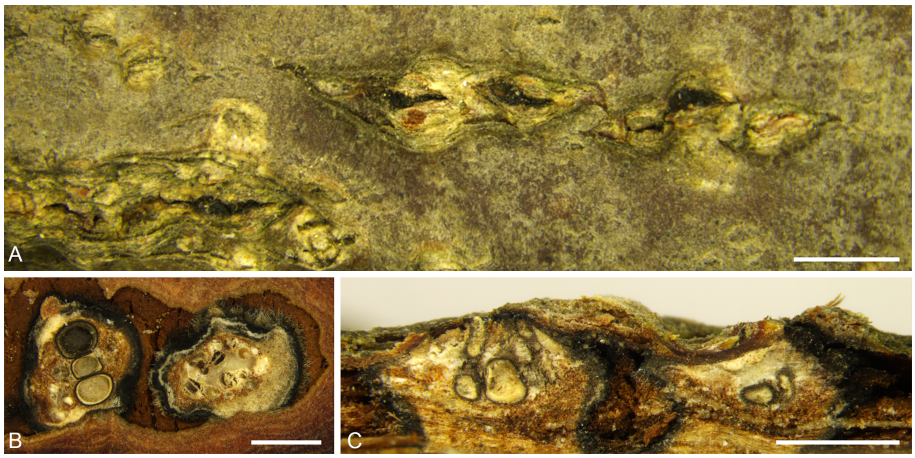

FIGURE 6 | *Cytospora leucostoma* from *Corylus heterophylla*. **(A)** Habit of ascomata on twig. **(B)** Transverse section of ascomata. **(C)** Longitudinal section through ascomata. Scale bars: 2 mm **(A)**; 1 mm **(B, C)**.

Culture characteristics: Colonies are white initially and change to dark green to dark after seven days. Growing up to 80 mm after three days. Colonies have a uniform texture, thick, aerial mycelium lacked (**Figure 9C**).

Specimens examined: CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′15.67″E, 40°52′40.18″N, from branches of *Corylus heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210117), living culture CFCC 54680 and CFCC 54681; CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′36.65″E, 40°52′32.14″N, from branches of *C. heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF2021118), living culture CFCC 54682 and CFCC 54683.

Notes: *Cytospora leucostoma* commonly causes branch canker in Rosaceae in China (Fan et al., 2020; Pan et al., 2020). In the current study, two specimens were collected from the infected branches of the Chinese hazelnut, and fruiting bodies were observed. However, the specimens were not fresh, and little information is available on the micromorphology. Thus, we identified the strains as *C. leucostoma*, based on ascostromata with conceptacle, culture characteristics, and DNA data.

Diaporthe corylicola H. Gao & X.L. Fan, sp. nov. **Figure 7**.

MycoBank MB 838644.

Holotype: CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′18.55″E, 40°52′38.27″N, from stems of *Corylus heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (**holotype** BJFC CF20210121), ex-type living culture CFCC 53986.

Etymology: Named after the host genus on which it was collected, *Corylus*.

Description: Necrotic tissues on stems and branches of *Corylus heterophylla*. Asexual morph: Stromata immersed in bark. Pycnidial stromata ostiolated, scattered, discoid to conical, erumpent slightly through the surface of bark at maturity, with single locule. Conceptacle absent, 750–1,300 (av. = 991, n = 30) μm in diameter. Ectostromatic disc buff or orange with only one ostiole in center, covered by orange discharged conidial masses, discoid, circular to ovoid, 175–270 (av. = 206, n = 30) μm in diameter. Ostiole dark-brown, conspicuous, at the same level as the disc surface, 130–150 (av. = 141, n = 30) μm in diameter. Conidiogenous cells enteroblastic, phialidic, subcylindrical to cylindrical, 16.0–24.0 × 1.5–2.5 (av. = 19.4 ± 2.5 × 2.0 ± 0.2, n = 30) μm. Alpha conidia are aseptate, hyaline, fusiform, multi-guttulate, rarely 2 guttulate and smooth, 11.0–16.5 × 2.0–3.5 (av. = 13.8 ± 1.3 × 2.8 ± 0.3, n = 50) μm, L/W ratio 4.58–5.41 (av. = 4.87 ± 0.19). Gamma conidia hyaline, multi-guttulate, subcylindrical with a nearly rounded apex, 13.0–19.5 × 1.5–2.5 (15.3 ± 1.1 × 1.7 ± 0.2, n = 50) μm, L/W ratio 6.68–12.61 (av. = 8.92 ± 1.28). Beta conidia undetermined.

Culture characteristics: Colonies are white initially, only 25 mm after three days and going to buff after 15 days. Colonies are felty with thick texture, aerial mycelium lacked and conidiomata are randomly distributed fat the marginal area, with orange conidial drops oozing out of the ostioles (**Figures 9D, E**).

Other specimens examined: CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′13.81″E, 40°52′58.11″N, from stems of *Corylus heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210122), living culture CFCC 53987; *ibid.* BJFC CF20210123, living culture CFCC 53988 to 53990; BJFC CF20210134, living culture CFCC 54944 and CFCC 54945; CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′25.25″E, 40°52′29.83″N, from stems of *C. heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210124), living culture CFCC 53991 to CFCC 53993; *ibid.* BJFC CF20210125, living culture CFCC 53994 and CFCC 54704; BJFC CF20210126, living culture CFCC 53995; CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′21.21″E, 40°52′38.02″N, from branches of *C. heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210127), living culture CFCC 53996 to 53998; *ibid.* BJFC CF20210128, living culture CFCC 554696 and CFCC 54697; BJFC CF20210129, living culture CFCC 54698 to CFCC 54701;

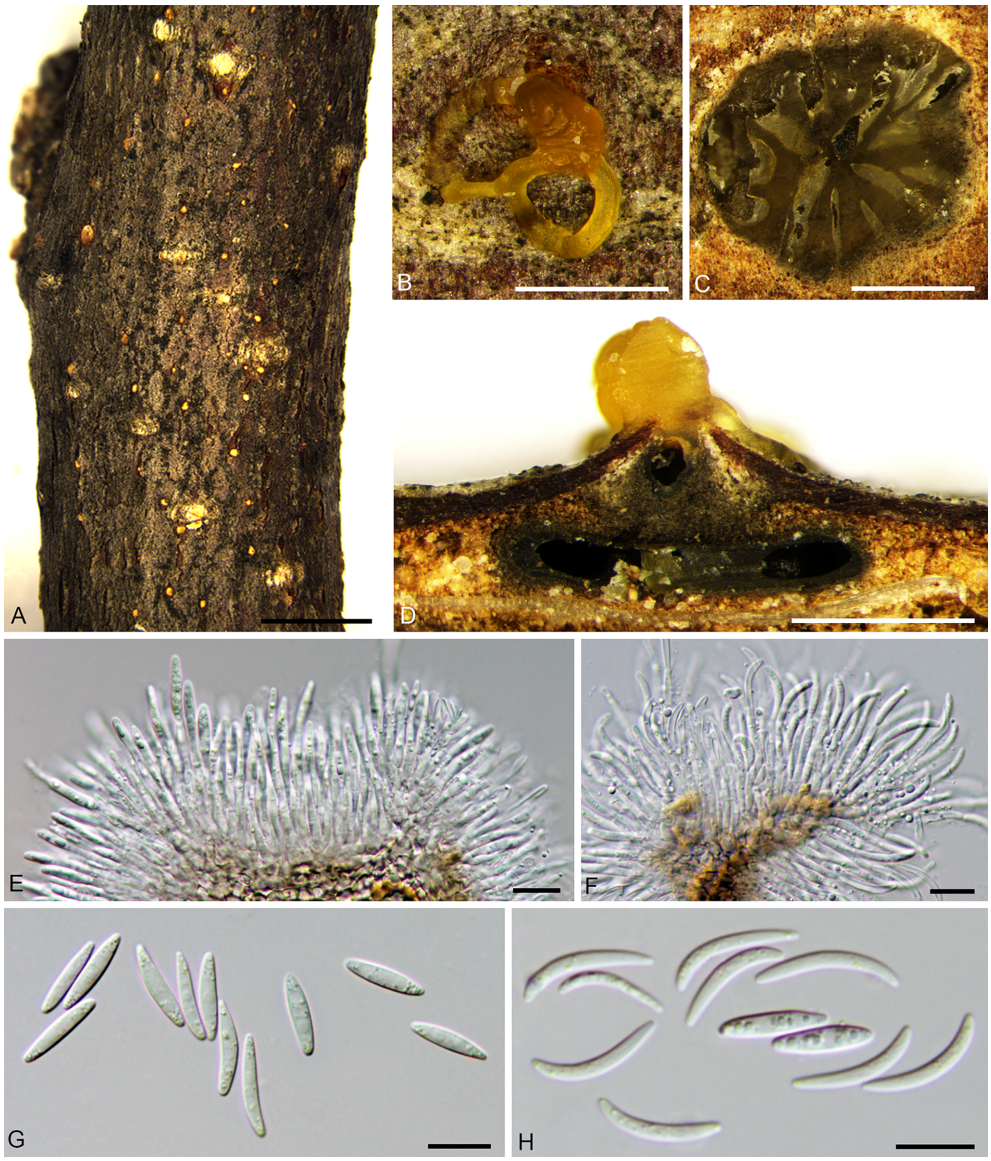

FIGURE 7 | *Diaporthe corylicola* from *Corylus heterophylla*. **(A, B)** Habit of conidiomata on twig. **(C)** Transverse section of conidiomata. **(D)** Longitudinal section through conidioma. **(E, F)** Conidiogenous cells and conidia. **(G, H)** Alpha and gamma conidia. Scale bars: 3 mm **(A)**; 500 μm **(B–D)**; 10 μm **(E–H)**.

BJFC CF20210130, living culture CFCC 54702 to CFCC 54705; CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′28.67″E, 40°52′36.72″N, from stems of *C. heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210131), living culture CFCC 54706 to CFCC 54708; *ibid.* BJFC CF20210132, living culture CFCC 54709 to CFCC 54712; BJFC CF20210133, living culture CFCC 54713.

Notes: *Diaporthe corylicola* was isolated from *Corylus heterophylla* in Beijing, China. The cluster represented a single lineage with high support (MP/ML/BI = 100/100/1). The culture morphology was similar to that of *D. coryli*, which was isolated from *Corylus mandshurica*, but alpha conidia were longer and thinner (11.0–16.5 × 2.0–3.5 *vs*. 11.5–13 × 3–3.5 μm) (Yang et al., 2020). Phylogenetically, there was high contrast between *Diaporthe coryli* and *D. corylicola*, with 36/610 for ITS, 77/584 for *cal*, 63/575 for *his3*, 61/642 for *tef1-α*, and 61/556 for *tub2*. Thus, we describe this species as novel.

Diaporthe eres Nitschke, Pyrenomyc. Germ. 2: 245, 1870. **Figure 8**.

Synonyms are listed in Yang et al. (2018).

Description: Necrotic tissues on stems and branches of *Corylus heterophylla*. Sexual morph: not observed. Asexual morph: Stromata immersed in bark. Pycnidial stromata ostiolated, scattered or serried, discoid to conical, erumpent slightly through the bark surface at maturity, with single locule. Conceptacle absent. 140–380 (av. = 221, n = 30) μm in

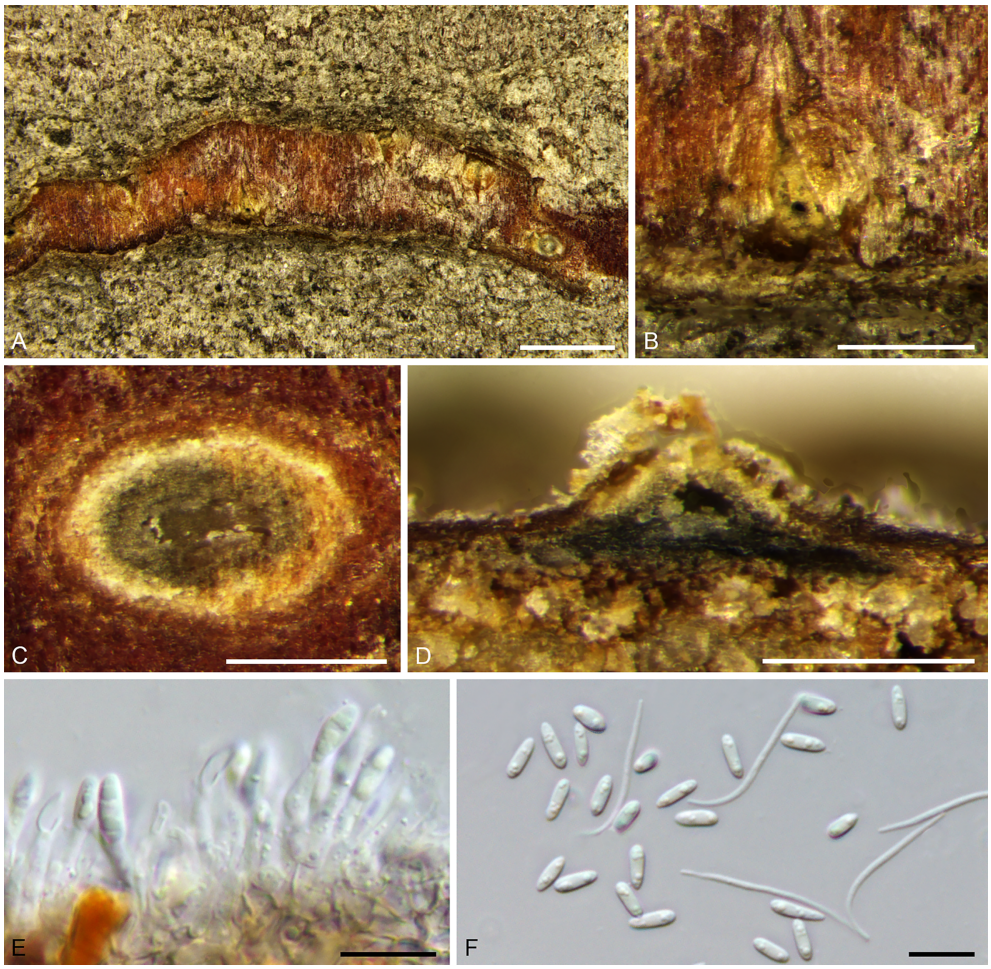

FIGURE 8 | *Diaprthe eres* from *Corylus heterophylla*. **(A, B)** Habit of conidiomata on twig. **(C)** Transverse section of conidioma. **(D)** Longitudinal section through conidioma. **(E)** Conidiogenous cells and conidia. **(F)** Alpha and beta conidia. Scale bars: 2 mm **(A)**; 500 μm **(B–D)**; 10 μm **(E, F)**.

diameter. Ectostromatic disc brown to black with only one ostiole in center, discoid, circular to ovoid, 120–270 (av. = 163, n = 30) μm in diameter. Ostiole dark-grey, conspicuous, at the same level as the disc surface, 20–80 (av. = 56, n = 30) μm in diameter. Conidiogenous cells phialidic, cylindrical, terminal, 7.5–16.0 × 2.0–3.0 (av. = 12.2 ± 2.5 × 2.6 ± 0.3, n = 30) mm. Alpha conidia hyaline, aseptate, ellipsoidal, one guttulate at each end, 5.5–8.5 × 1.5–2.5 (av. = 6.5 ± 0.6 × 2.1 ± 0.2, n = 50) μm, L/W ratio, 2.58–4.75 (av. = 3.14 ± 0.41). Beta conidia hyaline, lanceolate to linear, 12.5–30.5 × 1.0–1.5 (av. = 23.9 ± 4.0 × 1.2 ± 0.2, n = 50) μm, L/W ratio 8.99–32.06 (av. = 19.91 ± 4.23).

Culture characteristics: Colonies with felty aerial mycelium, white, changing to compact at center later and sparse at surrounding. Growing up to 45 mm after three days incubation. Conidiomata sparse, black, distributed irregularly (**Figure 9F**).

Specimens examined: CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′35.53″E, 40°52′47.18″N, from branches of *Corylus heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210119), living culture CFCC 53999 and CFCC 54714; CHINA, Beijing City, Huairou District, Forestry Center of Beijing University of Agriculture, 116°26′41.03″E, 40°52′29.63″N, from stems of *C. heterophylla*, June, 2019, *H. Gao* & *X.L. Fan* (BJFC CF20210120), living culture CFCC 54715 to 54716.

Notes: *Diaporthe eres* were defined first by Nitschke (1870), collected from *Ulmus* sp. in Germany. Udayanga et al. (2014b) described them as a complex and provided a phylogram of seven genes. Phenotypic plasticity and host affiliations have been used for species identification of this complex, but have shown little significance (Udayanga et al., 2014a; Udayanga et al., 2014b; Udayanga et al., 2015; Du et al., 2016; Gao et al., 2016; Gao et al., 2017; Dissanayake et al., 2017). Fan et al. (2018) investigated this complex using a three (*cal*, *tef1-α*, and *tub2*) data matrix and further identified the species. *Diaporthe eres* were identified as pathogens on hazel trees in Oregon (Battilani et al., 2018). The current results indicate four strains of *D. eres* from hazel trees in China.

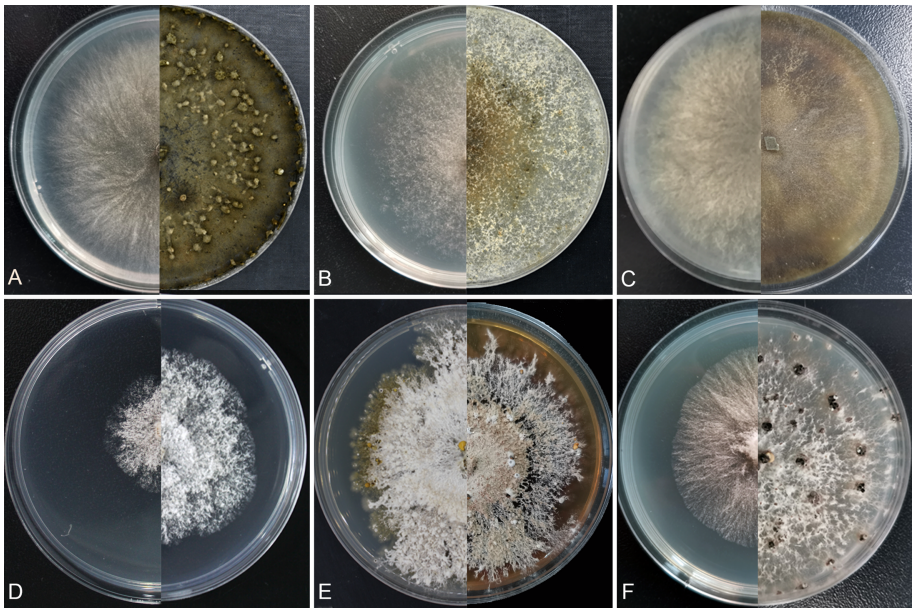

FIGURE 9 | Cultures on potato dextrose agar (PDA). **(A)** *Cytospora corylina*. **(B)** *Cytospora curvispora*. **(C)** *Cytospora leucostoma*. **(D, E)** *Diaporthe corylicola*. **(F)** *Diaporthe eres*. Days of incubation: three days at left and 30 days at right **(A–C)**, **(F)**; three days at left and seven days at right **(D)**; 15 days at left and 30 days at right **(E)**.

Effects of Temperature, pH, and Carbon Source on Mycelial Growth

Effects on Mycelial Growth of *Cytospora corylina*

Cytospora corylina (CFCC 54684) expressed high adaptability to the three conditions and fast growth rate. Colonies grew from 5 to 30°C but not at 0, 35, or 40°C after 96 h of incubation (**Figure 10A**). The maximal growth occurred at 25°C after 24 h, at which point colonies grew up to 45 mm diameter, reaching 90 mm after 48 h. After 72 h, colonies reached 90 mm at 20°C and 70 mm at 30°C, and after 96 h, the colony diameter reached 81 mm. Colonies grew on PDA in the pH range 3.0–11.0 but not at 2.0 or 12.0 (**Figure 10B**). Mycelium had the highest growth rate at pH 6.0, reaching diameters of 25 mm after 24 h and 90 mm after 72 h, followed by pH 4.0 and 8.0, which resulted in colonies of 75 mm and 51 mm diameters, respectively, after 72 h. However, on the fourth day, colonies showed fast growth in pH 5.0, reaching up to 90 mm diameter. The slowest growth occurred at pH 11.0, with colonies reaching only seven millimeters in diameter after four days of incubation. In general, *C. corylina* is more suitable for weakly acidic conditions ranging from pH 4.0 to 7.0.

Cytospora corylina grew on all six tested carbon sources (**Figure 10C**). The mycelia grew faster in dextrose- and fructose-supplemented media after 24 h than in media supplemented with the other carbon sources. After 48 h, mycelium reached 90 mm diameter on dextrose-supplemented medium, followed by fructose-supplemented medium; maltose-supplemented medium showed the least efficiency in terms of growth. The difference in the utilization of sucrose and other carbon sources gradually became apparent after 72 h: growth in sucrose-supplemented medium was significantly lower than that in media supplemented with the other carbon sources, all of which resulted in mycelium reaching 90 mm diameter after 96 h.

Effects on Mycelial Growth of *Cytospora curvispora*

Colonies of *Cytospora curvispora* (CFCC 54000) in the current study grew from 5 to 30°C but not at 0, 35, or 40°C after four days of dark incubation (**Figure 10D**). We observed the maximum total growth at 25°C after 24 h, and colonies grew up to 29 mm, followed by growth at 20 and 30°C. After 72 h, colonies reached 73 mm at 20 and 25°C and showed colony diameters of 90 mm after 96 h. *Cytospora curvispora* was highly adaptable to pH, the colonies of which could grow at pH 3.0–11.0 but not at 2.0 or 12.0 (**Figure 10E**). Growth was the fastest at pH 6.0, where mycelium reached 48 mm after 48 h and 90 mm after 96 h, followed by pH 7.0 in which a colony diameter of 78 mm was achieved. After 96 h, mycelial growth was the slowest at pH 11.0, reaching only 27 mm in diameter after four days of incubation.

Cytospora curvispora could utilize all six carbon sources (**Figure 10F**). After 24 h, the utility of the six different carbon sources was almost the same, except for sucrose. However, the difference in the utilization of these carbon sources gradually became apparent after 48 h. Growth on xylose- and fructose-supplemented media were significantly greater than that on the other carbon source-supplemented media, showing no difference after 96 h, with colonies of 90 mm diameter.

Effects on Mycelial Growth of *Cytospora leucostoma*

Cytospora leucostoma (CFCC 54680) expressed high adaptability to the three conditions. Colonies of it in the current study grew

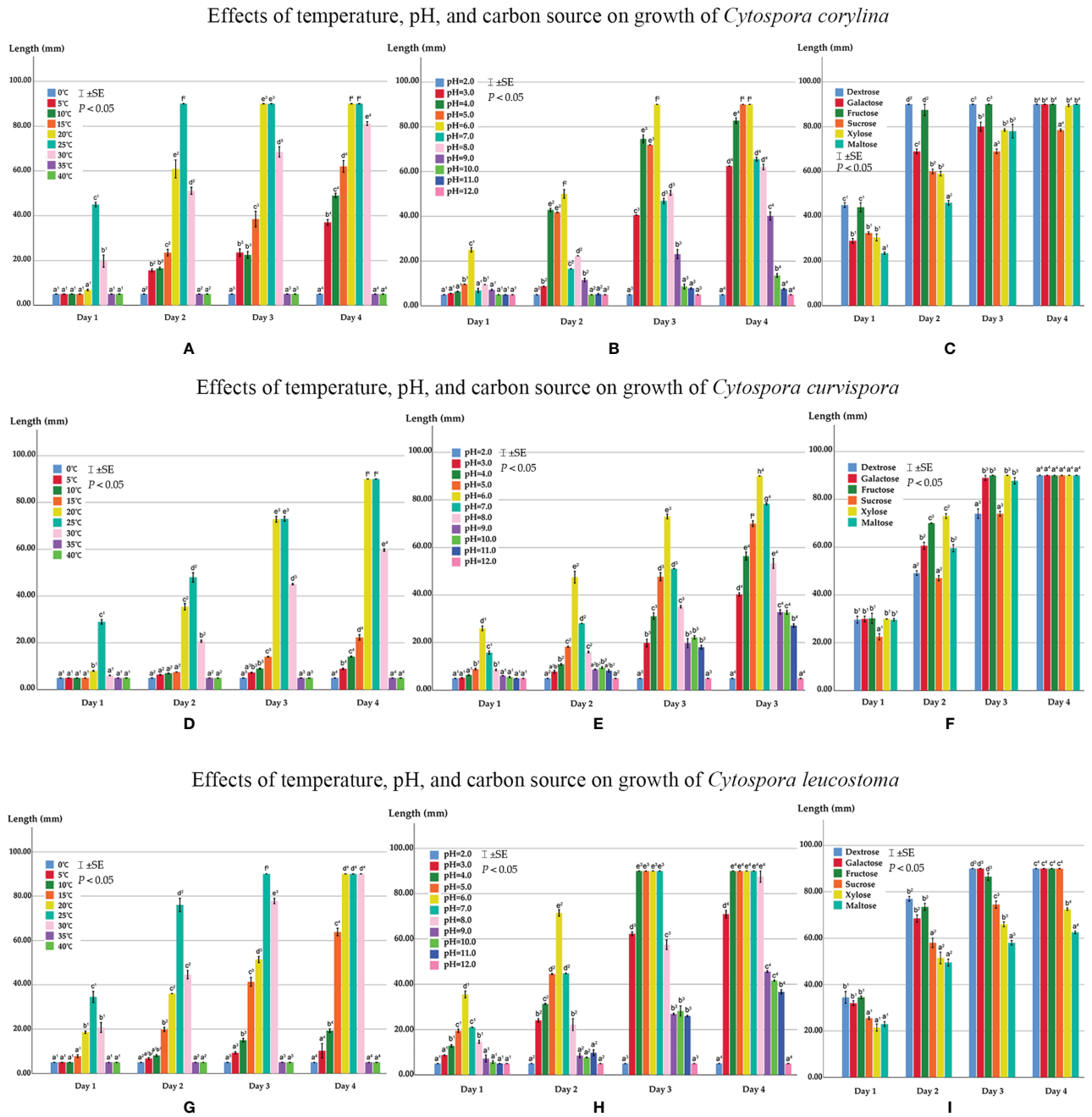

FIGURE 10 | **(A)** Effects of temperature on growth of *Cytospora corylina*. **(B)** Effects of pH on growth of *Cytospora corylina*. **(C)** Effects of carbon source on growth of *Cytospora corylina*. **(D)** Effects of temperature on growth of *Cytospora curvispora*. **(E)** Effects of pH on growth of *Cytospora curvispora*. **(F)** Effects of carbon source on growth of *Cytospora curvispora*. **(G)** Effects of temperature on growth of *Cytospora leucostoma*. **(H)** Effects of pH on growth of *Cytospora leucostoma*. **(I)** Effects of carbon source on growth of *Cytospora leucostoma*. Bars represent ± SE. Mycelium length not connected by the same letter in a group are apparently different (p <0.05) for that condition.

from 5–30°C but not at 0, 35, or 40°C after four days of incubation (**Figure 10G**). The maximum growth rate was observed at 25°C. Colonies reached 90 mm diameter at 25 and 30°C and nearly 80 mm in diameter after 72 h. At 20, 25, and 30°C, mycelium grew up to 90 mm after 96 h. Colonies could grow at a pH range of 3.0–11.0 but not at 2.0 and 12.0 (**Figure 10H**). We observed the maximum growth rate at pH 6.0, with diameters reaching 35 mm after 24 h and 72 mm after 48 h, followed by pH 5.0 and 7.0, both

of which resulted in diameters of 45 mm after 48 h. After 96 h, colonies grew up to 90 mm diameter under pH 4.0, 5.0, 6.0, and 7.0 and reached 88 mm at pH 8.0.

Cytospora leucostoma could utilize six carbon sources (**Figure 10I**). The utility of dextrose, galactose, and fructose was greater than that of sucrose, xylose, and maltose after 96 h of incubation. After 72 h, mycelia growing on dextrose- and galactose-supplemented media were the first to reach 90 mm diameter, followed by colonies growing on fructose- and sucrose-supplemented media. Colonies grew slower on maltose-supplemented medium, reaching only 63 mm after four days.

Effects on Mycelial Growth of *Diaporthe corylicola*

Colonies of *Diaporthe corylicola* (CFCC 53986) in the current study grew on 10–30°C (**Figure 11A**). The maximum growth rate occurred at 25°C, but almost no growth was observed on the first day. *Diaporthe corylicola* grew slowly, and the colony diameter only reached 35 mm at 25°C after 96 h, followed by 18 mm diameter at 20°C. Colonies grew on PDA in the pH range of 3.0–11.0 but not at 2.0 or 12.0 (**Figure 11B**). The maximum mycelial growth was achieved at pH 6.0, reaching 35 mm after 96 h, followed by pH 5.0 and 4.0, and growth was slower at the other pH conditions. All six carbon sources tested could be metabolized by *D. corylicola* (**Figure 11C**). In the first three days, no significant difference was observed among the growth of colonies in media supplemented with all carbon sources, except sucrose. After 96 h of incubation, the utilization of dextrose and galactose was apparently greater, while that of the other four carbon sources reached nearly the same level.

Effects on Mycelial Growth of *Diaporthe eres*

Colonies of *Diaporthe eres* (CFCC 53999) grew at 5–30°C but not at 0°C, 35°C, or 40°C after four days. At 25°C, the maximum growth rate was achieved at 24 h, while at 30°C, the maximum growth rate was achieved at 96 h (**Figure 11D**). Colony growth was comparable at 20–30°C. After 96 h, colonies reached 65 mm diameter at 30°C, followed by 61 mm and 56 mm, respectively, at 25 and 20°C, while at the other temperatures, a slow growth rate was observed with no more than 25 mm diameter. *Diaporthe eres* were highly adaptable to

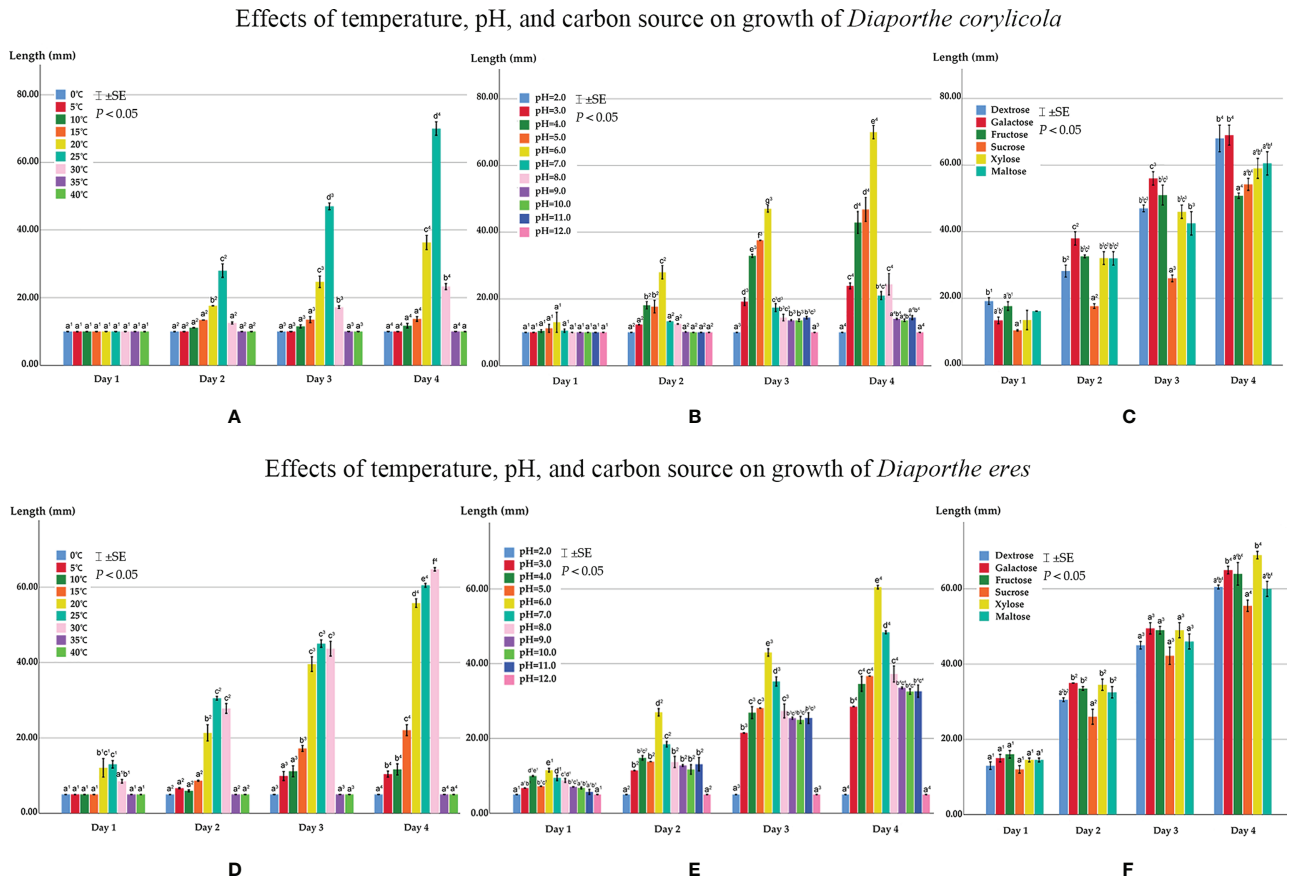

FIGURE 11 | **(A)** Effects of temperature on growth of *Diaporthe corylicola*. **(B)** Effects of pH on growth of *Diaporthe corylicola*. **(C)** Effects of carbon source on growth of *Diaporthe corylicola*. **(D)** Effects of temperature on growth of *Diaporthe eres*. **(E)** Effects of pH on growth of *Diaporthe eres*. **(F)** Effects of carbon source on growth of *Diaporthe eres*. Bars represent ± SE. Mycelium length not connected by the same letter in a group are apparently different (p <0.05) for that condition.

pH, the colonies of which grew on PDA at a pH range of 3.0–11.0 but not at 2.0 or 12.0 (**Figure 11E**). Mycelium grew the fastest at pH 6.0, reaching 61 mm after 96 h, followed by pH 7.0, reaching 43 mm diameter after 96 h. In the other pH environments, although the growth rate was quite different from that at pH 6.0 and 7.0, it was maintained at a relatively high level.

All six carbon sources were utilized by *D. eres* (**Figure 11F**). After 24 h, the mycelial length was the same for colonies grown on media containing the six different carbon sources and was maintained at a similar level over 96 h. The medium containing xylose was utilized the best, and that containing sucrose was utilized slightly less than media containing the other carbon sources.

DISCUSSION

Identification of *Cytospora* Species

Members of *Cytospora* have been reported as plant pathogens in forest and urban trees, including Anacardiaceae, Elaeagnaceae, Fabaceae, Juglandaceae, Myrtaceae, Rosaceae, Salicaceae, and Ulmaceae (Ehrenberg, 1818; Adams et al., 2002; Adams et al., 2005; Adams et al., 2006; Mehrabi et al., 2011; Wang et al., 2011; Fan et al., 2014a; Fan et al., 2014b; Fan et al., 2015a; Fan et al., 2015b; Lawrence et al., 2017; Fan et al., 2020). Previously, the identification of *Cytospora* relied on morphology and host affiliation. However, host affiliation is not always stable, and some species have similar morphology and uninformative illustrations and descriptions (Teng, 1963; Tai, 1979; Wei, 1979; Adams et al., 2002; Adams et al., 2005; Wang et al., 2011; Fan et al., 2014a; Fan et al., 2014b; Lawrence et al., 2017; Fan et al., 2020; Pan et al., 2020). ITS sequences were first used in *Cytospora* species identification by Adams et al. (2002), with six *Cytospora* groups suggested. Twenty-eight *Cytospora* species have been described from *Eucalyptus*, and 144 strains representing 20 species of *Cytospora* were subsequently collected from Iran (Adams et al., 2005; Fotouhifar et al., 2010). Recently, morphology combined with multi-locus phylogeny has been used for species identification, and has revealed many cryptic species (Fan et al., 2014a; Fan et al., 2015a; Fan et al., 2015b; Lawrence et al., 2017; Lawrence et al., 2018; Norphanphoun et al., 2018; Shang et al., 2020).

Fan et al. (2020) used six genes (ITS, LSU, *act*, *rpb2*, *tef1-α*, and *tub2*) to summarize 52 *Cytospora* species in China. Nevertheless, hidden fungal diversity has been revealed continuously in some special plant hosts (Pan et al., 2020; Pan et al., 2021). The present study revealed three species associated with *Corylus heterophylla*, i.e., *Cytospora corylina*, *C. curvispora*, and *C. leucostoma*. As the LSU gene is only available for a few species, we adapted a five-gene sequence of ITS, *act*, *rpb2*, *tef1-α*, and *tub2* in the phylogeny analyses. Moreover, although *C. leucostoma* has been reported as a common species that causes canker in plants of Rosaceae, we have a poor understanding of its host specificity and pathogenicity (Pan et al., 2020). This study represents an attempt to enrich the study of *Cytospora* in China.

Identification of *Diaporthe* Species

Diaporthe was established by Nitschke (1870) and has been extensively studied by Udayanga et al. (2011; 2012a) in recent years. Species of this genus, including endophytes, saprobes, and plant pathogens, are widely distributed in natural ecosystems (Udayanga et al., 2011; Udayanga et al., 2012a).

The species were initially determined based on host affiliations and morphological features (Aa et al., 1990). However, in terms of phylogenetic relationships, morphology and host association usually showed little siginificance (Brayford, 1990; Rehner and Uecker, 1994; Udayanga et al., 2014a; Udayanga et al., 2014b; Udayanga et al., 2015). Molecular techniques have been utilized in the latest taxonomic methods to define *Diaporthe* species (Santos and Phillips, 2009; Santos et al., 2010; Udayanga et al., 2011; Udayanga et al., 2012a; Udayanga et al., 2012b; Gomes et al., 2013; Udayanga et al., 2014a; Udayanga et al., 2014b; Udayanga et al., 2015). Since these revolutionary studies, more than 50 novel *Diaporthe* species have been identified in China (Huang et al., 2013; Huang et al., 2015; Gao et al., 2016; Gao et al., 2017; Yang et al., 2017a; Yang et al., 2017b; Yang et al., 2018; Fan et al., 2018; Yang et al., 2020). New records and species have been reported on the basis of molecular evidence (Rossman et al., 2015; Dissanayake et al., 2017; Guarnaccia and Crous, 2017; Perera et al., 2018; Tibpromma et al., 2018; Wanasinghe et al., 2018; Wrona et al., 2020).

In the current study, based on multi-locus sequences (ITS, *cal*, *his3*, *tef1-α*, and *tub2*), we identified two *Diaporthe* species associated with hazelnut. The known species, *D. eres*, has been widely reported as a plant pathogen. The other was identified as a new species, *D. corylicola*, with highly supported clades and holomorphic morphology.

Fungal Diversity Associated With Hazelnut

A great number of fungal pathogens associated with many *Corylus* species, especially *C. avellana*, have been identified based on molecular data and morphological characteristics in recent studies (Pinkerton et al., 1992; Chen et al., 2007; Guerrero and Pérez, 2013a; Guerrero and Pérez, 2013b; Linaldeddu et al., 2016; Wiman et al., 2019; Yang et al., 2020; Zhu et al., 2020). A broad list of fungi has been reported on different parts of hazel trees. For example, *Anisogramma*, *Anthostoma*, *Diaporthe*, *Diaporthella*, *Diplodia*, *Dothiorella*, and *Gnomoniopsis* are common fungi that inhabit branches in hazel trees in America, Chile, Italy, and Turkey (Gottwald and Cameron, 1980; Guerrero and Pérez, 2013a; Linaldeddu et al., 2016). *Alternaria*, *Aspergillus*, *Botryosphaeria*, *Colletotrichum*, *Diaporthe*, *Fusarium*, *Pestalotiopsis*, and *Phoma* are often isolated from fruits, especially in Turkey and the Caucasus region (Sezer and Dolar, 2016; Battilani et al., 2018; Arciuolo et al., 2020; Arciuolo et al., 2021). Although little information is available, *Cytospora corylicola* generally occurs in hazelnut growing areas in Europe (Salerno, 1961). These studies indicate great similarities among fungi in hazelnut cultivating areas in different geographical locations and environmental conditions. A recent report introduced *Elsinoë coryli* (*Sphaceloma coryli*) as a re-emerging pathogen on hazel trees in southern Italy, and nine other species were collected from symptomatic branches in Sardinia (Italy),

causing serious economic losses (Lamichhane et al., 2014; Linaldeddu et al., 2016; Minutolo et al., 2016; Fan et al., 2017). Thus, several studies have confirmed the high fungal diversity associated with European hazelnut.

In the current study, 51 strains were isolated from the stems and branches of *Corylus heterophylla* collected from Beijing, China. Among them, 14 were identified as *Cytospora* and the other 37 as *Diaporthe*. Among them, *Cytospora corylina* and *C. leucostoma* were only isolated from branches and other three species were observed both on stems and branches. These results indicate that the diversity of fungi associated with hazelnut canker and dieback disease is greater than previously recognized. All species in this study, except *Diaporthe eres*, were discovered for the first time on hazel trees. Previously, *Cytospora coryli* and *Diaporthe coryli* were reported as pathogens on branches of *Corylus mandshurica*, which is related to and generally grows naturally alongside *C. heterophylla* (Yang et al., 2020; Zhu et al., 2020). Thus, we speculate that *C. heterophylla* has great potential to be infected by *Cytospora coryli* and *Diaporthe coryli*. Although many *Cytospora* and *Diaporthe* species are endophytes and saprobes, it is still unclear whether opportunistic species would transform into pathogens on new hosts or in different environmental conditions, or adapt to climate change (Wang et al., 2020). Furthermore, *Cytospora leucostoma* and *Diaporthe eres* are plant pathogens that have been collected from a wide range of woody hosts, and *Diaporthe* species have been reported as the main cause of hazelnut defects in the Caucasus region (Battilani et al., 2018; Wiman et al., 2019). Thus, extensive investigations and pathogenicity tests on the five species need a further study.

Optimum Environment for Culturing Isolates

Most fungi can grow at 10–35°C, with the most suitable range between 20 and 30°C. The most suitable pH for mycelial growth is 5.0–6.5 (Elfar et al., 2013). For instance, the optimal growth temperature of *Diaporthe* sp. is 22°C (Strausbaugh and Dugan, 2017), and that of *Cytospora hadianensis* is 19.8°C (Zhou et al., 2020). Helton and Konicek (1962) isolated six *Cytospora* species and indicated that the optimum pH is approximately 4.5, with a temperature of 20–35°C. Similar results were obtained in this study; all five species tested grew on PDA at 5–30°C and a pH of 3.0–11.0, with an optimum temperature of 20–30°C and pH value of 4.0–7.0. However, *Cytospora corylina* incubated at 15 and 20°C showed special growth characteristics on PDA. It formed very fine mycelial strands, which radiated outward from the central portion of the plate, and did not have the same physical appearance as the rest of the mycelium. The special growth characteristics of *Cytospora* species have been observed by Konicek and Helton (1962a), who provided a reference to include these fine strands in the measurement. As a result, the optimum conditions for *Cytospora* species were 20–30°C and pH 4.0–7.0, and those for *Diaporthe* species were 20–30°C and pH 5.0–7.0. If one unusual factor alone is removed, such as the aberrant marginal growth of *C. corylina* on PDA, optimum temperature for *Cytospora* is 25–30°C.

The six carbon sources tested in the current study were efficiently utilized, although the utilization of sucrose by *Cytospora corylina*, *C. curvispora*, and *Diaporthe eres* was less than that of the other sources. Zhou et al. (2020) reported that utilization of galactose by *Cytospora hadianensis* is low, and Zhao et al. (2019) reported that the utilization of xylose by *Lasiodiplodia vaccinii* and *L. theobromae* is the lowest. The best overall growth results of the six *Cytospora* species tested were obtained with maltose. However, none of the species in our study had the best overall growth on maltose media, but all had a high ability to use galactose and xylose, except *Cytospora leucostoma* for xylose.

Cultured isolates of different species differ subtly in mycelial growth and pathogenicity under a given condition, and one species may have different characteristics under different conditions (Konicek and Helton, 1962a; Konicek and Helton, 1962b; Wang et al., 2020). The different growth characteristics and high adaptability to the environmental conditions of these *Cytospora* and *Diaporthe* species seem to justify their widespread occurrence. Biological characterization of these strains, combined with their host distribution, may be used for species identification and distribution prediction. However, as there are currently few details regarding pathogen biology, it is necessary to evaluate the effects of environmental conditions, such as temperature, pH, and carbon sources, on mycelium growth and pathogenicity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

All authors have made extensive contributions to the work presented in the article. XF and CT: contributed to conception of the experiment. HG and MP: completed the experiment. HG: conducted the data analyses. HG: wrote the original manuscript. XF: reviewed and edited the draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.664366/full#supplementary-material

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REVIEW
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Fungal Pathogens in Grasslands

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Grasslands are major primary producers and function as major components of important watersheds. Although a concise definition of grasslands cannot be given using a physiognomic or structural approach, grasslands can be described as vegetation communities experiencing periodical droughts and with canopies dominated by grasses and grass-like plants. Grasslands have a cosmopolitan distribution except for the Antarctic region. Fungal interactions with grasses can be pathogenic or symbiotic. Herbivorous mammals, insects, other grassland animals, and fungal pathogens are known to play important roles in maintaining the biomass and biodiversity of grasslands. Although most pathogenicity studies on the members of Poaceae have been focused on economically important crops, the plant-fungal pathogenic interactions involved can extend to the full range of ecological circumstances that exist in nature. Hence, it is important to delineate the fungal pathogen communities and their interactions in man-made monoculture systems and highly diverse natural ecosystems. A better understanding of the key fungal players can be achieved by combining modern techniques such as next-generation sequencing (NGS) together with studies involving classic phytopathology, taxonomy, and phylogeny. It is of utmost importance to develop experimental designs that account for the ecological complexity of the relationships between grasses and fungi, both above and below ground. In grasslands, loss in species diversity increases interactions such as herbivory, mutualism, predation or infectious disease transmission. Host species density and the presence of heterospecific host species, also affect the disease dynamics in grasslands. Many studies have shown that lower species diversity increases the severity as well as the transmission rate of fungal

diseases. Moreover, communities that were once highly diverse but have experienced decreased species richness and dominancy have also shown higher pathogenicity load due to the relaxed competition, although this effect is lower in natural communities. This review addresses the taxonomy, phylogeny, and ecology of grassland fungal pathogens and their interactions in grassland ecosystems.

Keywords: Ascomycetes, foliar diseases, graminicolous fungi, grassland ecology, human and plant disease, phytopathogens, soil-borne diseases

INTRODUCTION

There is no concise and unambiguous definition for grasslands (Gibson, 2009). The definition of a grassland could be based on the absence of specific vegetation features (Milner and Hughes, 1969) when using a physiognomic or structural approach (Bazzaz and Parrish, 1982; Sims, 1988). A more promising definition, however, was given by Risser (1988), who indicated that grasslands are "types of vegetation that are subject to periodic drought, that have a canopy dominated by grass and grass-like species, and that grow where there are fewer than 10 to 15 trees per hectare". Grasslands are distributed throughout the world's land area except on the continent of Antarctica (Gibson, 2009). Based on the "The Pilot Analysis of Global Ecosystems (PAGE)" classification, grasslands cover 52,544,000 km^2 or 40.5% of the world's land mass (excluding urban areas according to night time lights) (White et al., 2000; Kassam, 2002). According to the PAGE classification, grasslands cover more land area than the other major vegetation cover types (White et al., 2000). For example, forests cover 28.97×10^6 km^2 and agriculture covers 36.23×10^6 km^2 (White et al., 2000). Furthermore, grasslands are the second largest land type inhabited by humans (nearly 800×10^6 people), second only to agricultural land, which holds 2.8×10^9 people according to 1995 estimates (White et al., 2000; Gibson, 2009). Grasslands also occupy comparatively large areas of many major watersheds in the world (Gibson, 2009).

The effect of species diversity on the productivity of a community has been explained by two mechanisms. They are: (i) the sampling effects, which state that the probability of finding key-trait species in a community is reduced due to lower species richness (Aarssen, 1997; Huston, 1997; Tilman et al., 1997) and (ii) the niche complementarity hypothesis, which states that less diverse communities with competing species utilize resources incompletely (Naeem et al., 1994; Tilman et al., 1996; Hector et al., 1999). Furthermore, the loss of interactive competition and loss of species diversity have been shown to increase mutualism, predation, herbivory, and infectious disease transmission (Bond, 1993; McNaughton, 1994; Chapin et al., 1997; Chapin et al., 2000). Mitchell et al. (2002) tested the long-standing hypothesis (Elton, 1958; van der Plank, 1963) that the lower diversity of plant species increases the severity of diseases, focusing mainly on specific pathogens. Several studies have previously suggested that this hypothesis applies only to a small number of plant species and genotypes (Garrett and Mundt, 1999; Zhu et al., 2000). The relaxed interspecific competition due to the decreased plant species richness has been shown to increase the abundance of one or more species existing in a local community, which typically also increases the abundance of one or more host species for specialist pathogens (Aarssen, 1997; Huston, 1997; Tilman et al., 1997). The basic mechanism of the diversity-disease hypothesis is that a decreasing number of plant species allows for an increased local abundance of other singular species, which then facilitates the spread of diseases specific to that species within the community (Burdon and Chilvers, 1976; Knops et al., 1999; Chapin et al., 2000). The extent of the differences among different species is correlated with the ecological effects of species diversity (Tilman and Lehman, 2014); hence, the susceptibility of different species for a particular disease must vary. Thus, the local mechanisms of the diversity-disease hypothesis vary highly, as the host abundance depends on numerous biotic and abiotic factors in communities (Mitchell et al., 2002). Apart from host abundance, many other factors such as microclimate and the competitive ability of host plants also influence the disease level in ecosystems with decreasing diversity (Boudreau and Mundt, 1992; Boudreau and Mundt, 1994; Zhu et al., 2000; Boudreau and Mundt, 2018).

Most studies examining the diversity-disease hypothesis have been focused on agriculture or silviculture (Mitchell et al., 2002). However, studies by Kranz (1990) revealed that natural, communities with higher diversity did not necessarily have low disease levels, whereby the diversity of forest and pasture communities was higher while in meadow and agricultural field communities it was lower (Kranz, 1990). As such, species diversity is related to species composition, microclimates, and many other factors (Kranz, 1990; Mitchell et al., 2002). Agronomic intercropping, with increased species diversity, subsequently decreases the prevalence of diseases (Mitchell et al., 2002), especially fungal diseases. Experimental studies on the diversity-disease hypothesis with regards to intercropping are fewer (Boudreau and Mundt, 2018), but intercropping can change the microclimate and the competitive nature of the crops and all these factors together can either increase or decrease disease severity (Boudreau and Mundt, 1992; Boudreau and Mundt, 1994; Zhu et al., 2000; Boudreau and Mundt, 2018).

It has been shown that cultivating a variety mixture or multiline with multiple genotypes of one crop reduced the severity of airborne fungal diseases when compared with its intercrop cultivation (Wolfe, 1985; Finckh and Wolfe, 1997; Garrett and Mundt, 1999; Zhu et al., 2000; Boudreau and Mundt, 2018). In agricultural systems, the main mechanism of

reducing the spread of the disease is through reducing the host abundance (Burdon and Chilvers, 1977; Burdon and Chilvers, 1982; Chin and Wolfe, 1984; Wolfe, 1985; Alexander et al., 1986; Burdon, 1987; Garrett and Mundt, 1999; Zhu et al., 2000; Boudreau and Mundt, 2018). We can overlook highly diverse natural grassland ecosystems through the modern knowledge of agricultural multiline even though natural grasslands are more complex than the agricultural multiline.

Disease susceptibility and the dominant species present are two aggregated characteristics of a community that can influence the spread and severity of a disease (Mitchell et al., 2002). Mitchell et al. (2002) tested several hypotheses relating to disease levels in a community and found that (1) the loss of less susceptible species in a community increased the disease levels within the community more than the loss of highly susceptible species, (2) the loss of less susceptible species from a plant community increased the disease levels in that community, and (3) the loss of the dominant species increased the prevalence of species-specific pathogens more than the loss of rare species, when all other conditions were equal. However, in certain instances, the most abundant species can be more susceptible to disease, and in such cases the above hypotheses are not valid and the effect of losing the dominant, highly susceptible species is countervailing (Arneberg et al., 1998).

Grasslands are highly diverse ecosystems with many interactions. Plant communities in grasslands are comprised mainly of diverse species of grasses, including genotypes of the same species, and various dicots. However, the major part of the community is represented by grasses. Population structure of grasslands is crucial for the productivity as well as the resource utilization of grasslands. However, pathogens in grasslands play a crucial role in the productivity of these communities. Herein we investigate how fungal pathogens presence affect the dynamics of grasslands. Population dynamics of fungal pathogens are highly influenced by the diversity and the population structure of grassland is in question. Furthermore, the dynamics of specialist pathogens and generalist pathogens are contrasting. Increased host diversity of grasslands increases generalist pathogens while reducing specialist pathogens. However, this may be highly vary based on the many biotic and abiotic factors in grasslands. Hence, interactions between pathogens and their host in natural grasslands are complex.

This review brings together a vast amount of information on fungal pathogens in grasslands and then discusses their role within and effects on grassland ecosystems. This also describes the pathogenic fungal communities in grasslands and discusses well-studied pathogenic fungal species reported on host grasses.

PATHOGENIC FUNGAL COMMUNITIES IN GRASSLANDS AND THEIR INTERACTIONS

Pathogenic fungi significantly affect the population biology of grasses and their contribution to plant communities by affecting the physiology and chemical composition of those grasses (Gibson, 2009). This in turn affects the population ecology of grassland communities and ecosystems, especially when they affect the dominant species of those communities (Burdon et al., 2006). However, the pathogenic effects of fungi cannot be studied by considering only the fungi themselves; they manifest their effects *via* complex species interactions (Ampt et al., 2019).

Ecologists have observed that two main aspects drive the interactions between plant species and their natural enemies in natural ecosystems—host-specificity and density-dependency (Janzen, 1970; Connell, 1971; Bever et al., 2015). According to the "pathogen hypothesis", the negative effects from a pathogen on a host must either be species-specific affecting certain species only, or a generalist which can have negative effects on multiple host species (Hersh et al., 2012; Ampt et al., 2019). Mommer et al. (2018) studied monocultures of four grass species and four forbs found in natural grasslands. In this study, the Internal transcribed spacer (ITS) based Next-Generation Sequencing (NGS) analysis of soil-borne fungal species showed a clear difference in the host specificities of fungal communities among grasses and forbs. In addition, host specificity has been apparent in plant-soil feedback studies, where the same species or the functional group, has been grown and the plant growth was reduced (Petermann et al., 2008; Mangan et al., 2010; Hendriks et al., 2013; Cortois et al., 2016). It is important to use NGS complemented analysis in conjunction with traditional pathology knowledge to gain a proper knowledge of the interactions between soil-borne fungal pathogens and their plant hosts (Mommer et al., 2018). An insight into the host specificity of soil-borne fungal pathogens in natural grasslands has been provided by Klironomos (2002). Klironomos (2002) isolated fungi from roots of several rare plants found in Canadian meadows, which were later identified as species of *Cylindrocarpon*, *Fusarium*, and *Verticillium*. Although identification of these fungi was only to the level of the genus, in the ecological context host specificity was clearly demonstrated upon inoculation to the field as there was a reduction in plant growth as a result of fungi isolated from conspecific roots but not from heterospecific roots (Klironomos, 2002). Consequently, this demonstrated that those fungi had only a negative impact on their preferred host (Gibson, 2009).

Biodiversity experiments in natural grasslands have also demonstrated pathogen dilution in foliar (Knops et al., 1999; Mitchell et al., 2002; Rottstock et al., 2014) and soil-borne fungi (Mommer et al., 2018). Rottstock et al. (2014) showed that foliar pathogen incidence and severity were reduced with increased species diversity. However, Ampt et al. (2019) suggested that the results might be due to a sampling error. Another study examined the effect of plant density on soil-borne disease dynamics in relation to seedling mortality in agricultural systems (Otten et al., 2004; Otten et al., 2005; Hiddink, 2008). During experiments on Wageningen grassland biodiversity (Van Ruijven et al., 2003; Van Ruijven and Berendse, 2005; Van Ruijven and Berendse, 2009; Cong et al., 2014), nearly 50% of the variation in fungal community composition could be explained by the density of the below ground host components. Hence, host density has a major impact on the fungal community. Moreover, above ground fungal pathogens play

a major role in biomass of the grasslands (Cappelli et al., 2020). The recent studies by Cappelli et al. (2020) showed that the infection incidence is highly affected by trade-off between plant growths and defines with the consideration of diverse grasslands and generalist pathogens as the generalist pathogens can spread out easily within a highly diverse grassland.

Although host specificity and host density both play major roles in the disease dynamics within a community, heterospecific neighbors also have an impact (Otten et al., 2005; Eppinga et al., 2006). Increased heterospecific neighbor plants, or the host diversity, reduce the host density of the specialist pathogens while increasing the density of generalist pathogens (Petermann et al., 2008). This evidence was observed in agricultural intercropping, where the presence of maize reduced Phytophthora blight severity and spread in pepper plants (Yang et al., 2014). The heterospecific neighbor effect can be explained in two ways by considering the below ground disease dynamics (Ampt et al., 2019). First, there are direct neighbor effects occurring through plant traits and root exudates. Second, there are indirect neighbor effects through the root microbiome (Ampt et al., 2019). Heterospecific neighbors can either act as barriers or vectors for diseases (Otten et al., 2005; Ampt et al., 2019). Host plants surrounded by non-host plants are less likely to become infected, and hosts that are surrounded by the hetero-specific hosts or symptomless hosts are susceptible to becoming infected (Malcolm et al., 2013). Furthermore, phylogenetically distant hosts are less likely to infect other hosts, and thereby plant communities with phylogenetically diverse hosts are less likely to spread disease within themselves (Gilbert and Webb, 2007; Haas et al., 2011; Wehner et al., 2014; Gilbert and Parker, 2016). The root system of different species may also either enhance or reduce disease transmission by physical or chemical means (Newsham et al., 1995). Intercropping of maize and pepper reduced the spread of Phytophthora blight due to the higher degree of root intermingling (Yang et al., 2014). Similarly, the dense and tightly intermingled roots of natural grasslands reduce the spread of disease (Kesanakurti et al., 2011; Ravenek et al., 2014; Frank et al., 2015). Moreover, complex below ground chemical composition and chemical communication has an impact on disease dispersal as well, but these mechanisms are not yet well explained (Ampt et al., 2019). Indirect neighbor effects *via* the root microbiome have been shown in natural grasslands inhabited by species of *Streptomyces* (Bakker et al., 2013; LeBlanc et al., 2015).

In natural grasslands, hosts, fungi, and pathogens are highly correlated. Below-ground host components shape total fungal composition while above-ground pathogens shape the biomass of grasslands. Moreover, generalist pathogens are highly dispersed in grasslands characterized with rich diversity. Hence, in grasslands, disease incidence highly relies on the plant growth, diversity of grasslands, and generalist pathogens in grasslands. Host specificity, host density, and host diversity play huge roles in the dynamics of grasslands. Heterospecific neighbors disturb the spread of specialist pathogens in grasslands. However, the presence of heterospecific neighbors increases the spread of generalist pathogens. However, heterospecific hosts can be either barriers or vectors if the host is symptomless. The close phylogenetic relatedness of grasses can increase disease spread among grasses with shared traits. Heterospecific neighbors can affect the spread of diseases through plant traits or root microbiomes. However, the tightly intermingled roots of heterospecific neighbors reduce specialist pathogens in grasslands.

SOIL-BORNE FUNGAL PATHOGENS IN GRASSLANDS

The majority of studies on grassland pathogens have been focused on above ground systems and monocultures for a few primary reasons (Ampt et al., 2019). The air-borne dispersal and pest-based dispersal of above ground pathogens attributes to fast spread of the pathogen and can be detected much more easily, especially under epidemics (Mitchell et al., 2002; Rottstock et al., 2014). Particularly in monoculture systems, the diseased areas can be visually seen as gaps in cultivation drawing immediate attention. Furthermore, much of the research attention in this area has been focused on monocultures, as they are often a part of economically important agricultural systems (Ampt et al., 2019).

Plant species richness has been shown to increase plant productivity, and soil-borne fungal pathogens play an important role in plant productivity (Maron et al., 2011; Schnitzer et al., 2011; Cardinale et al., 2012; Mommer et al., 2018). Experiments on how biodiversity loss affects ecosystem functioning in experimental grasslands has shown that monocultures perform less well than polycultures in measures of plant productivity (Tilman et al., 1997; Hector et al., 1999; Tilman et al., 2001; Van Ruijven and Berendse, 2005). However, the mechanism responsible for this scenario is not clear (Cardinale et al., 2012). The most promising strategy for explaining the difference in plant productivity has been by focusing on plant-plant interactions, resource partitioning, and facilitation between plants (Mueller et al., 2013; Ravenek et al., 2014; Wright et al., 2017; Jesch et al., 2018). Furthermore, interactions between the plants and soil biota are important contributor for positive biodiversity effects. The main hypothesis regarding the effect of soil-borne pathogens is that their negative impact is stronger in plant monocultures and weaker in mixed-plant ecosystems (Maron et al., 2011; Schnitzer et al., 2011; de Kroon et al., 2012; Mommer et al., 2018). Ecologists have investigated the "pathogen hypothesis" using a black box method, such as comparing plant growth on soil with and without the soil biota (Kos et al., 2013; Mccarthy-Neumann and Nez, 2013; Hendriks et al., 2015; Cortois et al., 2016; Wubs and Bezemer, 2018). Schnitzer et al. (2011) grew plant communities in both field and sterile soil. The result showed that on sterilized soil, the positive effects of plant species richness on plant productivity disappeared. Sterilization enhanced the productivity at lower biodiversity but did not affect biodiversity of soil with high plant species richness. Again, Schnitzer et al. (2011) re-inoculated the sterilized soil using a soil wash containing soil-borne fungi and observed that the productivity for the lower diversity group was reduced. This emphasized the importance of soil-borne fungi on biodiversity patterns. Maron et al. (2011) experimented on the relationship between plant

diversity and above-ground plant biomass in fungicide treated and non-treated soils. Application of systemic fungicides thiophanate ethyl and mefenoxam adversely affected this relationship indicating the positive effect exerted by soil fungi on plant diversity and productivity (Maron et al., 2011). The experiments of Maron et al. (2011) and Schnitzer et al. (2011) demonstrated the importance of host specific microorganisms in determining the diversity-productivity relationship. Hence it can be stated that a deep understanding of the roles played by soil-borne fungi in grasslands is essential in defining the exact roles of grassland associated fungi.

The most studied soil-borne pathogens of grasses are the ones that have been known to cause severe diseases for many hosts, especially for agriculturally important crops (Ampt et al., 2019). For example, *Fusarium oxysporum* causes Fusarium wilt on nearly 100 monocot and dicot hosts (Michielse and Rep, 2009). Soil-borne fungal diseases have been reported from commercially grown medicinal herbs and turf grass species found in natural grasslands (Smiley et al., 2005). Furthermore, soil-borne fungal diseases have also been identified in commercial monocultures of dicots found in grasslands (Gaetán et al., 2004). However, this knowledge has not been found to provide very much insight into fungal-plant interactions of highly diverse grassland ecosystems (Ampt et al., 2019).

Natural grasslands are diverse ecosystems housing a variety of monocot and dicot plants (Herben et al., 1993; Van Der and Sykes, 1993). Hence, grassland ecosystems are highly static yet show a wide variety of spatiotemporal dynamics (Herben et al., 1993; Van Der and Sykes, 1993). Soil-borne pathogens play a major role in these spatiotemporal dynamics (Olff et al., 2000). Soil-borne fungal diseases in natural grasslands are rarely observed, either because the disease incidence is low in higher diversity communities or other species replace the poorly performing species before they have a significant effect (Gilbert, 2002; Burdon et al., 2006; Alexander, 2010). Soil-borne pathogens in grasslands are highly diverse due to the overall high species diversity and the genotype diversity for a given species (Termorshuizen, 2014; Dassen et al., 2017; Yang et al., 2017; Bach et al., 2018). Although plant root systems in grasslands are colonized by a wide variety of fungi, only a few have been isolated and had their pathogenicity thoroughly examined through Koch's postulates (Vandenkoornhuyse et al., 2002). Mills and Bever (1998) isolated five species of *Pythium* from two types of perennial grass, *Danthonia spicata* and *Panicum sphaerocarpon*, from a 50-year-old grassland. They reintroduced four species of *Pythium*, and it reduced the biomass of *D. spicata* and *P. sphaerocarpon*, while *Anthoxanthum odoratum* (Poaceae) and *Plantago lanceolata* (Plantaginaceae) were not adversely affected by the four reintroduced species of *Pythium*. In the most recent study of grassland biodiversity by Mommer et al. (2018), the authors isolated 27 species of fungi from symptomatic roots of *Anthoxanthum odoratum* (Poaceae) and the forb *Leucanthemum vulgare* (Asteraceae). Among the isolates, *Magnaporthiopsis panicorum* and *Paraphoma chrysanthemicola* caused host specific adverse effects on seedlings (Mommer et al., 2018). Hence, root inhabiting pathogens can cause a considerable loss on the biomass of natural grasslands (Ampt et al., 2019).

Although the mechanisms of soil-borne plant pathogenic fungi affecting the spatiotemporal dynamics of natural grassland ecosystems are not well known, three main pieces of experimental based evidence have been identified (Ampt et al., 2019). First, next generation sequencing (NGS) has been used to identify a very high diversity of soil-borne fungal community growing on roots of grassland plants. Second, some soil-borne fungi have been observed to negatively affect plant growth in bioassays. Third, the relationship between plant diversity and plant productivity is affected by the sterilization of the soil and by application of fungicides (Ampt et al., 2019). However, the interactions of soil-borne fungi and their effects on plants within the larger context of biodiversity are yet to be discovered.

In natural grasslands, strategies that account for different productivities are plant-plant interactions, resource partitioning, and facilitation between plants. In species-rich grasslands, soil-borne fungi play an immense role in increasing the productivity of grasslands. However, in less-diverse grasslands, soil-borne fungi reduce productivity. Hence, stable grasslands are in proper equilibrium with host species and soil-borne fungal communities. In highly diverse grasslands soil-borne fungal diseases are rarely observed due to the low disease incidence and the natural replacement of poorly performing species. However, in general, high species or genotypic diversity of grasses leads to high diversity of soil-borne pathogens, although disease expression depends on the traits of host plants. Furthermore, the monocots and dicots in grasslands share the same soil-borne pathogens.

The studies on above ground fungal pathogens of grasses mentioned above were primarily studies based on agricultural monocultures. Above ground fungal pathogens in complex natural grassland ecosystems are much less studied. In the following sections, several well-studied fungal pathogens found in agricultural monocultures will be discussed.

SELECTED FUNGAL PATHOGENS ON GRASSES

There are many studies of pathogens on grasses. Although the pathogen-related studies are mainly focused on economically important monocultures. They provide invaluable information about aspects of populations involved as well as their genomes. As such, in this section we describe several studies on selected well studied pathogens to understand how important it is to carry out a proper study on pathogenic fungal population in natural grasslands. Herein we are describing four well studied species: *Bipolaris sorokiniana, Colletotrichum graminicola, Fusarium graminearum*, and *Pyrenophora tritici-repentis*. Each species was selected in order to explain significant characteristics of fungal pathogens relevant to the Poaceae. The race structure of *Pyrenophora tritici-repentis* and its relatedness to economically important crops as well as certain instances where it is relevant to the natural grassland systems along with data on genomes relevant to the pathogenicity have been intensively studied. *Fusarium graminearum* has been well studied for its mycotoxin production

and its involvement with pathogenicity. *Bipolaris sorokiniana* is a well studied pathogen noted for its variations in pathogenicity, DNA polymorphism, and adaptability. *Colletotrichum graminicola* has been well studied for the infection mechanism and involvement of proteins in inducing diseases. Some of the common details of those pathogens are given in **Table 1**.

Pyrenophora tritici-repentis

Pyrenophora tritici-repentis (Died.) Drechsler is one of the most widely studied pleosporalean fungi that causes an economically important disease (Ciuffetti et al., 2014) (**Table 1**). From this point onwards, *Pyrenophora tritici-repentis* is abbreviated as *Ptr*. *Pyrenophora tritici-repentis* shows the homothallic nature of sexual reproduction with the presence of MAT genes MAT1-1 and MAT1-2 in a single locus (Lepoint et al., 2010).

Pyrenophora tritici-repentis has been isolated from cereal grasses (*Avena sativa*, *Bromus* spp., *Dactylis glomerata*, *Hordeum vulgare*, *Secale cereale*, *Triticum aestivum*, and *T. turgidum*) along with noncereal grasses including native prairie grasses and other species of grasses (Hosford, 1971; Krupinsky, 1986; Krupinsky, 1992a; Ali and Francl, 2003; Bockus et al., 2010) (**Figures 1**, **2**). With the studies of monocultures, it has been found that *Ptr* can overwinter saprophytically as a sexual stage with the asexual stage appearing with the growing stage of the host (Krupinsky, 1992b). Humidity induces the conidial production of *Ptr* and conidia disperse by wind (Schilder and Bergstrom, 1992). The latter is responsible for the multiple inoculations of all stages of the host which defines the symptom severity on mature leaves. Furthermore, the conidia remain viable up to three years inside the infected seeds (Schilder and Bergstrom, 1995; Bergstrom and Schilder, 1998).

The studies of *Ptr* have been focused primarily on tan spot disease of wheat (Ciuffetti et al., 2014). Based on Hosford (1971), the resistance to tan spot differs for different genotypes of wheat. Early knowledge of the physiological specializations of tan spot disease was based on quantitative parameters (Ciuffetti et al., 2014). Additional qualitative studies of tan spot symptoms revealed that necrosis and chlorosis are the most reliable signs to define the physiologic specialization of *Ptr* with the host (Lamari and Bernier, 1989a; Lamari, 1991). Accordingly, Lamari and Bernier (1989a), recognized four pathotypes based on the presence or absence of chlorosis and necrosis on the host tissues. These are pathotype 1 (necrosis+ chlorosis+), pathotype 2 (nec+ chl-), pathotype 3 (nec- chl+), and pathotype 4 (nec- chl-) (Ciuffetti et al., 2014). Later, a race based classification was introduced to characterize pathogenic diversity of *Ptr* (Lamari et al., 1995; Lamari and Strelkov, 2010).

The population studies of *Ptr* have focused primarily on agricultural monocultures and little information is avaiable on natural grasslands. Consequently, herein, we discuss the population and race structure of *Ptr* based on agricultural monocultures. The race structure of *Ptr* has been characterized mainly on the basis of spore inoculation and the host-selective toxins (HSTs) produced on the three wheat genotypes: 'Glenlea', 6B365 and 6B662 (Strelkov and Lamari, 2003; Lamari and Strelkov, 2010). Three HSTs in *Ptr* have been recoginzed thus far. They are Ptr ToxA (syn. Ptr necrosis toxin, Ptr toxin, and ToxA) (Ballance et al., 1989; Tomas, 1990; Tuori et al., 1995; Zhang et al., 1997; Ciuffetti et al., 1998), Ptr ToxB (syn. Ptr chlorosis toxin) (Orolaza et al., 1995; Ciuffetti et al., 1998; Strelkov et al., 1999) and Ptr ToxC (syn. Ptr chlorosis toxin) (Ciuffetti et al., 1998; Effertz et al., 2002). Thus far, eight races of

TABLE 1 | Host, distribution factors and overwintering stages of selected fungal pathogens.

Pathogen	Disease	Host	Distribution factors	Overwintering stages	References
Pyrenophora tritici-repentis	Tan spot	Cereal grasses (*Avena sativa*, *Bromus* spp., *Dactylis glomerata*, *Hordeum vulgare*, Secale cereale, *Triticum aestivum* and *T. turgidum*), noncereal grasses including native prairie grasses and other species of grasses	Humidity induces the conidial production of *Ptr* and conidia disperse by wind	Saprophytic stage on wheat stubble as pseudothecia	Hosford, 1971; Krupinsky, 1986; Krupinsky, 1992a; Schilder and Bergstrom, 1992; Ali and Francl, 2003; Bockus et al., 2010; Ciuffetti et al., 2014
Fusarium graminearum	Head blight Ear rot and stalk rot	Wheat, barley, oats, and many other small grain cereal crops Maize	Ascospores and macroconidia can be disseminated by wind, rain, and insects to host plants and deposited on or inside of spike tissues. anthers are suggested as vulnerable sites for primary infection	Perithecia at spring	Sutton, 1982; Guo and Ma, 2014
Bipolaris sorokiniana	foliar spot blotch, root rot, and black points	Cereal crops such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*)	Contaminated soil and contaminated plant debries	Dormant mycelium or conidia in infected plant tissues, thatch, and in plant debris	Kumar et al., 2002; Dai et al., 2011; Dicklow 2011; Kang et al., 2021;
Colletotrichum graminicola	Anthracnose, causing stalk rot and seedling blight	Maize	Wind and raindrop splashes	Saprophytically on dead plant material to over winter (Nordzieke et al., 2019)	Bergstrom and Nicholson, 1999; Nordzieke et al., 2019

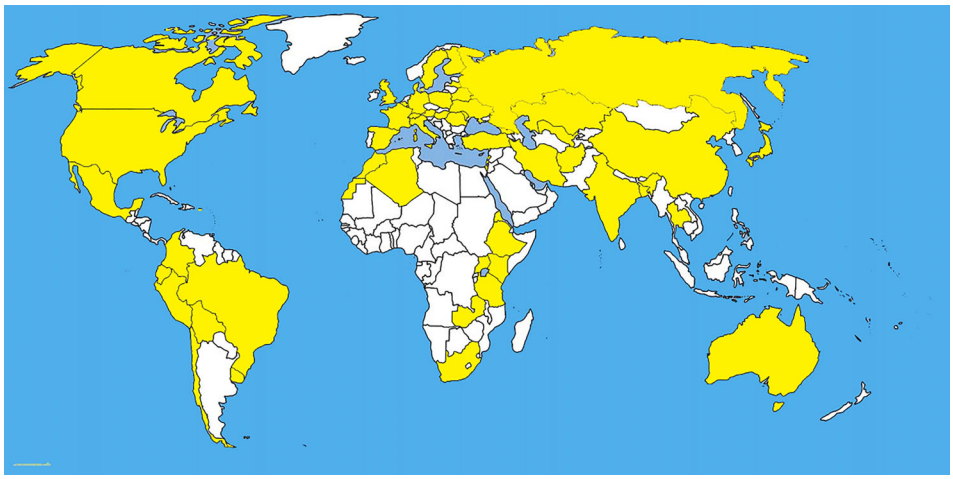

FIGURE 1 | The distribution map of *Pyrenophora tritici-repentis*. The areas where infection have been reported are indicated in yellow (www.cabi.org/isc. Accessed on 25/03/2021).

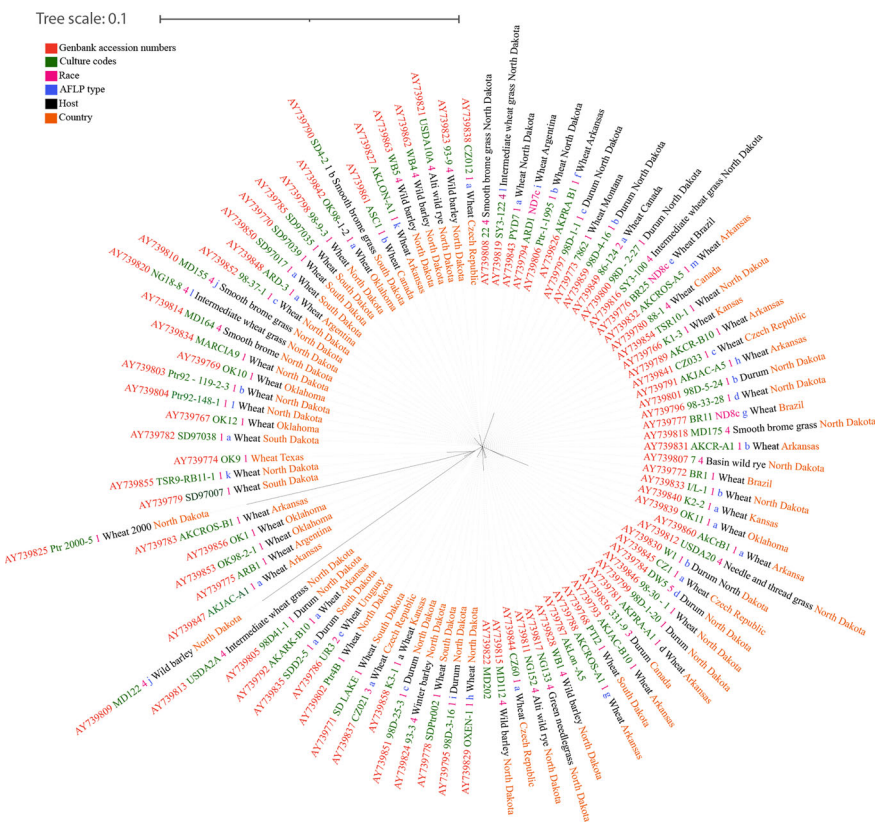

FIGURE 2 | The unrooted RAxML bipartion unrooted phylogenetic tree of *Pyrenophora tritici-repentis* based on Friesen et al. (2005).

Ptr have been identified and are described in **Table 2**. Races 3 and 4 are less frequently found on wheat, although they are more prominent in noncereal grasses in the Great Plains (Ali and Francl, 2003). Furthermore, races 7 and 8 were reported from Algeria (Benslimane et al., 2011). In Australian *Ptr*, *ToxB* gene is absent, while *ToxA* is more ubiquotious. As such, races 1 and/or 2 are present in the continent (Antoni et al., 2010; Moolhuijzen et al., 2019).

TABLE 2 | *Ptr* races, structure and distribution.

Race	HSTs	Patho type	Predominant regions
Race 1	ToxA and ToxC	1 and 2	North Africa (Benslimane et al., 2011)
Race 2	ToxA	1 and 2	Great Plains of North America (Lamari et al., 1995; Lamari et al., 1998; Ali and Francl, 2003; Singh, 2007; Aboukhaddour et al., 2013) and the Southern Cone Region of South America (Singh, 2007)
Race 3	ToxC	3	North America (Lamari et al., 1998; Ali and Francl, 2003; Singh, 2007; Aboukhaddour et al., 2013) and the Caucasus region (Lamari et al., 2005)
Race 4	No HTSs	4	North America (Lamari et al., 1995; Lamari et al., 1998; Ali and Francl, 2003; Singh, 2007; Aboukhaddour et al., 2013) and North Africa (Benslimane et al., 2011)
Race 5	ToxB		Algeria (Lamari et al., 1995), the United States (Ali et al., 1999), Canada (Ali et al., 1999), Azerbaijan, and Syria (Lamari et al., 2005) but is rarely found in North America
Race 6	ToxB and ToxC		Algeria (Strelkov et al., 2002)
Race 7	ToxA and ToxB		middle east and Caucasus regions (Lamari et al., 2003; Lamari et al., 2005)
Race 8	ToxA, ToxB, and ToxC		middle east and Caucasus regions (Lamari et al., 2003; Lamari et al., 2005)

Apart from the eight well characterized races of *Ptr*, reports have suggested the presence of more races (Ciuffetti et al., 2014). Proper PCR-based analyses are needed to confirm the presence of *ToxA* and *ToxB* genes (Andrie et al., 2007). There are no molecular tests available for *ToxC*, and classification has to rely on the reaction of the differential line 6B365 (Ciuffetti et al., 2014). Ali et al. (2010) reported Ptr strains which induce necrosis even in the absance of the *ToxA* gene, which was known to be the necrosis-inducing toxin. Benslimane et al. (2011) isolated five strains causing disease on tetraploid but not hexaploid wheat. This study suggested the presence of novel races. Hence, all these facts suggest that even considering the monocultures, defining the races requires more collections and molecular level studies. This confirms the complexity of studying the *Ptr* in natural grasslands and the importance of uncovering novel races to develop a proper understanding on race distribution and population dynamics.

One of the earliest studies on genetic diversity of *Ptr* was an effort to develop an understanding about the relationship between isolates from North America and North Africa using random amplified polymorphic DNA (RAPD) markers (Aung, 2001). In that study, a significant difference was shown between the isolates of race 1 and 2 which produce the necrosis-inducing ToxA and necrosis noninducing races 3, 5, and 6, which do not produce ToxA. Later, the studies by Aboukhaddour et al. (2011), who used simple sequence repeat (SSR) markers, yielded data that agreed with what Aung (2001) had reported, with significant genetic differentiation detected. This differentiation grouped the known eight races of *Ptr* into four distinct populations based on their region of origin (Aboukhaddour et al., 2011). Interestingly, Aboukhaddour et al. (2011) revealed that ToxA nonproducing and ToxA-producing isolates are to be distantly related. Moreover, it was suggested that the host-specificity imposed by the Ptr toxins may lead to differentiation among isolates of *Ptr* (Aboukhaddour et al., 2011). Based on amplified fragment length polymorphism (AFLP) analysis, Friesen et al. (2005) concluded that the *Ptr* population is preferentially outcrossing and that spread of the pathogen is recent or constant, and also cosmopolitan. The disagreement between Aboukhaddour et al. (2011) and Aung (2001) with Friesen et al. (2005) suggests the possibility of the differential origin of the isolates (Ciuffetti et al., 2014). The results obtained by Aboukhaddour et al. (2011) are more reliable regarding the diversity of the entire *Ptr* genome because some of the SSR loci located more than 2 Mb apart even on the same chromosome. A broad study of 12 SSR markers showed moderate to high population differentiation between continents (Gurung et al., 2013). Furthermore, Aung (2001) reported 26% similarity between pathogenic and non-pathogenic isolates, while Aboukhaddour et al. (2011) reported 25%. Mating type locus (MAT) based phylogenetic analysis of *Ptr* races between 1-5 has found that the latter represent two distinct phylogenetic groups where one group is characterized by a higher homogeneity of typical tan spot producing strains, while the second group with a higher heterogeneity, including race 4 with small lesion producing strains from wheat and other hosts (Lepoint et al., 2010). Higher levels of genomic diversity (Lichter et al., 2002; Aboukhaddour et al., 2009) and karyotype polymorphisms between pathogenic and nonpathogenic strains were observed in the chromosome-based characterization of *Ptr* isolates by Lichter et al. (2002). These studies revealed the genetic diversity and the significant genomic difference between pathogenic and non pathogenic *Ptr* diversity among monocultures and in certain cases among natural grasslands.

The interaction between wheat pathogenic *Ptr* and the host is inverse of the classical gene-for-gene system observed in host-biotroph systems (Wolpert et al., 2002; Strelkov and Lamari, 2003; Friesen et al., 2008; Lamari and Strelkov, 2010). Hence, the genetic locus that conditions the HST sensitivity is known as the susceptibility locus (Ciuffetti et al., 2014). The *Tsn1* gene (*Tan spot necrosis*) is sensitive to ToxA. *Tsc1* and *Tsc2* genes (*Tan spot chlorosis*) sensitive for ToxC and ToxB (Ciuffetti et al., 2014). Furthermore, several more genes have been recognized through conidial inoculations which are not associated with HSTs and have been named as 'Tsr' (Tan spot resistance) (Singh and Hughes, 2006; Tadesse et al., 2006a; Tadesse et al., 2006b; McIntosh and Yamazaki, 2008; Singh et al., 2008). Toxin

sensitivity was shown to be controlled by the *Tsn1* gene (Lamari and Bernier, 1989b; Faris et al., 1996). Sensitivity to ToxA does not always completely define susceptibility alone (Friesen et al., 2003; Cheong et al., 2004; Faris and Friesen, 2005; Chu et al., 2008; Singh et al., 2008; Chu et al., 2010; Faris et al., 2012) but does influence disease severity to varying degrees depending on the genetic background of the host and the toxin compliment of the pathogen (Ciuffetti et al., 2014). The single dominant gene *Tsc2* produces ToxB on *Ptr* race 5 (Orolaza et al., 1995; Friesen and Faris, 2004). The *Tsc1* gene is responsible for ToxC and *Tsc1*–ToxC system is much complex than the previous two connections (Ciuffetti et al., 2014).

Plant and microbe interactions involve diverse signaling mechanisms which in turn cause changes in gene expressions both in the host and the signal-producing microbe. *Pyrenophora tritici-repentis* races have both saprobic and pathogenic forms. The pathogenic form of the *Ptr* is known as a necrotrophic pathogen due to the formation of HSTs (Ciuffetti et al., 2014). The infection process of *Ptr* is more complex than the infection process of general nectrotrophs where, the infection of *Ptr* includes the necrotrophic form and a reduced biotrophic phase (Larez, 1986; Lamari and Bernier, 1989a; Dushnicky et al., 1996; Dushnicky et al., 1998b; Dushnicky et al., 1998a). The infection phase of *Ptr* starts from spore germination and the penetration of the epidermal cells is completed within 24 hrs following the formation of the appressorium. This leads to the formation of cell wall depositions on epidermal cells due to the compatible/susceptible and incompatible/resistant genotypes suggestive to the biotroph interactions. With the susceptible-resistant interaction, the intercellular hyphal growth in mesophyll cells occurs and leads to the chlorosis and necrosis of the tissue exhibiting the characteristic tanspot symptoms (Ciuffetti et al., 2014). In *Ptr*, HSTs are known to be involved in pathogenesis because the HSTs are toxic only for the susceptible hosts but not to resistant plants or nonhosts (Ciuffetti et al., 2014). The purpose of HSTs in *Ptr* is to induce the necrosis to benefit the fungi. The response of ToxA is rapid as initiating the necrosis within nine hours of HST treatment and the signaling events initiated by toxin perception induce the changes in gene expression, which leads to cell death (Kwon et al., 1998; Rasmussen et al., 2004; Manning and Ciuffetti, 2005; Pandelova et al., 2012).

Broad genomic studies carried out for *Ptr* and karyotype analyses of currently well known races from diverse geographic regions have shown a range of sizes and defernet numbers of chromosomes, both between races and within the same race (Lichter et al., 2002; Martinez et al., 2004; Aboukhaddour et al., 2009). Genome sizes were estimated to range from 25.5 to 48 Mb with 8 to 11 chromosomes (Ciuffetti et al., 2014). The correlation between genome size and race has not been well established (Ciuffetti et al., 2014), although nonpathogenic isolates trended toward smaller genome sizes and chromosome numbers (Ciuffetti et al., 2014).

Pathogenic fungi in grasslands play a major role in shaping the dynamics of these communities. *Pyrenophora tritici-repentis* is a good example to show how certain fungal pathogens behave in a host community. Different genotypes of hosts respond differently to pathogens. Different races of the same fungi cause different symptoms or non-symptomatic conditions for the same host. It has been suggested that the host-specificity imposed by the *Ptr* toxins may lead to differentiation among isolates of *Ptr*. The studies of *Ptr* show moderate to high population differentiation between continents and karyotype polymorphisms between pathogenic and nonpathogenic strains were observed. *Pyrenophora tritici-repentis*, HSTs are known to be involved in pathogenesis because the HSTs are toxic only for the susceptible hosts but not to the resistant plants or nonhosts. Natural grasslands contain different host genotypes. Each genotype behaves differently for the same pathogen. Furthermore, through this differentiation, the different genotypes together with non-host plants can occur as heterospecific neighbors and reduce the disease spread. In addition, the same fungal pathogen species can exist as different races. Hence, the presence of a fungal pathogen in a grassland community may not be apparent unless the proper response is given by the host species or the host genotype. However, in this concern, grasslands can reserve certain pathogens inertly without showing pathogenicity in grassland communities.

Fusarium graminearum

Fusarium graminearum Schwabe, is an important cosmopolitan pathogen (**Figure 3** and **Table 1**) and produces mycotoxins which are carcinogenic to humans. Like many pathogens, *Fusarium graminearum* is self-fertile and shows facultative outcrossing (Leslie and Summerell, 2007). The primary inoculum of *F. graminearum* causes a head blight infection of wheat and barley (Guo and Ma, 2014).

Fusarium graminearum has the unique feature of compartmentalized genome structure (Guo and Ma, 2014). The subtelomeric regions of *F. graminearum* chromosomes are highly polymorphic among different isolates in the population (Guo and Ma, 2014). Furthermore, *F. graminearum* chromosomes also show a high single nucleotide polymorphism (SNP) rate (Guo and Ma, 2014). Those regions are enhanced for genes that are responsible in plant–fungus interactions, including the secretion of proteins and the actual genes expressed (Cuomo et al., 2007). Another remarkable feature in *F. graminearum* is the lack of repetitive sequences (Guo and Ma, 2014).

Fusarium graminearum is homothallic (Guo and Ma, 2014). The genome encodes for both the MAT1-1 and the MAT1-2 loci, while other fertile species of *Fusarium* are heterothallic, harboring either MAT1-1 or MAT1-2 and strains having MAT genes that are different and sexually compatible (Guo and Ma, 2014). The homothallic lifestyle of *F. graminearum* might be developed from a self-sterile ancestor. Perithecia serve as a source of inoculum for the stem root rot of pepper caused by *F. solani* f. sp. *piperis* (sexual morph: *Nectria haematococca* f. sp. *piperis*) (Cuomo et al., 2007).

To overcome host defenses, *Fusarium graminearum* must have evolved an effective reserve in order to establish infection. In fungi, G protein coupled receptors (GPCRs) act as sensors for environmental cues on the cell membrane, which then activate the signaling pathways, and in *F. graminearum* 84 GPCR were predicted (Cuomo et al., 2007). Plant tissue penetration is a

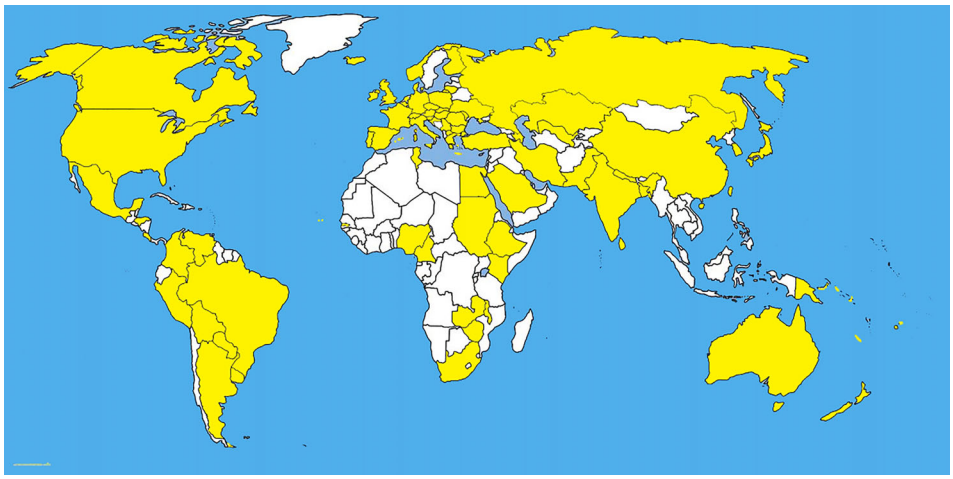

FIGURE 3 | The distribution map of *Fusarium graminearum*. The areas where infection have been reported are indicated in yellow (www.cabi.org/isc. Accessed on 25/03/2021).

crucial step in the early stage of infection (Guo and Ma, 2014). The genome of *F. graminearum* is enriched with cutinase genes, which are responsible in cuticle degradation and penetration of the epidermis by the pathogen (Guo and Ma, 2014). The virulence of *F. graminearum* on both wheat and maize is determined by Lipase FGL1 (Cuomo et al., 2007). This lipase FGL1, along with MAPKGPMK1, is regulated by the RAS2 protein in *F. graminearum* (Bluhm et al., 2007). The process is clearly demonstrated by the MAPK cascade and signal RAS proteins (Bluhm et al., 2007). Mycotoxin DON is known to be the virulence factor in *F. graminearum* as the DON nonproducing strains do not show virulence or reduced virulence (Proctor et al., 1995). However, Tri5 mutants form appressoria-like structures similar to those of the wild-type strain (Boenisch and Schäfer, 2011). The production of DON depends on tissue types during the infection of wheat heads by *F. graminearum* (Guo and Ma, 2014). In addition, mutants that have increased sensitivity to environmental stresses such as oxidation, heavy metals, and antifungal compounds tend to have attenuated virulence as these mutants are likely to be vulnerable to the plant defense actions (Guo and Ma, 2014).

Results of studies on *Fusarium graminearum* shows how the fungus overcomes the host defense and establishes the disease. In highly diverse and highly competitive grassland populations, *F. graminearum* is well adapted to implement the disease by overcoming the host defense. Furthermore, *F. graminearum* shows high adaptability for environmental stresses.

Bipolaris sorokiniana

Bipolaris species are mostly found on grasses (Manamgoda et al., 2014; Bhunjun et al., 2020) and in this example we deal with *Bipolaris sorokiniana*. Although the population, pathogenicity, and DNA polymorphism of *Bipolaris sorokiniana* Shoemaker in natural grasslands have not been studied, but the behaviour of *B. sorokiniana* was well studied as a pathogen of economically important crops. Most recent studies on *B. sorokiniana* provides a good understanding about pathogenicity variations and DNA polymorphism of pathogenic fungal communities in a monoculture (Kang et al., 2021). (**Figures 4**, **5**) in diverse geographical areas. Several fungal pathogens are associated with wheat black points in different regions (Dai et al., 2011). *Bipolaris sorokiniana* shows a cosmopolitain distribution (Kang et al., 2021) with highly varied pathogenicities and genetic diversities (**Figures 4**, **5**) (**Table 1**). Aghemiri et al. (2015) has studied 46 samples of *B. sorokiniana* in Iran and it showed that *B. sorokiniana* grouped into highly distinct clusters representing different geographical regions. Hence, it is obvious that *B. sorokiniana* has higher adaptability towards different geographical conditions, which denotes that *B. sorokiniana* populations in different grassland communities might be varied with the physical conditions that the fungal community faces. In another study, 95 isolates of *B. sorokiniana* collected from various plant parts and cultivars of spring barley showed remarkable differences in pathogenicity (Baturo-Ciesniewska, 2011). Furthermore, no significant correlation among pathogenicity and origin of isolates was observed (Baturo-Ciesniewska, 2011). Population genetic variations among two different populations in two consecutive years were assesed by a most recent study of Kang et al. (2021). It revealed significant polymorphisms between isolates and proportional connectivity between geographic distance and genetic diversity. Moreover, the study revealed that the pathogenic variations in *B. sorokiniana* has no relevance to its geographical origins. Cross assays of *B. sorokiniana* among wheat and maize roots showed the ability of causing disease in both hosts. Clustering analyses of different isolates from the same area indicated the variability of genetic populations in *B. sorokiniana*. More interestingly, isolates from the same tissue sample showed higher variations. Leisovasvobodova et al. (2012) revealed that the limits of gene exchange were around 80–100 km, and beyond that gene exchange rates became very low. The studies relevant to the host infection are controversial. Yan et al. (2012) found that the

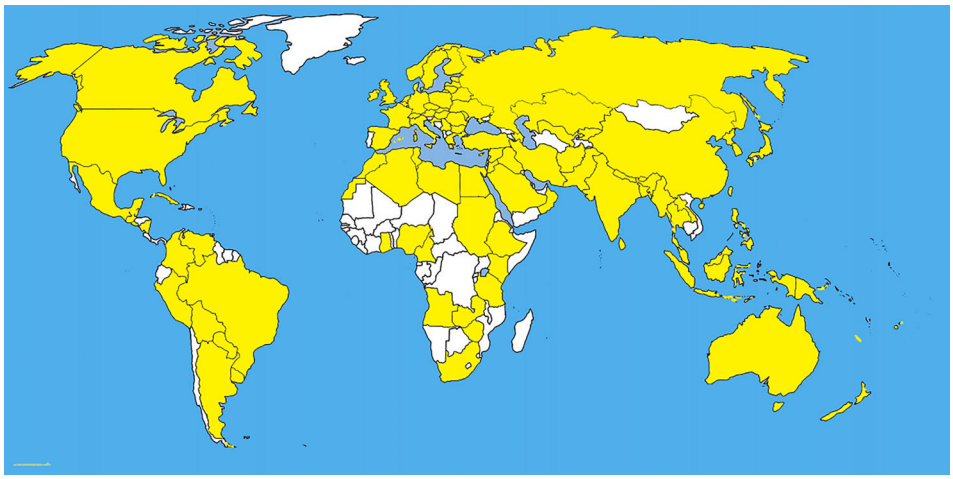

FIGURE 4 | The dIstribution map of *Bipolaris sorokiniana*. The areas where infection have been reported are indicated in yellow (www.cabi.org/isc. Accessed on 25/03/2021).

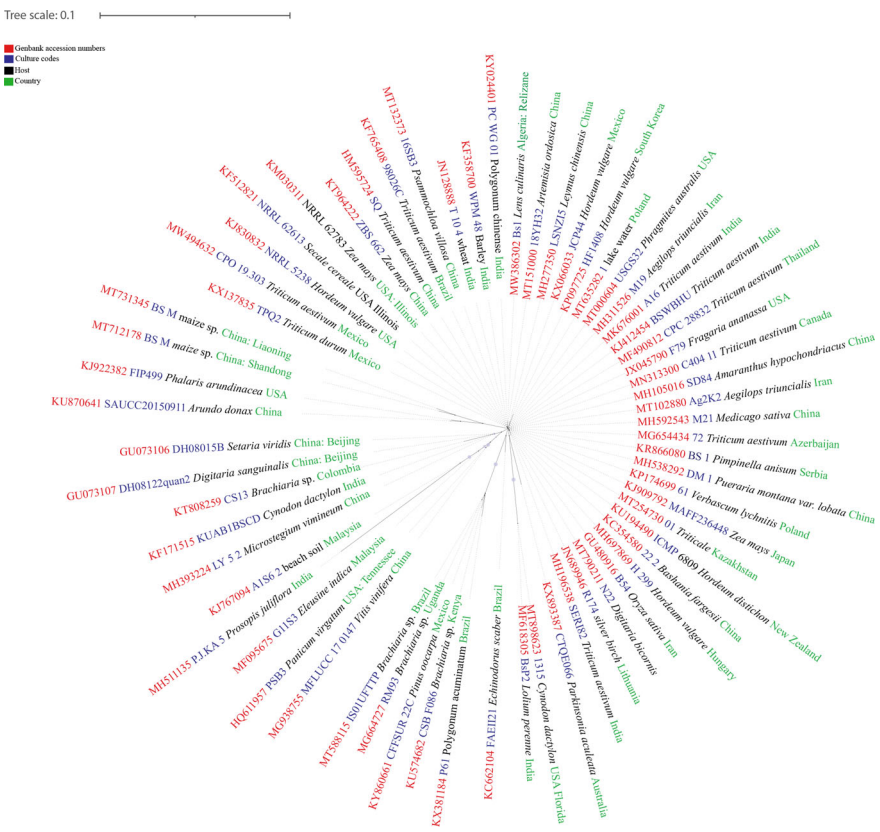

FIGURE 5 | The unrooted RAxML bipartion unrooted phylogenetic tree of *Bipolaris sorokiniana* built from sequences retrieved from NCBI GenBank.

systematic generation of infection is from root to leaf. However, Gyawali et al. (2012) revealed that isolates from root and leaf on the same host show differentiation of infections.

Studies of *Bipolaris sorokiniana* have revealed its higher adaptability to various environmental conditions. Even though all previous studies were carried out in monocultures,

experiments such as those involving cross infection assays indicate that grassland comunities into some extent. *Bipolaris sorokiniana* can infect several grass species and has considerably variable infection mechanisms, which reveals the ability of retaining it successfully in natural grasslands. Hence, this reveals that *B. sorokiniana* in different grasslands have different pathogenicities and significantly different polymorphism.

Colletotrichum graminicola

Colletotrichum being a major pathogen causes diseases on grasses as well (Jayawardena et al., 2016; Bhunjun et al., 2021). *Colletotrichum graminicola* (Ces.) G. W. Wils. which is now considered to be in *Colletotrichum graminicola-caudatum* complex with 25 species (Bhunjun et al., 2021). *Colletotrichum graminicola* (Ces.) G. W. Wils. causes anthracnose disease in maize, which causes stalk rot and seedling blight worldwide (Bergstrom and Nicholson, 1999) (**Figure 6**). Although, the effect of *C. graminicola* on roots is not well known, several studies have shown that the infection spreads through residues of soil and progresses to upper aerial sections (Leonard and Thompson, 1976; Keller and Bergstrom, 1988). The studies by Leonard and Thompson (1976), and Keller and Bergstrom (1988) are not based on molecular phylogeny. Hence it is not known the exact species they were dealing with, as the *C. graminicola* is a species complex. Based on ultrastructural studies, it is clear that anthracnose infection penetrates through the formation of appressorium with penetration peg (Eisermann et al., 2019).

According to the studies of Sunko et al. (2008), *Colletotrichum graminicola* shows morphological characteristics of root pathogens and spreads out to aerial plant organs. Nordzieke et al. (2019) studied infection stratergies of two different spore types of *C. graminicola.* Falcate conidia form in necrotic lesions and have to capability of forming appressoria. Hence, falcate conidia are key disease spreading propagules from plant to plant while oval conidia formed in vascular system of infected plants cause the distribution of disease within the plant. Furthermore, Nordzieke et al. (2019) showed that germinating falcate conidia are controlled by self-inhibitor mycosporine-glutamine, whereas it is absent in oval conidia. Moreover, studies revealed that oval conidia undergo germling fusions while falcate conidia do not. However, plant infection experiments revealed efficient leaf infections by oval conidia (Nordzieke et al., 2019). With such specifications on conidia, *C. graminicola* has the ability to infect various plant tissues, such as leaves, stems, and roots as well as grows saprophytically on dead plant material to over winter (Nordzieke et al., 2019).

At the onset of infection, *Colletotrichum graminicola* secretes proteins to avoid pathogen-associated molecular pattern recognition by the host (Eisermann et al., 2019). Eisermann et al. (2019) revealed that among the proteins secreted by *C. graminicola*, only a few are essential for fungal virulence. Among small clusters of *C. graminicola*, only a single protein is involved in infection process. There is a probable involvement of other proteins, even though it might not be able to detect experimentally. In the infection process, targeted deletion of 26 individual candidate genes and seven gene clusters with 32 genes of *C. graminicola* are known as a pathogenicity cluster (CLU5) of five co-linear genes. With the exception of CLU5b, encode secreted proteins CLU5a and CLU5d are required for full appressorial penetration.

Studies of *Colletotrichum graminicola* have demonstrated fungal adaptability on fungal infection, implementation of the disease and how the disease spreads through the plant. These are critical aspects in highly diverse grasslands. Current knowledge of *C. graminicola* is mainly based on monocultures and effects on infection mechanisms in highly diverse environments are not well known.

DISEASES OF HUMANS AND OTHER ANIMALS

The natural grasslands are complex ecosystems of plants and animals (Gibson, 2009). Their interactions are shown to be

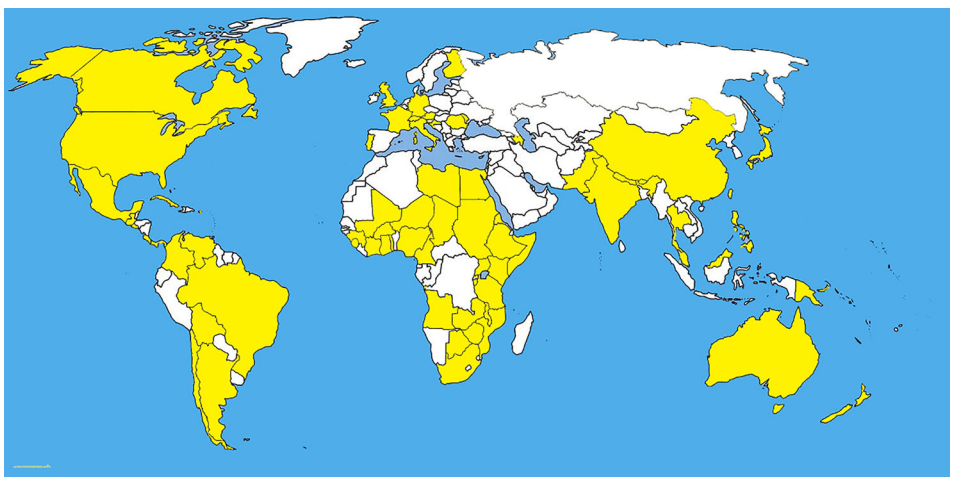

FIGURE 6 | The distribution map of *Colletotrichum graminicola sensu lato.* The areas where infection have been reported are indicated in yellow (www.cabi.org/isc. Accessed on 25/03/2021).

highly variable (Gibson, 2009). Hence, the interactions between animals and grassland ecosystems cannot be neglected. Grasslands shelters an immense number of vertebrate and invertebrate animals and their interactions are vital for the grasslands (Gibson, 2009). Therefore, identification and understanding of fungal diseases on animals living in grasslands are important.

A direct study regarding fungal pathogens on grassland animals is not reported. Hence the linkage of current knowledge regarding domestic and industrial farms such as cattle farms can be taken to get an insight. A well-studied disease chronic facial eczema on sheep is caused by a grassland fungus *Pithomyces chartarum*. *Pithomyces chartarum* is a well-known saprobe in monocots including grasses (Laven et al., 2020; Munday et al., 2020) and it causes facial eczemas in ruminants and camelids (Laven et al., 2020). *Pithomyces chartarum* produces a mycotoxin known as sporidesmin which causes edema, ulceration and crusting dermatitis on face and ears in ruminants and camelids (Munday et al., 2020). Sporidesmin concentrates in bile and causes damages to liver through the bile duct (Laven et al., 2020).

Fungal diseases on humans relevant to grasslands have not studied directly and no published data is available on direct fungal infections on humans caused specifically by grassland fungi. But diseases caused by mycotoxins through the cattle industry was reported (van Egmond, 1983; Kemboi et al., 2020). Mycotoxins are hazardous to cattle as well as humans who consume dairy products (van Egmond, 1983; Kemboi et al., 2020). Main mycotoxin categories relevant to the dairy industry are Aflatoxins (AF) produced by *Aspergillus* species, deoxynivalenol (DON) produced by *Fusarium* species, fumonisins (FUM), ochratoxin A (OTA) produced by *Penicillium* and *Aspergillus* species, T-2 toxin (T-2) and zearalenone (ZEN) (Kemboi et al., 2020). The well-known aflatoxin M1 is a carcinogen (van Egmond, 1983; Kemboi et al., 2020).

Fungal pathogens of humans and other animals in grasslands are least studied, although, such pathogens are economically highly important. There should be emerging animal and human pathogens in grasslands around the world. Hence comprehensive studies on pathogens in grasslands are important.

CONCLUSIONS

Natural grasslands are a vital component of several different types of terrestrial ecosystems but are not yet sufficiently studied (Gibson, 2009). Grasslands are highly complex ecosystems comprised of perennials and are dominated by members of the family Poaceae (Risser, 1988). With the high density of plants and the high density of below ground root systems, grasslands provide an ambient environment for microbial growth (Mommer et al., 2018; Ampt et al., 2019). The composition of the flora of the grasslands in various regions is affected by unique biological and ecological factors (Risser, 1988; Gibson, 2009). Hence, the interaction between the microbiota and their hosts becomes unique in each grassland system based on the floral composition.

There is a wide range of fungal lifestyles in nature, but our concern in this paper was focused on the pathogenic lifestyle of the fungi associated with natural grasslands. The fungal- plant interactions in grasslands can be described using two approaches —host specificity based and density dependent based. Research on fungal pathogens can be based on either phylogeny or pathogenicity (Janzen, 1970; Connell, 1971; Bever et al., 2015). Currently, there have been many taxonomy and phylogeny based studies on the fungi associated with grasses in different ecosystems, which are subject to change since many novel taxa are continuously being introduced. This situation provides the basis for developing an insight for resolving the taxonomic placement of identified and unidentified fungal taxa. In many cases these data cannot be used in accordance with the pathogenicity related studies unless the life mode is confirmed, specifically through the confirmation of the pathogenicity through the Koch postulate. Grasslands also cause disease of humans and other animals.

Grassland ecosystems are characterized by complex interactions between pathogens and their hosts. Although the majority of the grassland plants are monocots, there are dicots present among the monocots plants (Herben et al., 1993; Van Der and Sykes, 1993; Gibson, 2009). Furthermore, there is a high diversity of species in the family Poaceae. Hence, grasslands cannot be considered as a monoculture, and the interactions between monocots as well as between monocots and dicots need to be considered (Herben et al., 1993; Van Der and Sykes, 1993).

The fungal community below ground and above ground are highly diverse and contrasting. Furthermore, the distribution of fungal pathogens above and below ground in grasslands are complex (Van Ruijven et al., 2003; Van Ruijven and Berendse, 2005; Van Ruijven and Berendse, 2009; Cong et al., 2014; Cappelli et al., 2020). Comparatively speaking, the ecology-based studies of fungal pathogens below ground are much more common than the ecology based studies of fungi above ground in natural grasslands. Studies of the fungal pathogens of members of the Poaceae are mainly focused on agricultural monocultures. Hence, there is a dearth of information on above ground fungal pathogens rather than those below ground. In certain cases, these data are applicable to natural grasslands as far as the same species is concerned. However, the effects of complex ecological intereactions in the natural grasslands cannot be neglected.

Fungal pathogens of animals in grasslands are poorly studied. Natural grasslands provide grazing sites for large ruminants (Gibson, 2009). In many instances those grazing ruminants directly or indirectly involve human activities and the economy. Hence it is important to study relationships between grassland animals and fungi. Chronic facial eczema caused by *Pithomyces chartarum* shows how important it is to understand fungal communities in grasslands (Laven et al., 2020; Munday et al., 2020). In a way, grasslands can be a reservoir of fungal pathogens.

In this review, we have provided four examples where a complete study has been carried out on the pathogenicity and genomics of a particular pathogen. They are based on agricultural monocultures with the help of natural grassland based data. There is a necessity of exploring the natural grasslands to identify novel fungal species. In the meantime, confirming the pathogenicity is also very important. Agronomically, it is important to study the pathogenic community in grasslands as a reservoir of fungal pathogens and understanding the dynamics of the fungal community further helps to understand

the effects of fungal pathogens in agricultural multi-lines. The study of *Pyrenophora tritici-repentis* demonstrated just how important it is to perform a thorough study even on a well-known pathogen, since there are many races with different life modes and attributes. The behavior of the pathogen in natural grassland is also important to observe in order to understand changes in gene expression and pathogenicity.

Plant diversity of grasslands is highly variable around the world. However, the stable natural grasslands are rich in host diversity. The belowground host components shapes the total fungal community and the above ground fungal community highly affects the productivity and the biomass of the grassland. Hence, the stability of grasslands is highly dependent upon the balanced interactions of those factors together with other biotic and abiotic factors. The behavior of specialist pathogens and generalist pathogens in grasslands is crucial for the stability of these communities. Heterospecific neighbors in grasslands disturbs the spread of specialist pathogens but also could facilitate the spread of generalist pathogens. However, fungal disease spread in grasslands depend on huge number of biotic and abiotic factors. Different fungal pathogens act differently on the hosts. Meantime, host respond is highly variable. Studies of *Pyrenophora tritici-repentis* on grasses provide good insight on how pathogenic response of different host genotypes vary towards the same pathogen. In addition, different hosts or genotypes of grasses can induce host-fungal pathogens without causing symptoms. Hence, in a way, grasslands can act as a reservoir for fungal pathogens. Even though pathogenic mechanisms of fungi have been well explained from monocultures, the virulence of the fungus varies with the high diversity in grass lands. Many factors such as heterospecific neighbors, tightly and closely arranged roots, effects of root exudates and root microbiomes, and different host traits can control disease spread.

The knowledge of host-pathogen interactions in grasslands can be used for agricultural purposes. Strategic grasslands can be used among the crop fields to control the spread of diseases. Grasslands in between crop fields can act as dense heterospecific host sites for many pathogens and this can immensely reduce the disease spread among the adjusent crop fields. Furthermore, the presence of highly diverse grasslands can reduce the disease insidence of the specialist pathogens. Grass heterospecific neighbor effects of the soil-borne fungal pathogens can be explained in two ways. First, neighbor effects though plant traits or root exudates. Second, neighbor effects through root microbiome. However the loss of dominant plant species in grasslands can increase the extent of disease in the system (Mitchell et al., 2002). Hence, proper understanding of the dominant species in the strategic grasslands and maintainance of the dominant species is important for disease control.

Grasslands are an ecologically and economically important component of the earth's vegitation. Fungal communities in grasslands play a huge role on the stability of grasslands. Having more complete knowledge of fungal pathogens in grass lands is important for developing an understanding of grassland ecology. In addition, understanding behaviour of fungal pathogens in highly diverse grasslands may provide novel insights towards being able to control the diseases in commercial crop fields. In this review, we address the effects of fungal pathogens in grasslands and discussed their complex interactions.

AUTHOR CONTRIBUTIONS

AK, ST, KH and SCK designed the review. SL, JK and SK provided the grant. AK wrote the manuscript with ST, CN, SCK and SLS. RJ, SA, JX, KH, JK and SL reviewed and edited the manuscript. All authors reviewed and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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