

Fungi in Pine Wilt Disease

Subjects: Mycology | Forestry

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Pine wilt disease (PWD) is a complex disease that severely affects the biodiversity and economy of Eurasian coniferous forests. Three factors are described as the main elements of the disease: the pinewood nematode (PWN) *Bursaphelenchus xylophilus*, the insect-vector *Monochamus* spp., and the host tree, mainly *Pinus* spp. Nonetheless, other microbial interactors have also been considered.

Keywords: biocontrol ; blue-stain fungi ; interactions ; mycobiome ; pine wood nematode

1. Introduction

Pine wilt disease (PWD) has become one of the most damaging diseases to conifers worldwide and is a risk nowadays for the sustainability and profitability of forest ecosystems. PWD was detected in Japan in 1905, later spreading to other Asian countries; namely China, Korea, and Taiwan in the 1980s [1], and to Europe (Portugal and Spain in 1999 and 2010, respectively) [2][3][4]. To date, no recorded outbreaks have been identified in other European countries, despite several scientific studies alert for a high vulnerability of northern European pine forests due to the oncoming effects of climate change and the reported susceptibility of the dominating pine species to PWD [5][6]. The PWD has gradually and consistently spread between continents as a result of an increase in the global trade of wood and derivative materials. Significant economic and ecological impacts were reported in the affected countries, including a reduction in productivity and an increase in the costs of management procedures for disease control, as well as a decrease in forest biodiversity [7][8].

Effective PWD management strategies have been difficult to achieve given the complex disease infection cycle, where several organisms contribute to the infection's overall development and severity, namely its causal agent, the pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Bühner) Nickle; the PWN's insect-vector *Monochamus* spp. ; and a susceptible host tree, commonly trees from the genus *Pinus* [1]. Recent studies have additionally identified a strong influence of the PWN-associated bacteria, and the microbiome associated with the susceptible pine, on PWD development [9][10][11]. While the description of microbial diversity has been established for the PWN, the insect-vector, and susceptible pine hosts—leading to the preliminary proposal of their functional roles in the PWD—information on the mycological diversity associated with these organisms is scarcer. In the present review, an up-to-date compilation of the published works reporting on naturally occurring fungi associated with the PWN, the host tree, and its insect-vector, is presented. A critical analysis of the summarized information further allows us to envision the functional role of associated fungi on PWD development and guide future research in this area.

2. The Complexity of PWD

The PWN is a small plant-parasitic nematode of about 500–1000 μm in length and 22 μm in width, capable of feeding on plant (phytophagous) and fungal tissues (mycophagous). Its life cycle is comprised of four juvenile stages (J1 to J4) and both adult males and females (Figure 1 a). In natural conditions, the PWN can quickly complete its life cycle, usually 4 days in summer conditions while, under in vitro conditions in laboratory cultured *Botrytis cinerea* Pers. mats, it can take between 4 to 6 days [1][12] (or slightly longer on aseptic co-cultures of in vitro pine with the PWN) [13]. Adverse environmental conditions and/or undernutrition induce morphological and physiological changes in the J3 stage, leading to the occurrence of the PWN dispersal stages, the third and fourth dispersal juvenile stages (J III and J IV). The J IV, known as the dauer juvenile stage, is characterized by an interruption of the feeding process, the establishment of large lipidic reserves, and the production of a thick protective layer around the PWN's body [1][14]. At this stage, the PWN is attracted to the juvenile longhorn beetles emerging from dead, or decaying, wood in forest ecosystems, invading beetles trachea. The PWN establishes these commensalism associations with members of the genus *Monochamus* (Coleoptera; Cerambycidae), considered the main PWN vector, with seven confirmed species vectoring the nematode in field and laboratory conditions [15][16][17], the most common being *M. alternatus* in East Asian forestlands [1], and *M. galloprovincialis* in Europe [18][19]. Colonized beetles can transmit the PWN by transporting them to (1) new beetle feeding sites during the beetle maturation stage, commonly in young branches of susceptible pine species (primary transmission), or (2) weakened or dead trees in the forest, where the matured female beetles oviposit (secondary transmission). The beetles benefit from an increase in reproduction sites at weakened or dead trees, where oviposition occurs, while the PWN takes advantage of the increase in beetle population, leading to higher rates of transmission (Figure 1 b) [20]. Once infection occurs in a susceptible tree, the PWN quickly multiplies and begins feeding on the epithelial parenchyma cells lining the pine resin ducts, inducing extensive damage that leads to a reduction in resin production and the release of volatile

terpenoids. As the infection progresses, the PWNs damage the tree's vascular system and an embolism phenomenon begins to interrupt water transport, partly due to the build-up of volatile terpenoids [20][21]. The typical symptoms of PWD (**Figure 2**) are visible at this stage, namely pine shoot wilting due to desiccation, chlorosis, and drooping. When external symptoms of the PWD are noticeable, the affected tree cannot recover and eventually dies. However, the symptoms caused by the PWN, including abrupt reduction in the production of resin and pine needle wilting, can be mistakenly attributed to other biotic or abiotic stress factors such as water stress [22]. In North America, endemic pines are mostly tolerant, with only exotic species expressing the strongest symptoms. In China, Japan, Korea, and Portugal, many pine species were found to be very susceptible, e.g., *Pinus densiflora* Siebold & Zucc. , *Pinus nigra* J.F. Arnold, *Pinus pinaster* Aiton, *Pinus radiata* D. Don, *Pinus sylvestris* L. or *Pinus thunbergii* Parl., which caused extensive economic and cultural repercussions [15][23].

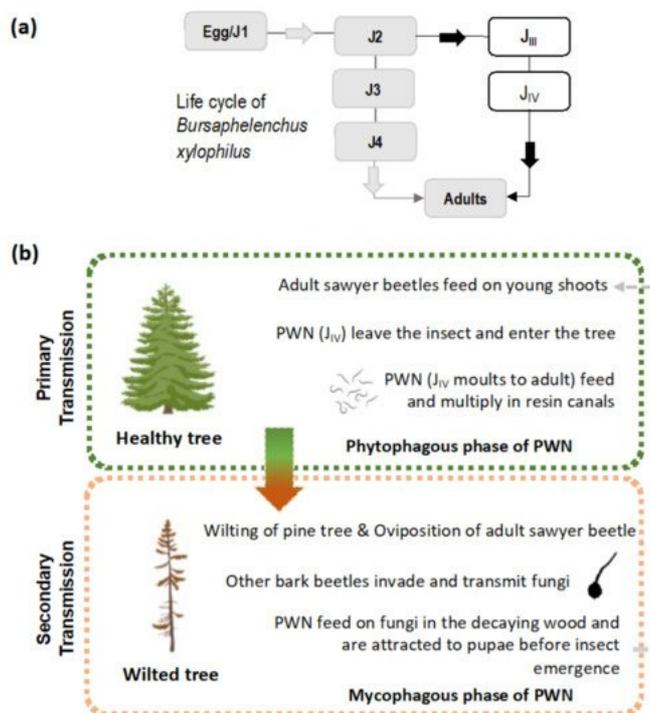


Figure 1. Pine Wilt Disease pathosystem. (a) Life cycle of the pinewood nematode *Bursaphelenchus xylophilus* (grey arrows indicate propagative stage; black arrows indicate dispersal stage); (b) Primary and secondary transmission of *B. xylophilus* by the sawyer beetle *Monochamus* sp. (grey arrow indicates interconnection between transmission modes). Image of a healthy and wilted tree, retrieved from Biorender®.



Figure 2. Symptoms of Pine Wilt Disease—wilted *Pinus pinaster* trees (pine trees with brown canopy) surrounded by asymptomatic *P. pinaster* (greenish canopy without PWD manifestation). Location: Companhia das Lezírias (Portugal; 38°49'17.6" N 8°52'20.5" W), in January 2020.

Despite not being able to induce PWD, the microbiome (bacteria and fungi) associated with the PWD, and its elements is considered a major biotic factor influencing the disease's severity (further detailed in [9][10][11]). The isolation of fungi and bacteria dates back the 70s, when Tokushige and Kiyohara isolated microbial samples from dead pine trees and tested their pathogenicity in healthy pines [20]. Since then, several researchers attempted to correlate the pathogenicity of some bacterial species, like *Pseudomonas* spp. or *Bacillus* spp. , with the disease [24][25][26]. Still, the latest studies point to a less active role of the bacterial communities associated with PWD. These communities behave as opportunistic/saprophytes and/or endophytes expressing phenotypic plasticity in the PWN-wilted pine host [11][27][28][29][30][31]. The impact of fungal communities in the complexity of PWD and their elements is presented in detail in the next section.

3. Mycobiome Associated with PWD Complex

Forest trees harbour extremely complex fungal communities that play important roles on ecosystem multifunctionality and equilibrium [32]. Most of these communities are shaped by intrinsic factors of the host, like host genotype, condition, and/or development, and by external factors such as geographical location, seasonality, or even surrounding vegetation [33]. Under the presence of non-native pathogens, these ecosystems suffer disequilibrium situations that can eventually lead to considerable spatial and temporal community variation [34].

Fungal communities associated with PWD have been described since the early 80s. These culture-dependent studies worked with different culture conditions, such as different growth media (e.g., potato dextrose agar or malt extract agar) or supplementation of antibiotics (Table 1). The most recent reports in fungal communities associated with the disease combine morphology with molecular identification based on fungal DNA barcode markers (i.e., primary marker ITS, internal transcribed spacer; and secondary marker TEF1- α , translational elongation factor 1 alpha) [35][36] and other protein-coding genes (i.e., beta-tubulin BT or calmodulin CAL) [37]. These conventional culturing methods are biased towards fast-growing species rather than the more specialized fungi [38], suggesting that only a limited fraction of the fungal community's diversity has been uncovered in this complex disease. Three main phyla were identified within the Fungi kingdom, namely Ascomycota, Basidiomycota, and Mucoromycota. The most predominant phylum, Ascomycota, was represented by 6 classes (Blastomycetes, Dothideomycetes, Eurotiomycetes, Leotiomycetes, Orbiliomycetes, and Sordariomycetes) and nearly 30 families (Table 1). Most of the described taxa are common saprophytes and probably not specific to the disease or associated with the PWN. This may be the case of *Penicillium* (Ascomycota, Eurotiomycetes, Trichocomaceae), *Trichoderma* (Ascomycota, Sordariomycetes, Hypocreaceae) and *Aspergillus* (Ascomycota, Eurotiomycetes, Aspergillaceae) among others, which are ubiquitous to all existing environments and detected in all PWD elements (Table 1). The description of fungal communities has also been made in different pine species and insect-vectors from different geographical locations (e.g., China, Japan, Korea, Portugal, and the USA). The first isolations of mycoflora were conducted on symptomatic *P. thunbergii* (shoots, twigs, and woodchips), on the surface tissues of tunnels and pupal chambers bored by *Monochamus* larvae, and from the adult body of *M. alternatus* after its emergence [39][40]. These works reported that the genera *Ceratocystis* and *Verticicladiella* (synonym of *Leptographium* [41]), from Ceratocystidaceae and Ophiostomataceae families, respectively, were the only flora common to the three sampled locations apart from the saprophytic fungi *Trichoderma* and *Penicillium*. Later, Wingfield [42] isolated fungi from the cerambycid beetles *M. scutellatus* and *M. carolinenses*, also identifying *Ceratocystis* and *Ceratocystiopsis* as common genera associated with adult beetles and pupal chambers from *P. banksiana* and *P. resinosa*. Curiously, Wingfield [42] could also isolate the nematode-trapping fungi *Arthrobotrys cladodes* var. *cladodes* and *A. superba* in the PWN. Kuroda and Iko [43] isolated fungi from healthy and wilted *P. thunbergii* and reported that the same species were recovered from both pine trees (i.e., *Pestalotiopsis* spp., *Nigrospora* spp., *Cladosporium* spp., and *Phomopsis* spp.) and that *Ceratocystis* sp., was only detected after PWN inoculation. This study has also shown that the composition of fungal species varied slightly among seasons [43]. In 2007, Hyun et al. [44] characterized the fungal communities associated with the PWN; the insect- vector *M. alternatus* and *P. thunbergii* in Korea. Among the 15 genera identified, *Penicillium* and *Ophiostoma* were the most frequent genera in all elements, with PWNs and insect larvae showing a smaller number of associated fungi than insect adults or infected wood. In 2015, Inácio et al. [45] described fungal communities associated with *M. galloprovincialis*, native to Portugal. From a total of 100 insects, species of 18 genera of filamentous fungi were reported: *Acremonium*, *Alternaria*, *Arthrinium*, *Aspergillus*, *Beauveria*, *Bipolaris*, *Botryosphaeria*, *Botrytis*, *Cladosporium*, *Clonostachys*, *Eppicocum*, *Fusarium*, *Ophiostoma* s. l., *Paecilomyces* s. l., *Penicillium*, *Stemphylium*, *Trichoderma*, and *Trichothecium* (Table 1) [45]. A more detailed characterization of endophytic fungi associated with *P. pinaster* with, and without, PWN infection in Portugal was presented by Trindade [46]. Novel fungal species are also continuously described. Wang et al. [47] identified new species of Ophiostomatales associated with PWD in the pupal chambers of *M. alternatus* from infected *Pinus massoniana* Siebold & Zucc. and *P. thunbergii* in China. In this study, over 90% of all isolates were identified as *Ophiostoma ips*, with three novel species— *Ophiostoma album* sp. nov., *Ophiostoma massoniana* sp. nov. and *Sporothrix zhejiangensis* sp. nov.—and two species whose identities remained unclear; *Ophiostoma* cf. *deltoideosporum* and *Graphilbum* cf. *rectangulosporium* [47].

Table 1. Culture-dependent mycoflora isolated from the PWN, insect-vectors, and host pines. PWN, *Bursaphelenchus xylophilus*; Ma, *Monochamus alternatus*; Ms, *M. scutellatus*; Mg, *M. galloprovincialis* (A—Adult, L—Larvae); Pd, *Pinus densiflora*; Pm, *P. massoniana*; Pt, *P. thunbergii*; Pb, *P. banksiana*; Pr, *P. resinosa*; Pp, *P. pinaster* (W—wood, Pc, pupal chamber); JP, Japan; PT, Portugal; KR, Korea; CH, China; USA, United States of America.

The availability of high-throughput sequencing (HTS) technologies, such as amplicon metagenomics, has revolutionized ecological studies of fungal communities [37] allowing broader insight into the complexity of host-fungal interactions. The primary fungal DNA barcode ITS is commonly used on HTS-based metabarcoding. Still, caution should be taken when interpreting HTS results since, for several groups of important plant pathogens and endophytes, ITS provides insufficient resolution for species-level assignment [37]. It is recommended that researchers use the ITS2 subregion, less taxonomically biased, with lower length variation and more universal primer sites, or the full ITS region with greater taxonomic resolution and reduced amplification of dead organisms [37]. In the context of PWD, amplicon technologies were firstly applied to characterize bacterial communities (e.g., using the hypervariable regions of 16S rRNA molecule), revealing the presence of PWNs and PWD progression [54][55][52]. Chu et al. [56] described the impact of the disease on root-associated fungi (e.g., ectomycorrhizal fungi, ECMF; dark septate endophytic fungi, DSE; arbuscular mycorrhizal fungi, AMF) in different stands of *Pinus tabulaeformis* Carrière forest (undisturbed, moderate, and highly disturbed pine stands). The authors showed that fungal community richness and diversity, as well as soil hyphal density, decreased with the increase of disease disturbance. Basidiomycota and Ascomycota were the dominant root-associated fungi, with specific genera present in the different disturbed stands [56].

While exploring PWN growth and development by the fungi of non-infected and infected pine trees, several fungi species showed promising results in the control of nematode populations (**Table 2**). Naturally occurring Ascomycota fungi from the genera *Aureobasidium* , *Aspergillus* , *Cephalosporium* , *Fusarium* , *Gliocladium* , *Mucor* , *Mortierella* , *Penicillium* , *Rhizoctonia* , and some species of *Trichoderma* and *Verticillium* , affected PWN survivability in in vitro or in vivo bioassays [40][48][49][50][57][58]. To further study the effect of *Trichoderma* sp. on PWN suppression and transmission by *M. alternatus*, Maehara et al. [59] inoculated several isolates of *Trichoderma* spp. into wilt-killed *P. densiflora* logs. Beetles from logs treated with *Trichoderma* sp. 3 carried less than 1000 nematodes. The authors suggested that combining the use of this fungus for PWN control with the entomopathogenic fungus *Beauveria bassiana* for *M. alternatus* control could represent a potential biocontrol application in PWD [59]. The entomopathogenic fungus *B. pseudobassiana*, isolated from naturally infected *M. galloprovincialis* in Spain, has also showed potential as a natural insect population regulator [60], and it may also be feasible in combination with other PWN control agents.

Table 2. Nematode-fungi interactions in pine wilt disease (PWD). List of fungal species with a positive effect on pine wood nematode (PWN) multiplication and distribution, and potential agents for biocontrol applications with respect to PWD management.

Fungi in Interaction with PWNs	Mode of Action	References
<i>Botrytis cinerea</i>	Increase of PWN population growth in in vitro mycelial fungi	[49]
<i>Ceratocystis</i> sp.	Increase of PWN population growth in in vitro mycelial fungi and in pine segments of <i>Pinus densiflora</i>	[39][40][49]
<i>Diplodia</i> sp.	Increase of PWN population growth in in vitro mycelial fungi	[49]
<i>Pestalotia</i> sp.	Increase of PWN population growth in in vitro mycelial fungi and in pine segments of <i>Pinus thunbergii</i>	[39][40]
<i>Macrophoma</i> sp.	Increase of PWN population growth in in vitro mycelial fungi and in pine segments of <i>Pinus thunbergii</i>	[39][40][61]
<i>Fusarium</i> sp.	Increase of PWN population growth in in vitro mycelial fungi	[39][40]
<i>Ophiostoma minus</i>	Increase of PWN population growth in wood blocks of <i>Pinus thunbergii</i> ; Increase of PWN population growth in <i>Pinus densiflora</i> bolts; Increased no. of PWNs carried by emerging <i>Monochamus alternatus</i>	[57][58][61][62] [63][64]
Growth promoters		
<i>Leptographium</i> sp.	Increase of PWN population growth in logs of <i>Pinus densiflora</i> ; Increase of axenic PWN population on autoclaved cuttings of <i>Pinus thunbergii</i>	[48][49][62]
<i>Crytosporiopsis</i> sp.	Increase of PWN population growth in wood blocks of <i>Pinus densiflora</i>	[48]
<i>Sporothrix</i> sp.	Increase of PWN population growth in in vitro mycelial fungi	[65][64]
<i>Ophiostoma ips</i>	Increase of PWN population growth in in vitro mycelial fungi and in segments of <i>Pinus thunbergii</i>	[65][66][63]
<i>Leptographium pine-densiflorae</i>	Increase of PWN population growth in in vitro mycelial fungi	[64]
<i>Trichoderma</i> sp. 1	Increase of PWN population growth in in vitro mycelial fungi	[48]
<i>Trichaptum abietinum</i> <i>Arthrobotrys</i> sp. <i>Gloeophyllum striatum</i> <i>Cryptosporus volvatus</i>	Increase of PWN population growth in <i>Pinus densiflora</i> ; Increased no. of PWNs carried by emerging <i>Monochamus alternatus</i>	[61]

Fungi in Interaction with PWNs	Mode of Action	References
<i>Alternaria</i> sp. <i>Epicoccum</i> sp.	Decrease of PWN population growth in in vitro mycelial fungi	[49]
<i>Aureobasidium</i> sp. <i>Aspergillus</i> sp. <i>Glilocladium</i> sp. <i>Mucor</i> sp. <i>Mortierella</i> sp. <i>Penicillium</i> sp. <i>Rhizoctonia</i> sp.	Decrease of PWN population growth in in vitro mycelial fungi	[50]
<i>Cystidiophorus castaneus</i>	Decrease of PWN population growth in <i>Pinus densiflora</i> ; Decrease the no. of PWNs carried by emerging <i>Monochamus alternatus</i>	[61]
<i>Cephalosporium</i>	Decrease of PWN population growth in in vitro mycelial fungi	[49][50]
<i>Fusarium</i> sp.	Decrease of PWN population growth in in vitro mycelial fungi	[49][50]
<i>Pycnoporus coccineus</i>	Decrease of PWN population growth in <i>Pinus densiflora</i> ; Decrease in the no. of PWNs carried by emerging <i>Monochamus alternatus</i>	[61]
Potential for biocontrol applications	Decrease of PWN population growth in in vitro mycelial fungi and in wood blocks of <i>Pinus densiflora</i> and <i>P. thunbergii</i> ; Decrease in the no. of PWNs carried by emerging <i>Monochamus alternatus</i>	[49][57][58][61] [59]
<i>Trichoderma</i> sp.	Endoparasitic fungi; Decrease of PWN population growth in in vitro mycelial fungi and in wood blocks of <i>Pinus densiflora</i> and <i>P. thunbergii</i> ; Decrease in the no. of PWNs carried by emerging <i>Monochamus alternatus</i>	[58][61]
<i>Verticillium</i> sp.	Nematode-trapping fungi; Extracellular enzyme Ac1 with nematostatic effect on PWN	[67]
<i>Arthrobotrys conoides</i>	Nematode-trapping fungi	[68]
<i>Drechsleria dactyloides</i>	Endoparasitic fungi; Decrease of PWN population growth in in vitro feeding trials; Volatile compounds attractive to PWN	[68][69][70][71] [72]
<i>Esteya vermicola</i>	Endoparasitic fungi; Decrease of PWN population growth in in vitro feeding trials	[73]
<i>Esteya floridanum</i>	Nematicidal activity of caryospomycins A to C metabolites exhibit moderate killing of PWN	[74]
<i>Caryospora callicarpa</i>	Nematicidal activity against PWN	[75]
<i>Geotrichum</i> sp. AL4	Nematicidal activity against PWN	[76]
<i>Acronium</i> sp. BH0531		

4. Conclusions

Plant microbiome is considered a very propitious strategy for fostering plant protection against abiotic and biotic stressors [77]. Research on PWD-associated mycoflora has been slowly progressing since the first studies in the mid-seventies. HTS technologies have enriched the narrow vision of culturomic studies, and we now know that the presence of PWNs can affect the fungal diversity of the infected trees. Ophiostomatoid, or blue-stain fungi, often recovered from wilted pine trees, are considered the most determinative biotic factors for multiplication and distribution of PWNs inside the tree and in the insect-vector. Naturally occurring fungi, endophytic or nematophagous (e.g., nematode-trapping fungi *D. dactyloides* or the endoparasitic fungi *E. vermicola*), should be further explored as new tools for PWD management.

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