Decline of Mongolian Pine Forest by the Combinatory Effect of European Woodwasp Sirex noctilio (Hymenoptera: Siricidae) and Plant Pathogenic Fungi

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Decline of Mongolian Pine Forest by the Combinatory Effect of European woodwasp *Sirex noctilio* (Hymenoptera: Siricidae) and Plant Pathogenic Fungi

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Abstract

Interactions between the decline of Mongolian pine woodlands and fungal communities and invasive pests in northeast China, are poorly understood. In this study, we investigated the fungal communities occurring in healthy uninfested, unhealthy uninfested and *Sirex noctilio* infested Mongolian pine and analyzed the relationship between the decline of Mongolian pine and fungal communities and woodwasp. The population number of *S. noctilio* was very high in the declining Mongolian pine forest. 26 fungal species identified from three tree samples. Each tree sample harbored a fungal endophyte community with a unique structure. The invasion of woodwasp appear to be promoted by the fungal community in the Mongolian pine woodlands. Pathogenic fungi richness was four times higher in infested and unhealthy uninfested compared to healthy uninfested trees. *Sphaeropsis sapinea* was the most dominant pathogenic fungus in Mongolian pine forest, and infested healthy Mongolian pine without wounding, but a with lower incidence of without wounding (38.72%) than wound ones (83.22%). Collectively, these data indicated that the fungal disease may have caused the initial reason of the decline of the Mongolian pine forest, and also provided convenient conditions for the successful colonization of woodwasp. The woodwasp attacked stressed Mongolian pine trees and accelerated its decline.

Keywords: pathogenic fungi; declining Mongolian pine; endophyte communities; *Sphaeropsis sapinea*; *Sirex noctilio*

Introduction

Mongolian pine (*Pinus sylvestris* var. *mongolica*), a geographical variety of Scots pine (*P. sylvestris*), is naturally distributed in the Daxinganling mountains of China, in Honghuaerji of the Hulunbeier sandy plains of China, and in parts of Russia and Mongolia. It is often planted as an ornamental tree because of its height and greening characteristics. Also, this tree is characterized by cold hardiness, drought tolerance, strong adaptability, and rapid growth[1,2]. It is currently the main coniferous tree species utilized in the “3-North Shelter Forest Program” and the “Sand-Control Project” in China and plays an important

role in ecological construction and environmental restoration[3].

Over the last decades, as the area of Mongolian pine plantations grows year by year, a widespread decline phenomena and extensive mortality events of the Mongolian pine forest have been observed in several parts of northeast China, revealing the high vulnerability of these formations[4]. Severe decline and mortality events have the potential to drastically alter Mongolian pine ecosystems, with important implications for the plant community dynamics[5].

Sirex noctilio L. is a devastating killer of pines in the southern hemisphere. It was first discovered outside its native range of Europe, North Africa, and the Middle East in New Zealand[6]. Over the 20th century, it has invaded exotic pine plantations in New Zealand, Australia, South America, and South Africa successively[7], into the northern hemisphere in the northeastern United States and southeast Canada in 2004 and 2005, respectively, and in South America[7]. Interestingly, as a secondary pest of pine species, this species is not considered a pest in Europe[6,8], but in several countries in the southern hemisphere and North America, the wasp has attracted considerable attention due to its high invasibility and ability to kill a variety of pine species[9,10,12]. In August 2013, the woodwasps were first detected as a pest of Mongolian pine in the Duerbote Mongolian Autonomous County, Heilongjiang Province, China. To date, Mongolian pine plantations spanning 22 cities in northeast China are considered to be in danger because of the woodwasp[12,13]. *S. noctilio* damage trees by depositing an obligate symbiotic fungus, *Amylostereum areolatum* (Fr.) Boidon and a phytotoxic mucus, in the trees during oviposition. The toxic mucus affects tree defenses and assists the fungus in colonizing the host. The symbiotic fungus acts as an external gut of woodwasp larvae for the digestion of recalcitrant lignocellulosic compounds[14,15]. Thus, insects, toxins, and fungi act together to damage host trees.

Mongolian pine decline is commonly considered a multifactorial disease, in which many interacting abiotic and biotic factors such as drought, frost, insect pests and pathogens are involved. Among the biotic factors involved in the onset of Mongolian pine decline events, pathogenic fungi play a primary role. As of now, it is reported that there were more than 20 fungi diseases of Mongolian pine[4,5]. In particular, many independent surveys have demonstrated the involvement of some leaf blight agents such as *Lophodermium seditiosum*

Minter Stalay and Millar[16], *Coleosporium phellodendri* Kom, *Lophodermella sulcigenaa* (Link) Tubeuf[17], *Septoria pinipumilae* Sawada[18], and trunk parts agents such as *Cronartium quercuum*[19] (Berk.) Miyabe ex Shirai and *Cronartium flaccidum* (Alb. et Schw) Winter[20], and root rot agents such as *Rhizoctonia solani* Kühn[21], in the Mongolian pine decline processes. However, in recent years, it is shown that the colonization of some important invasive pests may contribute to accelerating the Mongolian pine decline. It is unknown whether the decline of *P. sylvestris var. mongolica* forest invaded by the European woodwasp *Sirex noctilio* is related to fungal communities in host trees.

The declining trees ware preferentially infested by the wasps, however, when the population density was high, they also infested the healthy trees[10]. A number of pathogenic fungi have been recognized as having a prominent role in Mongolian pine tree decline and mortality[5]. The most common method of the investigation was to cut down the declining pine trees to control woodwasp and has achieved great results. One drawback of many investigations, however, was from single, separate disciplines (e.g., climatologists, plant pathologists, entomologists, etc.), and led to broach only one possible cause at a time, without a comprehensive, holistic approach to the problem[22]. The result was in many instances a disjointed, and often incomplete, framework, which made it impossible to individuate the intertwined causes of tree declines.

S. sapinea is an important latent pathogen of *Pinus* spp. and widely distributed in *Pinus radiata* plantations in northern Spain, outbreaks of which have a considerable impact on plantations[23]. It was recognized as the most widespread necrotrophic ascomycete pathogen responsible for dramatic losses of pine trees across the continents[24]. *S. sapinea* has emerged as an aggressive fungal pathogen all over the world and could directly invade the young shoots of pine trees[25-27]. In the greenhouse experiment, Stanosz and Flowers proved that *S. sapinea* strains isolated from healthy and diseased pine tissues had high pathogenic potential [28,29]. Usually, numerous pycnidia of *S. sapinea* are present in forest stands occurring on twigs, needles branches and stems of pine. A high infection rate may pose a high risk to the forest when there are disease-triggering factors, e.g., hail or insect feeding or extreme weather conditions such as heat and drought, as in the years 2018 und 2019 in German[24].

The frequency of pine shoot blight on pine trees has significantly increased over the

past decades in southeast China, especially in mature pine forests[5], and the loss caused by *S. sapinea* is no less than that by *Bursaphelenchus xylophilus* (the most serious invasive species of conifer trees in China). Mongolian Pine in Northeast China have also been reports of infection with *S. sapinea*[30]. The reasons that cause the decline of the Mongolian pine forests invaded by *S. noctilio* in northeast China are unknown. The information was limited about the ecological interactions of both *S. noctilio* and pathogenic fungi that contribute to accelerating the Mongolian pine decline events. Knowledge of these interactions is important in understanding their impact on natural ecosystems and developing appropriate management strategies.

In this study, we hypothesized that the fungal disease was the initial reason for the decline of the Mongolian pine forest in Northeast China, the woodwasp attacked stressed trees and accelerated its decline. The current study was investigated the fungal species occurring in healthy uninfested trees, unhealthy uninfested and *S. noctilio* infested trees, and analyzed the relationship between the decline of pine trees and occurrence of pathogenic fungi and woodwasp colonization. In addition, we also counted the number of woodwasps in host tree and studied the pathogenicity of *S. sapinea* to healthy *P. sylvestris* var. *Mongolica.* The findings obtained in this study allowed us to characterize, for the first time, the relationship between the decline of Mongolian pine woodlands and fungal communities and invasive pest.

Results

Number of adult *S. noctilio* emergence

In total 141 *S. noctilio* individuals, including 57 females and 84 males, were observed from the infested trees, but no *S. noctilio* individual was found in healthy uninfested and unhealthy uninfested trees (Figure 1). Apart from the wasp *S. noctilio* which specifically attacks *P. sylvestris* var. *mongolica*, other wood-boring pests (all of them were bark beetles) were eventually found in sampled *P. sylvestris* var. *mongolica* trees. The number of bark beetles collected was 12 and 15 in unhealthy uninfested and infested trees, respectively. No insects were found in healthy uninfested trees.

In addition to the wasps *S. noctilio*, native bark beetles (*Ips sexdentatus*) were collected

not only present in infested trees by *S. noctilio*, but also in unhealthy uninfested trees. This result showed that the bark beetle was more widely distributed in the Mongolian pine forest, but the population number was lower than that of the woodwasp.

Structure of fungal communities from three tree samples

A total of 450 wood fragments of healthy uninfested, unhealthy uninfested and *S. noctilio* infested trees were evaluated for the occurrence of endophytic fungi. The colonization rates (CR) and isolation rates (IR) of endophytic fungi between the three tree conditions were significantly different (CR: $F = 10.64$, $df = 2$, $p < 0.05$; IR: $F = 8.7$, $df = 2$, $p < 0.01$) (Figure 2). There was no significant difference in the CRs and IRs between infested and unhealthy uninfested trees (CR: $F = 0.26$, $df = 1$, $p > 0.05$; IR: $F = 1.04$, $df = 1$, $p > 0.05$). In addition, the CRs and IRs of pathogenic fungi in *S. noctilio*-infested and unhealthy trees were significantly higher than that of healthy trees (CR: $F = 11.26$, $p < 0.05$; IR: $F = 7.52$, $p < 0.01$) (Supporting information Figure S1).

The isolated endophytic fungi (304 in total) were assigned to 26 species within 21 genera based on their ITS sequence data and morphological features (Table 2). Among the 21 genera, 19 genera (24 species) were within the phylum Ascomycota, and 2 genera (2 species) were within the phylum Basidiomycota. Among the 26 species (there were overlapping fungi species in different samples), 11 endophytic fungi species were isolated from healthy uninfested trees, including *Chaetomium globosum* (26.6%), *Sphaeropsis sapinea* (16.5%), *Alternaria alternata* (12.6%) and *Trichoderma atroviride* (11.4%). From unhealthy uninfested trees, 13 fungal species were isolated and the most frequent fungal isolates were *S. sapinea* (47.7%) and *T. atroviride* (14%) (Table 2). From *S. noctilio* infested trees, 16 fungi species were isolated, and the dominant fungi species were *S. sapinea* (37.3%), *Ophiostoma minus* (22%) and *T. atroviride* (11.9%) (Table 2).

Top-eight most prevalent fungi species (genera) accounted for 90% of all the isolates, ranging from 86.1% to 92.3% (Figure 3). The relative frequency of *S. sapinea* in healthy uninfested trees was lower than those of *S. noctilio* infested and unhealthy uninfested trees. The relative frequency of *Trichoderma* spp. was slightly higher in *S. noctilio* infested (15.3%) and unhealthy uninfested trees (14%) than that of healthy uninfested trees (11.4%),

and *Aspergillus* spp. and *Fusarium* spp. in healthy uninfested trees were higher than the other two tree samples.

Four fungal species, namely *Aspergillus niger*, *A. alternata*, *S. sapinea*, and *T. atroviride* were isolated from three tree samples, were isolated and common from three tree samples accounting for 15.3% of all the species. The highest overlap (Jc = 0.381) was observed for the fungal communities between *S. noctilio* infested and unhealthy uninfested trees (Figure 4). Some fungal species only existed in a single tree sample (healthy uninfested: 5 species; unhealthy uninfested: 4 species; infested: 7 species). The species *Leptographium lundbergii* and *O. minus* were isolated from *S. noctilio* infested and unhealthy uninfested trees, whereas *C. globosum* was only species isolated from healthy uninfested trees (Table 2; Figure 3).

A total of 11 pathogenic species were identified from three tree samples, including 2 pathogenic species from healthy uninfested trees, 8 pathogenic species from unhealthy uninfested trees and 8 pathogenic species from *S. noctilio* infested trees (Table 2; Supporting information Figure S1). The pathogenic fungi richness was four times higher in infested and unhealthy uninfested trees than in healthy uninfested trees. Some pathogenic fungi found in unhealthy trees were also isolated from healthy trees uninfested by *S. noctilio.* For example, *S. sapinea* (pathogen of pine shoot blight) was isolated from all three samples and the isolation rate was significantly higher compared to other fungi.

Diversities of the fungal community

The diversity indexes of endophytic fungal communities showed significant differences among the three tree samples (Shannon diversity index: $F = 6.72$, $df = 2$, $p < 0.05$; Simpson dominance index: $F = 43.47$, $df = 2$, $p < 0.05$; Richness index: $F = 21.25$, $df = 2$, $p < 0.05$). The Shannon diversity index was higher and the Richness index was lower for the fungal community associated with healthy uninfested trees than those from infested and unhealthy uninfested trees (Table 3). The Richness index was the highest in *S. noctilio* infested trees compared to the other two tree samples. For both communities (infested and unhealthy uninfested trees), high values of Simpson dominance index were obtained, demonstrating that fungal communities under these two conditions had a high concentration compared to healthy trees. In addition, the Simpson dominance index was slightly higher in

unhealthy uninfested trees than in the infested trees community ($F = 1.78$, $df = 1$, $p > 0.05$).

Infection ability of *S. sapinea* to healthy *P. sylvestris* var. *mongolica*

The incidence rates of infection to the needles of healthy *P. sylvestris var. mongolica* were significantly different in the two treatment groups (wounded+spore and nonwounded+spore) compared with the two negative control groups (wounded+water and nonwounded+water) ($F = 318.74$, $df = 3$, $p < 0.01$) (Table 4). *S. sapinea* showed strong pathogenicity to the wounded *P. sylvestris var. mongolica* needles (incidence, 83.22%), which eventually caused the needles to wither. However, *S. sapinea* could also penetrate *P. sylvestris* var. *mongolica* without wounding (incidence, 38.72%) but with lower incidences ($F = 122.99$, $df = 1$, $p < 0.01$). In the two negative control groups, the needles of healthy *P. sylvestris* var. *mongolica* could hardly be diseased, regardless of whether they were wounded or not. In addition, pathogenic fungi re-isolated from diseased needles were the same as in the inoculum used for the healthy needles previously (Table 4).

Discussion

The association of Mongolian pine decline with fungal communities has been shown previously[4]. Recently, the woodwasps have also been found in declining Mongolian pine woodlands in northeast China[12]. In this study, 141 woodwasps were collected only from *P. sylvestris* var. *Mongolica* that initially selected with signs of egg laying (Figure 1). Native bark beetles (*Ips sexdentatus*) were more widely distributed in the Mongolian pine forest than that of the woodwasp. However, the population number of the bark beetles was lower and not considered a pest in northeast China over the past several years[30]. As reported previously that this insect preferred to damage the declining pine species[31-34], we only found the woodwasps in unhealthy and *S. noctilio* infested *P. sylvestris* var. *Mongolica*. The woodwasp attacks stressed trees, particularly disease-stressed ones, which were their preferred hosts[35]. This accelerated the decline and even death of Mongolian pine trees. However, Mongolian pine woodlands had been declining before the invasion of *S. noctilio* in the Northeast China, which provides convenience for the invasion of *S. noctilio*[30]. This may be due to the fungal community in the Mongolian pine woodlands.

A total of 26 fungal species was isolated from three tree samples. Pathogenic fungi

richness was four times in *S. noctilio* infested and unhealthy uninfested trees compared to healthy uninfested trees (Table 2; Supporting information Figure S1). Some of common pathogens of pine needles were isolated from *S. noctilio* infested and unhealthy uninfested trees, such as *Phoma multriostrata*, *Botrytis cinerea*, *A. alternata*, *Sydowia polyspora* and *Fusarium tricinctum* in this study constitutes a danger to weakened pine stands[36]. *L. lundbergii*, *O. minus*, *Phoma multriostrata* and *Truncatella angustata* were exclusively in infested and unhealthy uninfested trees (Table 2). *O. minus* was isolated from *S. noctilio* infested and unhealthy uninfested trees and it was the second most common fungus in this study. Many cases found that wood colonized by *O. minus* dries more quickly[37]. Past work also showed that *L. lundbergii* and *O. minus* were considered blue stain fungus of different pine trees worldwide (Foelker *et al*., 2016), which is introduced to pine trees by the bark beetle[38,39], which was also found in unhealthy uninfested and infested by *S. noctilio* pin trees of this study. In contrast, *C. globosum*, the most frequent fungal isolates only from healthy uninfested trees, was a biocontrol fungus as it produces various secondary metabolites and enzymes capable of inhibiting the mycelia growth of pathogenic fungi[40,41]. *C. globosum* is known to be plurivorous and was found on twigs and branches of *Ginkgo* and *Populu*[41]. Recent research showed that *C. globosum* completely inhibited the mycelial growth of *Amylostereum areolatum*[42].

Previous studies have found that the species of endophytic fungi are closely related to the health level of trees[43]. *Trichoderma*, *Aspergillus* were the dominant genera of endophytes in different host plants[44,45,46] and similar result was obtained in this study (Table 2). The Richness index showed that endophytic fungi species in *S. noctilio* infested trees were the highest. The CR and IR values of endophytic fungi of healthy uninfested trees was the lowest compared with *S. noctilio* infested and unhealthy uninfested trees (Table 2; Figure 2). The highest similarity (0.38) was observed for the fungal communities between *S. noctilio* infested and unhealthy uninfested trees (Figure 4). However, *S. noctilio*-infested trees selected in this study were also unhealthy trees (Table 1). The results show that the fungal community structure is greatly affected by tree health degree[43].

On the other hand, the invasion of woodwasps accelerated host decay and promoted the colonization of saprophyte, such as *Fusarium solani*[40,47]. For example, the symbiotic fungus

was only isolated from Mongolian pine infested by *S. noctilio* in this study. Furthermore, no significant differences were observed in the CR or IR of unhealthy uninfested and infested by *S. noctilio* trees. The primary endophytic fungal species from unhealthy uninfested and *S. noctilio* infested trees were also similar, such as *S. sapinea*, *T. atroviride*, *O. minus*. The results of Simpson dominance index showed that fungal communities had a high concentration in unhealthy uninfested trees compared with the other two samples trees, and healthy uninfested trees has higher fungal diversity[24] (Table 3).

Sphaeropsis sapinea is the causal fungal agent of Diplodia tip blight disease of coniferous trees of relevance to forestry in the world (Supporting information Table S1). The severity of pathogenicity, the length of incubation period and propagation period of the fungi are related to the host tree vigor, tissue maturity and environmental conditions[44]. Palmer reported that *S. sapinea* strains from China could invade without wounding, while those from the United States could not[48]. However, Blodgett found that *S. sapinea* strains from the United States can also invade without wounding, but the incidence was low[49]. In this study, *S. sapinea* was the most abundant species obtained from tree trunk of infested (37.3%) and unhealthy uninfested (47.7%) trees (Table 2) and showed a very strong pathogenicity and could penetrate *P. sylvestris* var. *mongolica* without wounding (Table 4). The occurrence of *S. sapinea* in healthy pine trees of this study, measured in frequency of colonization, is higher than in other studies like by Zhou[50], Flowers[29], and Maresi[51]. In our opinion, *Pinus sylvestris* var. *mongolica* forests in Northeast China are being damaged by *S. sapinea* and other fungi, and these fungal diseases was getting worse year by year, causing the trees to decline[44]. Their infection may promote convenient conditions for the successful colonization of *S. noctilio*. Therefore, we considered the decline of Mongolian pine forests by the combinatory effect of *S. noctilio* and plant pathogenic fungi.

2. Materials and Methods

2.1 Study sites and wood sample collection.

The research site was in the Jun De Forest Farm (130° 17′ 47″ E, 47° 12′ 11″ N) in Hei longjiang Province, China, where the decline and damage of Mongolian pine woodlands by the wasp *S. noctilio* were previously reported[13,52]. The site was characterized by a cold climate with an average annual temperature of 3.7℃ and average annual precipitation of

600~650 mm. In April 2018, fifteen trees were randomly chosen from a pure *P. sylvestris* var. *mongolica* plantation, the distance between individual trees was at least 10 meters. The trees sampled are listed in Table 1, including 5 healthy uninfested trees, 5 unhealthy uninfested trees and 5 *S. noctilio* infested trees. The *S. noctilio* infestation of Mongolian pines was identified by typical oviposition symptoms (i.e., resin beads formed from each ovipositor insertion).

Fresh wood samples were collected from tree trunk segments of 2 m above ground[44] (Table 1). Briefly, a trunk disk (10 cm-thick cross-section) was cut off from the segment. A bark layer more than 1 cm thick was removed from the disk using a sterile knife. Next, a sample block (10×10×5 cm^3) was removed from each disk and sealed in a sterile vacuum bag. All sample blocks were transferred to the laboratory at Gansu Agriculture University and stored at 4°C (up to 2 weeks) until further analyses.

2.2 Collection of Adult *S. noctilio*

Fifteen Mongolian pine trees were cut into 1 m-long billets after the wood sample collection, excluding the bottom 1 m section, with a minimum 10 cm diameter. After sealing the cut ends with wax, sample logs were taken to the quarantine laboratory in Gansu Agriculture University. These logs from visually identified tree samples (healthy uninfested, unhealthy uninfested, infested) were individually placed in mesh cages in the rearing facility and maintained at 27 ± 3°C temperature and 65 ± 5% relative humidity (RH) until the adult *S. noctilio* emerged. The numbers of *S. noctilio* and other pests were counted from the tree samples.

2.3 Isolation and storage of endophytic fungi

Endophytic fungi were isolated from the sample blocks using a surface sterilization method[53]. Briefly, each sample block was cut with a sterile pruner into 30 fragments (size: 4~5 mm^3). The fragments were surface sterilized by dipping in a series of solutions (70% ethanol for 1 min, 12% sodium hypochlorite for 30s, and 70% ethanol for 1 min). They were then washed three times in sterile distilled water. Five surface-sterilized fragments were placed in a petri dish (90 mm) with potato dextrose agar (PDA: 200g potato, 20g glucose, 15g agar, and 1L distilled water) supplemented with 100 μg/mL ampicillin and 50 μg/mL chloramphenicol. All fragments were incubated at 25±1°C and 70±5% RH for 1~4 weeks or

until the emergence of fungal mycelium. Agar cubes (ca. 1 mm^2) were removed aseptically from the edge of fungal colonies and transferred to fresh PDA plates. Each fungal colony was transferred at least three times until a well-defined uniform culture was obtained. Purified fungal isolates were sub-cultured with half-strength PDA in 60-mm Petri dishes and kept on the laboratory bench at about 20~25°C, where they received indirect sunlight to enhance sporulation. The fungal isolates were initially grouped as representative isolates and classified by their macro- and micro-morphological features, such as colony appearance, size, and shape of spores with species descriptions available in the literature[54].

The fungal cultures were generated on PDA slants in centrifuge tubes and stored under sterile mineral oil at 4°C. For long-term preservation, the representative isolates of each taxon were transferred to 20% glycerol in ultra-clean distilled water (v/v) and stored at -80°C.

2.4. Molecular identification of isolates

For the determination at the species level, the representative isolates of each taxon identified by the morphological features above were grown on PDA and incubated at 25℃ in the dark using InstaGene Matrix (BioRad Laboratories, Hercules, CA, USA). Genomic DNAs were extracted from 5-day-old cultures. The primers ITS1 and ITS4[55] were used to amplify the internal transcribed spacer (*ITS*) regions by PCR. The PCR reactions were carried out in a volume of 25μL using 23μL Golden Medal MIX (Thermo Scientific, USA), 1μL of each primer (10umol/L), and 1μL template DNA (50ug/mL). The PCR amplification was conducted using the following comditions: an initial denaturation step of 98℃ for 2 min; followed by 30 cycles of denaturation at 98℃ for 10s, annealing at 50°C for 15s, and polymerization at 72℃ for 15s; and then a final extension step of 5 min at 72°C.

The PCR products were separated by electrophoresis on 1% (w/v) agarose gels, stained with ethidium bromide for visual examination, and purified using the agarose gel DNA extraction kit (Takara, Japan) and sequenced at Qinke Biotech (Beijing, China). The sequences were submitted for BLAST search in the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The representative isolates were assigned to a species when their sequences were at least 99% identical to the sequence of a known species. Besides, morphological features of the representative isolates were also used an important

role to confirm the classification by the DNA sequence comparison. The following morphological features were evaluated: mycelium shape, mycelium surface texture, colony color, production of pigments and their diffusion in the medium, spore production, and mycelium growth rate on the PDA plates.

2.5 The pathogenicity test of *Sphaeropsis sapinea* to *P. sylvestris* var. *mongolica*

In this experiment, the *S. sapinea* (synonym: *Diplodia pinea*, pine shoot blight) that were isolated from Mongolian pine forest in the previous step was selected for pathogenicity test with *P. sylvestris* var. *mongolica*. Before inoculation, *S. sapinea* was cultured intermittently under black light (100 ~150 lx) for 14 h and in the dark for 10 h on PDA + M medium (PDA + sterilized powder of Mongolian pine needles) for 20 days at 25±1ºC and 70±5% RH to induce fungal sporulation[37], and then the spores were washed with sterile water and 50 spores were collected under a low power microscope (Wincom, China) to make into fungus suspension. Then, the inoculation experiment was conducted on the needles of 3-year-old healthy seedlings of *P. sylvestris* var. *mongolica* in the laboratory[30]. First, the needles of *P. sylvestris* var. *mongolica* seedlings were stabbed with a sterile knife at the base of the needles, with one wound per needle. The uninjured needles were used as a control treatment. Then, the fungus suspension, prepared as above, was smeared on the stabbed needles of *P. sylvestris* var. *mongolica* with a brush and bound with self-adhesive plastic film for 10 days. The uninjured and stabbed needles smeared with sterile water served as negative controls. In this experiment, the fungus was inoculated twice (once more after 10 days). Ten independents healthy seedlings of *P. sylvestris* var. *mongolica* were used for each of the four treatments (stabbed and uninjured needles smeared with fungus spores and with water) with 19~54 needles each seedling. The incidence rates of *S. sapinea* were investigated after 3 months post-inoculation. After the incidence rate of needle infections was determined, *S. sapinea* was re-isolated from 20 diseased needles randomly selected from each treatment group.

2.6 Data Analysis

The colonization rate (CR) was calculated as the number of tree fragments from which

one or more endophytic fungi were isolated, divided by the total number of incubated trees fragments[56]. The isolation rate (IR) was defined as the number of endophytic fungi isolated, divided by the total number of tree fragments incubated[57]. The incidence rate was calculated as the number of diseased needles, divided by the total number of inoculation needles. The CR and IR of endophytic fungi and the incidence of *S. sapinea* to healthy *P. sylvestris* var. *mongolica* were analyzed using one-way ANOVA. The differences between mean values were evaluated using Tukey's honestly significant differences (HSD) test. Pearson's chi-square test was applied to analyze the differences between pathogenic fungi and other fungi (remaining fungi except for pathogenic fungi) from each tree sample. The statistical analyses were performed using the IBM SPSS Statistics version 23.0 (Chicago, IL, USA). The relative frequency of the common fungi isolated from each tree sample was examined using the range diversity analysis[58].

The diversity of endophytic fungi isolated from each tree sample was evaluated using the Shannon–Weiner Index (H'), Simpson dominance index (D), and Margalef richness index (R)[59].

$$H' = -\Sigma\ (Pi \times \ln Pi)$$

$$D = 1\text{-}\Sigma Pi^2$$

$$R = (S - 1)/\ln N$$

$$Pi = Ni/N$$

where N is the total number of individuals; Ni refers to the number of individuals; and S indicates the total number of species. In addition, the similarity of fungal communities was evaluated using the Jaccard similarity coefficient (Jc)[60]. The similarities in fungal taxonomic richness between communities were summarized in Venn diagrams using GeneVenn software (http://genevenn.sourceforge.net/).

Informed consent

All experimental protocols were approved by Biocontrol Engineering Laboratory of Crop Diseases and Pests of Gansu Province, Gansu Agricultural University, Lanzhou, China

All the methods were carried out in accordance with the relevant guidelines and regulations.

Data availability statement

We declare that all the date in this study were available.

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Footnotes

Competing interests

The authors declare no competing interests.

Author Contributions

LXW and YQL conceptualization; LXW and CCL field survey and sample collection; LLR and LXW laboratory analysis and data elaboration; SSW and CCL draft writing; YQL, NL and JJZ review and manuscript editing.

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Figure legends

Figure 1. The number of adult *S. noctilio* emergence in different sampled trees. Numbers inside the bottoms of the bars are the number of *S. noctilio* or other borers. Healthy= Healthy uninfested, Unhealthy=Unhealthy uninfested.

Figure 2. The rates of isolation (**A**) and colonization (**B**) of endophytic fungi from three tree samples. Lowercase letters indicate a significant difference between the isolation rates or colonization rates in different tree samples at $P < 0.05$.

Figure 3. Composition of the most frequently isolated fungi from the different tree samples. The eight most frequently cultivated species (genera) were selected and the prevalence (%) of each species (genus) was determined per tree samples.

Figure 4. Venn diagram illustrating the unique and shared fungal taxa among healthy trees (red), unhealthy trees (yellow), and *Sirex noctilio* infested trees (green). Outside numbers are the Jaccard similarity coefficient.

Display Items

Table 1. Selection of tree samples

Tree samples	Diameter (cm)[a]	Height (m)	Dead branches and leaves (%)	Infestation[b]	Moisture content (%)
Healthy uninfested	16.91±1.42a	7.71±1.11a	6.20±3.8b	Without	72.13±4.11a
Unhealthy uninfested	16.78±1.27a	8.02±1.22a	50.00±5.7a	Without	61.97±3.25b
Infested	16.72±1.38a	7.83±0.65a	52.00±6.51a	Woodwasp	62.91±6.22b

[a] Diameter: The diameter of 2m above ground from each tree.

[b] Infestation: Whether insect infected the tree samples before sampling.

568 **Table 2. Colonization number and significance for forestry of fungal endophytes isolates obtained from three tree samples.**

Fungal taxa	Accession number	Closest species (Accession No.)	Similarity (%)	Health uninfested	Unhealthy uninfested	Infested	Assessment of the significance for forestry[a]
Aspergillus tubigensis	MT994717	*Aspergillus tubigensis* (GU595290)	99	2		1	–
Aspergillus niger	MT994716	*Aspergillus niger* (KP940593)	100	6	5	1	Endophyte; [54,55]
Alternaria alternata	MT994718	*Alternaria alternata* (KJ173524)	99	9	7	3	Generalist; [61]
Amylostereum areolatum	MT994715	*Amylostereum areolatum* (KC865582)	100			1	Symbiotic fungi of wasps, saprophyte; [6]
Bionectria ochroleuca	MT994719	*Bionectria ochroleuca* (HM037945)	99			2	Biocontrol; [62]
*Botrytis cinerea**	MT994722	*Botrytis cinereal* (MH860108)	100		2		Pathogen; [16,63]
Chaetomium globosum	MT994720	*Chaetomium globosum* (KM268644)	99	21			Biocontrol, typical endophyte of Mongolian Pine; [51,63]
Epicoccum nigrum	MT994725	*Epicoccum nigrum* (AF455403)	99			3	Generalist; [64]
*Fusarium tricinctum**	MT994723	*Fusarium tricinctum* (EF611089)	100	8	3		Endophyte, potential pathogen, potential pathogen; [31, 54]
*Fusarium chlamydosporum**	MT994724	*Fusarium chlamydosporum* (MG857338)	99			1	Potential pathogen; [54]
Fusarium solani complex*	MT994721	*Fusarium solani* (EU719658)	99			7	Pathogen, saprophyte; [65]
Gliomastix sp.	MT994727	*Gliomastix polychrome* (AB540566)	97	1			–
*Leptographium lundbergii**	MT994733	*Leptographium lundbergii* (DQ062031)	99		5	8	Blue stain of wood, pathogen;

							[66]
Nectria haematococca	MT994726	*Nectria haematococca* (MH729023)	99		1		_
*Ophiostoma floccosum**	MT994728	*Ophiostoma floccosum* (KF854000)	99			1	Pathogen; [70]
*Ophiostoma minus**	MT994729	*Ophiostoma minus* (GU134172)	100		11	26	Pathogen, associated fungi of bark beetles, blue stain of wood; [48]
Penicillium glabrum	MT994735	*Penicillium glabrum* (HG326279)	99	7			_
Peyronellaea sp.	MT994734	*Peyronellaea* sp. (KF293765)	99	2		-	_
*Phoma multriostrata**	MT994731	*Phoma multriostrata* (EF585395)	100		2	1	Pathogen; [31]
Pestalotiopsis sp.	MT994730	*Pestalotionpsis lespedezae* (FJ467379)	99		1		_
*Sphaeropsis sapinea**	MT994737	*Sphaeropsis sapinea* (HM467670)	99	13	51	44	Typical pathogen of pine shoot blight, saprophyte; [25,27,28]
Schizophyllum commune	MT994736	*Schizophyllum commune* (MK910781)	99	1			Saprophyte; [67]
*Sydowia polyspora**	MT994732	*Sydowia polyspora* (KU319069)	99		1		Typical endophyte of *P. sylvestris* twigs, Potential pathogen; [68]
*Truncatella angustata**	MT994738	*Truncatella angustata* (KU319069)	99		3	1	Saprophyte, weakness pathogen; [47]
Trichoderma atroviride	MT994739	*Trichoderma atroviride* (HM037962)	100	9	15	14	Endophyte; [69]
Trichoderma viride	MT994740	*Trichoderma viride* (HM037962)	100			4	Endophyte; [32]

* Plant pathogen fungi

[a] The numbers in the column that assessment of the significance for forestry were references number.

Table 3. Diversity indices of fungal communities from three tree samples.

Index	Healthy uninfested	Unhealthy uninfested	Infested
Shannon diversity index	2.123±0.14a	1.807±0.08b	1.958±0.11ab
Simpson dominance index	0.145±0.05b	0.269±0.07a	0.213±0.03a
Richness index	2.517±0.08b	2.568±0.12b	3.144±0.15a

The data were analyzed by one-way ANOVA followed by HSD test. The results are expressed as the mean ± SD. The results followed by different letters are significantly different according to the HSD test ($p < 0.05$)

Table 4. Pathogenicity of *S. sapinea* to healthy *P. sylvestris* var. *mongolica*

Inoculation treatments	Number of inoculation needles	Number of diseased needles	Incidence (%)	Re-isolated from diseased needles	Same as inoculated fungus
Wounded + spore	304	253	83.22 ± 10.45 a	20	20
Nonwounded + spore	359	139	38.72 ± 8.14 b	20	20
Wounded + water	267	6	2.25 ± 4.46 c	−	−
Nonwounded + water	380	0	0.00 ± 0.00 c	−	−

Incidence followed by different letters were significantly different according to the HSD test ($P < 0.05$).

Figure 1


160
120
80
40
0
Woodwasp number
Others
Woodwasp
12
15
141
Healthy
Unhealthy
Infested

Figure 2

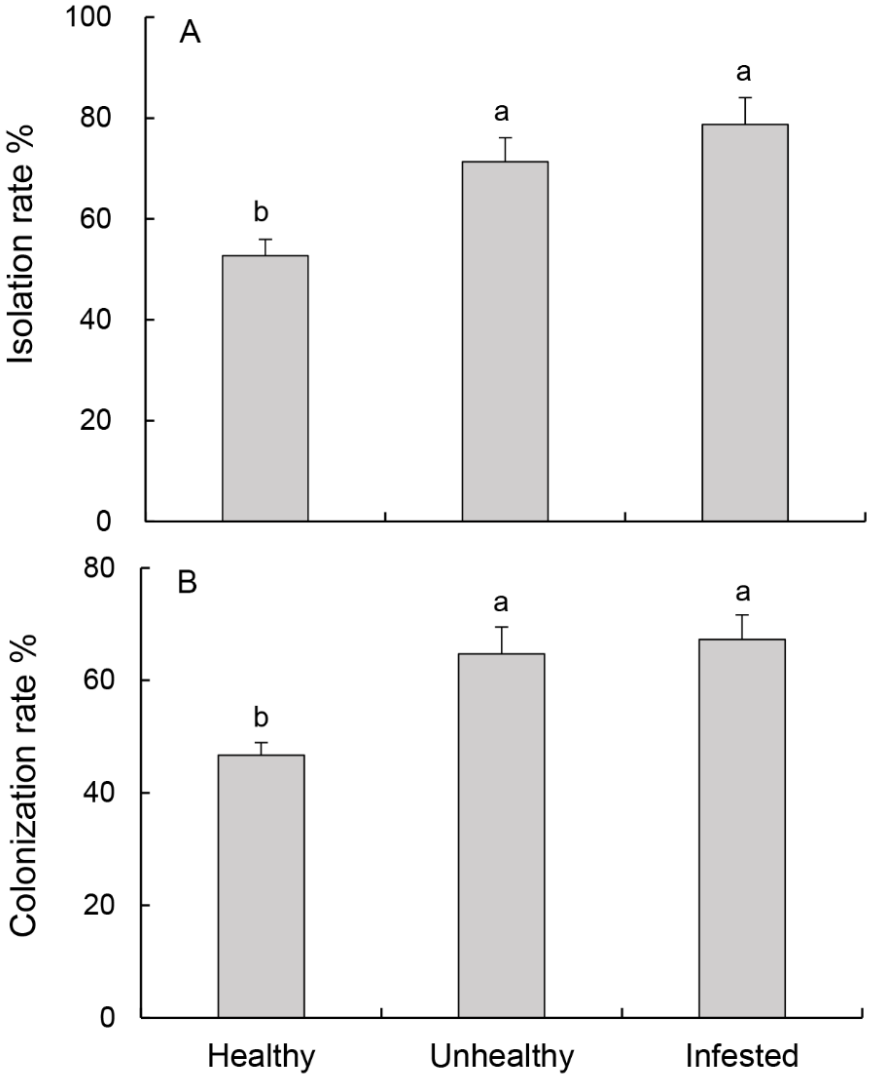

A
Isolation rate %
100
80
60
40
20
0
b
a
a
B
Colonization rate %
80
60
40
20
0
b
a
a
Healthy
Unhealthy
Infested

Figure 3

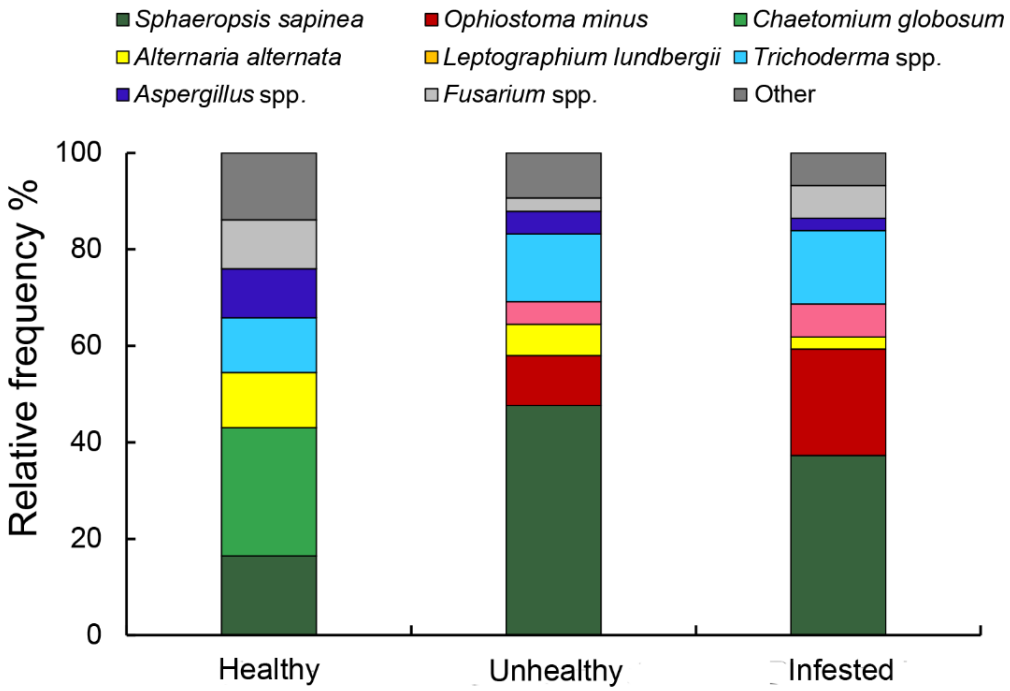
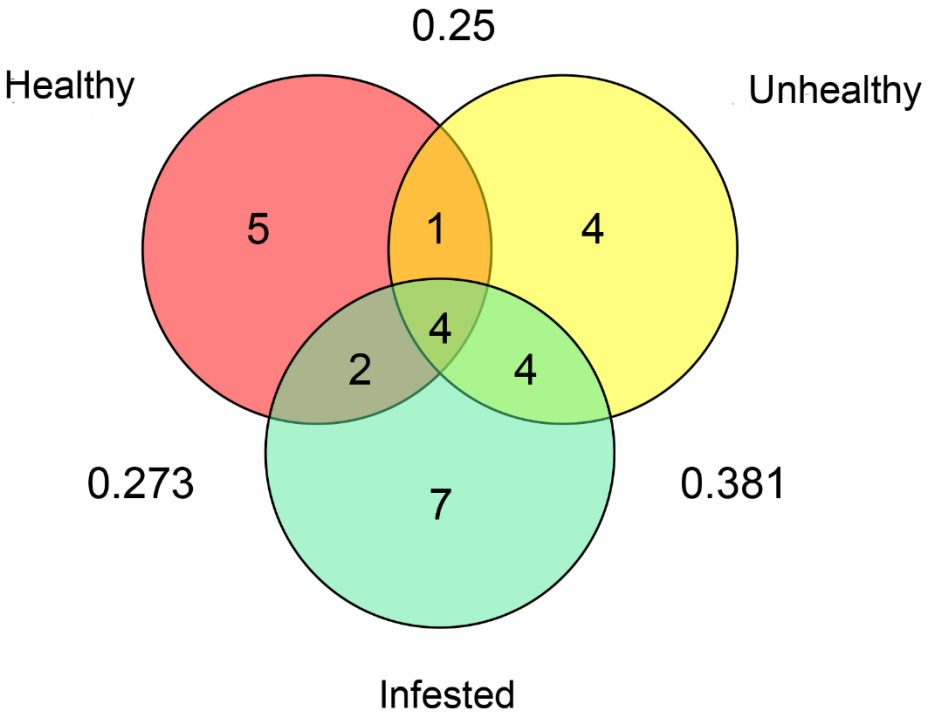

Sphaeropsis sapinea
Ophiostoma minus
Chaetomium globosum
Alternaria alternata
Leptographium lundbergii
Trichoderma spp.
Aspergillus spp.
Fusarium spp.
Other
Relative frequency %
100
80
60
40
20
0
Healthy
Unhealthy
Infested

Figure 4



Figures

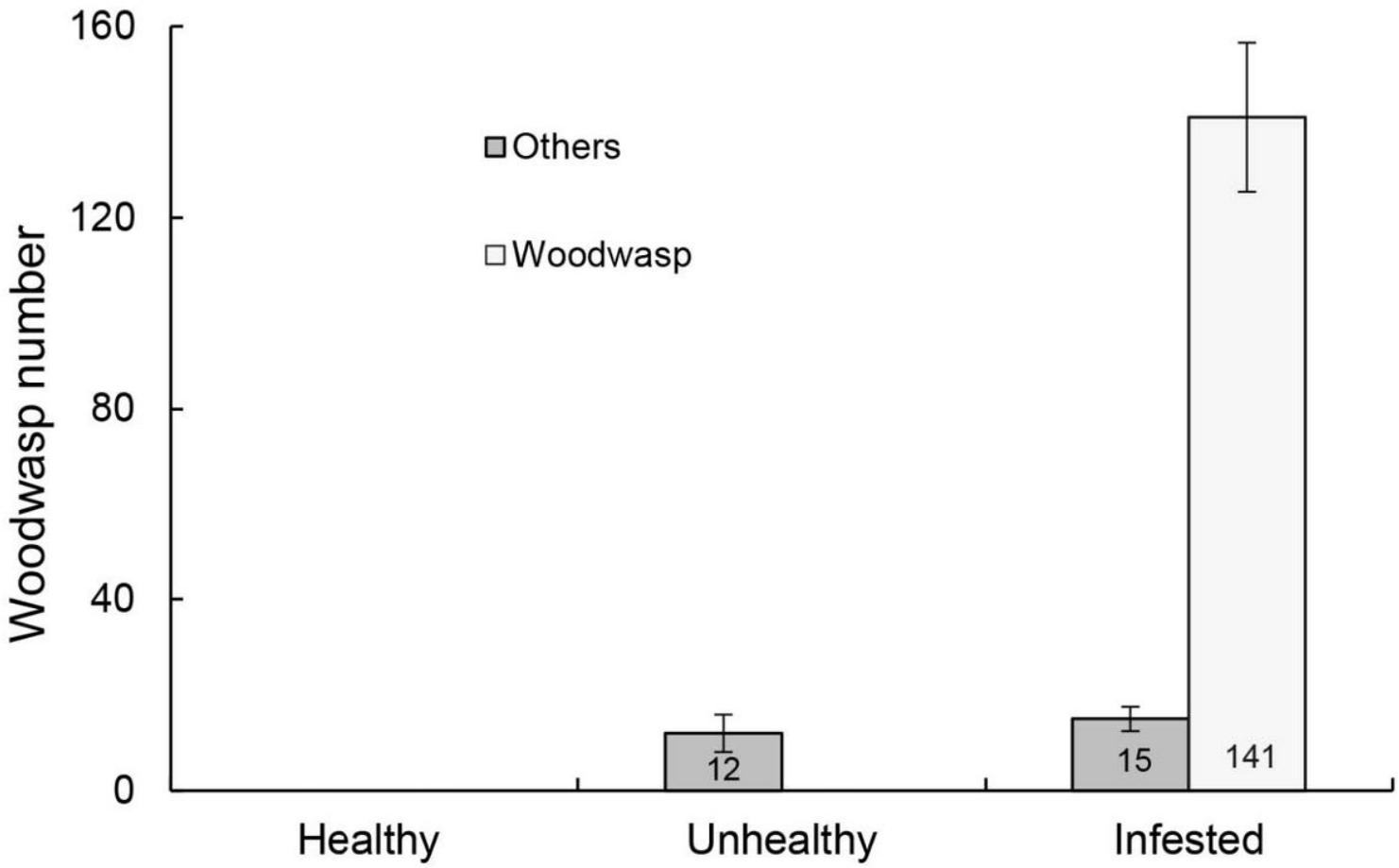


Figure 1

The number of adult S. noctilio emergence in different sampled trees. Numbers inside the bottoms of the bars are the number of S. noctilio or other borers. Healthy= Healthy uninfested, Unhealthy=Unhealthy uninfested.

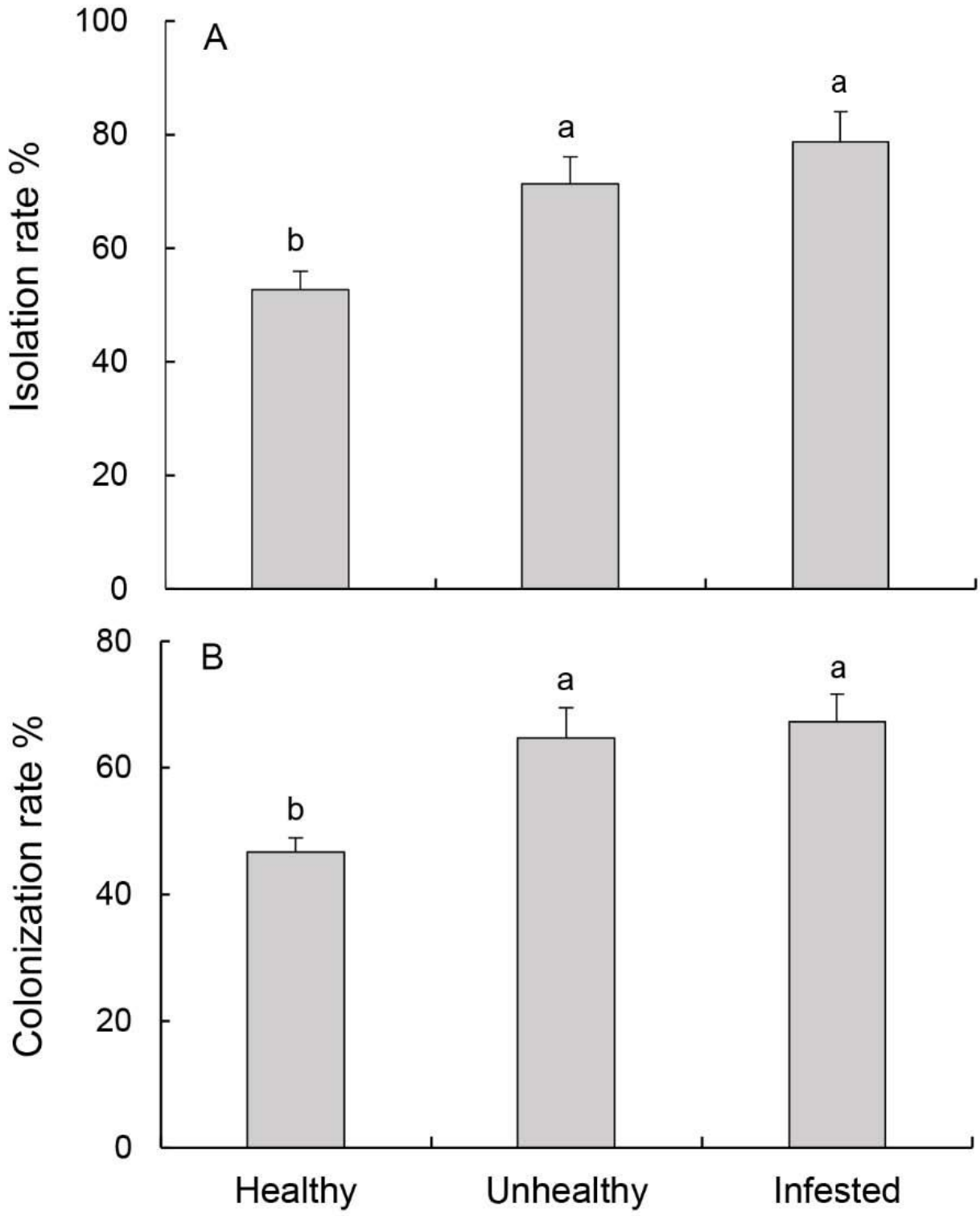


Figure 2

The rates of isolation (A) and colonization (B) of endophytic fungi from three tree samples. Lowercase letters indicate a significant difference between the isolation rates or colonization rates in different tree samples at $P < 0.05$.

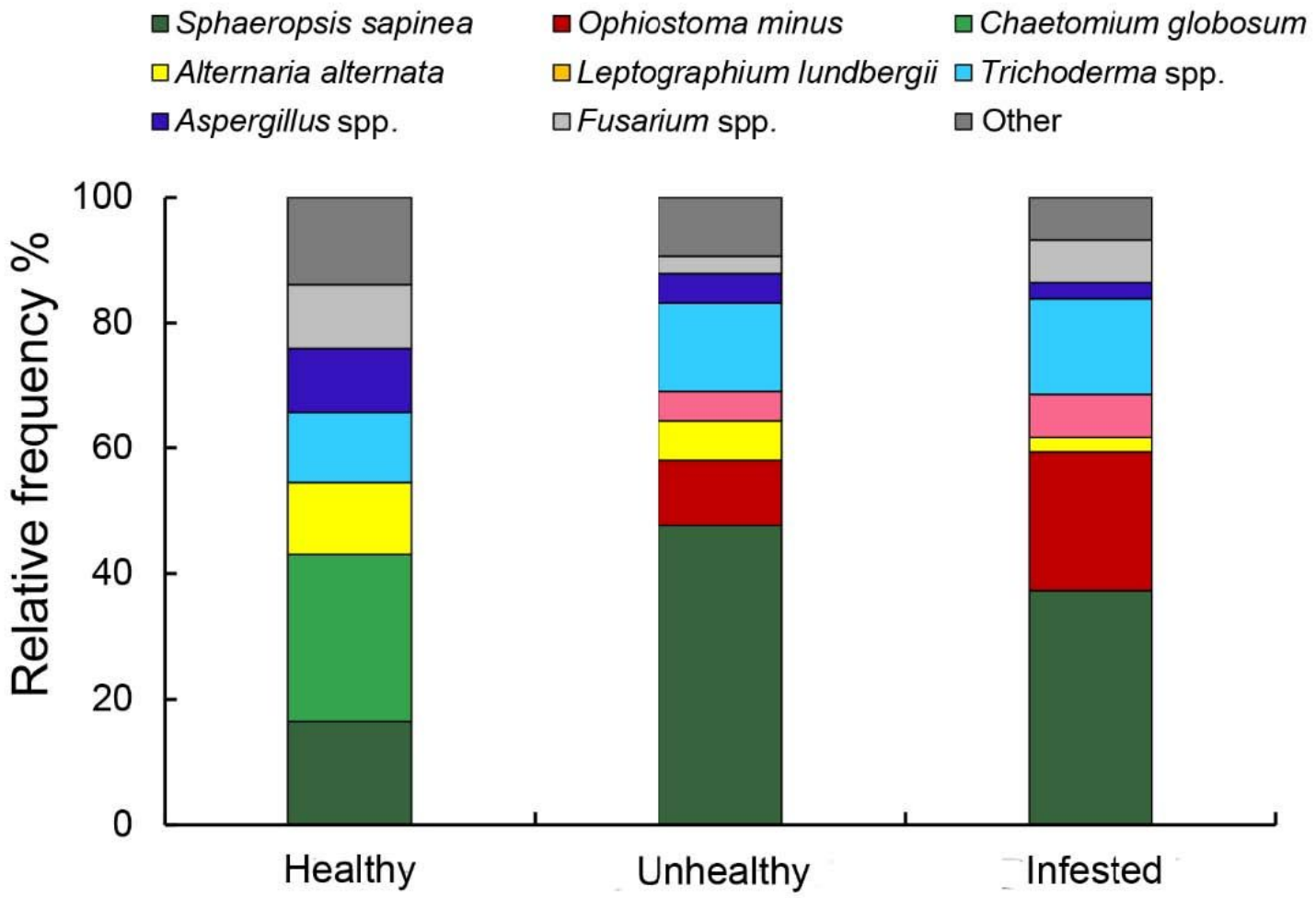


Figure 3

Composition of the most frequently isolated fungi from the different tree samples. The eight most frequently cultivated species (genera) were selected and the prevalence (%) of each species (genus) was determined per tree samples.

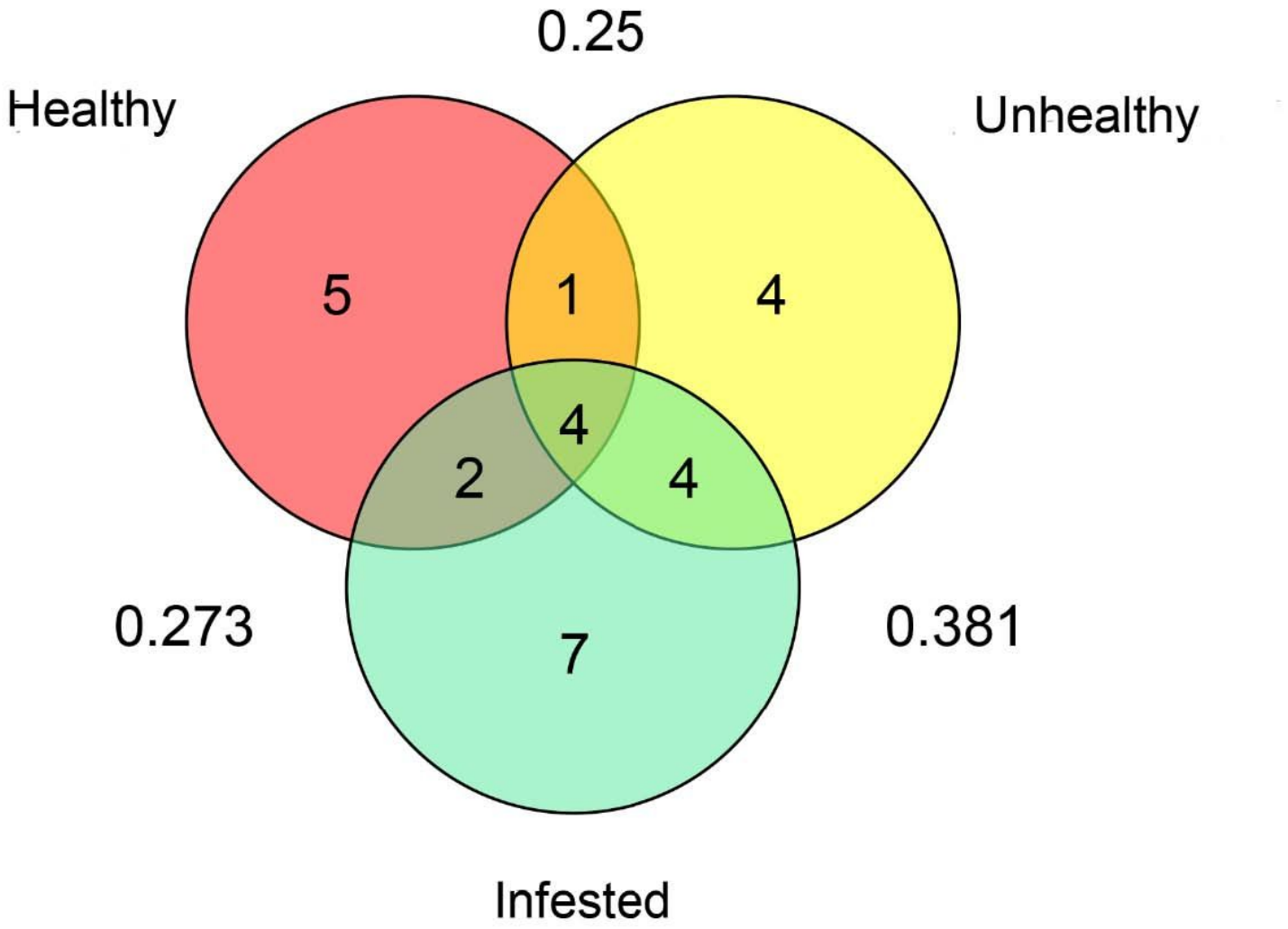


Figure 4

Venn diagram illustrating the unique and shared fungal taxa among healthy trees (red), unhealthy trees (yellow), and Sirex noctilio infested trees (green). Outside numbers are the Jaccard similarity coefficient.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupportingInformation.pdf