Article

Fungal Communities Associated with Bark Beetles in *Pinus radiata* Plantations in Northern Spain Affected by Pine Pitch Canker, with Special Focus on *Fusarium* Species

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Abstract: *Fusarium* spp., as well as other endophytic or pathogenic fungi that form communities, have been reported to be phoretically associated with bark beetles (Coleoptera; Scolytinae) worldwide. This applies to *Fusarium circinatum* Nirenberg and O'Donnell, the causal agent of pine pitch canker (PPC), which threatens *Pinus radiata* D. Don plantations in northern Spain. The main objective of this study was to study the fungal communities associated with bark beetles and their galleries in stands affected by PPC, with special attention given to *Fusarium* species. Funnel traps and logs were placed in a *P. radiata* plot known to be affected by *F. circinatum*. The traps were baited with different attractants: four with (E)-pityol and six with ethanol and α-Pinene. In addition, fresh green shoots with *Tomicus piniperda* L. feeding galleries were collected from the ground in 25 *P. radiata* plots affected by PPC. Extracts of whole insects and gallery tissues were plated on agar medium to isolate and identify the associated fungi. A total of 24 different fungal species were isolated from the bark beetle galleries constructed in logs and shoots, while 18 were isolated from the insect exoskeletons. Ten different *Fusarium* species were isolated from tissue and insects. *Fusarium circinatum* was isolated from bark beetle exoskeletons (1.05% of the *Pityophthorus pubescens* Marsham specimens harboured *F. circinatum*) and from the galleries (3.5% of the *T. piniperda* feeding galleries harboured the pathogen). The findings provide information about the fungal communities associated with bark beetles in *P. radiata* stands in northern Spain.

Keywords: *Fusarium circinatum*; bark beetles; Monterey pine; endophytes

1. Introduction

Fungal endophyte species form fungal communities, together with saprotrophic and pathogenic species, in forests. Knowledge about the species composition and the factors influencing the presence of

different fungal communities is important for understanding the role that fungi play in regulating other organisms [1]. Bark beetles are known to be closely associated with fungi worldwide. They are well known for the associations that they form with endophytic, plant-pathogenic and entomopathogenic fungi, such as *Fusarium* species, which are widespread and abundant in living and dead plants [2].

The genus *Fusarium* includes important plant pathogens that affect both forest and agricultural species [3] by producing different types of wall-degrading enzymes (e.g., cellulases, glucanases and glucosidases) and mycotoxins such as beauvericin and fumonisins [4,5]. Some of the species in this genus, such as the *Fusarium oxysporum* spp. complex, cause disease symptoms in a large number of vegetable crops [6] and forest trees. The pathogenic fungi *Fusarium circinatum* Nirenberg and O'Donnell (teleomorph = *Gibberella circinata*) is an ascomycete fungus that causes pine pitch canker (PPC) [7]. The main symptom of this disease is the presence of pitch-soaked cankers which can girdle both trunks and large branches in adult trees [8]. *Fusarium circinatum* is currently threatening pine plantations and natural stands throughout the world [9], especially *Pinus radiata* D. Don, a highly susceptible pine species [10]. Up to 60 *Pinus* species and *Pseudotsuga menziesii* (Mirb.) Franco [11,12] are also susceptible to the pathogen. In Spain, the disease caused by *F. circinatum* was reported for the first time in 2005 [13] and has led to important ecological and economic losses in forest plantations and nurseries. Regarding the interaction between *F. circinatum* and other *Fusarium* spp., introduction of the non-pathogenic *Fusarium lateritium* Nees as a potential biocontrol agent was found to inhibit the pathogen growth when introduced as a pioneer [14].

The fungal communities that inhabit *P. radiata* trees may strongly influence the distribution of PPC in Spain. Endophytic species which do not cause damage to the host [1], such as *Trichoderma viride* Bissett, could be used for biological control of *Fusarium* spp. [15–17]. The antagonistic properties of species of the genus *Trichoderma* are mediated in five different ways: antibiotic production, competition, production of enzymes (chitinases and/or glucanases), induction of host plant defence mechanisms and parasitism [18]. The last of these occurs when the hyphae of *Trichoderma* coil round the hyphae of other fungi and eventually penetrate the tissues [19]. Other fungal species like *Diplodia sapinea* (Desm.) Kickx, which is a latent pathogen in pine trees, have been associated with PPC in *P. radiata* trees, causing shoot dieback and the presence of resin drops and necrotic stem lesions [20].

Some bark beetles have been reported to be phoretically associated with *F. circinatum* in *P. radiata* plantations in northern Spain: *Pityophthorus pubescens* (Marsh.), *Ips sexdentatus* (Börner) and *Tomicus piniperda* L. [21–23]. In California, the importance of *Pityophthorus* spp. as the main vectors of *F. circinatum* has also been demonstrated [24], although in northern Spain *P. pubescens* was found to be only weakly associated with the pathogen [23]. Differences in bioecology may determine spread of fungal infections; for example, *Hylastes* spp. feed on roots or trunks of declining trees whereas *T. piniperda* feeds on the shoots of healthy crowns [25]. Moreover, there is a risk that bark beetle populations may increase from endemic to epidemic levels [26]. For example, *I. sexdentatus* populations may increase as a result of forest disturbances, leading to subsequent attacks on healthy trees [25].

We hypothesize that different bark beetle species living in these plantations play specific roles in the spread of *Fusarium* spp. Moreover, the fungal composition of the associated mycobiota may determine the distribution of the pathogens. The main objective of this study was to characterize the fungal communities associated with bark beetles and their galleries in stands affected by PPC, with special focus on *Fusarium* species.

2. Materials and Methods

2.1. Sample Collection

This study was carried out in a *P. radiata* plot affected by *F. circinatum*, located in Vejorís (Cantabria, Spain). Two types of traps were set up in the plots: piles of bait logs and funnel traps. The bait logs consisted of 6 piles of branches (diameter: 6.4–16.9 cm) and 6 piles of thick logs from trunks (diameter: 16–31 cm), all obtained from healthy *P. radiata* trees collected from a *F. circinatum*-free plot. Plant tissues

(xylem and phloem) and insects were collected from the breeding galleries weekly between June and October 2010.

In addition, four funnel traps baited with (E)-pityol and six traps baited with ethanol-α-Pinene (Econex) were placed within the plantation. (E)-pityol, an aggregation pheromone produced by *P. pubescens*, attracts both males and female beetles [27]. Insects were collected weekly between June and October 2010.

To examine the association between *T. piniperda* and *F. circinatum*, fresh fallen green shoots with *T. piniperda* feeding galleries were collected from 25 *P. radiata* plots affected by *F. circinatum*. A total of 285 fallen *P. radiata* shoots with a *T. piniperda* entrance hole and feeding gallery were collected from the ground between June and October 2010. Twenty-seven shoots were collected during the summer (from June to August) and 258 were collected in autumn (September and October).

2.2. Molecular and Morphological Identification of Fungi

Shoots and xylem and phloem from beetle galleries in logs were plated on PDAS (potato dextrose agar with 0.3 g/L of streptomycin sulfate) culture medium previously treated by surface sterilization (1 min tap water, 1 min ethanol 70%, 1 min sodium hypochlorite 20% and 1 min distilled sterilized water). In addition, a total of 438 bark beetles collected from logs and funnel traps belonging to 11 different species (Table 1) were plated by pressing the whole bodies onto *Fusarium* selective media (FSM: 15 g bactone peptone, 1 g KH_2PO_4 monobasic, 0.5 g $MgSO_4$-$7H_2O$, 20 g agar, 0.2 g of pentachloronitrobenzene (PCNB) and 0.3 g streptomycin sulfate per liter) to avoid bacterial and soil fungi contamination. All specimens were processed in this way, except those collected from the shoots, which were used for molecular identification.

Table 1. Insect species collected from logs, funnel traps and shoots.

Insect Species	Total Number	Logs	Ethanol	(E)-Pityol	Shoots
Ips sexdentatus	116	116	0	0	0
Pityophthorus pubescens	97	0	0	97	0
Hylastes attenuatus Erichson	86	83	2	0	1
Orthotomicus erosus (Wollaston)	30	30	0	0	0
Crypturgus mediterraneus (Eichhoff)	26	26	0	0	0
Hylastes ater (Paykull)	25	20	5	0	0
Hylastes angustatus (Herbest)	23	23	0	0	0
Xyleborinus saxeseni Ratzenburg	22	1	21	0	0
Tomicus piniperda	19	0	0	0	19
Hylurgops palliatus (Gyllenhal)	9	9	0	0	0
Xyleborus dispar F.	4	0	4	0	0
Hylastes linearis Erichson	1	1	0	0	0
Total	458	309	32	97	20

Fungi isolated from plant material and insects were classified into morphological units, i.e., colonial morphotypes (CMs), on the basis of cultural characteristics [28]. Thus, fungi obtained from plant tissues were grouped into 30 CMs according to macromorphological features of the colony growing on the PDAS. Fungi derived from insects and isolated on FSM were grouped into 17 different CMs. One isolate from each CM was selected for molecular identification. However, 29 isolates of reddish, orange, yellowish, violet or pinkish colonies, which probably belonged to the genus *Fusarium*, were selected. Regarding the identification of *F. circinatum*, 16 isolates were selected on the basis of their macroscopic features for specific molecular identification. Single hyphae cultures were grown prior to molecular identification.

DNA extraction was carried out on the fungal culture following the protocol described by Vainio et al. [29]. Once the DNA was extracted, the internal transcribed spacer (ITS) region of the rDNA was amplified by polymerase chain reaction (PCR), with primers ITS-1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) [30]. The

thermal cycling program for amplification was as follows: 10 min denaturation at 95 °C followed by 13 cycles of 35 s at 95 °C, 55 s at 55 °C and 45 s at 72 °C; 13 cycles of 35 s at 95 °C, 55 s at 55 °C and 2 min at 72 °C; 9 cycles of 35 s at 95 °C, 55 s at 55 °C and 3 min at 72 °C; and a final elongation 7 min at 72 °C. The purified PCR product (NucleoSpin Gel and PCR Clean up, Macherey Nagel) was sent to a commercial sequencing service (SecuGen, Madrid, Spain). The ITS region sequences were revised with the Geneious Pro 5.6.5 software package for Blast search in the GenBank database.

As the ITS region is not a suitable molecular marker for identifying *Fusarium* spp. to species level, microscopic morphological and morphometric identification was carried out together with molecular identification using specific molecular markers. Thus, 29 isolated fungi belonging to the genus *Fusarium*, according to the ITS region, were plated on Spezieller Nahrstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA), both specifically used for identification of *Fusarium* species [31]. After 10 to 20 days, SNA diagnostic characters including the shape of the macroconidia, the presence or absence of microconidia, the shape and mode of aggregation of microconidia, the shape of conidiogenous cells, and the presence or absence of coiled sterile hyphae and chlamydospores were observed. The colour and size of the sporodochia were observed on CLA. These samples were amplified with the primers EF1 (forward primer; 5′-ATGGGTAAGGA(A/G)GACAAGAC-3′) and EF2 (reverse primer; 5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′) [32] for sequencing the translation elongation factor (α-TEF) region, which encodes an essential part of the protein translation machinery and is highly informative at the species level in *Fusarium* [33]. PCR reactions were conducted in 50 μL volumes containing 1 μL template DNA, 1× reaction buffer, 200 mM dNTPs, 0.4 μM forward and reverse primers, 1U of Kapa Taq DNA polymerase. The thermal profile of PCR was one cycle of 10 min at 94 °C followed by 36 cycles of 30 s at 94 °C, 55 s at 62 °C and 1 min at 72 °C and a final 10 min extension at 72 °C (modified from Pérez-Sierra et al. [34]). The α-TEF region was sequenced and the sequences were corrected with Geneious Pro Software (V 5.6.5) and blasted in the GenBank and FUSARIUM ID sequence databases.

Multiple sequence alignment (ClustalW) of the α-TEF region sequences and evolutionary analyses were conducted with MEGA 6 [35]. The phylogenetic tree was constructed according to the neighbor-joining statistical method [36]. The bootstrap method (1000 replicates) was used to represent the phylogenetic history of the taxa analyzed [37]. The evolutionary distances were computed using the Kimura 2-parameter method [38]. The rate of variation between sites was modeled with the gamma distribution (Shape parameter = 1). All positions containing gaps and missing data were eliminated. *Fusarium equiseti* (Corda) was used as a outgroup (GenBank accession number: AJ543571.1).

Those fungi morphologically classified as *F. circinatum* were identified with the specific primer pair CIRC-1A (5′-CTTGGCTCGAGAAGGG-3′)/CIRC-4A (5′-ACCTACCCTACACCTCTCACT-3′) as described by Schweigkofler et al. [39]. Gel electrophoresis was used to detect the diagnostic 360 bp band in 1% agarose gel 1× TAE buffer (40 mM Tris base, 0.114% glacial acetic acid and 1 mM EDTA (pH = 8)) and the gel was stained with 3× GelRedTM solution (Biotium), following the manufacturer's instructions.

2.3. Molecular Identification of Insects

Tomicus destruens (Woll.) and *T. piniperda* are morphologically difficult to distinguish, although there are some differences between their life cycles [33,34]. In order to confirm the identification, 17 specimens randomly collected from the feeding galleries were sent to the Department of Animal Biology (University of Murcia) for molecular identification following the protocol described by Gallego and Galian [40].

2.4. Statistical Analysis

Analysis of variance (ANOVA) and multiple comparison procedures were used to test the effect of insect species from the insect sampling and to examine how fungal species richness was affected by the season of shoot sampling. Robust methods were applied as the data did not fulfil two of the ANOVA

assumptions (normality and homoscedasticity) [41]. Specifically, one-way fixed-effects ANOVAs were performed under the assumption of non-normality and inequality of variances using the generalized Welch procedure and a 0.2 trimmed mean transformation. The ANOVAs were carried out using the 'Wilcox' Robust Statistics (WRS)' package (Version 2014 [42]) implemented in the R software environment (V 3.4.3), R Foundation for Statistical Computing, Vienna, Austria). A paired-sample Wilcoxon test was carried out to determine whether fungal species richness varied according to the tissue surveyed, particularly xylem and phloem. Pearson's correlation coefficient was calculated to examine the association between the frequency of appearance of *T. harzianum* and *F. circinatum*, in those samples that were positive for the pathogen.

Non-metric multidimensional scaling (NMDS) and the multiple response permutation procedure (MRPP) were conducted with the VEGAN package (Version 2015) [43] implemented in the R software environment in order to analyze the fungal communities associated with (1) the insect bodies, (2) the logs, depending on the tissue and the insect species, and (3) the shoots, depending on the season. NMDS was conducted using Bray–Curtis as the distance metric and the multivariate ordination was created using the metaMDS results. MRPP was also performed using Bray–Curtis dissimilarity with 1000 permutations.

3. Results

A total of 24 different fungal species were obtained from the bark beetle galleries constructed in logs and shoots, and 18 fungal species were obtained from the insect exoskeletons. The bark beetle species collected during the sampling are listed in Table 1.

3.1. Fungal Communities from Insect Galleries

Fourteen fungal species from the logs (xylem and phloem) were identified: five species of *Fusarium*, two species of *Pestalotiopsis*, two *Trichoderma* spp., *Mucor* sp., *Trichoderma harzianum* Rifai, *Diplodia sapinea* (Desm.), *Peniophora pini* (Schleich.) Boidin and *Penicillium glabrum* (Wehmer) Westling. Six other fungal species remained unidentified (Table 2). The species richness did not differ depending on the log tissue (xylem and phloem) ($V = 4757.5$, $p = 0.36$).

As *F. circinatum* and *T. harzianum* were negatively correlated ($r = -0.8143875$, $p < 0.1$), higher frequencies of *T. harzianum* corresponded to the lowest frequencies of *F. circinatum*.

Table 2. Percentage of plant tissue samples containing each fungal species.

Species	Shoots	Logs												Accession Number
	Tomicus piniperda	*Ips sexdentatus*		*Hylastes attenuatus*		*Orthotomicus erosus*		*Hylastes ater*		*Hylastes angustatus*		*Hylurgops palliatus*		
		Xylem	**Phloem**	**Xylem**	**Phloem**	**Xylem**	**Phloem**	**Xylem**	**Phloem**	**Xylem**	**Phloem**	**Xylem**	**Phloem**	
Diplodia sapinea	88.4	62.2	74.1	54.8	67.1	100.0	-	45.0	50.0	84.2	89.4	60.0	100.0	KP900724
Pestalotiopsis sp.	24.5	2.5	5.4	4.11	2.7	-	-	10.0	5.0	21.05	-	-	-	KP900723
Mucor sp.	3.8	0.0	1.8	15.1	6.9	-	-	50.0	15.0	5.26	10.5	-	16.6	KP900722
Trichoderma spp.	9.1	19.3	21.4	11.0	15.0	-	-	15.0	10.0	10.53	15.7	-	-	KP900738
Fusarium spp.	21.05	2.5	9.8	30.1	13.7	100.0	-	40.0	10.5	26.32	15.78	20.0	16.6	-
Fusarium circinatum	3.5	-	0.9	-	1.3	-	-	-	-	5.2	5.5	-	-	-
Penicillium glabrum	11.2	3.4	-	16.4	23.3	-	-	15.0	-	26.3	21.0	-	16.6	KP900733
Trichoderma harzianum	11.9	46.2	52.7	20.5	20.5	-	-	5.0	30.0	-	10.5	-	-	KP900736
Trichoderma atroviride Bissett	1.4	-	-	-	-	-	-	-	-	-	-	-	-	KP900725
Peniophora sp.	-	1.7	-	-	-	-	-	-	-	-	-	-	-	KP900735
Gliocladium roseum Bainier	12.2	-	-	-	-	-	-	-	-	-	-	-	-	KP900726
Botrytis cinerea Pers.	6.3	-	-	-	-	-	-	-	-	-	-	-	-	KP900730
Epicoccum nigrum Link	1.7	-	-	-	-	-	-	-	-	-	-	-	-	KP900729
M5	11.93	16.0	17.0	27.4	22.0	-	-	30.0	20.0	21.5	21.0	60.0	33.0	-
M10	3.16	0.8	10.7	12.33	4.1	-	100.0	15.0	25.0	10.53	26.0	60.0	-	-
M16	1.4	0.8	-	-	1.4	-	-	-	-	-	-	-	-	-
M17	3.51	2.5	-	2.74	5.5	-	-	-	5.0	10.53	5.3	-	-	-
M22	2.81	3.4	1.8	-	-	-	-	-	-	-	-	-	-	-
M27	8.77	0.8	-	-	-	-	-	-	-	-	-	-	-	-

The prefix M indicates unidentified species.

Regarding the fungal communities associated with the bark beetle galleries, three groups commonly associated with the insects were observed: one associated with *Orthotomicus erosus* (Wollaston), one associated with those of *I. sexdentatus* and one with the three species of *Hylastes* (Figure 1).

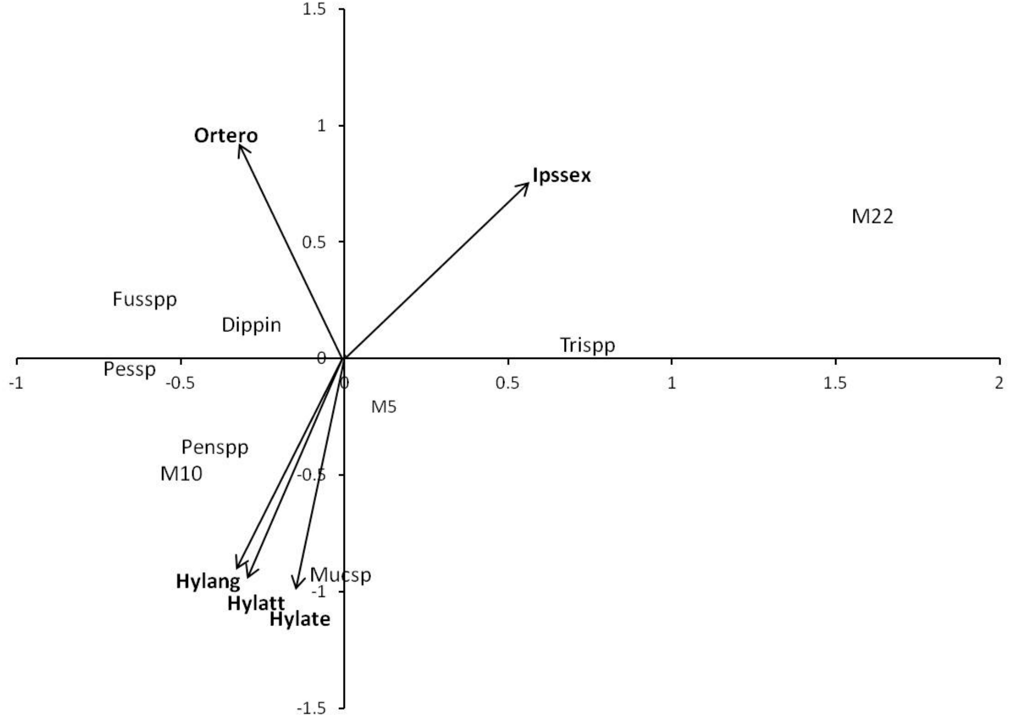

Figure 1. Non-metric multidimensional scaling (NMDS) at insect species level (Ortero = *O. erosus*, Ipssex = *I. sexdentatus*, Hylang = *H. angustatus*, Hylatt = *H. attenuatus*, Hylate = *H. ater*). Fungal species (Fusspp = *Fusarium* spp., Dippin = *D. sapinea*, Trispp = *Trichoderma* spp., Pessp = *Pestalotopsis* sp., Penspp = Peniophora spp., Mucsp = Mucor sp.) correspond to those isolated from the insect galleries on *Pinus radiata* logs. Dissimilarity distance = Bray–Curtis.

Eighteen fungal species were isolated from shoots and identified. The proportions (%) of shoots containing these fungi were as follows: *D. sapinea* (88.4%), three species of *Pestalotiopsis* (24.5%), five species of *Fusarium* (21.05%), *Gliocadium roseum* Bainier (12.2%), *T. harzianum* (11.9%), two other species of *Trichoderma* (9.1%), *Penicillium glabrum* (11.2%), *Mucor* sp. (3.8%), *Botritys cinerea* (6.3%), *Epicoccum nigrum* Link (1.7%) and *Trichoderma atroviride* Bissett (1.4%). However, six fungal species remained unidentified (Table 2). The species richness in the shoots differed significantly depending on the season in which they were collected (higher in autumn than in summer) ($F_{We} = 42.1$, $p < 0.001$). Likewise, statistically significant differences in fungal communities in shoots were observed according to the season (summer/autumn) ($A = 0.04833$, $p < 0.001$). *Fusarium* spp. were more abundant in the plots sampled in autumn and the same tendency was found for *D. sapinea* and *P. pini* (Figure 2).

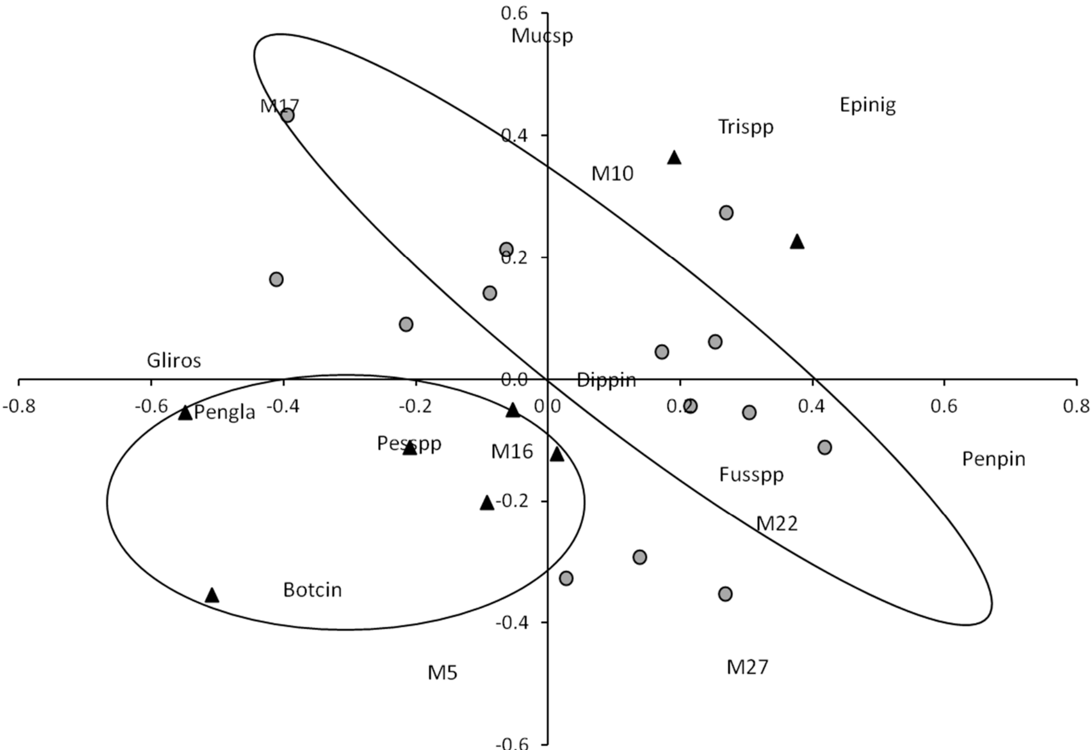

Figure 2. Multivariate analysis for fitting seasonal variables to NMDS ordination plots. Fungal species (Mucsp = *Mucor* sp., Epinig = *E. nigrum*, Trispp = *Trichoderma* spp., Gliros = *G. roseum*, Dippin = *D. sapinea*, Pengla = *P. glabrum*, Pesspp = *Pestalotiopsis* spp., Fusspp = *Fusarium* spp., Penpin = *P. pini*, Botcin = *B. cinerea*) correspond to those isolated from the galleries in the shoots. Symbols represent the sites (● = plots sampled during autumn, ▲ = plots sampled during summer).

3.2. Fungal Communities from Insect Exoskeletons

Eighteen fungal species were obtained from the insect exoskeletons. Six *Fusarium* spp., *Candida fructus* Nakase *Neonectria radicicola* (Gerlach and L. Nilsson) Mantiri and Samuels, *Penicillium* sp., *T. atroviride* and *G. roseum* were obtained, together with seven other species that remained unidentified (Table 3).

Table 3. Percentage of bark beetles harbouring each fungal species.

Species	*Pityophthorus pubescens*	*Ips sexdentatus*	*Hylastes attenuatus*	*Orthotomicus erosus*	*Crypturgus mediterraneus*	*Hylastes ater*	*Hylastes angustatus*	*Xyleborinus saxeseni*	*Hylurgops palliatus*	*Xileborus dispar*	Accesion Number
Candida fructus	25.7	0.9	8.1	10	-	8	4.3	27.3	11.1	25	KP900741
Fusarium spp.	20.6	34.48	32.5	56.6	12	64	39.1	31.8	55.5	25	
Fusarium circinatum	1.05	0.9	1.6	-	-	-	-	-	-	-	
Gliocadium roseum	-	-	2.3	-	-	-	-	-	-	-	KP900740
Neonectria radicícola	5.1	-	2.3	-	-	-	4.3	22.3	-	-	KP900737
Penicillium sp.	16.5	-	-	-	-	-	8.7	4.5	-	-	KP900731
Trichoderma atroviride	1.03	0.9	3.48	3.3	8	-	4.3	-	-	-	KP900728
Mi1	25.7	0.9	8.1	10	-	8	4.3	27.3	11.1	25	-
Mi2	1.03	-	11.6	-	-	-	8.7	-	-	-	-
Mi5	-	-	4.6	-	-	8	4.3	-	11.1	-	-
Mi6	1.03	1.7	1.16	-	-	-	-	-	-	-	-
Mi9	1.03	1.7	6.96	-	-	-	8.7	-	-	-	-
Mi14	-	1.7	2.3	6.6	-	4	-	4.5	-	-	-
Mi16	2.06	0	5.81	-	4	-	-	4.5	-	-	-

The prefix Mi indicates unidentified species.

The species richness on the insect exoskeletons differed significantly depending on the insect species (F_{We} = 4.8, $p < 0.001$) (Figure 3). Species richness was highest for *X. saxaseni* Ratzenburg and *P. pubescens* and was significantly different from the values associated with *I. sexdenatus* and *C. mediterraneus*. The fungal communities present on the insect bodies were clustered in two different groups depending on the insect species (Figure 4): one group was associated with *Hylastes* spp. and another one with *P. pubescens*.

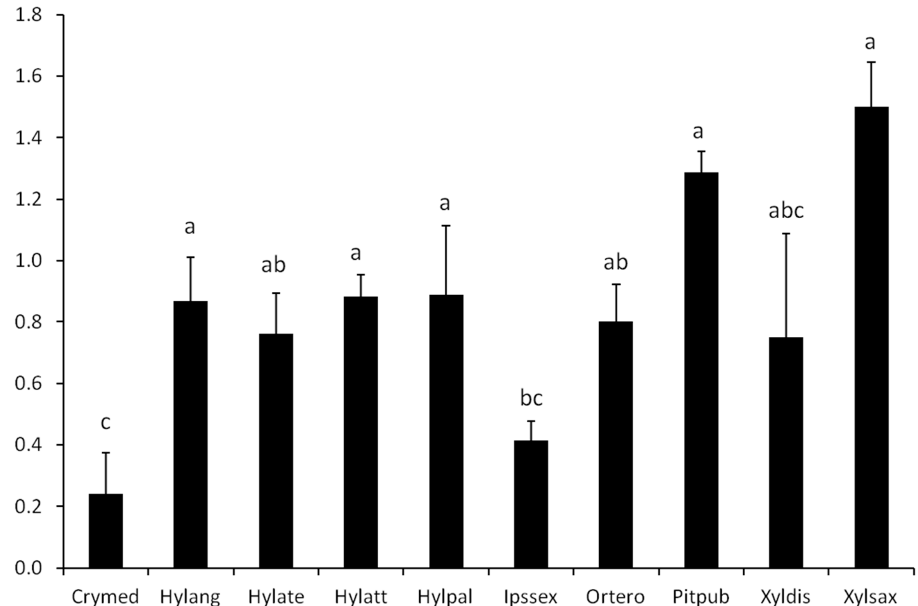

Figure 3. Fungal species richness on the exoskeleton of the different bark beetle species (Crymed = *C. mediterraneus*, Hylang = *H. angustatus*, Hylate = *H. ater*, Hylatt = *H. attenuatus*, Hylpal = *H. palliatus*, Ipssex = *I. sexdentatus*, Ortero = *O. erosus*, Pitpub = *P. pubescens*, Xyldis = *X. dispar*, Xylsax = *X. saxeseni*). Different letters represent significant differences ($p < 0.05$). Error bars represent standard errors.

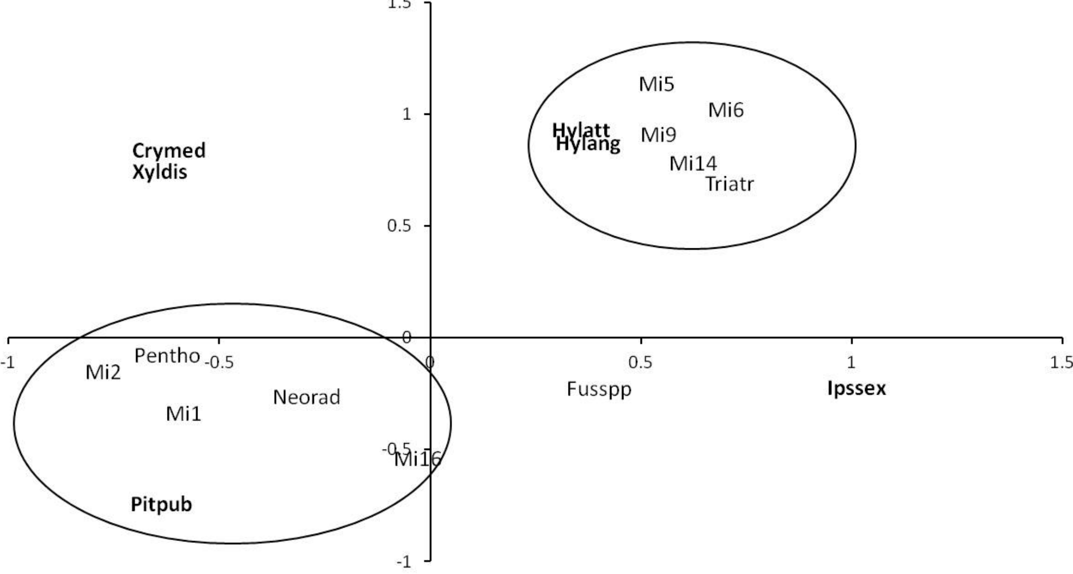

Figure 4. NMDS at insect species level. Fungal species (Triatr = *T. atroviride*, Pentho = *P. thomentosum*, Neorad = *N. radicicola*, Fusspp = *Fusarium* spp.) correspond to those isolated from insects collected from funnels and logs (Crymed = *C. mediterraneus*, Hylang = *H. angustatus*, Hylatt = *H. attenuatus*, Ipssex = *I. sexdentatus*, Pitpub = *P. pubescens*, Xyldis = *X. dispar*). Dissimilarity distance = Bray–Curtis.

Fungal communities from the insects collected from logs differed significantly (A = 0.3538, $p < 0.001$) from those associated with log tissues. Thus, two distinct groups of fungi were observed: one associated with the xylem and phloem and the other related to the insect exoskeletons. *Fusarium* spp. were associated with both types of samples although they appeared to be more closely related to the insect exoskeletons (Figure 5).

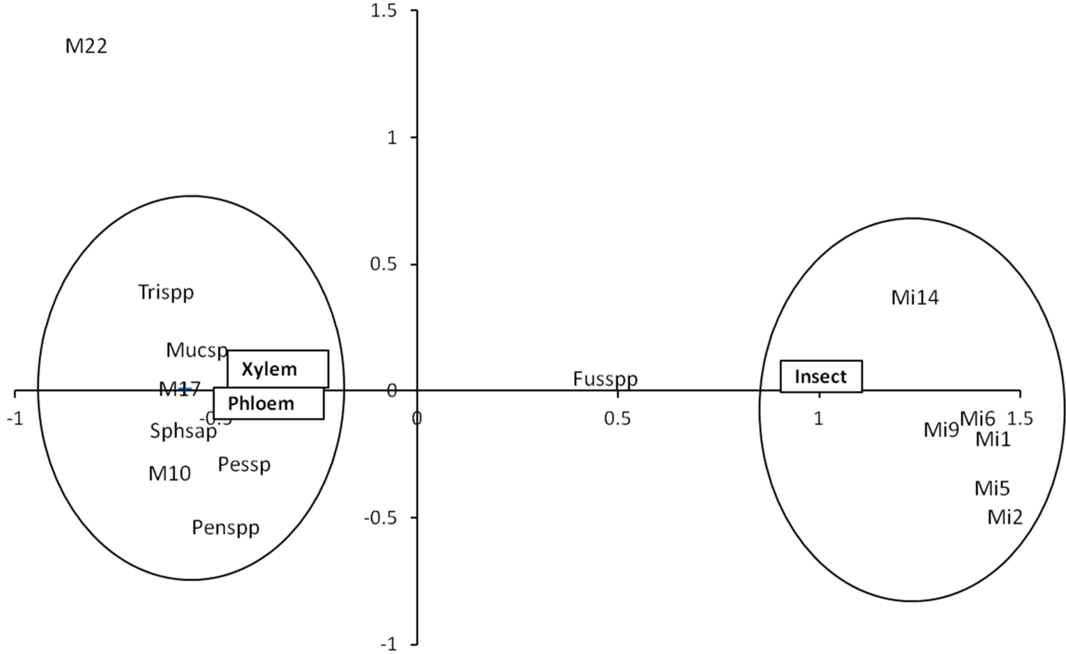

Figure 5. NMDS at sample level. Fungal species (Trispp = *Trichoderma* spp., Mucsp = *Mucor* sp., Sphsap = *S. sapinea*, Pessp = *Pestalotiopsis* sp., Penspp = *Penicillium* spp., Fusspp = *Fusarium* spp.) correspond to those isolated from logs (xylem and phloem) and from insects collected from the logs. Dissimilarity distance = Bray–Curtis.

3.3. Fusarium *spp. from Insect Galleries*

Fusarium species were isolated from both xylem and phloem from logs. A total of 11 isolates were identified: two belonged to the *F. oxysporum* species complex, one was identified as *Fusarium sporotrichioides* Sherbakoff, two were identified as *Fusarium beomiforme* (Nelson, Toussoun and Burgess), three belonged to *Fusarium avenaceum* (Fries) Saccardo, and finally three were identified as to *F. circinatum* (Tables 2 and 4). *Fusarium circinatum* appeared in 0.85% and 0.43% of the phloem and xylem samples, respectively. In the *Hylastes angustatus* (Herbst) galleries, *F. circinatum* appeared in 5.2% and 5.5% of the xylem and phloem samples respectively, whereas *F. circinatum* was found in only 1.36% of the phloem samples from the *H. attenuatus* galleries (Table 2).

Table 4. Isolates of *Fusarium* species identified by different methods.

Isolate	Origin	Collected From	ITS Region	Morphology	TEF Region	Consensus Species	Accession Number TEF
1	Phloem	Logs I.s	*Fusarium* sp.	*F. sporotrichioides*	*F. sporotrichioides*	*F. sporotrichioides*	KR002044
2	Phloem	Logs H. att	*Fusarium* sp.	*F. beomiforme*	-	*F. beomiforme*	-
3	Phloem	Logs I.s	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002062
4	Phloem	Logs H. ang	-	*F. circinatum*	*F. circinatum*	*F. circinatum*	KR002060
5	Xylem	Logs H. att	*Fusarium* sp.	*F. beomiforme*	-	*F. beomiforme*	-
6	Xylem	Logs H.att	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002050
7	Xylem	Logs H. a	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002057
8	Xylem	Logs H. a	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002049
9	Xylem	Logs Hy. p	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002058
10	Shoot	Shoots T.p.	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002063
11	Shoot	Shoots T.p.	*Fusarium* sp.	*F. tricinctum*	*F. tricinctum*	*F. tricinctum*	KR002064
12	Shoot	Shoots T.p.	*Fusarium* sp.	*F. cortaderiae*	*F. cortaderiae*	*F. cortaderiae*	KR002048
13	Shoot	Shoots T.p.	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002046
14	Shoot	Shoots T.p.	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002051
15	Shoot	Shoots T.p.	*Fusarium* sp.	*F. tricinctum*	*F. tricinctum*	*F. tricinctum*	KR002054
16	Shoot	Shoots T.p.	*Fusarium* sp.	*F. tricinctum*	*F. tricinctum*	*F. tricinctum*	KR002047
17	Shoot	Shoots T.p.	*Fusarium* sp.	*F. sporotrichioides*	*F. sporotrichioides*	*F. sporotrichioides*	-
18	*Hylastes ater*	Logs	*F. lateritium*	*F. avenaceum*	-	*F. avenaceum*	KR002059
19	*Hylastes attenuatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002052
20	*H. attenuatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	-
21	*H. attenuatus*	Logs	*Fusarium* sp.	*F. anthophilum*	*F. avenaceum*	*Fusarium sp.*	KR002055
22	*H. attenuatus*	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002065
23	*Ips sexdentatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002056
24	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002046
25	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	-
26	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. tricinctum*	-	*F. tricinctum*	-
27	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002066
28	*Orthotomicus erosus*	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002043
29	*Pityophthorus pubescens*	Funnel	*Fusarium* sp.	*F. sambucinum*	*F. sambucinum*	*F. sambucinum*	KR002053
30	*Xyleborinus saxesni*	Funnel	*Fusarium* sp.	*F. konzum*	-	*F. konzum*	-

I.s = *Ips sexdenatus*, H. att = *Hylastes attenuatus*, H. ang = *H. angustatus*, H.a = *H. ater*, Hy.p = *Hylurgops palliates*, T.p = *Tomicus piniperda*, ITS = internal transcribed spacer, TEF = translation elongation factor.

In the *T. piniperda* shoot feeding galleries, *Fusarium* species were isolated from 21.05% of the shoots collected (Table 2). Eighteen isolates were found to correspond to five different *Fusarium* species: one was identified as *Fusarium cortaderiae* (=*Fusarium graminearum* clade), one as *F. sporotrichioides*, three as *F. avenaceum*, three as *Fusarium tricinctum* (Corda) Saccardo (Table 4) and 10 as *F. circinatum*, corresponding to 3.5% of the feeding galleries in shoots (Tables 2 and 4).

3.4. Fusarium *spp. on Insect Exoskeletons*

A high percentage of *Fusarium* spp. appeared on the insect exoskeletons, showing that the bark beetle species usually carried at least one *Fusarium* species (Table 3). In some cases, one insect species was associated with more than one *Fusarium* species (Table 4). A total of seven *Fusarium* species were identified: four isolates were identified as *F. oxysporum* spp. complex, three isolates as *F. circinatum*, one as *Fusarium anthophilum* (A. Braun) Wollenw, six as *F. avenaceum*, one as *Fusarium sambucinum* Fuckel, one as *F. tricinctum* and one as *Fusarium konzum*. Of those isolates identified as *F. circinatum*, one was isolated from *H. attenuatus* collected from logs, one from *P. pubescens*, and one from *I. sexdentatus*.

Phylogenetic analysis of the α-TEF region indicated that all *Fusarium* species identified were clustered together (Figure 6) regardless of the type of sample (insect, logs or shoots). Moreover, the results of the sequence analysis support the molecular identification as the isolates that were identified as the same species clustered together.

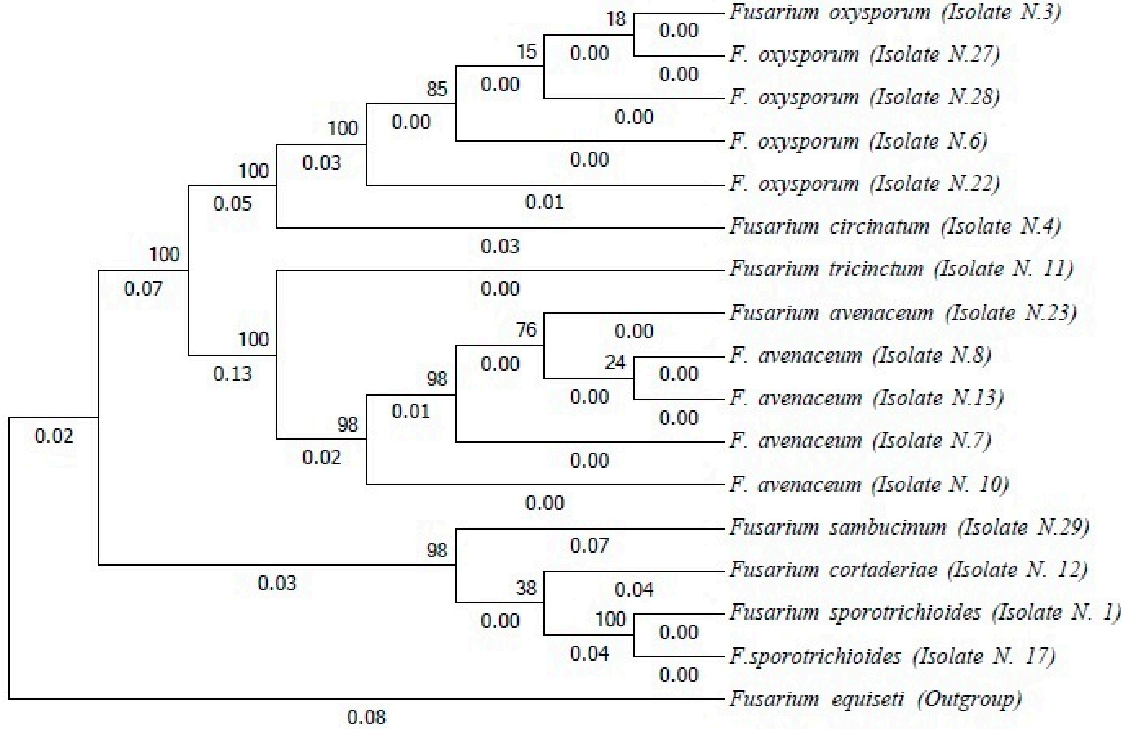

Figure 6. Phylogenetic tree inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances are in the units of the number of base substitutions per site.

4. Discussion

Amongst the fungal species isolated from the shoots and log tissues, we can highlight the presence of *D. sapinea*, *Penicillium* spp., *Pestalotiopsis* spp., *Trichoderma* spp. and *Fusarium* spp. for their pathogenicity, parasitism and potential as biocontrol agents. *Diplodia sapinea* was the species most frequently isolated in the plant tissue samples, although it did not appear on the insect bodies. This fungus, which is responsible for shoot blight and dieback on pine trees, has previously been found in association with bark beetles [44]. *Penicillium* spp. have previously been isolated as

saprotrophs in pine species, although they may also occur as endophytes in healthy tissues [45]. Four *Penicillium* species (*P. glabrum*, P. *Penicillium polonicum* K.M. Zalessk, *Penicillium minioluteum* Dierckx and *Penicillium melinii* Thom) were found to be associated with healthy *Pinus sylvestriss* L. twigs in Spain [46]. However in the present study, only one species of this genus was found in plant tissues. *Pestalotiopsis* is a ubiquitous genus that acts as an endophyte, saprotroph and pathogen in different hosts worldwide [47]. It has also been found in healthy tissues of pine species in Spain [45], although some species such as *Pestalotiopsis funerea* can cause damping off in other conifers [48]. Four CMs were identified as *Trichoderma* spp. in this study, but two of them could not be identified to species level from the ITS region. *Trichoderma harzianum*, *T. atroviride* and *T. viride* have been proposed as effective biological control agents for pathogenic *Fusarium* spp. [3,49], indicating the importance of determining the endophytic species that inhabit *P. radiata* trees. Our findings seem to indicate a negative correlation between *T. harzianum* and *F. circinatum*. Thus, *T. harzianum* reached a maximum of 52.7% of the phloem samples from *I. sexdentatus* galleries, while *F. circinatum* occurred in only 0.9% of such samples (Table 2). In addition, the higher rates of occurrence of *F. circinatum* in plant tissue (3.5% in shoots and 5.2%–5.5% in *H. angustatus* galleries) were associated with lower rates of occurrence of *T. harzianum* (11.9%, 0% and 10.5%, respectively). Although in the present study *T. atroviride* did not appear in high proportions, this fungus has been shown to parasitize a large variety of phytopathogenic fungi due to the production of hydrolytic enzymes, which has led to its use as a biological control agent [50].

Regarding the fungal communities associated with *T. piniperda*-colonized shoots, *D. sapinea* was the species most frequently isolated, as it appeared in 88.4% of the samples. Seasonal variation in the fungal species isolated from shoots was observed, and *D. sapinea* was more frequently found in samples collected during autumn. *Fusarium* spp. also appeared more frequently in those plots sampled during autumn. This is consistent with findings of a previous study [22], in which we isolated higher percentages of *F. circinatum* during autumn and winter than during the rest of the year.

Bark beetles are known to be associated with endophytic, phytopathogenic and entomopathogenic fungi. In this study, 18 species were isolated from insect bodies. Among these, the ascomycete yeast *Candida fructus* was identified in nine insect species. *Candida* species have been reported to be associated with several bark beetle species as entomopathogens, although many yeasts appear in symbiosis with bark beetles [51]. *Trichoderma atroviride* appeared on the bodies of six different insect species, mainly on *C. mediterraneus* (8% of the samples), coinciding with the lower rate of appearance of *Fusarium* spp. (12% of the samples). In order to evaluate the importance of *Trichoderma* spp. as an antagonist of *Fusarium* spp., more detailed studies of the role of bark beetles are required. Other fungi, such as *Neonectria radicicola*, were isolated from four bark beetle species. In Norway, this fungal species has been observed as an endophyte in pine roots without causing any damage [52]. However, other species of the genus *Neonectria* are known to cause neonectria canker disease on subalpine fir in Denmark [53] and stem cankers on *P. radiata* in Chile [54]. The species richness and fungal communities differed according to the bark beetle species. This difference may be due to the presence of specialized structures for carrying spores such as mycangia on some species (e.g., *H. ater*, *I. sexdentatus* and *Xyleborinus saxesenii*). The fungal communities associated with the insect exoskeletons and their galleries also differed significantly, although *Fusarium* spp. appeared to be present in both types of samples. This may indicate the importance of bark beetles in spreading *Fusarium* species.

Ten species of *Fusarium* were identified in this study by both molecular and morphological methods. These species were associated with the insect exoskeletons and galleries. The rate of occurrence of *Fusarium* spp. in plant tissue samples reached maximum levels as the fungi appeared in all of the xylem samples from galleries constructed by *O. erosus*, and this insect species also showed a high rate of phoresy for these fungal species (56.6% of the specimens). *Fusarium* spp. comprise a polyphyletic group that can act as endophytes or as a plant pathogens depending on the species and on the plant host, according to host specificity for plant–pathogen interactions. Moreover, these species are widely distributed, infecting a wide range of organisms worldwide. Several *Fusarium*

spp. have mutualistic associations with insects, e.g., *Fusarium solani* (Martius) which has a symbiotic relationship with *Hypothenemus hampei* (Ferrari) while colonizing coffee beans [55] and with *Xyleborus ferrugineus* (Fabricius) when colonizing dead insects as saprophytes or entomopathogens. In general, the *Fusarium* species associated with beetles are weakly entomopathogenic, although infection of the pine beetle *Dendroctonus frontalis* (Zimmerman) with *F. solani* resulted in the death of 90% the insects in 5 days [2]. In the present study, *Fusarium avenaceum* was the most commonly identified species and was directly isolated from *I. sexdentatus*, *H. attenuatus* and *H. ater* specimens and their galleries. Moreover, it also appeared on *T. piniperda*-infested shoots. *Fusarium avenaceum* has been previously isolated from *P. radiata* in New Zealand, where it was associated with dieback caused by physical injury [56]. However, the *F. graminearum* clade and *F. avenaceum* are commonly associated with crops [57] and cause *Fusarium* head blight (FHB) on wheat [58]. Molecular identification of *Fusarium* species was necessary to distinguish isolate number 21 (Table 4) and the DNA sequence corresponded to *F. avenaceum*, although the presence of napiform microconidia in this isolate had identified it morphologically as *Fusarium anthophilum*. *Fusarium tricinctum* was isolated from *I. sexdentatus* and from *T. piniperda* feeding galleries. This fungus usually acts as a saprophyte or a weak parasite in Europe and North America [31]. The *Fusarium oxysporum* spp. complex was found to be associated with *O. erosus*, *H. attenuatus* and detected in the *I. sexdentatus* galleries in logs (both, xylem and phloem). This is a saprophyte and soil pathogen species complex with a wide range of plant hosts divided into many formae specialis depending on the host specificity. *Fusarium sambucinum*, which was isolated from *P. pubescens*, causes *Fusarium* dry rot in potatoes [59] but has also been isolated from *P. radiata* in New Zealand and found to be associated with dieback and root rot [56]. Other species isolated in this study, such as *Fusarium sporotrichioides*, *F. konzum* and *F. beomiforme*, have not been previously described as plant pathogens or are known only as very weak pathogens [26].

Fusarium circinatum, the causal agent of PPC, appeared in 3.5% of the fallen shoots occupied by *T. piniperda*. This insect species has been proposed as a plausible vector of the disease in the study region, where 12% of the shoots collected from the ground were found to be associated with the pathogen in plantations affected by PPC [22]. However, the pathogen was not found in association with specimens of *T. piniperda* and *T. destruens* analyzed by Muñoz-Adalia et al. [60] in the same study area, although other *Fusarium* spp. were detected. The singularity of this association lies in the shoot-feeding maturation behavior of *T. piniperda* in the crowns of healthy pine trees [61], which may enable transmission of the fungal spores into healthy crowns. This insect has also been associated with some other highly phytopathogenic fungi such as *Leptographium wingfieldii* Morelet in Europe and North America [62,63]. These species have caused significant economic losses due to blue staining of infected wood. Brood galleries of *Hylastes* spp. also appeared to harbour to *F. circinatum* in this study. These insects have a similar way of interacting with sapwood fungi in several pine species [64], including *P. radiata*, although no ophiostomatoid fungi were found in the present study. *Hylastes angustatus* galleries frequently harboured *F. circinatum* (5.2% of xylem samples and 5.5% of phloem samples). However, *Hylastes* spp. are secondary pests, attacking weakened trees and roots, which suggests that they are not able to inoculate healthy trees with the pathogen as in the case of *T. piniperda*. In addition, some of the specimens of the bark beetle *P. pubescens* (1.05%) captured carried *F. circinatum*. The importance of other species of this genus has been noted in several countries. For example, in California the potential role of *Pityophthorus carmeli* Swaine and *Pityophthorus setosus* Blackman in spreading *F. circinatum* was shown by the wounding behaviour of these twig beetles while they tested the suitability of *P. radiata* branches as hosts [24]. Bonello et al. [65] reported that *Pityophthorus* spp. discriminated between healthy and pitch canker-diseased branches, preferring symptomatic branches due to the increased emission of ethylene. However, *P. pubescens* is a secondary species in the present study area, affecting small branches from weakened trees [23], and it does not seem to have an important role as a vector. On the other hand, *F. circinatum* was present in 0.9% of the *I. sexdentatus* specimens. Although *Ips sexdentatus* is a secondary bark beetle, it can act as a primary parasite when the population reaches epidemic levels [66] and could thus inoculate healthy trees with the pathogen.

In the Basque Country (Spain), 8.57% of the *I. sexdentatus* analyzed [21] carried the pathogen. Likewise, in California (USA) other species such as *Ips mexicanus* (Hopkins) and *Ips paraconfusus* Lanier, have been reported to be vectors of the pitch canker fungus [67]. *Fusarium circinatum* spores may be harboured on the exoskeletons of bark beetles when they feed on or breed in pitch canker diseased trees. Insects carrying the spores could then inoculate healthy pines. Some authors report that the association between insects and pathogenic fungi leads to stimulation of tree resistance [68]. However, as epidemic levels of bark beetles can kill healthy trees without carrying any phytopathogenic fungi, other authors state that insect–pathogen interactions could only benefit the fungal species, thus helping them expand their range to trees that they would otherwise not reach without this association [69]. Bark beetles are also principal wounding agents, contributing to the infections caused by airborne spores, although the insects themselves do not carry the pathogen [70]. The presence of the airborne inoculum of the causal agent of PPC has been recently studied by Dvorak et al. [71], who demonstrated that spores are present in the air throughout the year.

5. Conclusions

In conclusion, the species richness and also the fungal communities associated with the bodies of the bark beetles and their galleries varied depending on different factors such as insect species, type of plant tissue and season. The interactions between different fungal species associated with insects galleries has been shown to be a relevant factor determining the composition of the mycobiota, as the presence of *T. harzianum* was negatively correlated with a the frequency of *F. circinatum*. Moreover insect–fungi interactions are important for the distribution of fungal species. In this study, an association was found between the *Fusarium* species and bark beetles and their galleries, reaching the maximum rate in *H. ater* samples, 64% of which harboured *Fusarium* species. The importance of bark beetles in the distribution of *Fusarium* spp. may be related to the season (as observed in this study) as well as to the population levels, as bark beetles can reach epidemic levels after forest disturbances as fires, storms or outbreaks of pathogens. Specifically, our results showed that bark beetles appeared at a low frequency as phoretic agents of the PPC pathogen, suggesting that they may not be the main means of spreading of the disease in the study area. This should be considered together with the fact that the bark beetles were found at endemic levels, and if the population increased, this association would become more important. However, as *F. circinatum* is a quarantine pathogen, all pathways by which it can be introduced to new areas represent a potential risk to forest health. Further studies are required to identify the specific associations between different bark beetle species and fungal communities, in particular to clarify the role of the different *Scolytinae* species in the distribution of *Fusarium* spp.

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References

1. Arnold, A.E. Understanding the diversity of foliar endophytic fungi: Progress, challenges, and frontiers. *Fungal Biol. Rev.* **2007**, *21*, 51–66. [CrossRef]
2. Teetor-Barsch, G.H.; Roberts, D.W. Entomogenous *Fusarium* species. *Mycopatbologia* **1983**, *84*, 3–16. [CrossRef]
3. Summerell, B.; Leslie, J. Introducing the genus *Fusarium*. In *Control of Fusarium Species*; Álves-Santos, F., Diez Casero, J., Eds.; Research Point: Kerala, India, 2012.
4. Mirete, S.; Patiño, B.; Vázquez, C.; Jiménez, M.; Hinojo, M.J.; Soldevilla, C.; González-Jaén, M.T. Fumonisin production by *Gibberella fujikuroi* strains from *Pinus* species. *Int. J. Food Microbiol.* **2003**, *89*, 213–221. [CrossRef]
5. Summerell, B.; Leslie, J. Introducing the genus *Fusarium*. In *Control of Fusarium Diseases*; Alves-Santos, F., Diez, J., Eds.; Research Signpoin: Kerala, India, 2012; pp. 1–16.
6. Román-Avilés, B.; Lewis, J.; Kelly, J. Fusarium genetic control: A long term strategy. In *Control of Fusarium Diseases*; Alves-Santos, F., Díez, J., Eds.; Research Sing Post: Kerala, India, 2011; pp. 65–108.
7. Nirenberg, H.I.; O'Donnell, K. New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. *Mycologia* **1998**, *90*, 434–458. [CrossRef]
8. Wikler, K.; Storer, A.J.; Newman, W.; Gordon, T.R.; Wood, D.L. The dynamics of an introduced pathogen in a native Monterey pine (*Pinus radiata*) forest. *For. Ecol. Manag.* **2003**, *179*, 209–221. [CrossRef]
9. Wingfield, M.J.; Hammerbacher, A.; Ganley, R.J.; Steenkamp, E.T.; Gordon, T.R.; Wingfield, B.D.; Coutinho, T.A. Pitch canker caused by *Fusarium circinatum*—A growing threat to pine plantations and forests worldwide. *Australas. Plant Pathol.* **2008**, *37*, 319–334. [CrossRef]
10. Viljoen, A.; Wingfield, M.J.; Kemp, G.H.J.; Marasas, W.F.O. Susceptibility of pines in South Africa to the pitch canker fungus *Fusarium subglutinans* f.sp. *pini*. *Plant Pathol.* **2018**, *44*, 877–882. [CrossRef]
11. Bezos, D.; Martínez-Alvarez, P.; Fernández, M.; Diez, J.J. Epidemiology and management of pine pitch canker disease in Europe—A review. *Balt. For.* **2017**, *23*, 279–293.
12. Gordon, T.R.; Storer, A.J.; Wood, D.L. The pitch canker epidemic in California. *Plant Dis.* **2001**, *85*, 1128–1139. [CrossRef]
13. Landeras, E.; García, P.; Fernández, Y.; Braña, M.; Fernández-Alonso, O.; Méndez-Lodos, S.; Pérez-Sierra, A.; León, M.; Abad-Campos, P.; Berbegal, M.; et al. Outbreak of pitch canker caused by *Fusarium circinatum* on *Pinus* spp. in Northern Spain. *Plant Dis.* **2005**, *89*, 1015. [CrossRef]
14. Romón, P.; Troya, M.; Fernández de Gamarra, M.E.; Eguzkitza, A.; Iturrondobeitia, J.C.; Goldarazena, A. Fungal communities associated with pitch canker disease of *Pinus radiata* caused by *Fusarium circinatum* in northern Spain: Association with insects and pathogen-saprophyte antagonistic interactions. *Can. J. Plant Pathol.* **2008**, *30*, 241–253. [CrossRef]
15. Alves-Santos, F.; Diez, J. Biological control of *Fusarium*. In *Control of Fusarium Diseases*; Alves-Santos, F., Diez, J., Eds.; Research Singpoin: Kerala, India, 2012; pp. 131–158.
16. Martínez-Álvarez, P.; Manuel Alves-Santos, F.; Martínez-Álvarez, J.D. In vitro and in vivo interactions between *Trichoderma viride* and *Fusarium circinatum*. *Silva Fenn.* **2012**, *46*, 303–316. [CrossRef]
17. Martín-García, J.; Paraschiv, M.; Flores-Pacheco, J.A.; Chira, D.; Diez, J.J.; Fernández, M. Susceptibility of several northeastern conifers to *Fusarium circinatum* and strategies for biocontrol. *Forests* **2017**, *8*, 318. [CrossRef]
18. Howell, C.R. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. *Plant Dis.* **2003**, *87*, 4–10. [CrossRef]
19. Deacon, J.W. *Fungal Biology*; Blackwell Publishing: Malden, MA, USA, 2006.
20. García-Serna, I. *Diplodia pinea (Desm.) Kickx y Fusarium circinatum Niremberg & O'Donell*; Universidad del País Vasco: Lejona, Spain, 2011.
21. Romón, P.; Iturrondobeitia, J.C.; Gibson, K.; Lindgren, B.S.; Goldarazena, A. Quantitative association of bark beetles with pitch canker fungus and effects of verbenone on their semiochemical communication in monterey pine forests in Northern Spain. *Environ. Entomol.* **2007**, *36*, 743–750. [CrossRef] [PubMed]
22. Bezos, D.; Martínez-Álvarez, P.; Diez, J.J.; Fernández, M.M. The pine shoot beetle *Tomicus piniperda* as a plausible vector of *Fusarium circinatum* in northern Spain. *Ann. For. Sci.* **2015**, *72*, 1079–1088. [CrossRef]

23. Bezos, D.; Martínez-Álvarez, P.; Diez, J.J.; Fernández, M. Association levels between *Pityophthorus pubescens* and *Fusarium circinatum* in pitch canker disease affected plantations in northern Spain. *Entomol. Gen.* **2016**, *36*, 43–54. [CrossRef]
24. Sakamoto, J.M.; Gordon, T.R.; Storer, A.J.; Wood, D.L. The role of *Pityophthorus* spp. as vectors of pitch canker affecting *Pinus radiata*. *Can. Entomol.* **2007**, *139*, 864–871. [CrossRef]
25. López, S.; Ochoa, P.R.; Carlos Iturrondobeitia, J.; Goldarazena, A. *Los Escolítidos de las Coníferas del País Vasco*; Servicio Central de Publicaciones del Gobierno Vasco: Vitoria-Gasteiz, Spain, 2007; ISBN 978-84-457-2650-1.
26. Raffa, K.; Berryman, A. The role of host plant resistance in the colonization behaviour and ecology of bark beetles (Coleoptera: Scolitidae). *Ecol. Monogr.* **1983**, *53*, 27–49. [CrossRef]
27. López, S.; Quero, C.; Iturrondobeitia, J.C.; Guerrero, Á.; Goldarazena, A. Evidence for (E)-pityol as an aggregation pheromone of *Pityophthorus pubescens* (Coleoptera: Curculionidae: Scolytinae). *Can. Entomol.* **2011**, *143*, 447–454. [CrossRef]
28. Lacap, D.C.; Hyde, K.D.; Liew, E.C.Y. An evaluation of the fungal "morphotype" concept based on ribosomal DNA sequences. *Fungal Divers.* **2003**, *12*, 53–66.
29. Vainio, E.J.; Korhonen, K.; Hantule, J. Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. *Mycol. Res.* **1998**, *102*, 187–192. [CrossRef]
30. Gardes, M.; Burns, T. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rust. *Mol. Ecol.* **1993**, *2*, 113–118. [CrossRef] [PubMed]
31. Leslie, J.; Summerell, B. *The Fusarium Laboratory Manual*; Blackwell Publishing: Ames, IA, USA, 2006; ISBN 978-0-813-81919-8.
32. O'donnell, K.; Kistler, H.C.; Cigelnik, E.; Ploetz, R.C. Multiple evolutionary origins of the fungus causing panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2044–2049. [CrossRef] [PubMed]
33. Geiser, D.M.; Jiménez-Gasco, M.D.M.; Kang, S.; Makalowska, I.; Veeraraghavan, N.; Ward, T.J.; Zhang, N.; Kuldau, G.A.; O'donnell, K. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying Fusarium. *Eur. J. Plant Pathol.* **2004**, *110*, 473–479. [CrossRef]
34. Pérez-Sierra, A.; Landeras, E.; León, M.; Berbegal, M.; García-Jiménez, J.; Armengol, J. Characterization of *Fusarium circinatum* from *Pinus* spp. in northern Spain. *Mycol. Res.* **2007**, *111*, 832–839. [CrossRef] [PubMed]
35. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **2013**. [CrossRef] [PubMed]
36. Tamura, K.; Nei, M.; Kumar, S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA* **2004**. [CrossRef] [PubMed]
37. Felsenstein, J. Confidence Limits on phylogenies: An aproach using the botts trap. *Evolution* **1985**, *39*, 783–791. [CrossRef] [PubMed]
38. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**. [CrossRef]
39. Schweigkofler, W.; O'Donnell, K.; Garbelotto, M. Detection and quantification of airborne conidia of *Fusarium circinatum*, the causal agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple spore trapping method. *Appl. Environ. Microbiol.* **2004**, *70*, 3512–3520. [CrossRef] [PubMed]
40. Gallego, D.; Galián, J. The internal transcribed spacers (ITS1 and ITS2) of the rDNA differentiates the bark beetle forest pests *Tomicus destruens* and *T. piniperda*. *Insect Mol. Biol.* **2001**. [CrossRef]
41. García Pérez, A. *Métodos Avanzados De Estadística Aplicada: Métodos Robustos Y De Remuestreo*; UNED: Madrid, Spain, 2010.
42. Wilcox, R.R.; Schönbrodt, F.D. The WRS Package for Robust Statistics in R (Version 0.24). 2014. Available online: https://github.com/nicebread/WRS (accessed on 9 November 2018).
43. Oksanen, J.; Blanchet, F.G.; Kindt, R.; Legendre, P.; Minchin, P.R.; O'hara, R.B.; Simpson, G.L.; Solymos, P.; Henry, M.; Stevens, H.; et al. Package "vegan" Title Community Ecology Package. 2015. Available online: https://cran.r-project.org/web/packages/vegan/index.html (accessed on 9 November 2018).
44. Whitehill, J.G.A.; Lehman, J.S.; Bonello, P. *Ips pini* (Curculionidae: Scolytinae) is a vector of the fungal pathogen, *Sphaeropsis sapinea* (Coelomycetes), to Austrian Pines, *Pinus nigra* (Pinaceae). *Environ. Entomol.* **2007**, *36*, 114–120. [CrossRef]

45. Zamora, P.; Martínez-Ruiz, C.; Diez, J.J. Fungi in needles and twigs of pine plantations from northern Spain. *Fungal Divers.* **2008**, *30*, 171–184.
46. Sanz-Ros, A.V.; Müller, M.M.; San Martín, R.; Diez, J.J. Fungal endophytic communities on twigs of fast and slow growing Scots pine (*Pinus sylvestris* L.) in northern Spain. *Fungal Biol.* **2015**, *119*, 870–883. [CrossRef] [PubMed]
47. Jeewon, R.; Liew, E.; Hyde, K. Phylogenetic evaluation of species nomenclature of *Pestalotiopsis* in relation to host association. *Fungal Divers.* **2004**, *17*, 39–55.
48. Bajo, J.; Santamaría, O.; Diez, J.J. Cultural characteristics and pathogenicity of *Pestalotiopsis funerea* on *Cupressus arizonica*. *For. Pathol.* **2008**, *38*, 263–274. [CrossRef]
49. Pinto, P.M.; Alonso, J.A.P.; Fernández, V.P.; Casero, J.J.D. Fungi isolated from diseased nursery seedlings in Spain. *New For.* **2006**, *31*, 41–56. [CrossRef]
50. Olmedo-Monfil, V.; Mendoza-Mendoza, A.; Gómez, I.; Cortés, C.; Herrera-Estrella, A. Multiple environmental signals determine the transcriptional activation of the mycoparasitism related gene prb1 in *Trichoderma atroviride*. *Mol. Genet. Genom.* **2002**, *267*, 703–712. [CrossRef]
51. Hofstetter, R.W.; Dinkins-Bookwalter, J.; Davis, T.S.; Klepzig, K.D. Symbiotic Associations of Bark Beetles. In *Bark Beetles: Biology and Ecology of Native and Invasive Species*; Vega, F., Hostetter, R., Eds.; Academic Press: Cambridge, MA, USA, 2015; ISBN 9780124171565.
52. Ndobe, N.E. *Fungi Associated with Roots of Healthy-Looking Scots Pines and Norway Spruce Seedlings Grown in Nine Swedish Forest Nurseries*; SLU: Uppsala, Sweden, 2012.
53. Talgø, V.; Thomsen, I.M.; Nielsen, U.B.; Brurberg, M.B.; Stensvand, A. Neonectria canker on subalpine fir (*Abies lasiocarpa*) in Denmark. In Proceedings of the 10th International Christmas Tree Research & Extension Conference, Eichgraben, Austria, 21–27 August 2011; pp. 92–96.
54. Morales, R. Detección de *Neonectria fuckeliana* en Chile, asociado a cancros y malformaciones fustales en plantaciones de *Pinus radiata*. *Bosque* **2009**, *30*, 106–110. [CrossRef]
55. Morales-Ramos, J.A.; Rojas, M.G.; Sittertz-Bhatkar, H.; Saldaña, G.; Saldaña, S. Symbiotic Relationship Between *Hypothenemus hampei* (Coleoptera: Scolytidae) and *Fusarium solani* (Moniliales: Tuberculariaceae). *Ann. Entomol. Soc. Am.* **2000**, *93*, 541–547. [CrossRef]
56. Dick, M.A.; Dobbie, K. Species of *Fusarium* on *Pinus radiata* in New Zeland. *N. Z. Plant Prot.* **2002**, *55*, 58–62.
57. Satyaprasad, K.; Bateman, G.L.; Ward, E. Comparisons of isolates of *Fusarium avenaceum* from white lupin and other crops by pathogenicity tests, DNA analyses and vegetative compatibility tests. *J. Phytopathol.* **2000**, *148*, 211–219. [CrossRef]
58. Bottalico, A.; Perrone, G. Toxigenic Fusarium species and mycotoxins associated with head blight in small-grain cereals in Europe. *Mycotoxins Plant Dis.* **2002**, *108*, 611–624.
59. Niemira, B.A.; Hammerschmidt, R.; Safir, G.R. Postharvest suppression of potato dry rot (*Fusarium sambucinum*) in prenuclear minitubers by arbuscular mycorrhizal fungal inoculum. *Am. Potato J.* **1996**, *73*, 509–515. [CrossRef]
60. Muñoz-Adalia, E.J.; Sanz-Ros, A.V.; Flores-Pacheco, J.A.; Hantula, J.; Diez, J.J.; Vainio, E.J.; Fernández, M. *Sydowia polyspora* dominates fungal communities carried by two *Tomicus* species in pine plantations threatened by Fusarium circinatum. *Forests* **2017**, *8*, 127. [CrossRef]
61. Lieutier, F.; Langstrom, B.; Faccoli, M. The genus *Tomicus*. In *Bark Beetles: Biology and Ecology of Native and Invasive Species*; Vega, F., Hofstetter, R., Eds.; Academic Press: Cambridge, MA, USA, 2015; pp. 371–426. ISBN 9780124171565.
62. Jacobs, K.; Bergdahl, D.R.; Wingfield, M.J.; Halik, S.; Seifert, K.A.; Bright, D.E.; Wingfield, B.D. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycol. Res.* **2004**, *108*, 411–418. [CrossRef] [PubMed]
63. Lieutier, F.; Yart, A.; Garcia, J.; Ham, M.C.; Morelet, M.; Levieux, J. Champignons phytopathogènes associés à deux coléoptères scolytidae du pin sylvestre (*Pinus sylvestris* L.) et étude préliminaire de leur agressivité envers l'hôte. *Ann. Sci. For.* **1989**, *46*, 201–216. [CrossRef]
64. Reay, S.D.; Walsh, P.J.; Ram, A.; Farrell, R.L. The invasion of *Pinus radiata* seedlings by sapstain fungi, following attack by the black pine bark beetle, *Hylastes ater* (Coleoptera: Scolytidae). *For. Ecol. Manag.* **2002**, *165*, 47–56. [CrossRef]
65. Bonello, P.; Mcnee, W.R.; Storer, A.J.; Wood, D.L.; Gordon, T.R. The role of olfactory stimuli in the location of weakened hosts by twig-infesting *Pityophthorus* spp. *Ecol. Entomol.* **2001**, *26*, 8–15. [CrossRef]

66. Etxebeste, I.; Pajares, J.A. Verbenone protects pine trees from colonization by the six-toothed pine bark beetle, *Ips sexdentatus* Boern. (Col.: Scolytinae). *J. Appl. Entomol.* **2011**, *135*, 258–268. [CrossRef]
67. Fox, J.W.; Wood, D.L.; Koehler, C.S.; O'keefe, S.T. Engraver beetles (Scolytidae: *Ips* species) as vectors of the pitch canker fungus, *Fusarium subglutinans*. *Can. Entomol.* **1991**, *123*, 1355–1367. [CrossRef]
68. Lieutier, F.; Yart, A.; Salle, A. Stimulation of tree defenses by Ophiostomatoid fungi can explain attack success of bark beetles on conifers. *Ann. For. Sci.* **2009**, *66*, 801. [CrossRef]
69. Six, D.L.; Wingfield, M.J. The role of phytopathogenicity in bark beetle–fungus symbioses: A challenge to the classic paradigm. *Annu. Rev. Entomol.* **2011**. [CrossRef] [PubMed]
70. Baker, J.M.; Norris, D.M. A complex of fungi mutualistically involved in the nutrition of the ambrosia beetle *Xyleborus ferrugineus*. *J. Invertebr. Pathol.* **1968**, *11*, 246–250. [CrossRef]
71. Dvořák, M.; Janoš, P.; Botella, L.; Rotková, G.; Zas, R. Spore dispersal patterns of *Fusarium circinatum* on an infested monterey pine forest in north-western Spain. *Forests* **2017**, *8*, 432. [CrossRef]

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