Fungal endophytes in xylem of healthy Chilean trees and their possible role in early wood decay

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A survey of fungal endophytes associated with xylem of presumably healthy trees was conducted. Wood-inhabiting fungal endophytes of *Prumnopitys andina*, *Podocarpus saligna*, *Drimys winteri* and *Nothofagus obliqua* were isolated from surface sterilized xylem core samples. Five basidiomycetes (*Inonotus* sp., *Bjerkandera adusta* and three unknown strains), two ascomycetes (*Xylaria* sp., *Bipolaris* sp.) and one anamorphic strain were detected. *Xylaria* sp and *Bjerkandera adusta* were the most frequent fungal isolates. Ultrastructural observations of wood cores samples by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy showed the presence of fungal hyphae attached to the inner cell surface inhabiting xylem elements even before the induction of wood degradation. Evidence of latent infection was found mainly along the parenchyma rays indicating fungal colonization and distribution. Results showed simultaneous decay of all wood components, characterized by a thinning cell-wall from the cell lumen to the middle lamella and erosive wood degradation typical of non selective white rot. By using SEM, TEM and light microscopy it was possible to detect natural incidence of latent infections, to visualize spreading of colonization and the ability to produce wood degradation under suitable conditions.

Key words: endophytes, latent-infection, wood-rot, wood biodegradation

Introduction

Endophytic fungi are defined as micro-organisms that colonize healthy plant tissues inter-and/or intra-cellularly, persisting for the whole or part of its life cycle without causing disease symptoms in the host plant (Petrini, 1996; Saikkonen *et al.*, 1998; Bacon and White, 2000; Sánchez Márquez *et al.*, 2007). Contrary to previous assumptions concerning their aseptic conditions it is now widely accepted that plants and even lichen tissues commonly harbour cryptic fungal infections (Devarajan and Suryanarayanan, 2006; Duong *et al.*, 2006; Li *et al.*, 2007). These fungal endophytes are usually symptomless, but may produce effects ranging from beneficial to pathogenic (Strobel and Long, 1998; Photita *et al.*, 2001, 2004; Neubert *et al.*, 2006; Gonthier *et al.*, 2006; Wei *et al.*, 2007). Endophytes can be detected in diverse plant tissues including leaves, petioles, fruits, spines, seeds, bark and wood (Petrini, 1991) and have been found in almost all vascular plant species examined to date (Arnold *et al.*, 2000; Hu *et al.*, 2007; Sánchez Márquez *et al.*, 2007; Tejesvi *et al.*, 2007) and they are considered important components of fungal biodiversity which until now is believed to be an underestimate (Hawksworth and Rossman, 1997; Hawksworth, 2000; Arnold *et al.*, 2001; Mueller and Schmit, 2007).

The niches occupied by fungal endophytes in different plant tissues such as sapwood, deserves more attention (Hutchinson, 1999; Hoff *et al.*, 2004). Despite efforts, the full range of ecological functions of endophytic fungi of woody plants is poorly understood but it is likely to be correlated with species diversity (Purvis and Hector, 2000; Hendry *et*

al., 2002). A hypothesis for the role of endophytic *Xylaria* – which are understudied endophytes – proposes that fungi are simply waiting for their host to senesce at which time they can begin the decomposition of cell wall materials (Petrini and Petrini 1985; Whalley 1996). Endophytic fungi employing this strategy would have an advantage over competing saprobes, having "claimed" the substrate before decomposition begins (Carroll, 1995; Davis *et al.*, 2003). There is a need to further explore the role of wood-inhabiting endophytes especially in natural process such as wood biodegradation (Hoff *et al.*, 2004).

The Compartmentalization of Decay in Trees (CODIT) theory suggests the creation of barriers that resist invasion of microorganisms (Shigo and Marx, 1977; Shigo, 1984); however, these physical and morphological barriers do not make the tree immune to microbial colonization. As long as a wound remains open, its surface can be invaded by microorganisms. Compartments can fail when microorganisms become established, causing new barriers to be generated at greater depths and distances from the wound (Shigo and Marx, 1977; Shigo, 1984).

It has been proposed that the anatomical structure of wood in connection with physical phenomena at the wood surface (capillary action, changes in temperature and air pressure, rain splash) may be responsible for differences in the infection and colonization process of wood of different tree species (Hintikka, 1987). A single fungal infection could spread through the tree, via xylem sap, where cell vessels could be used as "highways" by endophytic fungi for long-distance dispersal within the xylem (Boddy, 1994).

In previous reports, it has been demonstrated that the incubation of fresh cut branches under various drying regimes allows the growth of active mycelia. Emergence of active mycelia is assumed to have originated from latent infections, which were naturally present in healthy tissues in the field (Chapela and Boddy, 1988 a,b). The water loss and the increasing oxygen concentration were proposed to trigger the switch from the latent to active phase of the mycelia (Rayner, 1986). The presence of inactive endophytic propagules such as dormant chlamydospores inside of living standing trees has been associated with wood decay (Schwarze *et al.*, 2000). Baum *et al.* (2003) isolated fungi from the xylem of European beech (*Fagus sylvatica*) immediately after cutting as well as after incubation at different periods of time. Only a few isolates were obtained from freshly cut wood, but after eight weeks of incubation under sterile conditions, a large number of isolates were recovered. Basidiomycetes required a longer incubation period than the Ascomycetes to be detected from the tissue samples.

An overall explanation for the mechanisms by which decay fungi invade and degrade wood in standing and dead trees is still incomplete (Eaton, 2000). It has been proposed that endophytic fungi could be involved in triggering the development of early stages of wood decay (Schwarze *et al.*, 2000; Baum *et al.*, 2003). In previous work, it was shown that fungal endophytes isolated from *Prumnopitys andina* (Poepp. Ex Endl.) de Laub and *Drimys winteri* J.R. et G. Foster had lignocellulolytic activity and also promoted wood biodegradation (Oses *et al.*, 2006).

The aim of the present study is to provide information about the natural occurrence and colonization of fungal endophytes associated with xylem sap of Chilean native trees with emphasis in wood decay fungi. Also, on the bases of ultrastructural observation of wood core samples by light microscopy, SEM and TEM, the role of fungal endophytes in natural wood biodegradation is discussed.

Materials and methods

Sampling, isolation and identification of fungal endophytes.

Samples were collected from six sampling units belonging to two different sites, Cordillera de Nahuelbuta (NAH), 900 masl; (37º35'S 73º10'W) and Termas de Chillán (TCH), 700 masl; (36º52'S 71º39'W). The size of the sampling areas was 5-6 ha in each site. During the 2003 winter season (June-August), core wood samples were collected from randomly selected healthy and symptomless gymnosperm (*Prumnopitys andina* and *Podocarpus saligna,* Podocarpaceae) and angiosperms (*Nothofagus obliqua* (Mirb.) Oerst., Fagaceae and *Drimys winteri* J.R. et G.

Forster, Winteraceae). The first were collected from TCH and the last from NAH. Within each sampling unit, wood cores were collected at breast height (BH) from the NE facing slope, using an increment borer (Suunto Oy, Vantaa, Finland), which was surface sterilized with ethanol:chlorine (1:5) after each use. Wood cores were sprayed with ethanol, and stored at 4ºC in a plastic bag. Each hole of sampled trees was sealed with synthetic resin to minimize post-sampling infections (Hoff *et al.*, 2004; Oses *et al.*, 2006). In the laboratory, wood cores were debarked, cut into fragments of ca 0.5 cm, washed with tap water to remove particles on the surface, submerged in 70% v/v ethanol, flame-sterilized (Mainfeld, 1998; Gorke, 1998) and placed into a 90 × 10 mm Petri dish containing selective medium BCS for basidiomycetes. The BCS media contained: malt extract (15 gl^{-1}), agar (15 g gl^{-1}), benomyl (40 mg gl^{-1}), streptomycin sulphate (100 mg gl^{-1}) and chloramphenicol (100 mg gl^{-1}) (Worrall and Harrington, 1993). Plates were incubated in the dark at 25ºC for eight weeks.

Wood fragments in which fungal growth was not observed in BCS medium were transferred within eight weeks to a non-selective, 2% malt extract agar (MEA), to facilitate the growth and isolation of fungi other than basidiomycetes that are sensitive to BCS medium. After eight weeks of incubation, fungal isolates were morphologically examined. Colonies growing from individual fragments were identified using specific keys for basidiomycetes (Stalpers, 1978) and anamorphic taxa (Kieffer and Morelet, 2000; Barnett and Hunter, 1998; von Arx, 1981). Fruiting bodies of basidiomycetes were induced using bags containing 400 g of sterilized hardwood sawdust (*N. dombeyi*) incubated for 30 days at 25ºC in a dark room at 80% relative humidity. Once mycelia covered the entire substrate, several holes in the plastic bag were made to allow the entry of oxygen and to induce carpogenesis (Croan *et al.*, 1997). Representative fungal isolates were stored at 4ºC in water.

Characterization of wood colonization and degradation by fungal endophytes

Growth stimulation of endophytic fungi within wood and the observation of wood decay were carried out as described by Chapela (1989). Sterilized wood core fragments (3-4 mm) of *Drimys winteri* were placed on water-agar solid media and incubated at 25ºC and 80% humidity in darkness for 25 days. For the same period of time a second group of wood core fragments were kept at 4ºC to be used as the control. After incubation, wood core fragments were observed. To study the fungal isolates, slides were prepared from pure cultures and stained with cotton blue/lactophenol-lactic acid solution and Melzer´s reagent. Morphological features such as hyphae, basidia/basidiospores, asci/ascospores and chlamydospores were observed for the different isolates. Permanent slides were prepared using Shurmount aqueous solution, examined in a light microscopy (Carl Zeiss, Axioskop, Germany) and digital photographs with a Nikon Coolpix 995 and 4500.

Wood decay and ultrastructural changes were observed using light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Transversal and tangential sections (10 μm) of wood core pieces were obtained using a microtome fitted with a steel knife. Sections were heat mounted on glass slides and stained directly with a drop of Melzer´s reagent.

Photographs of surfaces of each core fragment sample using SEM were obtained with an AUTOSCAN microscope. Before analysis, samples were dried in a desiccator and coated with gold in a vacuum evaporator system (Barrasa *et al.*, 1992).

For TEM, wood fragment samples were washed with 0.05 M phosphate buffer (pH 7.2), postfixed with 1% OsO$_4$ (24 hours, 4ºC), dehydrated in a series of ethanol-water and propylene oxide, embedded in hard formula Spurr polymerized 48 hours at 80ºC, sectioned with a diamond knife and stained with 0.1% lead citrate before microscopic observations (Barrasa *et al.*, 1992).

Results

Isolation and identification of fungal endophytes

A summary of the isolated fungal endophytes from xylem of different symptomless host trees is presented in Table 1. A total of 110 cores were evaluated and fungi

Table 1. Occurrence of single or multiple fungal isolated per host tree.

Species	nº trees		
	Sampled	With single isolate (%)	With multiple isolates (%)
Prumnopitys andina	10	2 (20.0)	1 (10.0)
Podocarpus saligna	10	1 (10.0)	0 (0.0)
Nothofagus obliqua	16	7 (43.8)	2 (12.4)
Drimys winteri	15	6 (40.0)	4 (26.7)
Total	**51**	**16 (31.6)**	**7 (13.7)**

were isolated from 51 samples. Twenty-three of the cores (45%) presented fungal xylem colonization. From these, sixteen had only one isolate, while multiple fungi were isolated from the other seven. From these wood cores, a total of 1,891 fragments were analyzed and fungal colonization was found in 95 samples (5%) (Fig. 1A; Table 2).
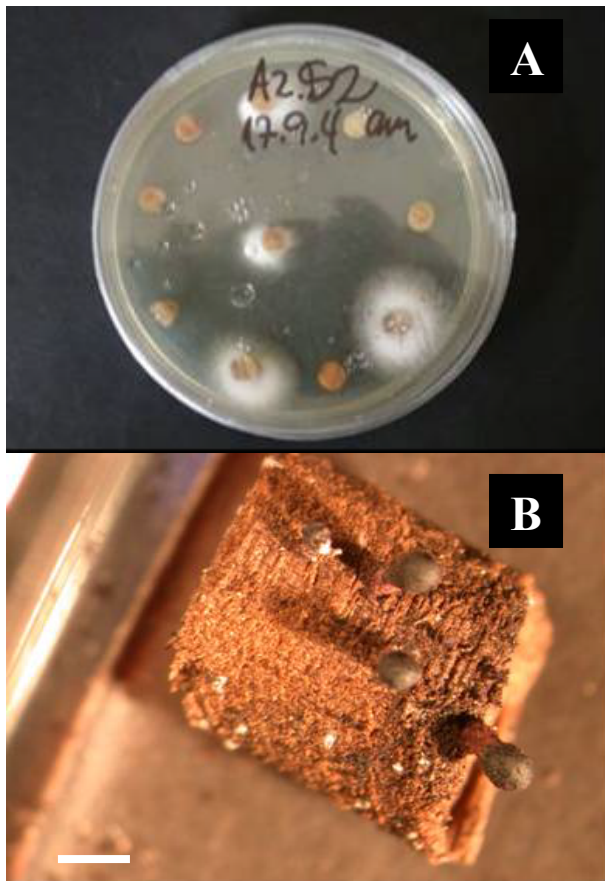
Fig. 1. Isolation and carpophore formation of endophyte fungi. Development of mycelium from wood core fragments after 30 days incubation on BCS media (A). Fruiting bodies formation of *Xylaria* sp. on *Nothofagus dombeyi* wood (B) (Scale bar = 2 mm).

The total number of trees from which fungal endophytes were isolated was higher in angiosperm species, *Nothofagus obliqua* and *Drimys winteri,* than in gymnosperms, *Prumnopitys andina* and *Prumnopitys andina* and *Podocarpus saligna* (Table 1).

Eight different fungal strains were isolated, corresponding to two ascomycetes, *Xylaria* sp. and *Bipolaris* sp; one strain of a anamorphic taxon and five basidiomycetes. The latter corresponded to *Inonotus* sp., *Bjerkandera adusta* and three unknown basidiomycetes PA-1, NO-1 and DW-2, isolated from *Prumnopitys andina, Nothofagus obliqua* and *Drimys winteri*, respectively. The most frequent fungal isolates were *Xylaria* sp (3.5%) and *Bjerkandera adusta* (4.7%) (Table 2).

The fungal endophyte *Bjerkandera adusta* completely covered the wood fragments of *Drimys winteri* during incubation. The aerial white mycelia had hyphae with a diam. between 2.5 and 6 μm and abundant clamp-connections near to the marginal zone (result not shown). The mycelia had a cotton-like aspect, dense in the border, whitish at the beginning then becoming yellowish at the end of third week.

Formations of fruiting bodies were observed during growth on sawdust under laboratory conditions. In exceptional cases fruiting bodies were observed *in situ* growing on core fragments as in the case of *Xylaria* sp., growing on *Nothofagus obliqua* fragments (Fig. 1B). Microscopic observations of the strains PA-1, NO-1 and DW-2, showed abundant clamp-connections and different growing patterns on solid media. The presence of tubes, groups of basidia and cheilocystidia in young carpophores allowed us to identify PA-1, NO-1 and DW-2 as *Aphyllophorales*. A *Bipolaris* sp found in *Prumnopitys andina* wood fragments was identified due to the presence of dark-brown melanized hyphae, conidia and intercalary chlamydospores.

Table 2. Relative isolation frequencies (RIF) of wood core fragments and isolated fungi.

Host tree	Analysed fragments (nº)	Colonized fragments (nº)	Relative isolation frequency RIF (%) [a]	Isolated fungi
Prumnopitys andina	385	11	2.9	Basidiomycete PA-1
		5	1.3	*Bipolaris* sp.
Podocarpus saligna	345	6	1.7	*Inonotus* sp
Nothofagus obliqua	736	26	3.5	*Xylaria* sp.
		9	1.2	Basidiomycete NO-1
Drimys winteri	425	20	4.7	*Bjerkandera adusta*
		5	1.2	Basidiomycete DW2
		13	3.1	Anamorphic taxa DW3
TOTAL	1891	95		

[a] The relative isolation frequencies (RIF) for each species are the percentages of infected fragments with respect number of fragments collected in each sample site.

Evidence of wood colonization and decay

Tangential and transverse sections of xylem samples of healthy *Drimys winteri* and *Nothofagus obliqua* cores were stained directly with Melzer´s reagent and then analyzed by light microscopy. Wood fragments had clear signs of fungal colonization and propagation after four weeks of incubation, present mainly along the xylem parenchyma (Figs 2A, B). Examination of slides from freshly cut wood did not show signs of decay. After incubation, decay of wood fragments resulted in a whitish, soft, fibrous and easily breakable material, in concordance with previous reports of white-rot (Blanchette, 1991, Blanchette *et al*., 1997).

Control samples of core fragment (Fig. 3A) shows a cross-field zone and aerolate pits with characteristic circular borders. The regular pits observed in the SEM of control samples became larger, irregular and in some cases connected with each other in the decayed samples. These were the main ultrastructural changes induced by fungal endophytes. The observed degradation pattern is non-selective because there is simultaneous damage in fibers as well as in the lamella media (Fig. 3B). There is evidence of abundant pits destroyed not only in the lumen but also in cross-field zone and complete degradation of the ray cell walls was also observed (Fig. 3B). Fungal endophytes caused both cavities and erosion at the lumen surface, and in some cases the erosion channels had serrated edges (Fig 3B)

Furthermore, transverse bore holes that become larger as the degree of decay increases, were frequently observed.

Ultrastructural changes in cell-wall by fungal endophytes were analyzed by TEM. Transversal sections of xylem core fragments of *Drimys winteri* used as control and wood decayed by fungal endophytes were analyzed by TEM after 30 days of incubation. Despite the presence of hyphal sheaths attached to the inner cell wall surface in the control sample (Fig. 4A), no damage in the xylem elements was observed. The decay process was characterized by tissue destruction in zones near to fungal growth with cell-wall thinning. Indeed, erosion channels beneath hyphae extended deeply into the secondary wall, degrading progressively the S1, S2 and S3 layers (Fig. 4B).

Discussion

The present work confirmed the presence of fungal colonization in the xylem of apparently healthy endemic trees, providing evidence that supports the "latent infection hypothesis" proposed by Boddy and Rayner (1983). Similar findings have been reported for different host tree species such as alder (Petrini and Fisher, 1990), conifers (Sieber, 1989; Kowalski and Kehr, 1992), beech and aspen (Chapela, 1989) and European beech (Baum *et al*., 2003). The fungal endophytes isolated were basidiomycetes and ascomycetes. The basidiomycetes were identified as *Bjerkandera adusta* and *Inonotus* sp., the ascomycete corresponded to *Xylaria* sp., all described as wood degraders. Of these, *Xylaria* species have been the most studied endophytes in woody plants (Petrini
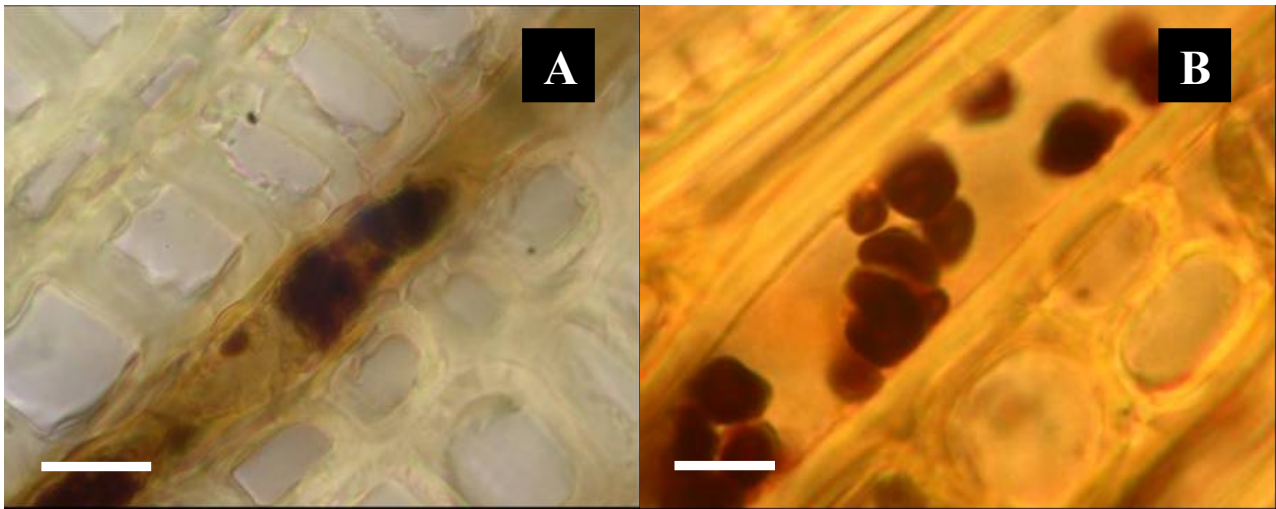
Fig. 2. Fungal propagules observed in wood core fragments of *Drimys winteri* (A) and *Nothofagus obliqua* (B). (Scale bar = 5 µm).
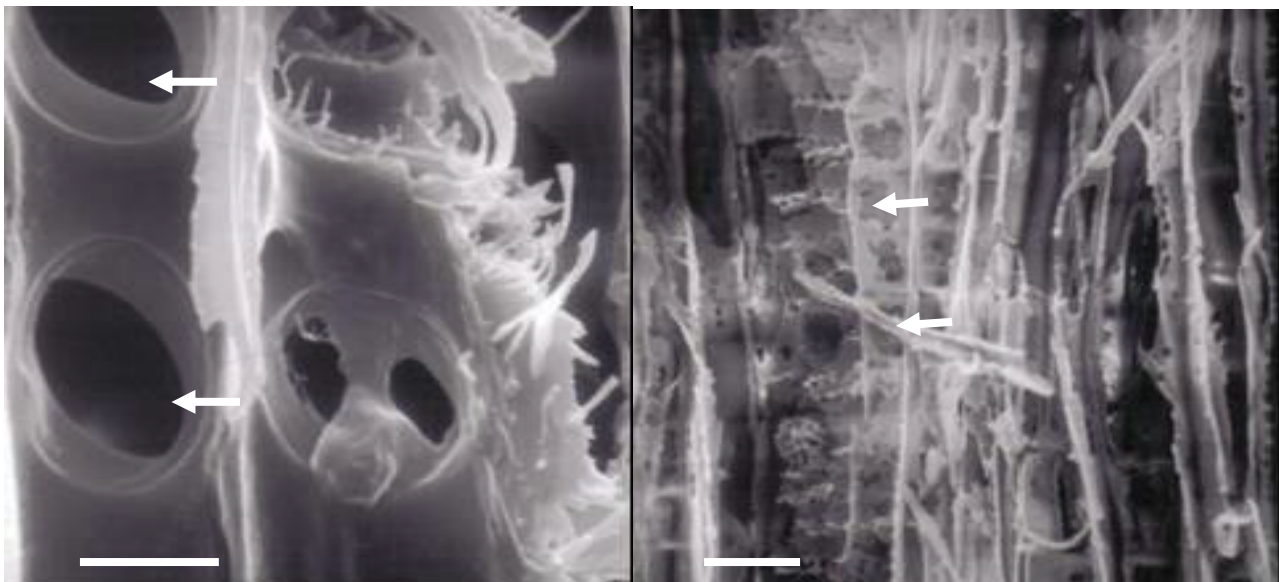
**Fig. 3**. SEM of *Drimys winteri*. Core fragment showing aerolate pits (arrows) in a non incubated sample (A) (scale bar = 4 µm). Degraded cross-field (multiserial medular ray) in a 30 day incubated wood core fragment (B) (longitudinal cut, scale bar = 60 µm).

and Petrini., 1985; Whalley, 1996). *Bjerkandera adusta* was isolated from *Drimys winteri* and has been reported as endophyte in the moss *Sphagnum fuscum* (Thormann *et al.*, 2002) produce wood decay in angiosperms (Oses *et al.*, 2006) and rarely in gymnosperms (Wright and Deschamps, 1972).

A *Bipolaris* sp. was isolated only from *Prumnopitys andina*. This genus has been recognized for its wide geographical distribution and the large number of species, which are mainly pathogens of subtropical and tropical plants (Arx, 1981; Kieffer and Morelet, 2000; Domsch *et al.*, 1980). *Bipolaris* sp. had pigmented hyphae, and could be related with a

staining process or weak dark brown necrotic lesions on woody tissues (Arx, 1981; Kieffer and Morelet, 2000).

Fungal colonization in the angiosperm species, *Nothofagus obliqua* and *Drimys winteri,* was different than in gymnosperms, *Prumnopitys andina* and *Podocarpus saligna*. Xylem colonization by fungal endophytes was observed as clamydospores in the lumen of all tree species studied**.** It has been suggested that passive entry and distribution of fungal spores into wood may be determined by anatomical structure of host and the interactions with physical phenomena such as changes in temperature and air pressure, capillary action
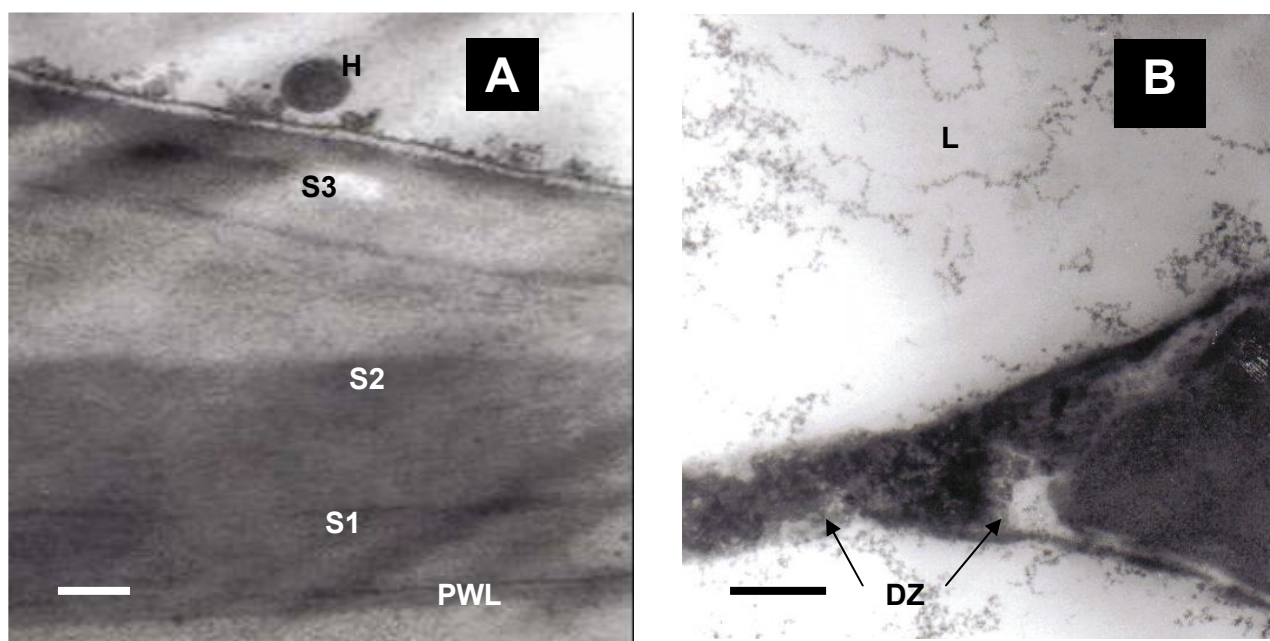
Fig. 4. Observation of fungal structures by TEM from a transverse sections of non incubated (A) and 30 days incubated wood core fragments of *Drimys winteri* (B) (L = lumen; PWL = primary wall layer; S1, S2 and S3 secondary cell layer; DZ = degraded zone; H = hypha). (Scale bar A = 200 nm, B = 20 μm).

and rain splash (Hintikka, 1987; Baum *et al.*, 2003). On the basis of these findings, it is possible to predict that when a freshly cut surface is exposed to spores or other type of propagules, the infection process varies in different tree species. In the case of gymnosperm wood, the spores remain at the outer surface, but in angiosperm, the wood may become infected throughout its length to several cm depth (Hintikka, 1987).

It has been suggested that adaptations such as a gelatinous layer on spores or propagules may be of advantage in penetrating the wood under suitable temperature and air pressures. It would be interesting to investigate whether the spore and propagules of certain wood decomposing species are adapted to the crossing of anatomical features, like perforation plates in different trees and its relation with the extensively and selectivity wood decay patterns performed by white rot and brown rot fungi (Hintikka, 1987; Baum *et al.*, 2003).

Baum and co-workers (2003) have pointed out that xylem infections could occur through the thin periderm, lenticels, leaf scars, or scars of bud scales, followed by a subsequent dormant phase. Early reports have shown that once inside the xylem, the mycelium either infects single cells or establishes small

propagules in a similar way to those formed by *Rhabdocline parkeri* Shrew in Douglas fir needles (Stone, 1987) or *Ophiostoma ulmi* (Buisman) Nannf (Oulette *et al.*, 1995).

Endophytic fungi could be involved in triggering the development of early stages of wood decay (Schwarze *et al.*, 2000; Baum *et al.*, 2003). Limited oxygen ratios and/or nutrient availability are suspected to control non-pathogenic behaviour of xylem endophytes such as *Fomes fomentarius* and *Nectria coccinea*, both considered forest pathogens (Sieber, 2007).

In future research, the ecological roles of fungal endophytes as harmless colonizers to the inner tissues of healthy plants should be evaluated. Some endophytes are potentially pathogenic but disease could only develop in combination with other, mostly unknown, triggering factors (Saikkonen, 2007; Sieber, 2007).

We were not able to identify all isolates to species or in some cases even genus. In future studies we should use molecular techniques to aid identification of isolates or even detect not cultural isolates in healthly xylem (see Promputtha *et al.*, 2005, 2007; Wei *et al.*, 2005; Duong *et al.*, 2006). The present work allowed us to visualize how and where fungal endophytes inhabit and spread through

the xylem of apparently healthy trees. Moreover, it has demonstrated the capacity of some fungal endophytes to be activated and to display wood decay machinery under suitable conditions. Several studies have shown that endophytes are capable of producing wood decay enzymes (Oses *et al*., 2006 and references therein). These aspects should be considered in future studies in order to complement the understanding of wood biodegradation in the topics including invasiveness ability, colonization strategies and degrading mechanisms displayed by fungal endophytes.

Acknowledgements

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