Mycobiota inhabiting sapwood of healthy and declining Scots pine (*Pinus sylvestris* L.) trees in the Alps

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In this study we examined the endophytic mycobiota of the outermost sapwood of Scots pine trees. We aimed at understanding whether endophytic fungal communities are affected by site and tree conditions, including the health status of trees. A total of 673 trees showing different degree of decline were sampled in four forests of the western Alps. Fungal endophytes were isolated from woody microcores and identified on the basis of their macroscopic and microscopic features. Molecular methods were used to identify sterile Basidiomycota and *Heterobasidion* isolates. A total of 143 fungal taxa were identified. Ascomycota and anamorphic fungi were dominant, followed by Basidiomycota and Zygomycota. Different fungal communities were identified in the sequence "healthy – intermediate declining – declining trees". Declining trees were found to harbour a higher number of taxa and are characterized by higher colonization frequencies than healthy-looking trees. Canonical Correspondence Analysis (CCA) indicates that fungal assemblages are significantly affected by the degree of decline of trees and to a lesser extent by their diameter and by presence of mistletoes. Our observations suggest that endophytic fungi inhabiting sapwood could play a secondary role in the decline processes of forest trees.

Key words: *Pinus sylvestris*, forest decline, sapwood, endophytes, fungi

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Introduction

Scots pine (*Pinus sylvestris* L.) is the most widespread pine species in Europe, covering more than 20 million hectares (Vacchiano *et al*., 2008). In the Alps, at the southern range of the species, Scots pine forests commonly cover the lowest slopes of dry inner valleys.

Since the beginning of the 1990s an increasing number of papers have been published about Scots pine decline in the Swiss Rhône valley (Rigling *et al*., 1999; Rigling *et al*., 2000) and in other dry inner valleys of the Austrian, French and Italian Alps (Vertui and Tagliaferro, 1998; Meier *et al*., 2000).

Forest decline is a complex phenomenon due to a series of interchangeable biotic and abiotic factors (Manion, 1981). Although Scots pine is known to be a drought-resistant species, drought has been considered the most important factor triggering tree decline (Rigling and Cherubini, 1999; Dobbertin *et al*., 2004b; Bigler *et al*., 2006).

Dry conditions have been reported to increase the susceptibility of pines to fungal pathogens (Mattson and Haack, 1987) and other parasites, such as mistletoes (*Viscum album* L.) (Dobbertin and Rigling, 2006). Furthermore, warm spring and hot summer temperatures may increase the outbreaks of bark beetles and pine wood nematodes (Wermelinger and Seifert, 1999; Dobbertin *et al*., 2004b; Bigler *et al*., 2006; Polomski *et al*., 2006; Wermelinger *et al*., 2008), often associated with pathogenic ophiostomatoid

fungi (Solheim *et al*., 2001; Sabbatini Peverieri *et al*., 2006; Rigling *et al*., 2006).

In addition to drought, pathogens and pests, the invasion of other competitive tree species (e.g. *Quercus pubescens* Willd.) may impose additional stress due to competition on the shade-intolerant Scots pine (Bigler *et al*., 2006; Rigling *et al*., 2006).

Endophytic fungal communities are important components of forest ecosystems and contribute significantly to their diversity (Saikkonen, 2007; Wang and Guo, 2007; Hyde and Soytong, 2008; Huang *et al*., 2008; Oses *et al*., 2008). Endophytic fungi are extremely widespread colonizers of various plant species with which they establish dynamic relationships (mutualism, commensalism and parasitism) (Danti *et al*., 2002; Schulz and Boyle, 2005; Sieber, 2007; Li *et al*., 2007; Sánchez Márquez *et al*., 2007, 2008; Wei *et al*., 2007). Endophytes may also become saprobes on dead trees (Oses *et al*., 2008) and possible antagonists against host pathogens (Rungjindamai *et al*., 2008).

The behaviour of pathogenic and mycorrhizal "endophytes" has been analysed in detail (Rinaldi *et al*., 2008; Tao *et al*., 2008). In contrast, little is known about symptomless endophytes with neutral, mutualistic behaviour affecting above-ground organs and roots of woody plants (Petrini, 1996; Gonthier *et al*., 2006). Endophytes living in trees often produce no symptoms for most of their life cycle (Oses *et al*., 2008). Evidence of their presence emerges only as a result of appropriate ecological or physiological stimuli. Under particular circumstances these endophytes can become pathogenic, causing damage to the trees (Ragazzi *et al*., 2003; Hyde and Soytong, 2008).

The distribution patterns of fungal endophytes have been described at different levels. For individual host species, the fungal communities of trees growing in the same area have been compared (Espinosa-Garcia and Langenheim, 1990; Huang *et al*., 2008), as well as those from plants living in ecologically different areas (Carroll and Carroll, 1978; Collado *et al*., 1999; Arnold *et al*., 2001; Hoffman and Arnold, 2008) or from plants growing in the same region but in different seasons throughout the year (Rodrigues, 1994). In a single host, leaves, twigs, stems and roots have been examined separately to test organ specificity of endophytes (Carroll and Carroll, 1978; Collado *et al*., 1996; Wang and Guo, 2007).

Despite an increasing knowledge on the presence of fungal endophytes in plants, very little is known about the interaction between endophytic fungi and tree decline. A few papers have been published on this topic (Ragazzi *et al*., 2001; Gennaro *et al*., 2003; Ragazzi *et al*., 2003; Lygis *et al*., 2005; Gonthier *et al*., 2006) and they all referred to endophytic mycobiota of leaves, twigs and stems of broadleaves. It has been shown that the water content in the functional sapwood and its integrity are major determinants for the initial development of fungi in the xylem of broadleaves (Boddy and Rayner, 1983; Chapela and Boddy, 1988a,b; Chapela, 1989). However, to our knowledge, there is very little documentation on whether the health status of trees affects endophytic fungal assemblages of outer sapwood, and this is particularly true for conifers.

In this study we examined the endophytic mycobiota of the outermost sapwood of Scots pine trees. We aimed at understanding whether endophytic fungal communities are affected by site and tree conditions, including the health status of trees. In this study the outer sapwood, included the cambium layer, tracheids and living parenchyma cells, is an important "physiological district" that could harbour endophytic fungi associated with the health status of the tree.

Materials and methods

Site description

The study was carried out in four Scots pine forests of the western Italian Alps. Three were declining forests located in the Aosta Valley, characterized by a continental climate, and the last one was a healthy-looking forest of Piedmont, characterized by a mesalpic climate. Main features of forests are shown in Table 1.

In each stand, mapping, measurement and sampling of trees were carried out in a 50 × 50 m (0.25 ha) plot. For every tree, the diameter at

Table 1. Main features of forest sites investigated.

Site	Coordinates (Lat-Long)[a]	Exposure	Elevation (m a.s.l.)	Slope (%)	Mean annual temperature (°C)	Mean annual rainfall (mm)	Density (trees ha^{-1})	DBH [b] (cm)	Tree age[c] (years)	Mean (and range) crown transparency (%)
Morgex (AO)	5069859 N 344753 E	S	1091	77	7.9	841	610	26.7	90	39.5 (5-100)
St. Denis (AO)	5068071 N 387870 E	SW	985	62	9.7	619	876	14.0	45	56.6 (15-100)
Challand S.A. (AO)	5062482 N 402270 E	W	1116	40	5.5	686	573	28.0	90	38.6 (5-100)
Toceno (VB)	5110964 N 458567 E	W	1050	80	7.4	1676	720	35.1	90-130	20.5 (5-40)

[a]Datum: WGS84.
[b]Diameter at breast height.
[c]Age of the stands was inferred from available documentation and forest management plans.

breast height (DBH) and social position were scored. The latter was defined as measure of the height of a tree relative to the surrounding trees; four classes were recognized: predominant, dominant, co-dominant and suppressed.

Trees were assessed for crown transparency as an indicator of needle loss and tree vitality using the methodology included in Müller and Stierlin (1990) as previously described (Dobbertin *et al*., 2004a). The assessed 5% crown transparency classes were grouped into four vitality classes: class I, < 25% transparency; class II, 26-50% transparency; class III, 51-75% transparency; and class IV, 76-100% transparency, including recently dead trees. Other crown symptoms associated with pine decline, such as chlorosis of needles and microphyllia, were annotated. Abundance of mistletoe (*Viscum album*) and damages caused by *Tomicus* bark beetles were also scored for each tree. Abundance of *Tomicus* spp. was estimated on the basis of number of damaged shoots, while abundance of mistletoe was estimated on the basis of percentage of colonized twigs.

Fungal isolation and identification

Sapwood samples were collected from all Scots pine trees within plots. Trees were sampled either in summer 2005 or in summer 2006. After bark removal, microcores of 15 mm in length and 2 mm in diameter were collected at 10-15 cm above the ground with a Trephor extractor (Rossi *et al*., 2006). Trephor extractor was surface sterilized with 70% ethanol after each use. Depending on tree DBH, either two or four microcores per tree were collected: two microcores (180° from each other) from trees up to 15 cm DBH and four microcores (90° from each other) from trees exceeding 15 cm DBH.

Microcores were singly placed in sterilized Eppendorf tubes and stored at 4°C before fungal isolations.

Microcores were surface sterilized using 30% hydrogen peroxide (H_2O_2) (Prospero *et al*., 2003; Lygis *et al*., 2005). Because of the small size of microcores, the duration and the procedure of sterilization was optimized in a preliminary study (data not shown). Microcores were surface sterilized in hydrogen peroxide for 8 seconds, and subsequently they were rinsed three times in sterile water for 10 seconds.

Two sections for each microcore were singly placed in 60 mm Petri plates containing 2% Malt Extract Agar (2% MEA; 20 g malt extract, 20 g agar, 1000 mL distilled water) supplemented with the antibiotic tetracycline (200 mg L^{-1}) to inhibit bacterial growth (Jankowiak, 2005, 2006), and a selective medium to isolate Basidiomycota (12 g malt extract, 15 g Bacto agar, 2 mg benomyl, 100 mg streptomycin and 1000 mL distilled water) (Prospero *et al*., 2003). Petri plates were incubated in the light at 20-25°C up to four months depending on the growth rates of the fungal colonies. Growing mycelia were transferred to 2% MEA.

Fungi were identified on the basis of their macroscopic and microscopic features. After the identification at the genus level (Domsch *et al*., 1980; Kiffer and Morelet, 1997; Arx, 1981), fungi were transferred to the media recommended by the authors of selected genus monographs for species identification.

Non-sporulating taxa were grouped as sterile mycelia (*sensu* Lacap *et al*., 2003) and divided into different morphotypes based on similar cultural characters. Among these, sterile Basidiomycota were detected by scoring mycelia for presence of clamp connections. For these sterile Basidiomycota, DNA regions comprising ITS1 and ITS2 were amplified and sequenced with universal fungal primers ITS1f and ITS4 (White *et al*., 1990; Gardes and Bruns, 1993). Sequences were compared with those of known fungi, using the National Center for Biotechnology Information's GenBank nucleotide BLAST search. We assumed the identification be accomplished at the species level when the match between our sequence and the most similar one in the database was greater than 98%. Matches with similarities comprised between 95% and 98% were deemed sufficient for an identification at the genus level (Sánchez Márquez *et al*., 2008).

Heterobasidion cultures were identified at the species level by Taxon-Specific Competitive-Priming (TSCP)-PCR combined with a PCR-mediated detection of species-specific DNA

insertions in the ML5-ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA) gene, as described by Gonthier *et al*. (2003).

Statistical data analyses

The occurrence of species in one microcore was scored as a single record regardless of the number of colonies developing on the two media.

The colonization frequency (F) was computed for each fungus as the percentage of infected trees on the total number of trees examined for each site. The colonization density (D) was computed as the percentage of microcores infected on the total number of microcores plated out for each tree. The differences in fungal colonization density were examined for significance by using the Tukey HSD test ($P < 0.05$) for unequal samples.

Chi-square test and contingency tables were used to test differences in the fungal colonization frequencies (all fungal taxa) between declining and healthy-looking trees.

The above statistical analyses were performed using StatSoft Inc. (1993).

Canonical Correspondence Analysis (CCA) of trees and fungal species was performed using CANOCO (ter Braak and Šmilauer, 2002). In this ordination analysis, trees and fungal species were constrained to optimize their linear relationship to the selected variables (ter Braak and Prentice, 1988). Three quantitative variables (DBH, crown transparency – CT, and mistletoe abundance – MIS) and one ordinal variable (social position – SP) were included. Following the results of Forward Selection, CCA ordination with the other variables was not significant (data not shown) and these variables (abundance of *Tomicus* and microphyllia) were excluded from the original matrix. Monte Carlo permutation test was run to check the validity of the ordination (ter Braak and Šmilauer, 2002). Only the most frequent fungi were subjected to this analysis: fungi isolated exclusively from one tree were excluded as they were deemed to be occasional. Similarly, plants hosting only one fungal endophyte in the original matrix data set were excluded.

Results

A total of 673 Scots pines were examined, 123 trees from Morgex, 283 from St. Denis, 91 from Challand St. Anselme and 176 from Toceno.

The 60% of the plated microcores (1207 out of 2024) resulted in fungal growth, while the remaining 40% was either uncolonized or colonized by bacteria.

Fungal colonization frequency was significantly lower in healthy-looking (crown transparency < 25%) than in declining trees (crown transparency > 26%) (164 infected trees out of 222 *vs* 389 infected trees out of 447) ($\chi^2 = 17.90$; $P < 0.01$; d.f. 1).

A total of 143 fungal taxa (Table 2) were identified from the four sites, of which 60 from Morgex, 52 from St. Denis, 54 from Challand St. Anselme and 28 from Toceno. Most taxa (80%) were isolated from one site. Twenty-nine taxa, including two dark sterile mycelia, were recovered from at least two sites. Of these only seven were isolated from all sites: *Alternaria alternata*, *Cladosporium chlorocephalum*, *C. cladosporioides*, *Epicoccum nigrum*, *Mucor plumbeus*, *Penicillium* spp. and *Trichoderma* spp.

The mycobiota was dominated by Ascomycota and anamorphic fungi (53 taxa, 37%), followed by Basidiomycota (17 taxa, 12%) and Zygomycota (8 taxa, 6%) (Table 2). Colonization frequency of the heterogeneous groups of dark, hyaline and pinkish sterile mycelia (45, 16 and 3 morphological types, respectively), whose sterility persisted in colonies grown on 2% MEA incubated at 20-25°C for several months, was comprised between 0.35% and 14.58%.

Of the most abundant taxa, *Penicillium* spp. displayed the highest colonization frequency (> 20%) in all stands and showed a significantly higher colonization density in Challand St. Anselme than in the other sites.

The next two most abundant taxa were *Alternaria alternata* and *Trichoderma* spp. These taxa were significantly more abundant in sites of Morgex and St. Denis, respectively. *Cladosporium chlorocephalum* was most frequent in the

site of Toceno, but no significant differences in colonization density were recorded between sites.

Heterobasidion annosum (Fr.) Bref. *sensu stricto* (*s.s.*) was significantly more abundant in the site of Morgex, where it was detected only in dead trees (3%), as confirmed by Taxon-Specific Competitive-Priming (TSCP)-PCR and by observation under a dissecting microscope (20X magnification) of its conidial stage (*Spiniger meineckellus*).

With the exception of *Heterobasidion annosum s.s.*, Basidiomycota showed low colonization frequencies at all sites. Zygomycota were mostly found in the two intermediate declining stands.

The CCA ordinations (Figs 1, 2) show the distributions of 318 trees and 43 fungal taxa in relation to the four selected variables. The first and second CCA axes (eigenvalues 0.21 and 0.11, respectively) explain the 2% of cumulative percentage variance of species data and the 76% of cumulative percentage variance of species-environment relationships. DBH was positively and significantly correlated with the first axis (Inter Set Correlation – ISC -0.52), while CT, SP and MIS were negatively correlated with this axis (ISC -0.34, -0.10, -0.36, respectively). Moreover, CT and SP were positively and significantly correlated with axis 2 (ISC 0.40, 0.23, respectively), while MIS and DBH were negatively correlated with this axis (ISC -0.18, -0.03). The last one displaying low ISC values.

The first axis and all axes are significant (Monte Carlo Permutation Test: F-ratio = 4.3, P-value = 0.002 for the first axis and F-ratio = 2.1, P-value = 0.002 for all canonical axes).

The distribution of trees (Fig. 1) showed a clear separation along the first axis. Declining trees were almost completely localized in the left part of the diagram showing that they were characterized by lower DBH, suppressed and mostly infested by mistletoe. Intermediate declining and healthy-looking trees were almost completely localized in the right part of the diagram and healthy-looking trees were mainly in the lower right quarter because of their low values of crown transparency.

The distribution of fungal taxa (Fig. 2) showed that four different groups of taxa may be identified in the diagram based on CT, DBH and MIS: group I including taxa associated with healthy-looking trees (CT up to 30% and mean DBH 30 cm); group II including taxa associated with intermediate declining trees (CT 30-70% and DBH 20-30 cm); group III including taxa associated with intermediate declining trees with high percentage of twigs infested by mistletoe; group IV including taxa associated with declining trees (CT exceeding 70% and mean DBH 20 cm). *Phialemonium curvatum* was associated with intermediate declining trees (group II) characterized by a very high DBH (mean DBH 48 cm).

Discussion

Fungal communities associated with Scots pine sapwood were mainly composed of a small number of dominant taxa accompanied by a cohort of rare isolates. Most of the taxa have previously been reported as colonizers (Lumley *et al*., 2001; Varese *et al*., 2003; Lygis *et al*., 2004, 2005; Menkis *et al*., 2006) or endophytes (Fisher and Petrini, 1990; Fisher *et al*., 1991; Ahlich and Sieber, 1996; Collado *et al*., 1999; Danti *et al*., 2002; Hoff *et al*., 2004; Sánchez Márquez *et al*., 2007, 2008; Huang *et al*., 2008) of wood in forest trees.

In total, 143 fungal taxa, belonging to the main systematic groups, were identified. This high diversity was unexpected. However, it is in agreement with results of a recently completed study on fungal communities inhabiting *Vitis vinifera* L. wood in Switzerland (Casieri *et al*., 2008).

A limited number of Basidiomycota was isolated, despite the use of a selective medium. Such finding supports the results of previous studies (Hoff *et al*., 2004; Lygis *et al*., 2005; Menkis *et al*., 2006) and reflects the low stress-tolerance of Basidiomycota in functional sapwood (Boddy and Rayner, 1983; Chapela and Boddy, 1988a). However it has been recently reported that Basidiomycota might be better detected through direct sequencing rather than by culture isolation (Arnold *et al*., 2007; Rungjindamai *et al*., 2008; Zhang *et al*., 2008).

Table 2. Colonization frequency (F) and mean colonization density (D) of the isolated fungal taxa. Density values followed by different letters differed significantly ($P < 0.05$, Tukey HSD test).

	Nucleotide sequence similarity (%)	TOCENO (T)		MORGEX (M)		CHALLAND S. A. (C)		ST. DENIS (S)	
		F	D	F	D	F	D	F	D
Basidiomycota									
1 *Bjerkandera adusta* (Willd.) P. Karst.	99	1.70	1.00	0.00	0.00	0.00	0.00	0.00	0.00
2 *Heterobasidion annosum* (Fr.) Bref. *sensu stricto* (*s.s.*)	n.s.[1]	0.00	0.00 a	3.03	2.10 b	0.00	0.00 a	0.00	0.00 a
3 *Peniophora* sp.	95	0.57	0.10	0.00	0.00	0.00	0.00	0.35	0.20
4 *Schizophyllum commune* Fr.	100	0.00	0.00	0.00	0.00	0.00	0.00	0.69	0.70
5 *Sistotrema coroniferum* (Höhn. & Litsch.) Donk	98	0.00	0.00	0.00	0.00	2.08	0.80	0.00	0.00
6 *Thanatephorus cucumeris* (A.B. Frank) Donk	99	0.00	0.00	0.00	0.00	0.00	0.00	0.69	0.30
7 *Trametes versicolor* (L.) Lloyd	99	1.14	0.70	0.00	0.00	0.00	0.00	0.00	0.00
8 Basidiomycetes with clamp connections (3 morphotypes)	n.s.	1.14	0.40	0.76	0.30	0.00	0.00	0.35	0.20
Ascomycota and anamorphic fungi									
9 *Acremonium pteridii* W. Gams & Frankland	n.s.	0.00	0.00	0.00	0.00	2.08	1.30	0.00	0.00
10 *Acremonium strictum* W. Gams	n.s.	0.00	0.00	0.76	0.20	2.08	0.50	0.35	0.10
11 *Alternaria alternata* (Fr.) Keissl.	n.s.	1.14	0.30 a	15.15	5.00 b	3.13	0.80 a	3.46	1.70 a
12 *Aspergillus flavus* Link	n.s.	1.70	0.70	0.00	0.00	0.00	0.00	0.00	0.00
13 *Aspergillus niger* Tiegh.	n.s.	0.57	0.10 a	4.55	1.70 b	1.04	0.30 a	0.00	0.00 a
14 *Aspergillus tubingensis* Mosseray	n.s.	0.00	0.00 a	3.03	1.60 b	1.04	0.30 ab	0.00	0.00 a
15 *Beauveria bassiana* (Bals.-Criv.) Vuill.	n.s.	0.00	0.00 a	0.00	0.00 a	3.13	0.80 b	0.00	0.00 a
16 *Chalara* sp.	n.s.	0.00	0.00	0.76	0.30	1.04	0.30	0.35	0.10
17 *Chaunopycnis pustulata* Bills, Polishook & J.F. White	n.s.	0.00	0.00	1.52	0.40	1.04	0.30	1.04	0.50
18 *Cladosporium chlorocephalum* (Fresen.) E.W. Mason & M.B. Ellis	n.s.	19.32	8.20	15.15	5.40	7.29	2.60	7.27	4.20
19 *Cladosporium cladosporioides* (Fresen.) G.A. de Vries	n.s.	2.27	1.10	3.79	1.10	1.04	0.50	3.81	2.20
20 *Cladosporium herbarum* (Pers.) Link	n.s.	0.00	0.00	1.52	0.40	1.04	0.30	0.35	0.10
21 *Epicoccum nigrum* Link	n.s.	2.84	0.90	3.79	1.50	2.08	0.50	1.38	0.70
22 *Exophiala / Phialophora* sp.	n.s.	0.00	0.00	1.52	0.70	0.00	0.00	0.00	0.00
23 *Fusarium* sp.	n.s.	0.00	0.00	1.52	0.40	0.00	0.00	0.00	0.00
24 *Gibberella avenacea* R.J. Cook	n.s.	0.00	0.00	0.76	0.20	0.00	0.00	1.04	0.70
25 *Gibberella fujikuroi* (Sawada) Wollenw.	n.s.	1.70	0.40	1.52	0.90	4.17	1.60	0.00	0.00
26 *Hypocrea lixii* Pat.	n.s.	0.00	0.00	0.00	0.00	0.00	0.00	0.69	0.30
27 *Isaria farinosa* (Holmsk.) Fr.	n.s.	0.57	0.10	0.76	0.40	2.08	0.80	0.00	0.00
28 *Penicillium* spp.	n.s.	23.86	9.40 a	59.85	29.30 b	71.88	45.70 c	34.95	24.50 b

Table 2 (continued). Colonization frequency (F) and mean colonization density (D) of the isolated fungal taxa. Density values followed by different letters differed significantly ($P < 0.05$, Tukey HSD test).

	Nucleotide sequence similarity (%)	TOCENO (T)		MORGEX (M)		CHALLAND S. A. (C)		ST. DENIS (S)	
		F	D	F	D	F	D	F	D
29 *Phialemonium curvatum* W. Gams & W.B. Cooke	n.s.	0.00	0.00 a	0.00	0.00 a	2.08	0.50 b	0.00	0.00 a
30 *Phoma eupyrena* Sacc.	n.s.	0.00	0.00	0.00	0.00	1.04	0.30	1.73	1.00
31 *Phoma herbarum* Westend.	n.s.	0.00	0.00	0.00	0.00	0.00	0.00	1.04	0.50
32 *Phoma leveillei* Boerema & G.J. Bollen	n.s.	0.00	0.00	0.00	0.00	1.04	0.30	1.73	1.40
33 *Rhinocladiella atrovirens* Nannf.	n.s.	0.00	0.00	3.79	1.60	4.17	1.30	1.04	0.50
34 *Trichoderma* spp.	n.s.	18.75	7.50 a	10.61	5.40 a	10.42	4.50 a	47.75	31.90 b
35 *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg	n.s.	0.00	0.00 a	3.03	1.10 b	0.00	0.00 a	0.00	0.00 a
36 *Trichoderma viride* Pers.	n.s.	0.00	0.00 a	12.12	5.10 b	22.92	11.50 c	0.35	0.30 a
37 Dark sterile mycelia (45 morphotypes)	n.s.	1.14	0.70 a	12.88	6.10 b	14.58	4.30 ab	7.61	3.50 ab
38 Hyaline sterile mycelia (16 morphotypes)	n.s.	1.14	0.70	6.82	2.10	8.33	2.30	1.73	0.80
39 Pinkish sterile mycelia (3 morphotypes)	n.s.	0.00	0.00	0.76	0.40	1.04	0.30	0.35	0.20
40 Unidentified Ascomycota	n.s.	0.00	0.00	0.00	0.00	2.08	0.80	0.00	0.00
Zygomycota									
41 *Mortierella globalpina* W. Gams & Veenb.-Rijks	n.s.	0.00	0.00	1.52	1.10	1.04	0.50	1.04	0.40
42 *Mortierella lignicola* (G.W. Martin) W. Gams & R. Moreau	n.s.	0.00	0.00	1.52	0.40	0.00	0.00	0.00	0.00
43 *Mucor hiemalis* Wehmer	n.s.	0.57	0.30	3.03	0.90	0.00	0.00	0.00	0.00
44 *Mucor plumbeus* Bonord.	n.s.	1.14	0.60	1.52	0.40	1.04	0.30	4.84	2.50
45 *Rhizopus stolonifer* (Ehrenb.) Vuill.	n.s.	0.57	0.40 a	12.12	6.90 b	3.13	1.30 a	0.00	0.00 a
46 *Umbelopsis isabellina* (Oudem.) W. Gams	n.s.	0.00	0.00 a	7.58	2.30 b	1.04	0.30 a	0.00	0.00 a
47 *Umbelopsis vinacea* (Dixon-Stew.) Arx	n.s.	0.57	0.10 a	7.58	2.70 b	1.04	0.50 a	0.00	0.00 a

n.s.[1] = non sequenced.

Taxa that could be isolated from only one tree, include *Acremonium kiliense* Grütz (T) (n.s.), *Aspergillus* sp. (C) (n.s.), *Cordana pauciseptata* Preuss (M) (n.s.), *Cordyceps confragosa* (Mains) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (M) (n.s.), *Devriesia staurophora* (W.B. Kendr.) Seifert & N.L. Nick. (M) (n.s.), *Emericella nidulans* (Eidam) Vuill. (T) (n.s.), *Eurotium amstelodami* L. Mangin (M) (n.s.), *Funalia trogii* (Berk.) Bondartsev & Singer (T) (%FASTA similarity – 100%), *Fusarium lateritium* Nees (S) (n.s.), *Gibberella moniliformis* Wineland (T) (n.s.), *Graphium fragrans* Math.-Käärik (C) (n.s.), *Hyphoderma praetermissum* (P. Karst.) J. Erikss. & Å. Strid (M) (%FASTA similarity – 99%), *Hypocrea koningii* Lieckf., Samuels & W. Gams (S) (n.s.), *Hypocrea pachibasioides* Yoshim. Doi (C) (n.s.), *Irpex lacteus* (Fr.) Fr. (S) (%FASTA similarity – 99%), *Monocillium indicum* S.B. Saksena (M) (n.s.), *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers (M) (n.s.), *Phoma glomerata* (Corda) Wollenw. & Hochapfel (S) (n.s.), *Phoma putaminum* Speg. (M) (n.s.), *Podospora* sp. (S) (n.s.), *Ramichloridium subulatum* de Hoog (M) (n.s.), *Sarea difformis* (Fr.) Fr. (C) (n.s.), *Sclerotinia sclerotiorum* (Lib.) de Bary (T) (n.s.), *Sistotrema coronilla* (Höhn. & Litsch.) Donk ex D.P. Rogers (M) (%FASTA similarity – 99%), *Steccherinum* sp. (S) (%FASTA similarity – 97%), *Stereum hirsutum* (Willd.) Pers. (T) (%FASTA similarity – 99%), *Trichaptum* sp. (M) (%FASTA similarity – 96%), *Trichoderma atroviride* P. Karst. (C) (n.s.), *Trichoderma koningii* Oudem. (M) (n.s.), *Tritirachium oryzae* (Vincens) de Hoog (C) (n.s.), *Trichothecium roseum* (Pers.) Link (T) (n.s.), *Umbelopsis ramanniana* (Möller) W. Gams (S) (n.s.), *Virgaria nigra* (Link) Nees (M) (n.s.).

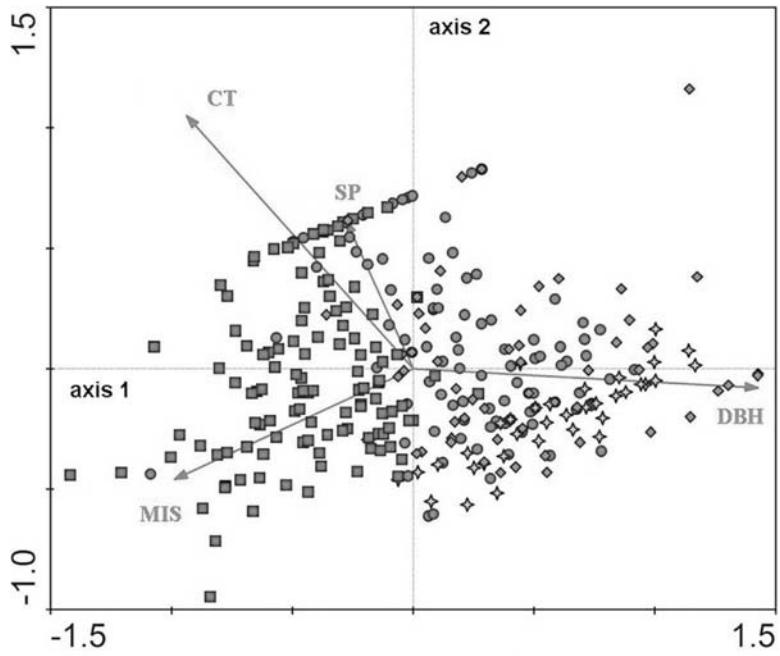

Fig. 1. Correlation biplot of trees (n = 318) based on CCA from four Scots pine forests. The first two axes are shown (eigenvalues: axis 1 = 0.213; axis 2 = 0.106). ● Morgex; ♦ Challand S. A.; ■ St. Denis; ✦ Toceno; DBH, diameter at breast height; CT, crown transparency; MIS, abundance of mistletoe; SP, social position.

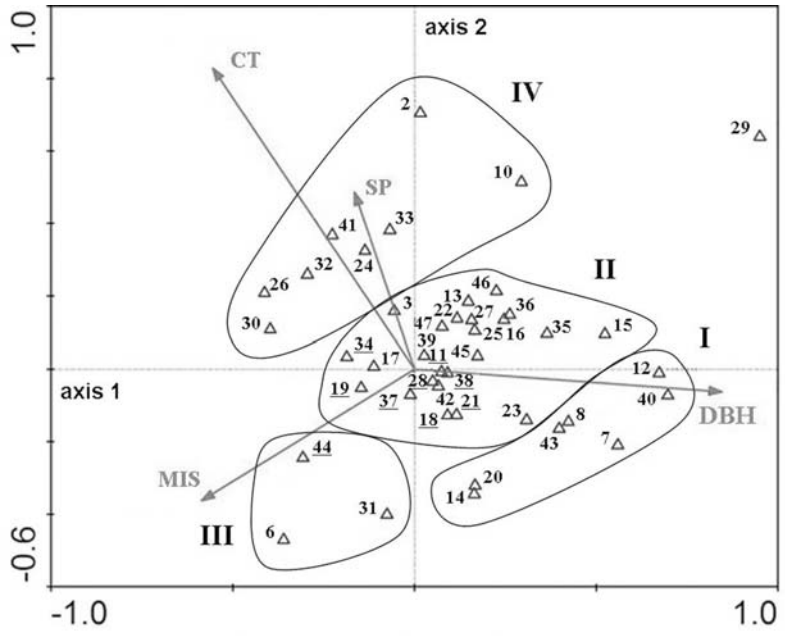

Fig. 2. Correlation biplot of fungal endophytic taxa (n = 43) based on CCA from Scots pine sapwood (numbers refer to taxa codes - Table 2). Underlined numbers indicate taxa present in all study sites. DBH, diameter at breast height; CT, crown transparency; MIS, abundance of mistletoe; SP, social position.

In this study most fungal taxa belong to Ascomycota and anamorphic fungi which is typical of most endophyte studies (Ragazzi *et al*., 2003; Lygis *et al*., 2005; Menkis *et al*., 2006; Sánchez Márquez *et al*., 2007; Huang *et al*., 2008). Several morphotypes of sterile mycelia were also isolated and this is consistent with previously reported results (Guo *et al*., 2003; Lacap *et al*., 2003).

Penicillium spp. were the most frequent taxa as previously described (Kowalski, 1991; Kaus *et al*., 1996; Kubátová, 2000; Seifert and Frisvad, 2000; Varese *et al*., 2003; Menkis *et al*., 2006). Kubátová (2000) found *Penicillium* species on healthy, intermediate declining and declining trees and usually a characteristic association of *Penicillium* species was recovered from the wood. We isolated Penicillia from twice as many trees from intermediate declining sites (Morgex and Challand St. Anselme) than from healthy (Toceno) and declining sites (St. Denis). The role of *Penicillium* on tree decline is still under question, and may deserve further investigations.

Unexpectedly eight species of Zygomycota, belonging to four genera, were obtained and these were mainly associated with intermediate declining stands. *Umbelopsis* spp. were commonly associated with woody roots of healthy Scots pine and Norway spruce (Fisher *et al*., 1991; Holdenrieder and Sieber, 1992; Hoff *et al*., 2004), but in our study this taxon was mainly associated with declining trees.

The ordination of fungal species in the CCA showed that declining, intermediate declining and healthy-looking trees are inhabited by different fungal communities. Similar results were previously reported by Lygis *et al*. (2004, 2005) that hypothesized that wood-inhabiting fungi in stems of living trees are likely to change along with changes in tree health conditions. The change in the species composition, in a sequence "healthy – declining – dead tree", could occur owing to less restricted external infections on the declining trees, where newcomers tend to replace species that were resident in apparently healthy stems.

Interestingly, declining trees were found to harbour a higher number of taxa and are characterized by higher colonization frequencies than healthy-looking trees. These results are consistent with the above model based on weakened defences to infections in declining trees and with the lowering of moisture content to a level allowing fungal development (Boddy and Rayner, 1983; Chapela and Boddy, 1988b). Similar results were reported by Ragazzi *et al*. (2003) and Lygis *et al*. (2005) on broadleaves.

Four different groups of taxa were identified based on CT, DBH, MIS and SP in the sequence "healthy – intermediate declining – declining trees".

Few species (Group I), such as *Aspergillus flavus*, *A. tubingensis*, *Cladosporium herbarum*, *Mucor hiemalis*, *Trametes versicolor*, were associated with healthy-looking trees (CT up to 30%) and high DBH (mean value 30 cm). In contrast, most taxa were associated with intermediate declining trees (Group II), including taxa that were characterized by higher colonization frequencies, such as *Alternaria alternata*, *Cladosporium chlorocephalum*, *C. cladosporioides*, *Epicoccum nigrum*, *Mucor plumbeus*, *Penicillium* spp. and *Trichoderma* spp.

Very few species, *Thanatephorus cucumeris*, *Phoma herbarum* and *Mucor plumbeus* (Group III) were selectively associated with the presence of the mistletoe. Mistletoe is recognized as an important component of forest ecosystems, far beyond its role as parasite. It influences growth and survival of individual trees by functioning as a carbon sink, but it also affects stand structure by causing significant crown losses and tree mortality (Cullings *et al*., 2005; Mueller and Gehring, 2006). Despite the importance of this parasite in the ecosystem structure and function, little attention has been turned to the effects of mistletoes on fungal communities inhabiting trees. Until now it has been reported that this parasite significantly affect the species diversity, composition and richness of the ectomycorrhizal communities (Cullings *et al*., 2005; Mueller and Gehring, 2006). To the best of our knowledge, this is the first report on the effect of this parasite on the endophytic mycoflora.

Several fungal taxa (group IV: mean CT > 70%) were associated with declining trees, such

as *Acremonium strictum*, *Gibberella avenacea*, *Heterobasidion annosum s.s.*, *Hypocrea lixii*, *Mortierella globalpina*, *Phoma eupyrena*, *P. leveillei*, *Rhinocladiella atrovirens*. Most of them were isolated from trees with small DBH (mean value 20 cm); only *Acremonium strictum* and *Heterobasidion annosum s.s.* were associated with trees exceeding 20 cm in DBH (mean values 28 and 22 cm, respectively). All these species have already been reported on declining trees (Franceschini *et al*., 2000; Ragazzi *et al*., 2003; Lygis *et al*., 2004, 2005; Menkis *et al*., 2006). Some of the above species, i.e. *Gibberella avenacea* and *Phoma leveillei*, have been reported as opportunistic pathogens (Domsch *et al*., 1980; Kinsey, 2002). Instead *Heterobasidion annosum s.s.* is a primary pathogen on Scots pine (Korhonen and Stenlid, 1998). The presence of mycelium of *Heterobasidion* under the bark and in the outer sapwood often results in tree death. This is the first report of tree death caused by *Heterobasidion* on Scots pine in the western Alps. Previously, Scots pine was considered resistant to *Heterobasidion* infections in the area (Gonthier *et al*., 2003).

Thus, declining Scots pine trees harbour in their sapwood an endophytic mycobiota that is significantly different from that of healthy trees. However, our data set does not allow us to draw conclusions on the role played by these fungi in the decline process. In fact, in order to determine if either the community of fungi within sapwood changes when trees decline or changes in the fungal community are driving changes in tree health, further experiments are needed.

Endophytic fungal assemblages of sapwood might play a role on Scots pine decline, as contributing factors, within the Manion's decline disease spiral model (Manion, 1981) and Houston's host-stress saprogen model (Houston, 1987). Such secondary role would be supported by the low isolation frequency, in this study, of ophiostomatoid fungi, that are often considered important contributing factors of forest decline (Rigling *et al*., 2006). Only two taxa belonging to this group were isolated, *Graphium fragrans* and *Chalara* sp. The first species was isolated from a dead tree; while the second was more widespread and was isolated from both healthy-looking and declining trees.

This research demonstrates that the outer sapwood of Scots pine is largely colonized by fungal endophytes. As we used culture-based techniques, we cannot exclude the number of endophytes be significantly higher than that documented in this study, which may have overlook the presence of unculturable or slow-growing species (Hyde and Soytong, 2008). Furthermore, non-sporulating fungi were not identified and they were ascribed to sterile mycelia. Molecular techniques such as direct sequencing of fungal DNA from plant tissues, both with or without a cloning step (Menkis *et al*., 2006; Arnold *et al*., 2007; Seena *et al*., 2008), Denaturating Gradient Gel Electrophoresis (DGGE) (Duong *et al*., 2006; Tao *et al*., 2008) or T-RFLP (Nikolcheva and Bärlocher, 2004, 2005) could successfully complement culture based methods in the investigations on endophytic mycobiota (Hyde and Soytong, 2008), thus providing additional chance to discover fungi involved in forest decline. In conclusion, based on our results, fungal assemblages of outer sapwood of Scots pine are significantly affected by the degree of decline of trees and to a lesser extent by their diameter or age and by presence of mistletoe. Our observations suggest that endophytic fungi inhabiting outer sapwood could play a secondary role in the decline processes of forest trees.

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