Root-endophytic fungi cause morphological and functional differences in Scots pine roots in contrast to ectomycorrhizal fungi

Journal:	<i>Botany</i>
Manuscript ID	cjb-2016-0161.R1
Manuscript Type:	Note
Date Submitted by the Author:	05-Sep-2016
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Keyword:	endophytic fungi, root, enzyme, pine, functional trait

1 **Root-endophytic fungi cause morphological and functional**
2 **differences in Scots pine roots in contrast to**
3 **ectomycorrhizal fungi**

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Abstract

Endophytic fungi and ectomycorrhizal fungi co-exist in the mycorrhizal root tips of boreal forest trees. However, very little is known about the functional role they play in their host's biology. The activity of enzymes responsible for important biochemical processes is used to unravel the functional role of root-associated mycorrhizal fungi. However, enzyme activity is never studied in the presence of endophytic fungi *in planta*. The aims of the present study were to investigate the effect of Scots pine root-isolated fungal endophytes on the host plant root morphology, to determine their functional effect using host root-excreted enzyme activity measurements and to compare them to roots colonized by decomposer and ectomycorrhizal fungal strains and non-colonised Scots pine root tips. Our results show that endophytic fungi did not damage the pine roots in contrast to the decomposer fungi. The endophytic fungi penetrated the cortical cells of the host plant. The roots colonised by endophytic fungi produce different exo-enzymes compared to those produced by roots colonized by other fungal groups or non-colonized control root tips. Our results indicate that endophytic fungi are clearly a distinctive ecological group of fungi that have functional traits different from those of ectomycorrhizal and decomposer fungi.

Keywords: endophytic fungi, root morphology, enzyme, functional trait, pine

1 Introduction

2 Plant roots contain a wide range of fungal endophytes (Vandenkoonhuyse et al. 2002; Addy et
3 al. 2005; Mandyam and Jumpponen 2005). Recent research on root-associated fungi has
4 focused on symbiotic mycorrhizal fungi, which were shown to play a crucial role in plant
5 performance in many different ecosystems (Van der Heijden et al. 1998; Smith and Read
6 2008). However, a large part of the root-associated fungi is endophytic, but not mycorrhizal
7 (Rodriguez et al. 2009). These endophytic fungi inhabit the root tissue but do not form
8 cellular interfaces with the plant partner using specialized structures (Rodriguez et al. 2009).
9 Such endophytic associations may be parasitic, saprotrophic, mutualistic, or neutral.
10 However, the ecological status of the association between the endophytic fungi and the host
11 plant is often unclear. The most studied root endophytes, the so called dark septate
12 endophytes (DSE), were earlier suggested to be functionally similar to symbiotic mycorrhizal
13 fungi (Jumpponen 2001). However, DSEs are recently defined as ‘not causing negative
14 effects on plant performance’ (Newsham et al. 2011), and may sometimes form symbiotic
15 structures like mycorrhizal fungi (Lukesova et al. 2015). Today, the definition of the DSE still
16 suffers from lack of taxonomic cohesion, the polyphyletic evolutionary origins of these fungi
17 and their variable ecological or physiological functions (Mandyam and Jumpponen 2014).
18 Root-inhabiting fungi other than DSEs are even less well studied, and their ecology is poorly
19 understood. The global diversity, abundance and functional roles of these root-colonising
20 fungi are also largely unknown (Addy et al. 2005; Mandyam and Jumpponen 2005) despite
21 their presence in roots being known for more than 100 years and reported in thousands of
22 scientific articles (Rodriguez et al. 2009).
23 Root endophytes are most commonly identified by direct agar plate culturing after surface
24 sterilization of roots. The identity of uncultured root endophytes may also be determined by
25 extracting DNA from the surface-sterilized roots. However, the PCR-amplification of the

1 internal transcribed spacer (ITS) region for example, may also amplify DNA from dead
2 fungal endophytes or epiphytic fungi found on the surface of the roots. Therefore, despite
3 advances in the development of molecular biological tools, the isolation of pure cultures of
4 root endophytes is invaluable when the targeted species is cultivable. It is possible to
5 determine their physiological properties and host effect of root endophytes only with pure
6 cultured endophytic fungal strains.

7 An abundance of endophytic fungi in the roots of woody plant species like Scots pine (*Pinus*
8 *sylvestris*, L.) is commonly observed (Sieber and Grünig 2013). Endophytic fungi also co-
9 exist with ectomycorrhizal (ECM) fungi in mycorrhizal root tips of boreal forest trees
10 (Kernaghan and Patriquin 2011; Reiningner and Sieber 2012). The role and function of
11 endophytes within mycorrhizas is not properly understood. Attempts to isolate pure cultures
12 of mycorrhizal fungi often lead to the co-isolation of endophytic fungi (e.g. Uroz and
13 Heinonsalo 2008).

14 Enzyme activity measurements have been used to estimate the capacity of soil microbial
15 communities to maintain functional diversity because specific enzymes drive most of the
16 biochemical reactions in soil (Caldwell, 2005; Talbot et al. 2014). Similarly, enzyme activity
17 measurements of tree root tips colonized by ectomycorrhizal fungi are used as a tool for
18 studying the functional diversity of ECM communities (Courty et al. 2005). The combination
19 of six functional markers (enzyme assays) allowed the classification of 26 fungal strains into
20 groups that include white, soft rot and brown rot fungi (Mathieu et al. 2013). The recently
21 developed fungal guild classification predicts the enzymatic role of fungi and links fungal
22 guilds to soil biogeochemical processes based on a combination of enzyme activity data,
23 biomass data and molecular and functional gene data (Talbot et al. 2015). Enzyme activity
24 measurements of host root-inoculated endophytic fungi has never been performed despite the

fact that enzyme activity profiling may produce significant new knowledge on the largely unknown functional roles of such endophytic fungi.

The first aim of the study was to investigate the association between eight root-isolated fungal strains and Scots pine roots using root section microscopy. The second and main aim was to study how these strains affect the secreted enzymes pattern of fungal-inoculated roots (Pritsch et al. 2004; Courty et al. 2005; Pritsch et al. 2011). The study includes the isolation of two ectomycorrhizal fungi, three endophytic fungi and three decomposer (later 'decay') fungi. We hypothesize that 1) fungal colonisation within plant roots alters the root morphology and the root-excreted enzyme activities and, 2) the endophytic fungi can be grouped into different ecological groups (fungal guilds) based on functional analysis of fungi in interaction with pine root, based on their enzyme activity profiles, and 3) ectomycorrhizal and endophytic fungi have more similar enzyme activity profiles than decay fungi, which supports the hypothesis that endophytic fungi have a mutualistic association with the host plant, supporting neutral or positive effects on plant performance.

Materials and methods

Seedling establishment, fungal strains, inoculation and growth conditions

Scots pine (*Pinus sylvestris* L.) seeds were surface-sterilized and germinated on glucose agar. Sterile germinated seeds were transferred to 20 mm diameter test tubes where they were grown until the first lateral short roots emerged after approximately three weeks. The seedlings (N=3) were inoculated with eight fungal strains (Table 1), selected to represent ectomycorrhizal (ECM), endophytic (ENDO) and decomposer (DECAY) fungi. The protocol for the fungal isolation is explained in details in Bäck et al. (2010). Non-inoculated seedlings were included as controls (CON). The seedlings were grown under stable growth conditions

(temperature +20°C, irradiation 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and day/night cycle of 18/6h) for three months (Heinonsalo et al. 2015) until harvested for microscopic, enzymatic and biomass analyses.

The strains were all originally isolated in Finland from Scots pine ectomycorrhiza except for the litter-decomposing fungal strain, which was isolated from a *Collybia dryophila* fruiting body. The taxonomic identity of the nine strains was determined by sequencing the ITS region of the rRNA operon using the ITS1 and ITS4 primer pair (Gardes and Bruns 1993). The sequences were deposited in GenBank and their accession numbers are given in Table 1. The strains were deposited as pure cultures in the Fungal Biotechnology Culture Collection (FBCC within HAMBI) at the University of Helsinki, Finland.

Root sections and microscopy

Seven root tips were randomly selected from the root system of inoculated and non-inoculated seedlings (N=3) and were prepared for microscopy by pre-fixing them at +4 °C overnight in 0.1 M phosphate buffer (pH 7.0) containing 2.5 % (v/v) glutaraldehyde. Subsequently, the root tips were rinsed three times for 10 min with phosphate buffer (pH 7.0) at +4 °C, followed by a fixing period of 3-4 hours in 1 % osmium tetroxide (OsO_4) phosphate buffer (pH 7.0). The samples were dehydrated after rinsing three times for 10 min with phosphate buffer (pH 7.0) at +4 °C. The samples were soaked twice in 50% ethanol, then in 70%, 94%, 100% ethanol and finally in 2% uranyl acetate in 100% ethanol for 10 min each. The samples were treated three times in propylene oxide for 10 min at +20 °C before eponification. Samples were incubated for 60 min in 3:1 mixture of propylene oxide and Epon resin (Ladd LX 112). The samples were then incubated for 120 min in 1:1 mixture of propylene oxide and Epon resin, 120 min in 1:3 mixture and finally in pure Epon resin overnight. The samples were

placed in pure Epon at +20°C prior to polymerization for one day at +37°C and three days at +60°C.

One μm sections were cut and stained with 0.1% toluidine blue for 30 min before examination with a Leica DMLB microscope (Leica®, Wetzlar, Germany). Microscope section photographs taken with a Zeiss Microscope with ZEN2012 blue edition software (Carl Zeiss Microscopy GmbH) were used to analyze the host-effect of fungal isolates.

Enzyme assays

The seven randomly selected root tips cut earlier from the roots of inoculated and non-inoculated seedlings for root sections were also used for the enzyme assays, as described in Courty et al. (2005). Seven of the enzyme assays performed were based on fluorescent detection of 4-methylumbelliferone (MU) or 7-amino-4-methylcoumarin (AMC) substrates and one on photometric detection of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) reaction products. The enzymes and corresponding substrates studied were acid phosphatase (EC 3.1.3.2; MU-phosphate), β -glucosidase (EC 3.2.1.3; MU- β -D-glucopyranoside), N-acetyl-glucosaminidase (EC 3.2.1.14; MU-N-acetyl- β -D-glucosaminide), glucuronidase, (EC 3.2.1.31; MU- β -D-glucuronide hydrate), xylosidase (EC 3.2.1.37; MU- β -D-xylopyranoside), cellobiohydrolase (EC 3.2.1.91; MU- β -D-cellobioside), leucine aminopeptidase (EC 3.4.11.1; L-leucine-AMC) and laccase (EC 1.10.3.2; ABTS). The enzyme activities are expressed as $\text{pmol min}^{-1} \text{mm}^{-2}$. The surface area of the analysed root tips was estimated using WinRhizo™ program (Regent Instruments, Quebec, Canada) and the results were calculated as previously described (Pritsch et al. 2011).

Biomass and shoot nitrogen content of seedlings

Total biomass was measured after collection of the root tips for the microscopic and enzyme assays. Total nitrogen and carbon content of the shoots was determined from dried and ground samples by using a varioMAX CN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Shoots weighing less than 50 mg DW were omitted from the N analysis because the total N measurement is sensitive to small samples.

Statistical analysis

The effects of different fungal treatments on enzyme activities and biomass were tested using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. The non-parametric Kruskal-Wallis test was used when assumptions of normality or equality of variances were not met. ANOVA and non-parametric tests were performed using IBM SPSS Statistics version 23.0.0.2. Endophytic, ectomycorrhizal and decay fungal colonised root tips were compared to control roots using a distance-based linear discriminant analysis (LDA) with fungal functional groups as grouping factor in order to investigate the multivariate patterns of enzyme activities (RStudio, Inc, Version 0.99.491, package MASS). The differences in the DA1 and DA2 axis scores in different functional groups were tested using ANOVA as described above.

Results

Effect of fungal inoculation on root structures

The effect of fungal inoculation on root structures was examined using light microscopy (Fig. 1). Non-inoculated Scots pine control root tips were intact (Fig. 1-A) and are used as a reference for fungal-inoculated root sections. The roots remained healthy after inoculation with the ECM fungi *Tomentellopsis echinospora* or *Suillus variegatus*. Multiseriate hyphae surrounded the root surface and formed a mantle in seedlings inoculated with *T. echinospora*

(Fig. 1-B). The root surface of seedlings inoculated with *Suillus variegatus* displayed only a thin mantle structure (Fig. 1-C). Well-developed Hartig net structures were observed throughout the cortex in both sets of seedlings, confirming the formation of ECM associations (Fig. 1 B, C).

The root section of seedlings inoculated with the endophyte *Phialocephala fortinii* displayed a thin layer of hyphae surrounding the root surface, and massive colonization by intercellular and intracellular hyphae were found in both cortical and cortex cells. The hyphae present in cortical cells formed coils and a deeply stained layer of tannin cells was also observed (Fig. 1-D). Intracellular hyphal coils were also observed in root sections of the endophytes *Metapochonia bulbillosa* and *Meliniomyces variabilis* (Fig. 1-E, F). However, the presence of clear intercellular hyphae, a tannin cell layer and the initial Hartig net-like structure was also observed in cortical layer in the sections of *Meliniomyces variabilis* (Fig. 1-F).

The root tissues of the seedlings inoculated with the decay fungi *Sistotrema brinkmannii*, *Conichaeta ligninaria* and *Gymnopilus penetrans* were clearly collapsed (Fig. 1-G, H, I) and the same signs of damage and empty cells were also observed in the roots of seedlings inoculated with the litter-decomposing *Collybia dryophila* (Fig. 1-J).

Enzyme activities

The catalytic activity for each enzyme assayed, and the sum of all enzyme activities per treatment are given in Table 2. The large variation in the measured enzyme activities between individual root tips resulted in just a few statistically significant differences between the treatments. Significant differences were observed in xylosidase, cellobiohydrolase, β -glucosidase and “chitinase” (N-acetyl- glucosaminidase) treatments. The sum of all enzyme activities differed significantly between the treatments (Table 2). No statistically significant

1 differences were observed for glucuronidase, acid phosphatase, leucine aminopeptidase and
2 laccase enzyme activities. The non-fungal control seedlings usually had the lowest enzyme
3 activities while seedlings inoculated with the endophytic *P. fortinii* had the highest activities.

4
5 The enzyme data analysed using linear discriminant analysis (LDA) showed that
6 ectomycorrhizal and endophytic fungal clusters were the most distant from each other while
7 controls and decay fungi fell in between these groups (Fig 2). The endophytic fungi differed
8 significantly from all other groups while ectomycorrhiza differed from decay fungi but not
9 from control according to the first discriminant axis (DA) that explained 78% of the variation
10 in the data (Table 3). The control seedlings differed from all fungal inoculated seedlings
11 according to the second DA that explained 22% of the variation in the data (Table 3).

12 13 *Seedling performance and fungal effect on host roots*

14 The shoot, root or total biomass or the roots/shoot ratio did not differ significantly between
15 the seedlings in different inoculation or control treatments. The average shoot biomass was
16 0.16-0.33 g DW, root biomass 0.21-0.81 g DW, total biomass 0.40-1.08 g DW, root/shoot
17 ratio 1.15-3.56 and needle nitrogen percentage 1.12-1.42 (data not shown).

18 19 **Discussion**

20 The ecological and functional role of most non-mycorrhizal root-inhabiting fungi is poorly
21 understood. There are indications that these endophytic fungi have mutualistic, parasitic,
22 pathogenic or neutral effects on the host plant (reviewed by Jumpponen et al. 1998, Mandyam
23 and Jumpponen 2005). It is often assumed that the presence of endophytic fungi is beneficial
24 to the host plant because the endophytic fungi often co-exist with ectomycorrhizal fungi in
25 apparently healthy roots (Kernaghan and Patriquin 2011) or that the co-colonization may even

1 promote plant growth (Reininger and Sieber, 2013). The aim of this study was to investigate
2 the morphological features and functional role of root-inhabiting fungi in axenic conditions in
3 the roots of their original Scots pine host. The effect of endophytic fungi on root-excreted
4 enzyme activities has not been previously studied.

5 The inoculated fungi had a clear effect on host root morphology and confirmed the predefined
6 fungal guild classification. The control and ectomycorrhizal roots were healthy and
7 undamaged with no intracellular hyphal penetration, whereas most decay fungi inflicted
8 serious damage on the root tissues and cell structures. The intracellular colonisation by the
9 endophytic fungi resulted in minimal damage to the host tissue and healthy root tissue
10 structures were observed. The presence of endophytes was associated with a thickening of the
11 cell wall and structures within or between cortical cells based on examination of the root
12 section images. This was particularly clear for *P. fortinii* where there was an increased
13 amount of dense material, similar to the 'electron opaque material' previously reported
14 (Peterson et al. 2008), between the outer cortical cells. The intracellular colonization by *P.*
15 *fortinii* was denser and more abundant than that of the two other endophytes, *Metapochonia*
16 *bulbillosa* and *Melinomyces variabilis*. A putative Hartig-net-like structure was observed in
17 the root tips, which were colonized by *Melinomyces variabilis*. This finding is inconsistent
18 with previous reports where *Melinomyces variabilis* modified the morphology of fine root tips
19 in a manner resembling early-stages of ectomycorrhizal development, but did not form typical
20 ectomycorrhizal structures, e.g. a mantle or a Hartig-net (Vohnik et al. 2007). *Melinomyces*
21 *variabilis* has been found to also colonize spruce root tip cortex (Vohnik et al. 2013). Our
22 findings suggest that endophytes like *Melinomyces variabilis* may have a flexible life-style
23 and lie at the crossroad between endophytism and mycorrhizal symbiosis.

24 The multi-enzyme assay, which was used for the first time in our study to investigate the
25 functioning of endophytic fungi, is widely used to study the functioning of ectomycorrhiza in

1 forest soils (Pritsch et al. 2004; Courty et al. 2005; Pritsch et al. 2011). The assay has been
2 shown to detect mainly fungal exo-enzyme activities that are stable enough to allow transport
3 of root samples from the field, for processing and analysis in the laboratory (Pritsch et al.
4 2011). We used the same assay in a novel way in order to detect the effect of non-mycorrhizal
5 root-inhabiting fungi on enzyme production in association with original host plant roots and
6 compare them to activities of non-colonised and mycorrhizal root tips. Our results
7 demonstrate surprisingly small differences in the root-excreted enzyme activities between
8 Scots pine roots colonised by different kinds of fungi that are supposed to be functionally
9 very different. Sterile Scots pine had the lowest activities in most cases. However, due to the
10 large variation between individual root tips, the enzyme activities induced by many of the
11 fungi did not differ from each other significantly. Our results indicate that all fungi inhabiting
12 the roots influenced the exo-enzymes produced by the plant-root association during our study
13 period. Furthermore, this result suggests that fungi primarily expressed or induced plants to
14 produce these exo-enzymes. Only the phosphatase enzyme appears to be expressed at similar
15 levels by the plant. Moreover, the roots colonized by the endophytic fungus *P. fortinii* had
16 very high enzymatic activities in comparison to the other fungal strains. These high levels of
17 activities could be related to the high density of *P. fortinii* hyphae within and around pine
18 roots as illustrated in our microscopic observations. Significant fungal species-related
19 differences in hemicellulase, cellulase and chitinase enzymatic activities, which are all
20 involved in cell-wall degradation, but not with other enzymes, were found among the fungi
21 tested. However, it is important to note that this assay does not provide any proof of nutrient
22 transfer from the fungal partner to the host, which is often the case in mycorrhizal symbiosis.
23 It is also noteworthy that the timing of the analysis may play a role as the plant-fungal
24 interaction may form a continuum along a gradient from mutualism to commensalism and to
25 parasitism (Peterson et al. 2008). Therefore, we conclude that the activities detected in the

1 root tip analysis must be interpreted with caution, if the functional role of root-associated
2 fungi is not known and the benefits to the host plant are uncertain.
3 Despite few significant differences in the activities of individual enzymes, the multivariate
4 discriminant analysis showed a separation between ectomycorrhizal, endophytic and decay
5 fungi when the score of the first discriminant axis was considered. The first axis explained
6 78% of the variation in the data. The second axis separated enzyme patterns of non-inoculated
7 root tips from all fungal-inoculated root tips. This is an important finding and shows that
8 endophytic fungi induce different enzymatic reactions in host plants compared to
9 ectomycorrhizal and decay fungi. Interestingly, Mathieu et al. (2013) used similar enzymatic
10 activity measurements and validated these functional traits to separate fungal ecological
11 groups within decaying fungi.

12
13 The biomass of the plants subjected to different treatments was not statistically different.
14 Differences in biomass were not expected as the aim of the experiment was to obtain active
15 and living root tips for root sectioning and enzyme activity analysis. Therefore the duration of
16 the experimental period compared to total plant growth period was intentionally short, which
17 resulted in only small changes in biomass. More seedlings would have probably become
18 unhealthy or died in this laboratory growth environment had a longer experimental period
19 been used and it would not have been possible to conduct the functional assay.

20 21 *Conclusions*

22 The root-isolated fungi included in this study clearly had different structures around or within
23 root tissues of the host plant. The non-inoculated control and ectomycorrhizal seedlings had
24 healthy-looking roots with intact cells, whereas endophytic fungi had intracellular penetration
25 and structures. The decay fungi tested damaged the root structures as expected. Our data

1 suggests that all fungi colonising the roots of the Scots pine plants change the rhizobiome
2 enzymatic pattern and that measured activities of exo-enzymes do not prove that the enzyme
3 exudation is beneficial to the plant. The enzyme activity pattern detected in root tip analysis
4 did not correlate with the root morphological characteristics of the seedling suggesting that
5 root tip exo-enzyme analysis is not suitable for assessing endophyte-plant nutrient transfer
6 patterns. Our results show for the first time that endophytic fungi have a different enzyme
7 activity pattern in roots compared to ectomycorrhizal and decay fungi. In particular, it seems
8 that *P. fortinii* significantly increases the production of enzymes in root tips but the
9 consequences of this increased activity for the host plant are not clear. Our results do not
10 support the hypothesis that endophytic and mycorrhizal fungi have similar effects on the host
11 plant. Our results indicate instead that endophytic fungi are clearly a distinctive ecological
12 group of fungi that have typical functional characteristics, different from those of
13 ectomycorrhizal and decay fungi.

15 Acknowledgements

16 We thank Dr. Karoliina Niemi for advice on root sample fixation. This research was
17 supported by grants from the Academy of Finland (project grants 263858 and 292699).

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Table 1. Fungal strains used in this study and GenBank accession numbers for the ITS region of the ribosomal rRNA operon.

Strain	Fungal species	Collection code	Functional group	Source of isolation	Accession no
JH5	<i>Tomentellopsis echinospora</i>	FBCC 1421	Ectomycorrhizal	Scots pine mycorrhiza, Finland	LK052757
JH35	<i>Suillus variegatus</i>	FBCC 1390	Ectomycorrhizal	Scots pine mycorrhiza, Finland	FN556986
JH8	<i>Phialocephala fortinii</i>	FBCC 1401	Endophytic	Scots pine mycorrhiza, Finland	AM905084
JH19	<i>Metapochonia bulbillosa</i>	FBCC 1400	Endophytic	Scots pine mycorrhiza, Finland	AB378551*
JH23	<i>Meliniomyces variabilis</i>	FBCC 1402	Endophytic	Scots pine mycorrhiza, Finland	AM905085
JH24	<i>Sistotrema brinkmannii</i>	FBCC 1416	Decomposer	Scots pine mycorrhiza, Finland	LK052763
JH37	<i>Coniochaeta ligniaria</i>	FBCC 1417	Decomposer	Scots pine mycorrhiza, Finland	LK052768
JH57	<i>Gymnopilus penetrans</i>	FBCC 1415	Decomposer	Scots pine mycorrhiza, Finland	AM905093
K209	<i>Collybia dryophila</i>	FBCC 626	Decomposer	Fruitbody, Finland	DQ068970

*closest similarity

Table 2. Enzyme activities measured in the root tips of control and fungal-inoculated seedlings (unit pmol min⁻¹ mm⁻²). Mean values with different letters are significantly (P< 0.05) different among the tested isolates.

		Glucuronidase		Xylosidase		Cellobio- hydrolase		b-Glucosidase		Chitinase		Acid phosphatase		Leucine aminopeptidase		Laccase		Sum of all enzyme activities	
		aver	stdev	aver	stdev	aver	stdev	aver	stdev	aver	stdev	aver	stdev	aver	stdev	aver	stdev	aver	stdev
Pine control	CON	0.00a	0.00	0.02ab	0.02	0.01a	0.01	0.04a	0.04	0.02a	0.05	1.73a	3.28	0.16a	0.07	0.05a	0.17	2.08a	3.35
JH5	ECM	0.01a	0.01	0.01ab	0.05	0.17ab	0.26	0.83ab	0.94	0.27ab	0.36	5.36a	7.66	0.35a	0.39	0.02a	0.03	7.05a	8.96
JH35	ECM	0.00a	0.00	0.00ab	0.05	0.33ab	0.35	0.90ab	0.90	1.67ab	1.51	2.92a	2.69	0.79a	0.53	0.05a	0.10	6.72a	5.15
JH8	ENDO	0.00a	0.029	1.41b	0.35	1.88b	0.38	4.18b	0.74	3.80b	0.88	4.03a	1.79	0.05a	0.03	0.06a	0.07	15.41b	3.26
JH19	ENDO	0.02a	0.00	0.05ab	0.021	0.12ab	0.08	0.79ab	0.58	0.22ab	0.15	1.64a	0.46	0.09a	0.04	0.01a	0.02	2.94a	1.06
JH23	ENDO	0.04a	0.08	0.22ab	0.10	0.20ab	0.11	0.57ab	0.34	0.48ab	0.29	1.96a	0.88	0.06a	0.12	0.04a	0.04	3.58a	1.65
JH24	DECAY	0.00a	0.00	0.00a	0.01	0.02ab	0.02	0.39ab	0.15	0.31ab	0.16	0.86a	0.20	0.17a	0.07	0.02a	0.05	1.78a	0.29
JH37	DECAY	0.01a	0.00	0.05ab	0.03	0.23ab	0.38	0.98ab	1.02	0.12ab	0.06	2.09a	1.20	0.02a	0.04	0.01a	0.03	3.51a	2.57
JH57	DECAY	0.13a	0.08	0.12ab	0.07	0.11ab	0.07	0.52ab	0.32	1.13ab	1.02	6.03a	5.71	0.27a	0.19	0.06a	0.08	8.38ab	5.83
C.dry	DECAY	0.05a	0.03	0.047ab	0.02	0.06ab	0.02	0.37ab	0.24	0.92ab	0.95	3.30a	1.64	0.00a	0.00	0.02a	0.21	4.82a	2.53
Statistical test		NPAR		NPAR		NPAR		NPAR		NPAR		NPAR		NPAR		NPAR		ANOVA	

Table 3. The scores of discriminant axis 1 and 2 (DA1 and DA2) that explained 78% and 22% of the variation in linear discriminant analysis of enzyme activities respectively. Standard deviation is shown in brackets. CON= non-inoculated control pine, ECM= pine inoculated with ectomycorrhizal, ENDO= with endophytic and DECAY= with decomposer fungi. Different letter indicate statistical difference ($p < 0.05$, ANOVA).

	DA1	DA2
CON	-0.72ab (± 1.69)	1.80a (± 0.75)
ECM	-2.34a (± 1.68)	-0.57b (± 1.15)
ENDO	1.68c (± 0.91)	-0.19b (± 1.00)
DECAY	-0.10b (± 0.76)	-0.07b (± 0.98)

1 **Figure captions**

2 Figure 1. Root sections of non-colonised control and fungal-colonised Scots pine root tips.
3 Scale bar in images 20 μm or 50 μm . A=Scots pine control, B= *Tomentellopsis echinospora*
4 ECM (JH5), C= *Suillus variegatus* ECM (JH35), D= *Phialocephala fortinii* ENDO (JH8), E=
5 *Metapochonia bulbillosa* ENDO (JH19), F= *Meliniomyces variabilis* ENDO (JH23), G=
6 *Sistotrema brinkmannii* DECAY (JH24), H= *Coniochaeta ligninaria* DECAY (JH37), I=
7 *Gymnopilus penetrans* DECAY (JH57), J= *Collybia dryophila* DECAY (K209)
8 Hyphae (h), Hyphal coil (hc), Tannin cells (tc), Hartig net (hn), putative Hartig net (phn),
9 Mantle (m).
10 Figure 2. Linear discriminant analysis plot analysed using enzyme activities and fungal
11 functional type as grouping factors. Discriminant axis DA1 and DA2 explains 78% and 22%
12 of the variation respectively. CON= non-inoculated Scots pine root, ECM= ectomycorrhizal,
13 ENDO= endophytic and DECAY= decomposer fungal colonised Scots pine root tips.