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Research

Growing evidence for facultative biotrophy in saprotrophic fungi: data from microcosm tests with 201 species of wood-decay basidiomycetes

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Summary

- Ectomycorrhizal (ECM) symbioses have evolved a minimum of 78 times independently from saprotrophic lineages, indicating the potential for functional overlap between ECM and saprotrophic fungi. ECM fungi have the capacity to decompose organic matter, and although there is increasing evidence that some saprotrophic fungi exhibit the capacity to enter into facultative biotrophic relationships with plant roots without causing disease symptoms, this subject is still not well studied.
- In order to determine the extent of biotrophic capacity in saprotrophic wood-decay fungi and which systems may be useful models, we investigated the colonization of conifer seedling roots *in vitro* using an array of 201 basidiomycete wood-decay fungi. Microtome sectioning, differential staining and fluorescence microscopy were used to visualize patterns of root colonization in microcosm systems containing *Picea abies* or *Pinus sylvestris* seedlings and each saprotrophic fungus.
- Thirty-four (16.9%) of the tested fungal species colonized the roots of at least one tree species. Two fungal species showed formation of a mantle and one showed Hartig net-like structures. These features suggest the possibility of an active functional symbiosis between fungus and plant.
- The data indicate that the capacity for facultative biotrophic relationships in free-living saprotrophic basidiomycetes may be greater than previously supposed.

Introduction

The ectomycorrhizal (ECM) symbiosis, involving a polyphyletic group of fungi spanning Basidiomycota, Ascomycota and Mucoromycotina (formerly Zygomycota) (Hibbett *et al.*, 2007; Tedersoo & Smith, 2013), is widespread and has important effects on ecosystem composition and function (van der Heijden *et al.*, 2015). In this association, fungi encase the root tips of host plants in a sheath of fungal material called a mantle, and proliferate in the apoplastic space between root cortical cells (Smith & Read, 2008). This proliferation between root cortical cells is called a Hartig net, and is the interface across which nutrients obtained by the fungus from the soil are traded from fungus to plant in return for photosynthetically fixed carbon (C) (Smith & Read, 2008). Although only *c.* 2% of terrestrial plant species form this type of association (Tedersoo *et al.*, 2010), the boreal and temperate forest biome that they dominate occupies a disproportionately large global area; ECM fungi and their associated plant species thus have a significant influence on global biogeochemical cycling (Averill *et al.*, 2014). In particular, many species in the obligately ECM (Briscoe, 1959; Tedersoo *et al.*, 2010) plant order *Pinaceae* are highly economically and ecologically important, as they are dominant members of forests in boreal and temperate zones (Liston *et al.*, 2003).

In contrast to ECM fungi, free-living saprotrophic wood-decay fungi derive C from dead organic material. Using extracellular enzymes and the nonenzymatic Fenton reaction (Eastwood *et al.*, 2011), they are able to access nutrients in recalcitrant forms such as those bound within the lignocellulosic matrix of wood. As primary decomposers of forest lignocellulose (Baldrian & Valásková, 2008), the most abundant organic substance in the world, as well as one of the most difficult to degrade (Tanesaka *et al.*, 1993), some of these fungi strongly influence the recycling of nutrients within forested ecosystems, as well as soil respiration (Osono, 2007). Saprotrophic fungi can also influence community composition of co-occurring bacteria (Folman *et al.*, 2008) and affect soil C storage through their interactions with ECM fungi (Averill *et al.*, 2014; Fernandez & Kennedy, 2016).

Despite the established ecological differences between ECM and saprotrophic fungi, evidence for functional overlap between the two groups continues to grow (Koide *et al.*, 2008). The capacity of ECM fungi to contribute to decomposition was first

theorized by Frank in 1894 (reviewed by Lindahl & Tunlid, 2015); their ability to oxidize organic matter is now supported by both laboratory (Rineau *et al.*, 2012; Vaario *et al.*, 2012) and field studies (Talbot *et al.*, 2013; Bödeker *et al.*, 2014; Phillips *et al.*, 2014). This is a hallmark of their evolutionary history (Shah *et al.*, 2016); the ECM life habit has evolved from saprotrophic lineages and persisted a minimum of 78 times, independently (Tedersoo & Smith, 2013).

This repeated convergent evolution of ECM symbiosis in fungi has resulted in an array of functionally similar but genetically diverse associations, illustrated by a diversity of retained genes coding for carbohydrate-active enzymes (Kohler *et al.*, 2015) and thus a diversity of enzymatic and nonenzymatic decomposition ability (Rineau *et al.*, 2012, 2013). Despite their differences, however, ECM fungi do share broad genetic commonalities, including increases in genes coding for nitrogen (N) and phosphorus (P) transporters (Martin *et al.*, 2010) and reductions in numbers of genes coding for plant cell wall degrading enzymes (PCWDEs) compared with saprotrophic relatives (Martin *et al.*, 2008, 2010; Nagendran *et al.*, 2009; Kohler *et al.*, 2015).

On the one hand, it may be expected that PCWDE loss helps to facilitate the adoption of symbiosis, because fungi expressing PCWDEs could trigger plant immune responses, precluding mutualism (Plett & Martin, 2011). Indeed, loss of a decomposition pathway has been implicated as the determining event in adoption of the ECM condition in *Amanita* (Wolfe *et al.*, 2012), and Eastwood *et al.* (2011) have also suggested that ECM transitions in *Boletales* may be correlated with diminished ligninolytic capabilities in brown-rot ancestors. On the other hand, the ECM life habit has been adopted many times independently (Hibbett *et al.*, 2000; Tedersoo & Smith, 2013), which suggests that to move from saprotrophy to ECM symbiosis is no great leap and that the genetic adaptations required in order to make the switch are relatively small. Some fungi that live both as necrotrophs and as saprotrophs have been shown to regulate gene expression, including that of genes coding for carbohydrate-active enzymes, according to the substrate on which they are growing (Olson *et al.*, 2012); thus, PCWDEs could simply be downregulated in symbiotic tissue, rather than lost. A further possibility is that neofunctionalization has occurred in some cases, resulting in moderate use of PCWDEs for plant cell wall remodeling in establishment of symbiotic structures. This hypothesis is supported by transcriptomic evidence from the model ECM fungus, *Laccaria bicolor* (Veneault-Fourrey *et al.*, 2014). Because ancestral saprotrophic abilities underlie the crucial capacity of ECM fungi to access organic nitrogen pools (Bödeker *et al.*, 2014; Shah *et al.*, 2016), complete loss of genes coding for associated enzymes could even be maladaptive. Hence, although establishment of symbiosis may require that PCWDEs not be highly expressed in symbiotic tissue, complete loss of PCWDEs is unlikely to be a necessity in early stages of adaption to an ECM lifestyle, and could even be detrimental.

In summary, many different lineages of saprotrophic fungi have independently adopted an ECM life habit throughout evolutionary history and the major known genetic differences between extant saprotrophic and ECM fungi do not appear to preclude development of biotrophic relationships between saprotrophic fungi and living plants. We therefore expect that extant saprotrophic fungi exhibit a range of capacity for facultative biotrophic relationships with plant roots, just as extant ECM fungi exhibit a range of capacity for facultative saprotrophy or decomposition (Colpaert & Laere, 1996; Vaario *et al.*, 2012; Bödeker *et al.*, 2014).

In support, some saprotrophic fungi can form functional orchid mycorrhizas in nature (Martos *et al.*, 2009; Lee *et al.*, 2015), and molecular studies using environmental samples sometimes recover saprotrophic DNA from mycorrhizal root tips (Tedersoo & Smith, 2013), even though these 'molecular scraps' are often discounted (Selosse *et al.*, 2010). Established wood-decay fungi have been found in the roots of apparently healthy trees both in forest nurseries (Menkis *et al.*, 2005) and in forest ecosystems (Menkis *et al.*, 2012), and laboratory experiments have confirmed colonization of living fine roots by several of these fungi (Vasiliauskas *et al.*, 2007). Because nearly a third of saprotrophic lineages sister to ECM lineages are wood-decay fungi (Tedersoo *et al.*, 2010), further research on this subject is likely to offer insight into the evolution of ECM symbioses as they now exist. Nevertheless, little work has been done to identify how common the ability of saprotrophic wood-decay fungi to enter into biotrophic relationships with plants is, nor which systems are likely to be most similar morphologically and anatomically to ECM fungi, and thus most relevant to the study of the evolution of ECM symbiosis. In order to identify the extent of biotrophic capacity in saprotrophic wood-decay fungi, and to identify patterns of root colonization, we undertook a survey of 201 species of saprotrophic wood-decay basidiomycetes. The fungi were grown in axenic culture with seedlings of two ECM host species, *Pinus sylvestris* and *Picea abies*, which are widespread and economically important tree species in north temperate and boreal zones. The colonized plant roots were examined using microtome sectioning, differential staining and fluorescence microscopy.

Materials and Methods

Establishment of microcosm systems

Stock cultures of 201 species of wood-decay basidiomycete fungi were obtained from the culture collection of the Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences (Table 1, Supporting Information Table S1), and were grown out on modified Melin–Norkrans (MMN) agar medium (Marx, 1969) in Petri dishes. Experimental 9-cm diameter microcosm systems were constructed based upon mycorrhizal synthesis methods first used by Duddridge (1986), with adjustments by Vasiliauskas *et al.* (2007). Briefly, sterile, 2-wk-old seedlings of *Pinus sylvestris* L. and *Picea abies* (L.) H. Karst were aseptically inoculated with 5 × 5 mm agar plugs from actively growing fungal cultures, and microcosms were established in Petri dishes filled with a solid growth substrate consisting of sterilized fine sphagnum peat : vermiculite : 1/10 strength liquid MMN mixture in the ratio 1 : 4 : 2 (v/v/v)

Table 1 List of wood-decay basidiomycetes that colonized roots in microcosm systems with *Pinus sylvestris* and/or *Picea abies* seedlings, and observed patterns of root colonization

	Pinus sylvestris				*Picea abies*			
	Pattern of root colonization				Pattern of root colonization			
Fungal species	Surface	Epidermis	Cortex	Vascular	Surface	Epidermis	Cortex	Vascular
Amylostereum ferreum	+	+	+	+	–	–	–	–
Amylostereum laevigatum	+	+	+	+	–	–	–	–
*Armillaria cepistipes**	+	na[†]	na	na	–	–	–	–
*Armillaria mellea**	+	+	+	+	–	–	–	–
*Bjerkandera adusta**	+	+	–	–	–	–	–	–
Ceratobasidium sp. 257*	+	+	–	–	–	–	–	–
*Chondrostereum purpureum**	+	+	–	–	+	+	+	–
*Collybia butyracea**	+	+	–	–	–	–	–	–
Coniophora cerebella	+	+	–	–	–	–	–	–
Creolophus cirrhatus	+	+	–	–	–	–	–	–
*Fomes fomentarius**	+	+	+	–	–	–	–	–
Grifola frondosa	+	+	+	+	–	–	–	–
Gymnopus sp. 406*	+	+	+	+	+	+	+	–
Heterobasidion parviporum	+	+	+	+	–	–	–	–
Hymenochaete tabacina[a]	+	+	+	–	–	–	–	–
*Hypholoma capnoides**	+	+	+	–	–	–	–	–
Hypholoma fasciculare	+	+	–	–	–	–	–	–
Lenzites betulina[b]	+	+	+	–	–	–	–	–
*Marasmius androsaceus**	+	+	+	–	–	–	–	–
*Marasmius scorodonius**	+	+	+	+	–	–	–	–
*Mycena abramsii**	+	+	+	–	–	–	–	–
*Mycena epipterygia**	+	+	+	–	–	–	–	–
*Mycena galopus**	+	+	+	–	–	–	–	–
***Mycena* sp. 480**[c]	+	+	+	–	–	–	–	–
*Phellinus chrysoloma**	+	+	–	–	–	–	–	–
*Phellinus igniarius**	+	+	+	+	+	+	+	–
*Phellinus nigricans**	+	+	+	–	–	–	–	–
Phellinus tremulae	–	–	–	–	+	–	–	–
Pholiota gummosa	+	+	+	–	–	–	–	–
Pholiota squarrosa	+	+	+	–	–	–	–	–
Pleurotus ostreatus	+	+	+	+	–	–	–	–
*Schizophyllum commune**	+	+	–	–	–	–	–	–
Stereum ostrea	+	–	–	–	–	–	–	–
*Stereum sanguinolentum**	–	–	–	–	+	+	+	–

Fungi in microcosms in which seedling mortality was observed are marked in bold; incidence of seedling mortality is indicated in the footnote as a proportion (%) of three replicates. Asterisks denote fungal species whose identity in microcosm systems was confirmed by internal transcribed spacer (ITS) rDNA sequencing from root tips. A list of tested fungi that did not colonize roots can be found in Supporting Information Table S1.
[a]*Picea abies* 66%; [b]*P. abies* 66%; [c]*P. abies* 33%; [†]data not available.

(Rosling *et al.*, 2004). The pH of this medium was 5.5. Three replicate microcosms were constructed for each tree species–fungus combination. Control systems were also established using sterile agar plugs. In order to keep the microcosms axenic after inoculation, holes for seedling shoots were sealed around the stem with sterile anhydrous lanolin and the system itself was wrapped with parafilm as in other microcosm studies (Duddridge, 1986; Finlay, 1989; Rosling *et al.*, 2004; Vasiliauskas *et al.*, 2007), ensuring inaccessibility to airborne spores and contaminants.

Monitoring of seedling growth and fungal colonization of roots

Inoculated microcosms were incubated under controlled conditions in a climate chamber at 16°C with a photoperiod of 16 h : 8 h, light : dark. The photosynthetic photon flux density within the climate chamber was *c.* 300 μmol m^{-2} s^{-1}; to ensure that the light affected only the shoot of the plant, microcosms were wrapped with aluminum foil. Microcosms were regularly monitored for changes in seedling vitality and for external features of fungal colonization of fine living roots.

The axenic microcosm systems were incubated for 6 months, and then subjected to a preliminary assessment under a Leica M165 FC dissection microscope (Wetzlar, Germany) for fungal affinity for roots. We define an affinity for roots here as hyphal growth on fine root tips as opposed to growth in the surrounding medium, as demonstrated by at least one microcosm replicate and at least three root tips. Systems showing fungal colonization of roots were frozen and stored at −20°C until further analysis.

Determination of root colonization patterns

From each selected system, colonized root tips (Fig. 1) were sampled under the Leica dissection microscope. A minimum of three such root tips, representing each selected fungus–plant system, were carefully excised and 5–10-μm cross-sections taken using a Leica CM1850 cryomicrotome. Staining was then carried out using Biotium CF™488A wheat germ agglutinin (Hayward, CA, USA), staining fungal tissue green, and propidium iodide, staining plant tissue red, according to the procedure of Doehlemann *et al.* (2009). Following staining, slides were examined using a Leica DM5500 B light microscope and photographed using fluorescence filter cube A4. Photographs were then assessed for patterns of hyphal colonization in specific, established root regions for each colonized fungus–tree combination: surface, epidermis, cortex and vascular tissues. Surface colonization included both aggregated and/or individual fungal hyphae on the outer surface of fine roots, visible in stained cross-sections. Epidermal or cortex colonization included the presence of fungal hyphae in each of these established root regions (Russell, 1977), and colonization within the endodermis was classified as that of vascular tissue. In order to minimize the possibility of erroneous classification, assessments were based upon presence or absence of clearly stained hyphae in these regions in multiple photographs per species. In addition, features present in roots that were stained green but deviated from known fungal morphology (e.g. crystalline or blotchy) were considered artifacts of the staining process rather than fungal tissue, and were not included in classification determinations. Finally, fungal tissue not integrated into cross-sections was also not included in classifications due to the possibility of the tissue having been displaced in the sectioning process. Fungi exhibiting morphology similar to that of ectomycorrhizal species were noted.

Molecular identification of fungal species

In order to confirm the identity of fungi that showed affinity for roots in microcosm systems, sequencing of the internal transcribed spacer of fungal rDNA (ITS rDNA) was carried out from root tips. Three individual root tips were collected in different parts of the microcosm systems. Extraction of fungal DNA from the roots, amplification and sequencing were performed as in Vasiliauskas *et al.* (2007).

Results

After 6 months of cultivation in the climate chamber, the majority of seedlings inoculated with wood-decay fungi and all non-inoculated control seedlings were healthy-looking: shoots and the majority of needles were green and without signs of decline, whereas root systems remained intact and free of visible decay. Examination under the dissection microscope confirmed that all control systems remained free of fungal colonization. Five (2.5%) fungi were associated with variable degrees of seedling mortality in *P. sylvestris* and six (3%) in *P. abies* (Tables 1, S1). In particular, the fungi *Kuehneromyces mutabilis* and *Phanerochaete rimosa* were associated with mortality in all three replicate seedlings of *P. sylvestris* (Table S1).

Of the 201 fungal species tested, 34 (16.9%) showed an affinity for roots (Table 1; Fig. 1). Among these, 29 (85.3%) colonized roots exclusively of *P. sylvestris*, two (5.9%) exclusively of *P. abies*, and three (8.8%) colonized roots of both tree species. Among the fungi that colonized roots of *P. sylvestris*, 32 (100%) showed colonization of the root surface, 30 (93.8%) colonization of the epidermis, 21 (65.6%) colonization of cortical tissue and 9 (28.1%) colonization of vascular tissue (Table 1; Fig. 2). Among the fungi that colonized roots of *P. abies*, five (100%) showed colonization of the surface, four (80.0%) colonization of epidermis and four (80.0%) colonization of the cortex; none showed colonization of vascular tissue (Table 1; Fig. 2). Although not scored, development of intracellular hyphae was sometimes observed (e.g. Fig. 2c,d; see also Figs S3–S6). Further photographic examples of root colonization by tested saprotrophic wood-decay fungi can be found in the Supporting Information (Figs S1–S6).

Two species, *Coniophora cerebella* and *Hypholoma capnoides*, formed mantle-like structures on *P. sylvestris* (Figs S2, 2b), which we define as thick proliferations of hyphae visible on the epidermal surface of a root cross-section. *Phellinus igniarius* developed intercellular hyphae resembling a Hartig net on *P. abies* (Fig. 2a). Dichotomously branching root tips, which are characteristic features of pines colonized by certain ECM fungi (Persson, 2002), were sometimes observed, but were not scored (e.g. Fig. S1).

The fungi that colonized roots of both tree species showed different patterns of root colonization for each tree species. For example, in *P. abies*, *Chondrostereum purpureum*, *Gymnopus* sp. 406 and *P. igniarius* colonized surface, epidermis and cortex. By contrast, in *P. sylvestris*, *C. purpureum* colonized only surface and epidermis, whereas *Gymnopus* sp. 406 and *P. igniarius* colonized surface, epidermis, cortex and vascular tissue.

Eighteen microcosm systems of *P. sylvestris* and four of *P. abies*, which were inoculated with different fungal species (Table 1), were used for sequencing of fungal ITS rDNA from the root tips. The remaining microcosm systems were not available as all root tips were used for cross-sectioning. Amplification was successful for between one and three root tips per microcosm system. Sequencing confirmed the identity of each inoculated fungus and no other fungi were detected, indicating that there was no fungal contamination. The sequences are available from GenBank under accession numbers KY352513–KY352531.

Discussion

Our data demonstrate that colonization of living fine tree roots by wood-decay fungi and formation of structures similar to those found in the ectomycorrhizal (ECM) symbiosis is a relatively rare phenomenon; colonization occurred in only 16.9% of our tested fungal species. Nevertheless, these results also reveal the potential for several wood-decay fungi to be used as model systems in studying the evolution of root symbioses and their functioning. In particular, the wood-decay fungus *Phellinus igniarius* showed development of intercellular hyphae around the cortical cells of *Picea abies* (Fig. 2a), similar in appearance to a Hartig net, the

Fig. 1 Patterns of fine root colonization of conifer seedlings by wood-decay fungi. (a) *Armillaria mellea* on *Pinus sylvestris*; (b) *Pholiota gummosa* on *P. sylvestris*; (c) *Lenzites betulina* on *P. sylvestris*; (d) *Heterobasidion parviporum* on *P. sylvestris*.

organ of nutrient exchange in the ECM symbiosis. Formation of such features in association with tree seedlings has, to the best of our knowledge, so far only been observed *in vitro* in one other saprotrophic wood-decay fungus, *Phlebiopsis gigantea* (Vasiliauskas *et al.*, 2007).

Even though the pure culture techniques used in our study are well-established methods of determining the mycorrhizal status of fungi (Duddridge, 1986; Finlay, 1989; Rosling *et al.*, 2004; many more reviewed by Vasiliauskas *et al.*, 2007), differences in important variables, such as pH and temperature, between natural and laboratory conditions can affect the ability of a fungus to form mycorrhizal associations (Riffle, 1973). Intraspecific genetic variation is a further factor that may influence the ability of saprotrophic fungi to colonize living fine roots; for example, although Eastwood *et al.* (2011) previously observed colonization of *Pinus sylvestris* roots by *Serpula lacrymans*, this species showed no affinity for roots in the present study (Table S1). Demonstration of mycorrhizal formation *in vitro* therefore does not necessarily prove that such interactions occur frequently *in vivo*, in the field, nor does an absence of interaction in laboratory microcosms preclude the possibility that they could occur under other conditions. Because multiple species of saprotrophic fungi previously shown to colonize living fine roots *in vitro* (Vasiliauskas *et al.*, 2007) have also been observed on tree roots and in rhizosphere soil both in tree nurseries and in forest ecosystems (Menkis *et al.*, 2005, 2012), the present relationships are unlikely to be restricted to laboratory conditions. Indeed, saprotrophic fungi from several of the genera that we have observed colonizing root tips in this study, such as *Gymnopus*, *Marasmius* and *Mycena*, also associate symbiotically with heterotrophic orchids in nature (Martos *et al.*, 2009; Lee *et al.*, 2015).

The effects of ecological interactions in axenic culture can also differ from those found in nature, as demonstrated in the present study by necrotrophs such as *Armillaria mellea* (Fig. 1a), *Chondrostereum purpureum* and *Fomes fomentarius*, which did not cause visible disease symptoms nor increased mortality in

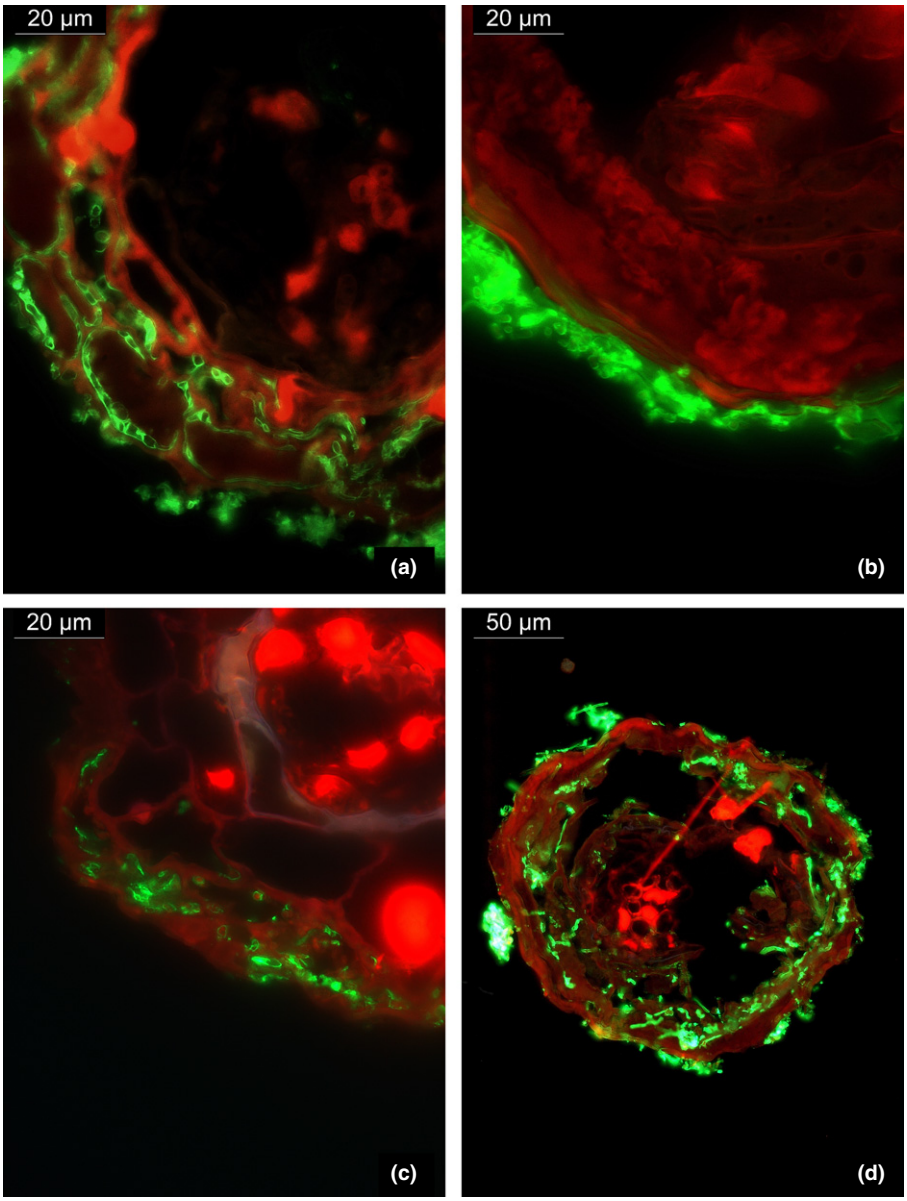

Fig. 2 Differentially stained fine root cross-sections of conifer seedlings grown for 6 months together with wood-decay fungi: fungal material is stained green and plant material red. (a) Intercellular colonization of cortical cells of *Picea abies* by *Phellinus igniarius*; (b) formation of mantle-like structures on fine root of *Pinus sylvestris* by *Hypholoma capnoides*; (c) colonization of fine root epidermis and outermost cortical cells of *P. abies* by *Stereum sanguinolentum*, (d) colonization of surface, epidermis, cortex, and vascular tissue of *P. sylvestris* by *Marasmius scorodonius*.

replicate host seedlings despite colonization of roots (Table 1). The possible mutualistic status of the observed symbioses can therefore be conclusively determined only by studies specifically quantifying nutrient and C transfer between fungus and plant.

Differences in colonization patterns show that a degree of host-specificity is present in the demonstrated relationships, and that the same saprotrophic fungus may develop qualitatively different symbioses on different hosts, just as the same mycorrhizal fungus may form different kinds of mycorrhizas on different hosts (Grelet *et al.*, 2010). Specific varieties of root colonization are therefore not inherent fungal traits, but rather emergent properties developed in interaction with plants. For example, although *P. igniarius* showed intercellular cortical colonization similar to an ECM Hartig net in association with *P. abies* (Fig. 2a), colonization of vascular tissue in association with *P. sylvestris* (Table 1) suggests a relationship different from those developed by mycorrhizal fungi, none of which are known to grow within living plant vascular tissue. Variation between plant species is also clear in the overall colonization patterns observed in *P. abies* and *P. sylvestris*: fewer fungal species associated with the former, colonization never extended into vascular tissue, and mortality of all three replicate seedlings was never observed. Although this would seem to suggest lower receptivity to fungal colonization in *P. abies* as compared with *P. sylvestris*, formation of Hartig net-like structures by wood-decay fungi has now been documented twice on the former (Fig. 2a; Vasiliauskas *et al.*, 2007), but never on the latter.

Only 34 of the 201 fungi tested showed any affinity for roots after 6 months, suggesting that despite the potential source of labile C represented by plant roots, sustained fungal responsiveness may not be universal. A further possibility is that the majority of fungal species were repelled by plant

immunity. Indeed, preventing colonization of living roots by pathogenic fungi is a primary role of plant immune responses, which can be triggered by chitin found in fungal cell walls (Zipfel, 2014). Saprotrophic enzymes, which normally do not facilitate entry into living cells, can also provoke an immune reaction (Plett & Martin, 2011) due to detection by the plant of endogenous byproducts of plant cell wall damage (Zamioudis & Pieterse, 2011). Our observance of intracellular hyphal development in colonized root tips of outwardly healthy plants is consistent with downregulated expression of such enzymes in symbiotic fungal tissue, a phenomenon similar to that observed in fungi such as *Heterobasidion* spp., which can live both necrotrophically and saprotrophically, regulating enzyme expression according to substrate (Olson *et al.*, 2012).

The sensitivity of plant immune systems to microbe-associated molecular patterns such as chitin (Zipfel, 2014), many of which are present both in pathogens and in mutualists, results in a requirement that symbiotic fungi, whether mutualistic or pathogenic, make use of effector proteins to attenuate plant immune response and facilitate establishment of symbiosis (Zamioudis & Pieterse, 2011). Thus, as plant health depends on simultaneously repelling microbial pathogens while accepting microbial mutualists, the formation of ECM root tips involves a complex co-evolved molecular exchange between fungus and host (Garcia *et al.*, 2015).

In particular, the development of the Hartig net by the ECM fungus *Laccaria bicolor* has been shown to require expression of an effector protein, mycorrhizal induced small secreted protein 7 (MiSSP7), in symbiotic fungal tissue (Plett *et al.*, 2011). Furthermore, mutants not expressing MiSSP7 are unable to engage in functional symbiosis (Plett *et al.*, 2011), underscoring the importance of the Hartig net for bidirectional nutrient transfer and demonstrating that its formation does not occur spontaneously but, rather, as part of a larger pattern of plant–fungus communication. The development of Hartig net-like structures by *P. igniarius* thus suggests the use of effectors such as MiSSP7 and potentially constitutes an independent ECM symbiotic origin event. Although secretomic analysis indicates that wood-decay fungi produce a reduced complement of effector proteins compared with obligate biotrophs (Kim *et al.*, 2016), *P. igniarius* in particular has not been examined, and to our knowledge no transcriptomic or secretomic analysis has been carried out using saprotrophic fungi grown with seedlings as in the present study.

Our results indicate that although facultative biotrophy is far from ubiquitous among saprotrophic basidiomycetes, it may be more common than previously supposed; this can be confirmed by future functional analysis. These findings thus support current hypotheses of multiple, independent ECM origin events throughout evolutionary history (Tedersoo & Smith, 2013) and extend scientific knowledge concerning the functional diversity of saprotrophic fungi. We also demonstrate here the potential for use of multitrophic nonmycorrhizal basidiomycetes as model systems in the study of ECM evolution and signaling. Coupled with modern genomic and transcriptomic tools, systems such as these can offer new insights into the evolution of this crucial and fundamental symbiosis. Our forthcoming studies examining the potential for bidirectional nutrient transfer in the most promising of these systems, such as those of *P. igniarius* and *P. gigantea* in association with *P. abies*, will build upon our present findings and further elucidate these phenomena.

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Author contributions

R.F., J.S., R.V. and A.M. designed the study; R.F., J.S. and R.V. contributed materials; G.R.S. and A.M. performed the research; G.R.S. and A.M. collected, analyzed and interpreted the data; and G.R.S., R.F., J.S., R.V. and A.M. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Dichotomously branching root tip of *Pinus sylvestris* colonized by *Marasmius androsaceus*.

Fig. S2 Cross-section of *Pinus sylvestris* root tip colonized by *Coniophora cerebella*.

Fig. S3 Cross-section of *Picea abies* root tip colonized by *Chondrostereum purpureum*.

Fig. S4 Cross-section of *Pinus sylvestris* root tip colonized by *Amylostereum laevigatum*.

Fig. S5 Cross-section of *Pinus sylvestris* root tip colonized by *Gymnopus* sp. 406.

Fig. S6 Cross-section of *Pinus sylvestris* root tip colonized by *Mycena abramsii*.

Table S1 List of tested fungal species that did not colonize roots of *Pinus sylvestris* nor of *Picea abies* in microcosm systems

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