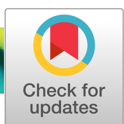

In vitro evidence of root colonization suggests ecological versatility in the genus *Mycena*

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Summary

- The root-associated habit has evolved on numerous occasions in different fungal lineages, suggesting a strong evolutionary pressure for saprotrophic fungi to switch to symbiotic associations with plants. Species within the ubiquitous, saprotrophic genus *Mycena* are frequently major components in molecular studies of root-associated fungal communities, suggesting that an evaluation of their trophic status is warranted. Here, we report on interactions between a range of *Mycena* species and the plant *Betula pendula*.
- In all, 17 *Mycena* species were inoculated onto *B. pendula* seedlings. Physical interactions between hyphae and fine roots were examined using differential staining and fluorescence microscopy. Physiological interactions were investigated using ^{14}C and ^{32}P to show potential transfer between symbionts.
- All *Mycena* species associated closely with fine roots, showing hyphal penetration into the roots, which in some cases were intracellular. Seven species formed mantle-like structures around root tips, but none formed a Hartig net. *Mycena pura* and *Mycena galopus* both enhanced seedling growth, with *M. pura* showing significant transfer of ^{32}P to the seedlings.
- Our results support the view that several *Mycena* species can associate closely with plant roots and some may potentially occupy a transitional state between saprotrophy and biotrophy.

Introduction

To understand ecosystem functioning, knowledge about species diversity, ecological niches and nutritional modes is crucial. For fungi, where a major part of their life cycle remains hidden below ground or within substrates, gaining this knowledge has been and still is problematic. High-throughput sequencing has enabled assessments of fungal diversity in a wide range of ecosystems and substrates, ranging from local (Pickles *et al.*, 2012; Anderson *et al.*, 2014) to global scales (Tedersoo *et al.*, 2010, 2014), and including substrates such as individual plant roots (Bahram *et al.*, 2011; Botnen *et al.*, 2014; Lorberau *et al.*, 2017), soils (Kyaschenko *et al.*, 2017; Sterkenburg *et al.*, 2018) and dead wood (Baldrian & Valášková, 2008). As information on habitat occupation by species accumulates, it is becoming clear that some taxa with supposedly defined niche distributions might occupy more than one niche, and that current ideas and beliefs concerning the ecology and function of some fungal taxa will require revision (Selosse *et al.*, 2009, 2010; Halbwachs *et al.*, 2018; Schneider-Maunoury *et al.*, 2018; Lofgren *et al.*, 2018; see also Selosse *et al.*, 2018).

Saprotrophic and biotrophic nutritional modes are generally thought to be shared among all species within fungal genera (Tedersoo & Smith, 2013), and nutritional modes are thus often assigned at genus level (Nguyen *et al.*, 2016). Indeed, the existence of mixed trophic modes within genera has been indicative of polyphyly, for instance *Paxillus* and *Tapinella* (Bresinsky *et al.*, 1999), which subsequent molecular phylogenies have substantiated (Binder & Hibbett, 2006). In addition to sporocarp morphology and molecular traits, nutritional modes often follow the defining traits in separating genera (Skrede *et al.*, 2011; Birkebak *et al.*, 2013).

The traditional view of how nutrients are acquired by mycorrhizal and saprotrophic fungi is that the former acquire reduced carbon (C) from their plant hosts, and in return provide benefits to the plant (van der Heijden *et al.*, 2015; Brundrett & Tedersoo, 2018), whereas saprotrophic fungi gain reduced C from the decomposition of organic material (Baldrian & Valášková, 2008). However, it is now clear that some ectomycorrhizal (EcM) fungi are actively involved in the decomposition of organic material for the acquisition of nitrogen and phosphorus (P), but not for C acquisition (Perez-Moreno & Read, 2000; Lindahl &

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Tunlid, 2015; Op De Beeck *et al*., 2018). In addition, most ericoid mycorrhizal (ErM) fungi have retained many of the genes involved in decomposition and are versatile saprotrophs as well as ErM symbionts (Martino *et al*., 2018).

The distinction between the two trophic modes has also been blurred from recent *in vitro* studies of interactions between mycelia of saprotrophic fungi and plant roots. Several known wood-decaying fungi can form mantle-like mycelial sheets around root tips, and intercellular hyphal networks similar to a Hartig net, which are reminiscent of structures formed by EcM fungi (Vasiliauskas *et al*., 2007; Eastwood *et al*., 2011; Kohler *et al*., 2015; Smith *et al*., 2017). Such interactions may represent positions on a saprotrophy–biotrophy continuum or, alternatively, high ecological versatility.

The transition from saprotrophic to biotrophic lifestyle has occurred several times independently during evolution, and at least 78 times for EcM fungi (Tedersoo & Smith, 2013; Kohler *et al*., 2015). Commonly, the transition to an EcM lifestyle is linked to loss of genes encoding for decay mechanisms; nevertheless a large set of symbiosis-upregulated genes have orthologues in white- and brown-rot species (Wolfe *et al*., 2012; Kohler *et al*., 2015). Thus, the evolutionary distance between saprotrophy and biotrophy may not be great, and whether this shift in ecology may occur more easily than previously thought for some species of fungi has been the subject of debate (see Selosse *et al*., 2010; Baldrian & Kohout, 2017).

The genus *Mycena* sensu stricto (Moncalvo *et al*., 2002) is among the most species-rich genera in the Agaricales, with over 500 described species (Kirk *et al*., 2008). Some species are known to have profound ecological importance in forest ecosystems as litter decomposers (Baldrian *et al*., 2012; Purahong *et al*., 2016; Kyaschenko *et al*., 2017; Kohout *et al*., 2018; Sterkenburg *et al*., 2018). However, in high-throughput sequencing studies of fungi associated with living plant roots, operational taxonomic units (OTUs) assigned to *Mycena* sensu lato are surprisingly common (Bjorbækmo *et al*., 2010; Liao *et al*., 2014; Botnen *et al*., 2014; Lorberau *et al*., 2017; Kohout *et al*., 2018). Moreover, the number of *Mycena* sequences may have been underestimated in some studies as recent evidence suggests that one of the commonly used metabarcoding primers for the ITS1 region has a mismatch for some *Mycena* species, and thus does not amplify the ITS1 region well (Tedersoo & Lindahl, 2016). In a study from Svalbard based on the ITS2 region, *Mycena* sensu lato was the most commonly recovered genus from the living roots of the ericaceous plant *Cassiope tetragonia*, and represented as much as 17% of the sequence reads from the plant roots (Lorberau *et al*., 2017). *Mycena* species have also been recovered from mRNA from healthy-looking EcM roots of *Pinus sylvestris* (Liao *et al*., 2014), showing that *Mycena* species may be functionally active when associated with living plant roots. In a study investigating the impacts of forest clear-cutting on fungal communities, *Mycena* species were relatively abundant in living roots at the beginning of the experiment, and then increased in abundance during the course of time after clear-cutting (Kohout *et al*., 2018). The authors suggested that some *Mycena* species may have been latent saprotrophs, living asymptomatically as root endophytes before clear-cutting, which would give them an advantage in accessing C as the roots senesce.

The recurrent findings of *Mycena* species in living plant roots raises a question over their ecology and challenges the current concept of the genus as being purely saprotrophic, acquiring C from dead organic material. In addition to these studies, others also suggest a possible biotrophic role of the genus: *M. galopus* was shown to form beneficial associations with *Vaccinium corymbosum* (Grelet *et al*., 2017); some *Mycena* species form associations with *Picea abies* roots *in vitro* (Smith *et al*., 2017), and other *Mycena* species have been shown to form associations with orchid roots (Martos *et al*., 2009; Ogura-Tsujita *et al*., 2009; Zhang *et al*., 2012). This raises questions about the roles of *Mycena* species when associating with plant roots, and whether or not they can act as biotrophs.

The present study investigated the interactions between a taxonomically diverse group of *Mycena* species and the living roots of an EcM plant. Birch (*Betula pendula* Roth.) was chosen as the model plant because it forms EcM with many different fungal species (generalist) in temperate regions and is easy to germinate and grow on agar plates. Interactions were examined *in vitro* where sterile seedlings were inoculated from axenic cultures of *Mycena* species. The extent and patterns of fungal colonization of fine roots were examined using differential staining and fluorescence microscopy. Physiological interactions between a selection of *Mycena* species and birch were investigated with radioactive isotopes to trace potential bidirectional flow of nutrients and C between the plant and the fungus.

Materials and Methods

A total of 17 species of *Mycena* were included in this study, with selection based partly on species previously reported from plant root studies, and on a need to cover the phylogenetic breadth of the genus (Fig. 1). Details of species and cultures are given in Table S1, and methods for obtaining and maintaining the fungal cultures, as well as molecular methods for generating ITS sequences and a phylogenetic analysis of the selected species (Fig. 1), are given in Methods S1. The *Mycena* ITS sequences are deposited to GenBank under accession numbers MT153125 – MT153149.

Inoculation and growth

Sterile seedlings of the *B. pendula* and axenic cultures of 17 *Mycena* species were grown together in microcosms for 8 wk. In total, 21 *Mycena* strains were included (see Table S1 for details). Seeds of *B. pendula* were obtained from the Norwegian Forest Seed Centre (seed batch no. F09-017; Skogfrøverket, Hamar, Norway), and sterile seedlings were germinated as described in Methods S1.

The microcosms were constructed in split, 90 mm Petri dishes, where one-half the dish contained 10% modified Melin–Norkrans medium (MMN; Marx, 1969), which was covered by a charcoal filter paper (90 mm in diameter, cut in half; Whatman® Schleicher & Schuell®, Maidstone, UK) to aid in visualization of

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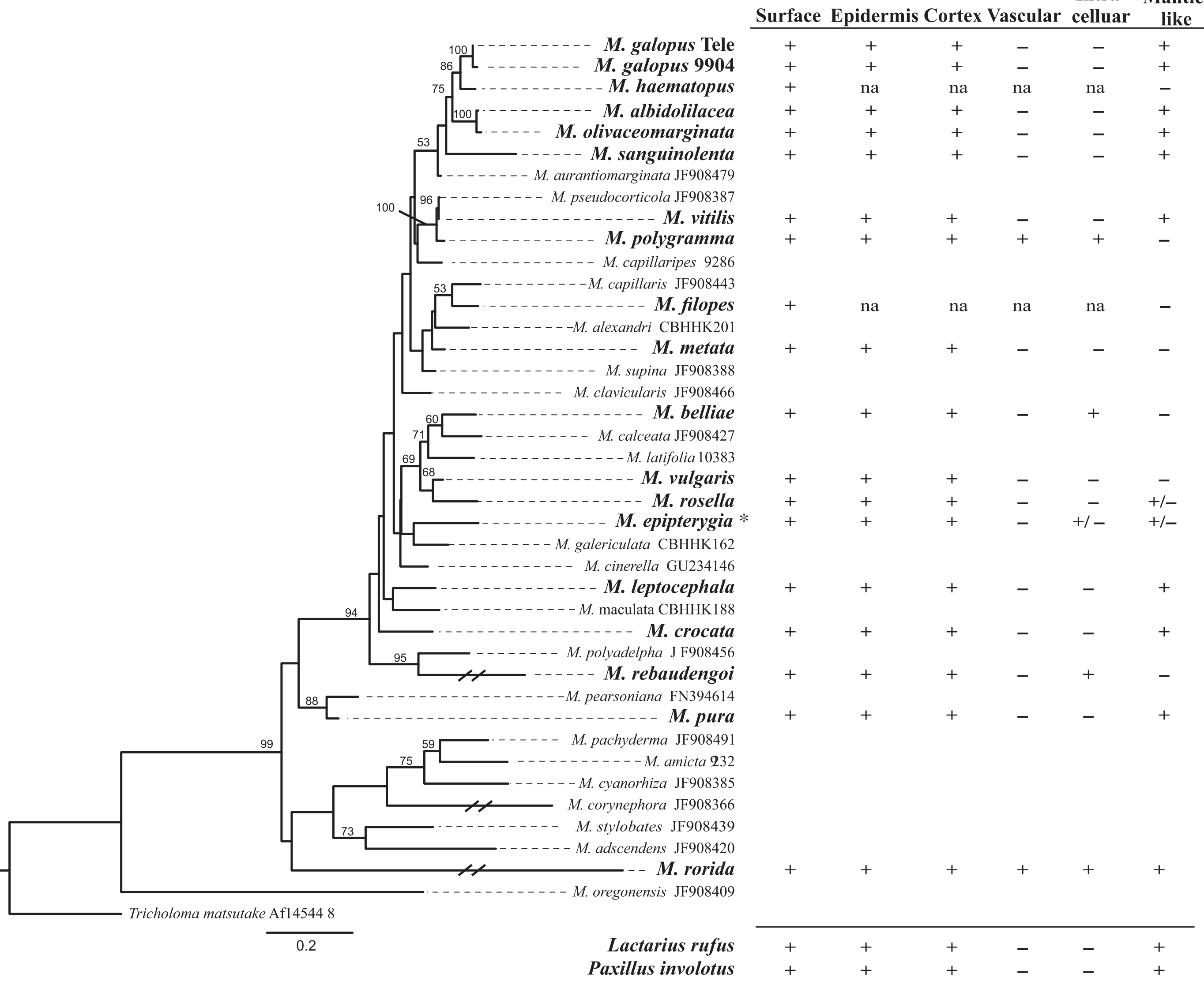

Fig. 1 Maximum likelihood phylogeny of a species selection of *Mycena* based on nuclear internal transcribed spacer (ITS) nucleotide sequences. All *Mycena* sections in Maas Geesteranus (1992) that had European representatives and available ITS sequence material from GenBank are included. Outcome of interactions between fungal mycelia of *Mycena* species and roots of *Betula pendula* seedlings in axenic microcosm systems are depicted in the table. Species included in the experiments are shown in bold. The pattern of fungal colonization was documented by the fluorescence microscope imaging in the root surface, epidermis, cortex and vascular tissues, scored as presence/absence. Additionally, intracellular colonization and mantle-like structures were scored as presence/absence. +, presence; –, absence; ±, fungal structures were present in some but not all cases; na, images could not be interpreted because of failed staining procedure. *For *M. epipterygia* the average of the four strains included in the experiment is scored on the right-hand side of the figure.

roots and mycelium, and to mimic dark soil conditions. A 5-mm-diameter plug of agar and fungal mycelium was transferred to the middle of the charcoal filter paper, and grown for 5 wk at 20°C in a Termaks KB8400L incubator (Termaks a/s, Bergen, Norway) in darkness before the addition of the seedling (Fig. 2a). In addition to the 21 *Mycena* strains, two EcM fungal species (*Lactarius rufus* and *Paxillus involutus*) were used as positive controls. Each host/fungal strain combination was replicated five times, and five microcosms with no fungus were included as negative controls, making a total of 24 treatments and 120 microcosms.

After 5 wk of fungal growth, one seedling of *B. pendula* was transferred to each microcosm. The entire seedling was inside the microcosm: the shoot was in the upper part of the Petri dish without agar, and roots were in the bottom half with agar (Fig. 2a). The microcosms were sealed with Parafilm (Sigma-Aldrich; now Merch KGaA, Darmstadt, Germany), and the lower half was covered with aluminium foil, to simulate dark soil conditions, and incubated vertically at 20°C for 8 wk (*c.* 12 000 Lux, 16 h : 8 h, light : dark) (Fig. 2a).

To record seedling health and growth, photos of the entire microcosms were obtained every second week in a controlled

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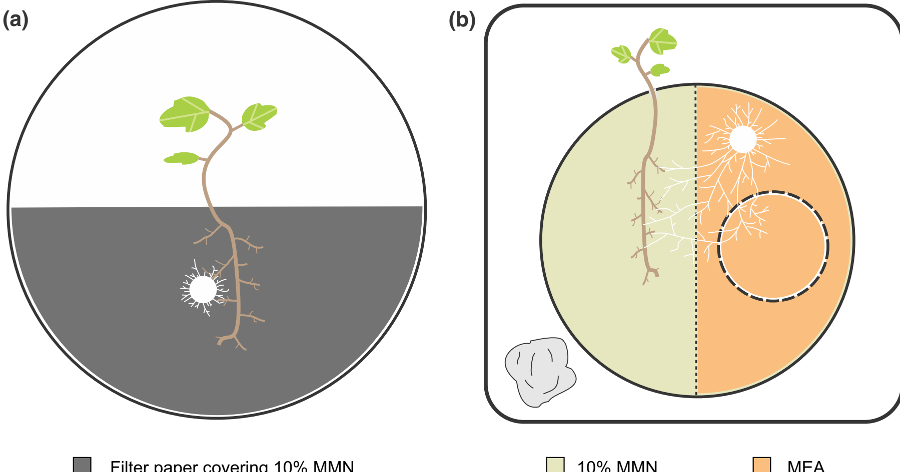

Fig. 2 Setup of microcosms for testing the interaction between *Betula pendula* seedlings and the fungal mycelia of *Mycena* species. (a) Seedlings were grown on a carbon filter paper covering 10% strength modified Melin–Norkrans (MMN) medium in the bottom half of a Petri dish and inoculated with fungal mycelia. (b) A fungal plug was placed on one side of a split Petri dish containing malt extract agar (MEA) medium and grown between 5 and 8 wk before a sterile *B. pendula* seedling was added to the system. A sterile, moistened cotton wool ball was added to the system to keep the seedlings from drying out (lower left of figure). ^{32}P tracer was added to a round chamber on the MEA medium side, and ^{14}C tracer was added to the chamber enclosing the shoot (Supporting Information Fig. S1).

light chamber. Images of each microcosm were taken using a Nikon D600 SLR camera (Minato, Tokyo, Japan). Roots were inspected biweekly under a dissection microscope to record fungal colonization. After 8 wk, close-up images of the root systems were taken using the SLR camera with a LM digital eyepiece adaptor (ID: 7895; Micro Tech Labs, Graz, Austria) for a Nikon SMZ745T dissection microscope at ×0.67 and ×5 magnification. After 8 wk, all microcosms were frozen at −18°C, and kept frozen for further analyses.

Fluorescent microscopy of fine roots

Fluorescent microscope imaging was used to visualize patterns and extent of fungal colonization of the fine roots. Procedures of root tip sectioning followed Smith *et al.* (2017) and staining followed that of Doehlemann *et al.* (2009), except 2 µl of propodium iodide was used to stain plant material red, and 7 µl of Biotium CFTM488A wheat germ agglutinin (Hayward, CA, USA) was used to stain fungal material green. Two to five root tips were selected from two to four microcosms of each *Mycena* strain/plant combination and from control seedlings without or without EcM fungi. Semithin transverse sections (5–10 µm in thickness) of root tips were prepared using a Leica CM1850 cryomicrotome (Leica Biosystems, Wetzlar, Germany), stained, and inspected and photographed using a Leica DM5500 B fluorescent microscope and an A4 fluorescent cube. Images were inspected for fungal colonization as described in Smith *et al.* (2017). Several sections from each root tip were inspected for greater accuracy. Intracellular hyphal growth was only scored as present if hyphae could clearly be seen within whole plant cells in several transverse sections from the same root tip. Mantle-like structures were only scored as present if layers of hyphae were present around all the examined root tip sections.

Data and statistical analyses

Photographs acquired at each time point were analysed using IMAGEJ software (Schneider *et al.*, 2012), where root and shoot growth were traced manually and measured, using the diameter of a 90 mm Petri dish as scale. The total growth of the seedlings (roots + shoot) measured in cm was used to assess differences in growth between the treatments. Initial analyses showed that growth was not dependent on initial size of the seedlings (data not shown), so analyses were conducted on the growth measured at the final time point (week 8).Total growth data were normalized by taking the square root before analysis using ANOVA and Tukey's honestly significant difference to compare means of total growth among treatments (the different fungal isolates). This was carried out using the AGRICOLAE (de Mendiburu, 2017) package in R v.3.4.3 (R Core Team, 2017), which also corrects for multiple comparisons (family-wise error rate).

Labelling with radioactive isotopes

A second experiment was set up based on results from the growth data obtained in the first experiment. Eight *Mycena* species representing poor, intermediate and good seedling growth (determined by seedling growth and appearance of the seedlings in the first experiment, Fig. 3) were selected and utilized with 10 replicates of each species (Table S2). *Paxillus involutus* was used as a positive control, and inoculation with a sterile agar plug was used as a negative control. This gave a total of 100 microcosms. Radioactive isotopes were used to investigate the transfer of nutrients between the birch seedlings and the fungus.

A detailed description of the construction of the microcosms can be found in Methods S1. In brief, 92 mm split Petri dishes (Sarstedt, Nümbrecht, Germany) were used to create a fungal

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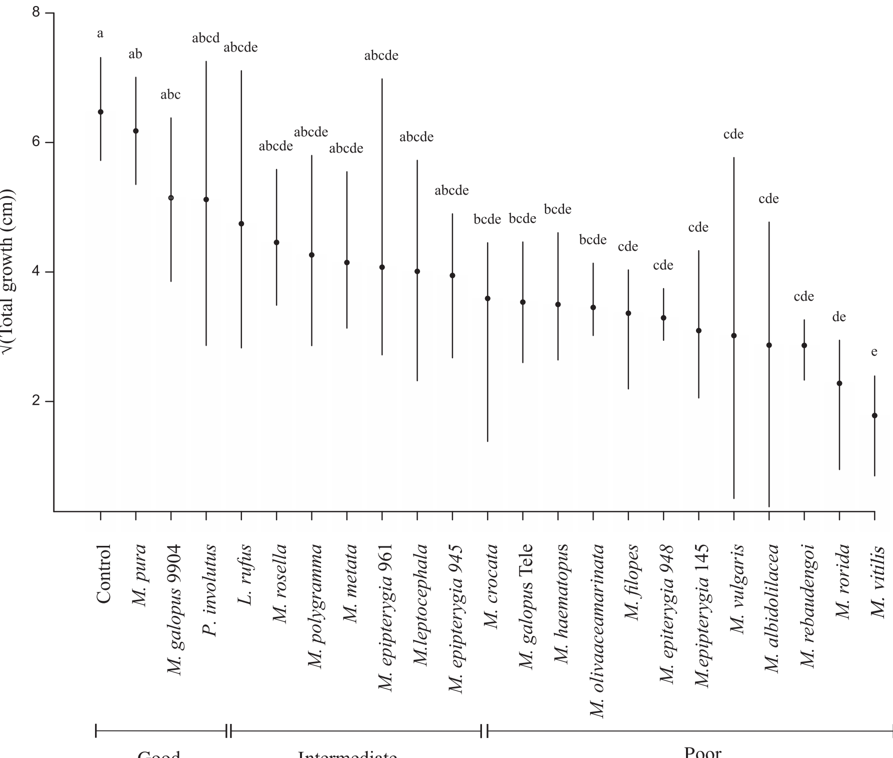

Fig. 3 Total length of roots and shoot (cm) of *Betula pendula* seedlings individually inoculated with *Mycena* species and two ectomycorrhizal fungi, *Lactarius rufus* and *Paxillus involutus.* The bars show the range of the total growth of the seedlings in each treatment, and the mean growth of each treatment is indicated by dots on the bars. Difference between means analysed using ANOVA of square-root-transformed data and Tukey's honestly significant difference test for multiple comparisons at significance level $\alpha < 0.05$. Significantly different groups and ranges are indicated by the letters a–e, that is, the groups sharing the same letter are not significantly different from each other. See Supporting Information Table S3 for details.

and a plant compartment in each microcosm (Fig. 2b), with a barrier to prevent leakage of tracers between the two compartments. On the fungal side of the microcosm, a small Petri dish (35 mm) was used as an extra barrier, preventing leakage of the tracers to the surrounding agar. One half of the dish and the small Petri dish were filled with malt extract agar. The plant side of the split dish was filled with 23 ml 10% MMN. After 5–8 wk of fungal growth (depending on the growth rates), sterile seedlings were added to the plant side of the microcosm, with shoots allowed to grow outside the Petri dish, and roots restricted to the plant side of the microcosm. Microcosms were maintained in incubators at Microlabs, University of Oslo, for 4 wk at 20°C, *c.* 12 000 lux, 16 h : 8 h, light : dark.

The potential for bidirectional transfer of nutrients between the plant and the fungus was studied using radioactive ^{14}C and ^{32}P tracers at the Department of Forest Mycology & Plant Pathology, SLU, Uppsala, Sweden. However, transportation from Oslo to Uppsala resulted in high seedling mortality and it was only possible to investigate a subset of the replicates and species. Half of the microcosms (five of each treatment) were subjected to either ^{14}C addition or ^{32}P addition (see Methods S1; Fig. S1; Table S2 for details).

To investigate whether the *Mycena* species could obtain photosynthesised C from the plant, the shoots of the seedlings were exposed to a pulse of ^{14}C, described in detail in Methods S1 and Fig S1(a). Radiographs were taken to assess uptake of ^{14}C in the seedlings and mycelium after 10 d. To obtain clear radiographs, microcosms were scanned destructively by dissecting out the medium from the Petri dish with both plant and mycelium intact. Radiographs were produced by placing a plastic foil covered Kodak Flexible Phosphor DirectView CR500 system screen (Carestream, Rochester, NY, USA) for radioactivity absorbance, directly onto the medium containing the seedling and mycelium for 4 h. The system was kept in the dark by covering it with aluminium foil. The radiographs were visualized using a Bio-Rad Personal Molecular Imager (Bio-Rad, Hercules, CA, USA), and the QUANTITY ONE 1-D ANALYSIS software program (Bio-Rad).

For the ^{32}P experiment, a 5 µl drop of 72 kBq carrier-free ^{32}P (PerkinElmer, Waltham, MA, USA) as orthophosphate was added directly to the mycelium in the 35 mm Petri dish. Controls (with no fungus) were included to account for passive leakage across the barrier. Systems were allowed to incorporate ^{32}P into the mycelium for up to 3 wk, as initial radiographs showed very slow transport of ^{32}P through the mycelium. After 3 wk, a destructive sampling was carried out, where the plant alone was carefully dissected out of the medium and radiographs were produced as described earlier. It was necessary to analyse the plant separately, as the signal from the fungal side of the plate was very strong, which concealed potential uptake by the plant in the radiographs.

Results

Seedling growth

All seedlings from the first experiment were alive after 8 wk of cultivation in the microcosm systems, with the exception of one that had fallen off the medium and dried out, making a total of 119 seedlings. In most microcosms, with the exception of

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microcosms with *M. pura*, *L. rufus* and negative control, seedlings showed symptoms of decline or stress, either loss of leaves and/or visible red coloration of stems, leaves and roots (Fig. 4c). This is a typical symptom of stress for plants, such as N limitation (Diaz *et al.*, 2006). Examination of the microcosms under a dissection microscope showed that in all microcosms (excluding controls with no fungus), hyphae were growing onto and surrounding the *B. pendula* seedling fine roots (Fig. 4a,b). In total, 117 microcosms were analysed for seedling growth and fungi–root interactions. Only two microcosms were discarded because of contamination. Both the EcM fungi *L. rufus* and *P. involutus*, which were used as positive controls of the inoculation system, had formed typical EcM root tips by week 2 (*P. involutus*) and week 4 (*L. rufus*, Fig. 4d). For one species, *M. bellia*, mycelial growth was covering the roots, and most of the seedling was entirely covered in mycelium, so measurements of seedling growth from photographs were not possible, and it was excluded from the downstream statistical analysis. The seedlings were, however, still alive at the end of the experiment.

The growth of *B. pendula* seedlings varied among inoculated fungal species (Fig. 3), both when accounting for differences in size at the start of the experiment and without standardization (data not shown). In general, the presence of the fungi impaired seedling growth compared with both control and positive control, with some exceptions. Seedlings grown together with *M. pura* grew better than with seven of the other species/strains (Fig. 3; Table S3). However, none of the *Mycena* strains were statistically different from the positive control *L. rufus* (Fig. 3; Table S3). Although not significant at a $\alpha = 0.05$ level, the seedlings in the control treatment (no fungus) had higher mean growth than any other treatment, and *M. pura* and *M. galopus* had a higher mean growth than both positive controls *L. rufus* and *P. involutus*.

Fig. 4 Outcomes of interaction between *Betula pendula* seedlings and *Mycena* species, and the ectomycorrhizal (EcM) fungus *Lactarius rufus*. (a) Root colonization by *Mycena epipterygia* causes no apparent change in root morphology. (b) Root colonization by *M. pura* results in altered root morphology and formation of a pink-coloured morphotype. (c) Root colonization by *M. vitilis* induces stress symptoms, that is, redness, senescence and dieback of leaves. (d) Formation of EcM root tips by *L. rufus*. Magnification of photographs: ×0.67 (a, c, d); ×5 (b).

Fungal colonization of roots

A total of 61 microcosms (two to four microcosms per treatment and two to five root tips per microcosm) were used to assess patterns of root colonization by *Mycena* spp. using cryomicrotome sectioning, differential staining and fluorescence microscopy (Fig. 5). In all images and sections that were inspected, hyphae were present on the surface of the root tips, either loosely attached or aggregated as mantle-like structures, as well as in the epidermis layer of the fine roots (Fig. 1). Hyphae were consistently found in the root cortex of 13 (76%) of species and were found intracellularly in six of the 17 (35%) combinations. This figure may be higher, as for some species the results were inconclusive as a result of failed staining or unsuccessful sectioning (Fig. 1). For one species, *M. rorida*, hyphae penetrated all the way into the vascular tissue of the plant root (Fig. 1). For *M. vitilis*, *M. pura*, *M. leptocephala*, *M. galopus*, *M. rorida*, *M. epipterygia* and some of the investigated root tips with *M. rosella*, mantle-like structures, defined as aggregated layers of hyphae on the surface of the root tips were visible in the transverse sections (Figs 1, 5a–d). For seedlings grown with *M. pura*, root tips covered by hyphae were consistently showing a change in coloration from white to pink, along with thickening and shortening of fine roots, (Figs 4b, S4). In no case was a Hartig-net like structure observed in transverse sections of the roots.

Transfer of ^{32}P and ^{14}C

For the nutrient transfer experiment, 100 initial microcosms were set up, including eight species of *Mycena*, a positive control using *P. involutus* and a negative control. For one treatment, *M. vitilis*, the fungus did not grow, leaving seven *Mycena* spp. However, in the course of the experiment, several seedlings died, as a result of contamination or stress/drying out (see Table S2 for seedling mortality). In total, incorporation of ^{14}C was assessed by radiographs of seedlings and mycelium for 22 microcosms, representing nine treatments. Three species, *M. pura* and *M. rosella*, and positive control with *P. involutus* showed extensive transportation of ^{14}C to root tips (Figs 6a, S2a,b). However, there was no evidence in any of microcosms for transfer of ^{14}C to fungal mycelium from colonized roots.

In the microcosms set up to examine transport of ^{32}P, the mycelium had, in all cases, crossed the barrier between the fungal and plant side of the system, but there were no roots in the

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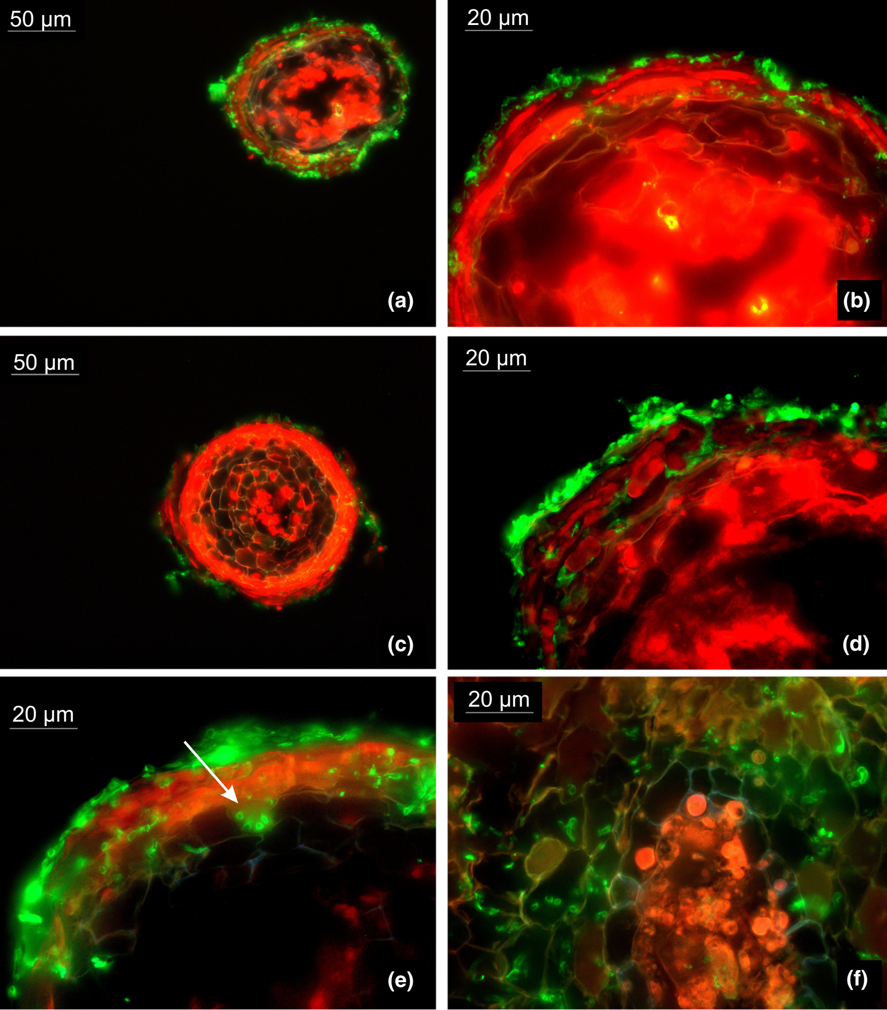

Fig. 5 Semithin transverse sections of *Betula pendula* fine roots colonized by *Mycena* species differentially stained using fluorescence dyes: plant structures stained red and fungal are stained green. (a, b) *Mycena pura* forms a mantle-like structures around the root tip and shows intercellular growth between plant epidermal cells. (c) *Mycena galopus* 9904 is restricted to outer layers of epidermis, but shows hyphal aggregation into mantle-like structure. (d) *Mycena vitilis* forms mantle-like structure, and has some intercellular growth in root epidermis and cortex. (e, f) *Mycena epipterygia* shows intracellular (arrow) and extensive intracellular growth in the cortex of examined fine root sections.

fungal-only compartments. Therefore, the uptake of ^{32}P would only be possible for plant roots through the transport of fungal mycelium. No leakage across the barrier was recorded in controls or outside the mycelium on the plant side of the dish for the treatments. Twenty-two samples, representing nine treatments, were destructively sampled after 3 wk (Tables 1, S2). Only *B. pendula* inoculated with *M. pura* showed uptake of ^{32}P within the plant roots, stem and leaves (Fig. 6c). There was some indication that a seedling grown with *M. rosella* had also taken up ^{32}P, but the signal was very weak (Fig. S3b).

Discussion

An increasing number of molecular studies on root-associated fungal communities are indicating that the taxonomic and functional diversity of these communities are extremely varied (Menkis *et al.*, 2005; Vasiliauskas *et al.*, 2007; Toju *et al.*, 2013; Kohout *et al.*, 2018; Schneider-Maunoury *et al.*, 2018). Surprisingly, species of *Mycena*, a genus known to be important in C and N cycling from plant litter and wood (Boberg *et al.*, 2008; Osono, 2020), are often amongst the most abundant taxa recovered from the roots of a range of plant species (Bougoure *et al.*, 2007; Blaalid *et al.*, 2012; Botnen *et al.*, 2014; Lorberau *et al.*, 2017), and several studies report orchid mycorrhizal associations by *Mycena* species (Martos *et al.*, 2009; Ogura-Tsujita *et al.*, 2009; Zhang *et al.*, 2012). This consistent association with plant roots has raised the idea that some species of *Mycena* may form biotrophic interactions with plant roots, and that the strict assignment of saprotrophy to all species may require a re-evaluation.

In this study, 17 species of *Mycena*, covering a broad range of the phylogenetic diversity within the genus, were inoculated onto the roots of the EcM plant *B. pendula*. We found that all investigated species of *Mycena* associated closely with the fine roots of birch, with all species colonizing tissues beyond the plant root surface (Fig. 1). Our findings are consistent with previous studies showing that *Mycena* species can be active colonizers of living roots (Martos *et al.*, 2009; Menkis *et al.*, 2012; Lorberau *et al.*, 2017; Smith *et al.*, 2017; Kohout *et al.*, 2018). However, they have probably been overlooked in many studies of fungal communities in roots as they would have been regarded as accidental contaminants (e.g. in Liao *et al.*, 2014; see also Selosse *et al.*, 2010, 2018). The results from the present study support the view that the occurrence of *Mycena* in the roots of field-collected plants is not coincidental, but indicate that many species may

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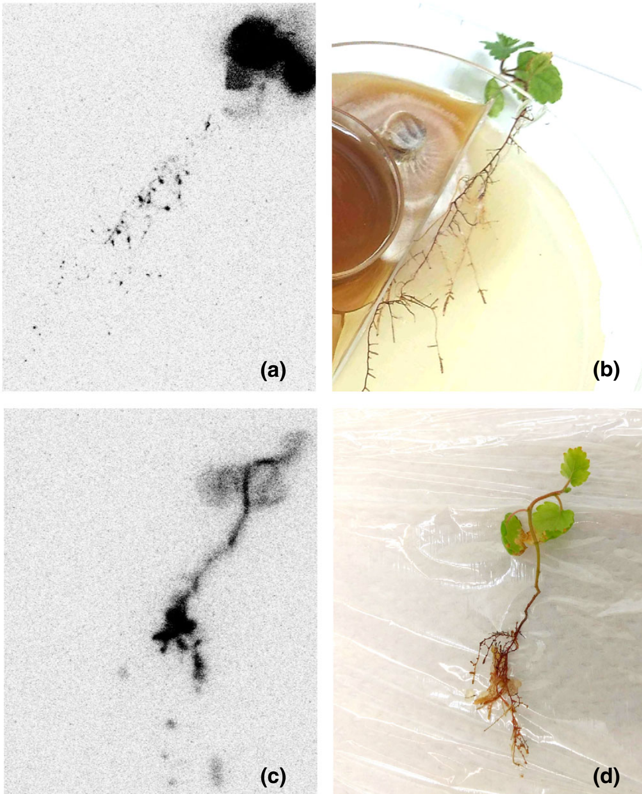

Fig. 6 Radiographs (a, c) and photographs (b, d) of *Betula pendula* seedlings inoculated with *Mycena pura* for 7 wk. (a, c) Radiographs were taken at 10 d after adding ^{14}C to the shoots (a) and 3 wk after adding ^{32}P to fungal mycelium (c). Photographs depict the seedlings immediately before they were radiographed (b, d) and the ^{14}C can be traced (i.e. dark areas in the radiograph) to leaves, stem and root tips (a), and ^{32}P was transferred to the entire seedling (i.e. dark areas in the radiograph, c).

Table 1 Outcome of experiment with *Mycena* species inoculated onto seedlings of *Betula pendula* to investigate transfer of nutrients between the plant and the fungus.

Species	Transfer of ^{14}C	Transfer of ^{32}P
Mycena crocata	–	–
Mycena galopus 9904	–	–
Mycena epipterygia 145	–	–
Mycena leptocephala	–	–
Mycena polygramma	–	–
Mycena pura	(+)	+
Mycena rosella	(+)	(+)
Paxillus involutus	(+)	na
Control	–	–

Nutrient transfer was scored based on radiographs taken 10 d (^{14}C) or 3 wk (^{32}P) after additions of the radiolabelled isotopes. +, confirmation of transfer of radiolabelled isotopes from one partner; –, unconfirmed transfer; (+), partial transfer (i.e. extensive transport to root tips for ^{14}C (Fig. 6a, Supporting Information Fig. S3a,b)) or weak signal for ^{32}P (Fig. S4); na, not investigated.

intimately associate with plant roots. Smith *et al.* (2017) also showed that the four *Mycena* species they investigated colonized *P. abies* roots all the way into the cortex of the root tip sections, but that overall root colonization by other wood-decay fungi was relatively rare (16.9% of the 201 species studied). Thus, a range of species of *Mycena* seem to be effective root colonizers, but their functional role is yet to be determined.

In the present study, several species of *Mycena* impaired seedling growth, in some cases severely (Fig. 3). The mechanism behind this effect is unknown, but could be direct if the species are acting as weak or strong pathogens, or indirect if the mycelia are absorbing nutrients to the extent that they are creating nutrient deficiencies in the seedlings. We did not observe degraded seedling roots, but we did observe hyphae all the way into the vascular tissues of seedlings grown with *M. rorida* (Fig. 1), where the seedlings performed very poorly (Fig. 3). Seedlings grown with *M. pura* and *M. galopus* had enhanced growth compared with other *Mycena* strains, and growth was equal to the known EcM fungi (positive control) (Fig. 3). However, the growth of the control seedlings without an inoculated fungus was as good as the positive control and had the highest mean growth of all, although not significantly (Fig. 3). This can most likely be attributed to competition between plant and fungus for nutrients in this nutrient limited setup (10% strength of MMN). Plant growth can be limited by associating with mycorrhizal fungi and is context-dependent, as Hoeksema *et al.,* (2010) concluded in their meta-study on the benefits of mycorrhizal associations. In 10% of all studies they assessed, plant growth was to some extent reduced by mycorrhizal fungi. The observed that *in vitro* effects of root colonization by *Mycena* species may differ from those *in vivo*, where nutrient availability and competition from other organisms will be important.

Although we found indications of growth benefits to the seedlings from associating with *M. pura* and *M. galopus*, the precise mechanism remains unknown and required further attention. Interestingly, *M. galopus* has been shown to enhance growth in *Vaccinium corymbosum* seedlings, while forming peg-like structures intracellularly (Grelet *et al.*, 2017). Growth benefits to *Pinus contorta* grown in association with *M. galopus* have also been shown, although in this case the authors argue that this was a result of increased recycling of nutrients in microcosms containing a saprotrophic fungus compared with EcM and negative controls (Dighton *et al.*, 1987). Interestingly, only one of the two *M. galopus* strains included in our study seemed to benefit the plant. Thus, there may be variation in interactions among different genotypes within species.

According to Brundrett (2006), the most significant difference distinguishing endophytic and mycorrhizal associations is the absence of substantial fungus-to-plant nutrient transfer in endophytic fungi, and the absence of synchronized development of plant and fungal structures, such as a Hartig net. Using this distinction would support the view that most of the *Mycena* species investigated in the present study were potential endophytes. Although no Hartig net was observed in any of the microcosms, seven *Mycena* species encapsulated fine roots of seedlings with hyphae, forming mantle-like structures characteristic for EcM root tips (Fig. 4, Fig. 5a–e). This was most evident for *M. pura*, where a change in coloration, from white to pink, as well as shortening and thickening of fine roots, was observed in all microcosms (Figs 4b, S4). More importantly, *M. pura* was

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capable of transferring ^{32}P to the seedlings (Fig. 6c), although it is hard to determine the physiological importance of the ^{32}P transfer, as the amount was not quantified. Taken together, these findings indicate potential benefits to the seedlings from *M. pura*, in addition to synchronized development of plant and fungal structures (root tips). Thus, *M. pura* does not act as an asymptomatic endophyte *in vitro* in this study *sensu* Brundrett (2006). Wilson (1995), on the other hand, defined endophytes as fungi or bacteria, which for all or part of their life cycle invade the tissues of plants without causing symptoms of disease. According to this definition, *M. pura* could be classified as an endophyte. The species' ecology clearly requires further investigation.

No evidence was found for ^{14}C transfer from the plant to the mycelia of any *Mycena* species. In contact with *M. pura* the plant transferred ^{14}C to the root tips (Fig. 6), similar to our results for the positive control *P. involutus*, but we could not detect the ^{14}C in the mycelia for either fungal species. This could be a result of too low resolution of the radiographs, and the fact that the ^{14}C was transferred but in concentrations too low to be detected. As an example, arbuscular mycorrhizal symbiosis can be established in roots of plants with very little C to donate to the fungus (Lekberg *et al*., 2010). If the seedlings were particularly stressed and/or nutrient-limited in the *in vitro* experimental setup this may have limited C transfer to the fungi.

Furthermore, in our study, the movement of ^{14}C and ^{32}P was not detectable until after several days or weeks. Previous studies have shown rapid movement (within 24 h) of ^{14}C to the roots in EcM symbiosis (Leake *et al*., 2001). This could suggest that the seedlings in our study are not donating the C to the mycelium, but rather move C-based compounds to the roots for growth, or for defence when interacting with an incompatible EcM partner (Malajczuk *et al*., 1982). *Betula pendula* was used in the present study and it may be possible that the observed interactions are between incompatible symbionts. While further research is necessary to resolve this question, it must be noted that the *M. pura* morphospecies has well-known generalist properties for substrate affinity (Harder *et al*., 2010, 2013). The *M. pura* culture used in the present study originated from a collection made near *Salix reticulata* above the treeline in subarctic Norway, yet shares an ITS similarity of 99–99.5% with the *M. pura* group 3 in Harder *et al.* (2010), which contains members that have been found growing on mixed litter (including *Betula*) as well as on *Pinus*, *Fagus* and *Quercus* leaves. Thus, there is little *a priori* indication of specialization, which could suggest higher potential compatibility with certain putative partners compared with others. An alternative explanation for our observations could simply be that the donation of ^{14}C to the seedlings was too low to detect in the radiographs.

In our study, we show that *Mycena* species readily colonize roots *in vitro*, but several nutritional modes are reported for this genus from the literature: species of *Mycena* have been shown to be saprotrophic (Ghosh *et al*., 2003), form orchid mycorrhizas (Ogura-Tsujita *et al*., 2009; Zhang *et al*., 2012), and associate with green tissues of bryophytes (Davey *et al*., 2013), healthy plant roots (Kernaghan & Patriquin, 2011; Menkis *et al*., 2012; Liao *et al*., 2014; Botnen *et al*., 2014; Lorberau *et al*., 2017; Smith *et al*., 2017), and even roots of nonmycorrhizal plants (Glynou *et al*., 2018). However, the rapid increase in relative abundance of *Mycena* in recently deceased plant roots underpins their important saprotrophic capabilities in natural ecosystems (Kohout *et al*., 2018). They may occupy roots as latent saprotrophs, or their mode of nutrition may change depending on substrate quality and or/state (i.e. dual niches as found in other fungal systems; Selosse *et al*., 2018).

We used a selection of species covering most of the genus *Mycena* in our study. Species phylogenies based only on ITS must be interpreted with caution (Harder *et al*., 2013; Stewart *et al*., 2014; Altermann *et al*., 2014); however, we found no apparent phylogenetic signal in fungal intracellular invasion or the formation of mantle-like structures (Fig. 1). Similarly, there is evidence of polyphyly (i.e. no phylogenetic signal) of the *Mycena* species involved in orchid mycorrhiza (Selosse *et al*., 2010). Thus, there may be a latent genetic ability to evolve opportunistic root invasion (and a wider nutritional range than saprotrophy) among the *Mycena* species.

Mycena species may be particularly adapted to exploit different nutritional modes in arctic and alpine areas. Interestingly, *Mycena* have been recovered in high abundances from plant roots at higher latitudes (Blaalid *et al*., 2012; Botnen *et al*., 2014; Lorberau *et al*., 2017). This could suggest that environmental stress as a result of extreme climatic conditions in cold and nutrient-limited environments may have provided a selective evolutionary pressure facilitating the ability of fungal partners to invade roots and, furthermore, that these harsh conditions may drive a stronger selection pressure for specialization or niche shifts. The *M. pura* culture used in the present study was sampled in an alpine habitat (Table S1) and was the only isolate to clearly show transport of nutrients to the seedling (Fig 6c).

Our study provides a glimpse into the versatile ecological roles of *Mycena*, where the emphasis has been on their capability to form interactions with plants roots *in vitro*. All species were able to colonize roots, but the degree of benefits in terms of seedling growth was highly variable and rarely beneficial. The transition from a saprotrophic to a mycorrhizal lifestyle has been hypothesised to have evolved from endophytic ancestors (Brundrett, 2002, 2006), often referred to as the 'waiting room hypothesis' (Selosse *et al*., 2009; van der Heijden *et al*., 2015). This hypothesis remains controversial, perhaps because it has not been easy to test directly in *in vitro* experiments. However, the hypothesis is supported by transitions from saprotrophy to endophytism to mycorrhizal nutrition within one fungal order, the Sebacinales (Weiß *et al*., 2016). The *Mycena* species included in the present study possess properties that allow for growth within plant roots, and in a few cases nutrient transfer from fungus to plant *in vitro*, and there are intraspecific differences in the interaction patterns (Fig. 3). While it remains unanswered whether these also occur in natural ecosystems, we suggest that *Mycena* may be useful in the attempt to evaluate this hypothesis more directly. Further resynthesis studies with several conspecific fungal strains, further isotopic measurements of nutrient exchange, and fluorescence *in situ* hybridization to visualize the hyphal position inside the roots should be attempted to test this hypothesis more directly.

Recent studies on the genomic mechanisms shaping the evolution of biotrophic interactions indicate some common features of

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biotrophic fungi, such as loss of carbohydrate active enzymes and expansion of secreted effector proteins (Raffaele & Kamoun, 2012; Kohler *et al.*, 2015). However, these studies also demonstrate large differences among the independently originated biotrophic lineages, suggesting that several genomic processes may lead to a biotrophic ecological lifestyle (Raffaele & Kamoun, 2012; Kohler *et al.*, 2015; Hess *et al.*, 2018). Genome sequencing of 21 *Mycena* species is currently in progress or completed at the Joint Genome Institute (JGI, Proposal ID: 1974, Deep Sequencing of Ecologically-relevant Dikarya), including 15 of the species in the present study. Knowledge of the variation of genome content and organization within *Mycena* will help to elucidate the potential diversity of nutritional mode in this genus. The ecology and nutritional modes of *Mycena* are probably far more complex, versatile and flexible than previously believed.

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Author contributions

CBH, HK, ET, AFST and IS designed the project; CBH provided fungal cultures; ET performed the experiments; ET, AM, UV, CBH and SSB, collected, analysed and interpreted the data; ET drafted the manuscript; ET, CBH, HK, UV, SSB, AFST, AM and IS wrote the paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Experimental setup of microcosms for testing the transfer of ^{14}C and ^{32}P between *Betula pendula* seedlings and the mycelia of *Mycena* species, showing how radioactive tracers were added to the microcosms.

Fig. S2 Radiographs of *Betula pendula* seedlings inoculated for 7 wk with the EcM fungus *Paxillus involutus* and *Mycena rosella.*

Fig. S3 A photograph and a radiograph of *Betula pendula* seedling inoculated with *Mycena rosella*, which was supplied with ^{32}P.

Fig. S4 Microscope images of root tips colonized by *Mycena pura* without and with fluorescent light, indicating that the pink coloration of root tips observed in microcosms where *Betula pendula* seedlings were inoculated with *M. pura* is of fungal origin.

Methods S1 Detailed descriptions of obtaining fungal cultures, molecular methods and experimental setup.

Table S1 Overview of all *Mycena* cultures and EcM positive control included in an inoculation experiment with *Betula pendula.*

Table S2 Overview of the *Mycena* species inoculated onto *Betula pendula* seedlings setup, an experiment with radioactive isotope (^{14}C and ^{32}P) labelling**.**

Table S3 Comparison of growth means using Tukey's HSD (honestly significant difference) test for *Betula pendula* seedlings grown with species of *Mycena* and positive controls, the ectomycorrhizal *Lactarius rufus* and *Paxillus involutus* and control (*B. pendula* seedlings with no fungus).

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