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Mycorrhizal syntheses between *Lactarius* spp. section *Deliciosi* and *Pinus* spp. and effects of grazing insects in Yunnan, China

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Draft

Abstract

Ectomycorrhizal fungi (EMF) in *Lactarius* sect. *Deliciosi* produce valuable edible mushrooms. Market supplies are harvested from natural populations. Sustainable cultivation could increase commercial crop production. The first step in EMF cultivation is the production of host seedlings well-colonised by the target species. The aim of this study was to compare the efficiency of vegetative versus spore inoculum for controlled mycorrhizal synthesis between *Lactarius* and *Pinus* species native to China. Inoculated seedlings were incubated in a glasshouse for up to 14 months. Mycorrhizae were synthesised, using vegetative inoculum, for 13 distinct combinations of five *Pinus* and four *Lactarius* species, 12 of these unprecedented. Spore inoculation was not successful. The successful mycorrhization presented here provides a foundation for establishing mushroom orchards, with *L. deliciosus* x *P. yunnanensis* or *P. radiata*, *L. hatsudake* x *P. yunnanensis* or *P. tabuliformis*, and *L. vividus* x *P. massoniana* or *P. radiata* appearing promising symbionts for cultivation. From 5 months following inoculation, mycorrhizal seedlings underwent extensive insect grazing. Adult forms of *Bradysia impatiens* were the most frequent insects caught on sticky traps, while their larvae were observed foraging through roots. The control of insects in the nursery will be critical to large-scale production of mycorrhizal seedlings.

Keywords

Lactarius section *Deliciosi*, *Pinus*, mycorrhizal synthesis, insect damage, *Bradysia impatiens*

Introduction

Forest mushrooms that belong to *Lactarius* section *Deliciosi* (Fr.: Fr.) Redeuilh, Verbeken & Walley are harvested worldwide for food and income. These fungi live in mycorrhizal symbiosis with a range of tree species, but are commonly associated with hosts in the Pinaceae family (Nuytinck et al. 2007). The species defining the section, *Lactarius deliciosus* (L.: Fr.) Gray, is the best known of all *Lactarius* spp., and is strictly associated with pines. It is consumed in many countries and is known by different local names, e.g. Lactaire délicieux (France), Pinetell (Spain), Reshik (Russia), Songrugu (China), and Saffron milk cap (Commonwealth nations). Worldwide, according to modern taxonomy and cultural knowledge, there are ~40 edible species listed in the section. These include *L. sanguifluus* (Paulet) Fr., *L. semisanguifluus* R. Heim & Leclair, *L. vinosus* (Quélet) Bataille, *L. akahatsu* Tanaka, *L. hatsudake* Nobuj. Tanaka, *L. indigo* (Schwein.) Fr., *L. subindigo* Verbeken & E. Horak and some recently described species in China, e.g. *L. vividus* X.H. Wang, Nuytinck & Verbeken sp. nov., *L. hengduanensis* X.H. Wang sp. nov., and *L. pseudohatsudake* X.H. Wang sp. nov. (Wang et al. 2015; Wang 2016). Apart from *L. deliciosus* and *L. sanguifluus*, which appear to be distributed both in Europe and Asia, intercontinental con-specificity is very low; America, Asia and Europe have their own endemic species in the section (Flores et al. 2005; Nuytinck et al. 2007). Asia may harbour the highest diversity, with about nine edible species from this section present on local markets in southwest China (Yunnan, Guizhou, Sichuan) including *L. deliciosus*, *L. hatsudake*, *L. pseudohatsudake*, *L. hengduanensis*, *L. abieticola* X.H. Wang, *L. subindigo*, *L. deterrimus-fennoscandicus* Verbeken & Vesterh complex, *L. vividus* and *L. akahatsu* (Wang XH, unpublished). Another two endemic species, one in Guangdong and one associated with *Picea* in Midwest China, have yet to be formally described (Wang XH, unpublished). Therefore, China alone may host over a quarter of the edible species in this section. Naturally distributed in the Northern Hemisphere, several species are now present in the Southern Hemisphere as a result of accidental (Sulzbacher et al. 2018) or controlled

introductions by humans, e.g. plantation of seedlings mycorrhized by *Lactarius deliciosus* that yielded fruiting-bodies (Wang et al. 2012).

Until now, edible mycorrhizal fungi (EMF) have been almost exclusively harvested from the wild (Wang and Hall 2004). They provide a valuable food and income in many rural areas, and *L. deliciosus* s. l. is no exception (Tomao et al. 2017). However, harvesting wild EMF may quickly become unsustainable in a competitive economic environment, and can lead to severe environmental issues such as over-harvesting and habitat damage (Hall et al. 2003). This results in a dramatic decline of mushroom yields, a loss of revenue for local communities, and can threaten the survival of fungal populations or species. However, the cultivation of EMF, and integration with myco-silviculture practices, has the potential to alleviate pressures on wild mushroom populations and on the environment (Savoie and Largeteau 2011). As in many other food sectors, the cultivation of commercially valuable species can increase overall production, in comparison to relying on natural systems exclusively (Guerin-Laguette et al. 2017). Furthermore, research on EMF cultivation advances the understanding of basic mushroom biology and ecology, which in turn can inform conservation efforts aimed at protecting fungi in their natural environment, by defining appropriate forest management methods.

Great progress has been made in the cultivation of EMF since the 1970s, when the development of truffle cultivation based on the production of mycorrhizal seedlings began in Europe (Chevalier and Grente 1978). Modifications of this approach are now used worldwide (Hall et al. 2007; Morcillo et al. 2015; Sourzat 2017). Improved mycorrhization techniques for other edible mycorrhizal mushrooms have also occurred, e.g. *L. deliciosus* (Guerin-Laguette et al. 2000a), *L. akahatsu* (Yamada et al. 2001), *L. hatsudake* (Tang et al. 2008), *L. indigo* (Flores et al. 2005), *Rhizopogon roseolus* (Visnovsky et al. 2010), *Tricholoma matsutake* (Yamada et al. 1999; Guerin-Laguette et al. 2000b), *Amanita caesareoides* (Endo et al. 2013), *Boletus edulis* (Endo et al. 2014), and *Cantharellus cibarius* (Ogawa et al. 2016). However, in contrast to truffles, only a handful of edible mushrooms have been

successfully cultivated in the field following the plantation of mycorrhizal seedlings, i.e. *Suillus granulatus* and *L. deliciosus* (Poitou et al. 1989), *R. rubescens* (Wang et al. 2002), *R. roseolus* (Visnovsky et al. 2010), and of these, only *L. deliciosus* has been the subject of continual monitoring of yields over time (Guerin-Laguette et al. 2014, 2017). New Zealand is the only country in the world where saffron milk cap is currently cultivated on private farms (<https://www.neudorfmushrooms.co.nz/>) and in trial plantations, supplying mushrooms to a wide range of customers (Guerin-Laguette et al. 2017). These recent achievements make it possible to envision multiple long-term benefits offered by efficient and sustainable cultivation of EMF: non-meat sources of protein, reforestation and CO₂ fixing, diversification and increased land value, income for local communities, improved tree growth and health, soil protection, habitats for species and recreational and educational activities, including myco-tourism (Büntgen et al. 2017). These early achievements also stress the necessity of supporting and expanding research aimed at developing commercially viable and sustainable farming technologies for EMF, including the cultivation of new species in different regions of the world.

The aim of this study was two-fold: (1) to assess the feasibility of controlled mycorrhizal synthesis between four native Chinese species of *Lactarius* sect. *Deliciosi*, i.e. *L. deliciosus*, *L. hatsudake*, *L. akahatsu* and *L. vividus* and five pine species, of which four are native to China (*P. yunnanensis*, *P. massoniana*, *P. armandii*, *P. tabuliformis*) and one is exotic (*P. radiata*), and (2) to acclimatise the synthesised mycorrhizae under glasshouse conditions, i.e. to ensure their continuous development and progressive colonisation of the roots until they appear abundant and dominant on the root systems. We used a vegetative inoculum approach for mycorrhizal synthesis, which was previously proven to be very effective (Wang et al. 2012). We also assessed whether spore inoculum, potentially a cost-effective technique, could produce mycorrhizae. The different *Pinus-Lactarius* species combinations used were selected based on natural associations observed in Yunnan and Guizhou. A *L. deliciosus* isolate grown in New Zealand and *Pinus radiata* were used as benchmark symbionts (Guerin-Laguette et al. 2014). Finally, we also report on the unexpected severity of insect

142 damage on mycorrhizae and roots detected five to six months after the inoculation and which
143 worsened during the rest of the acclimation stage, the growing period in the nursery during which
144 the synthesised mycorrhizae further develop over the whole root system. This phenomenon has
145 been poorly documented so far in the literature.

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Materials and methods

Mycorrhiza synthesis techniques and comparison of their efficacy

Most syntheses described in this study were obtained using vegetative or spore inoculum in nursery containers. Whenever possible, the same host/fungi combinations were tested in the same growing conditions, enabling us to compare the efficacy of both techniques. In addition, we used other, non-container, techniques based on vegetative inoculum, i.e. filter papers in growth pouches (Fortin et al. 1980) or square Petri dishes filled with growth medium. These techniques allowed us to monitor the outcome of inoculation continually and to increase the number of *Pinus-Lactarius* combinations tested. Non-inoculated seedlings were included as a control reference for non-mycorrhizal roots and, in case of containerised seedlings, as a means to assess the effect of *Lactarius* sect. *Deliciosi* mycorrhization on host plant growth.

Fungal material

Lactarius species and isolates used in this study were obtained from basidiomata purchased in markets or harvested from natural sites (Table 1). Whenever possible, specimen vouchers were made and conserved at the Kunming Institute of Botany (KIB) herbarium (Table 1). Morphological analyses followed the method described by Wang et al. (2009). Pure cultures were obtained by propagating the flesh of fresh basidiomata on m+p agar medium made by mixing equal volumes of Modified Melin-Norkrans (MMN, Marx 1969) and Potato Dextrose Broth (or PDA for agar medium), pH 5.6, with the following modifications/additions per L of MMN: 10 g glucose (instead of sucrose), 0.5 g yeast extract, 0.5 g malt extract, 0.15 g MgSO₄, 50 µg thiamine, and 1.5 mL of 1% ferric citrate in 1% citric acid (instead of FeCl₃). Pure fungal cultures were grown in the dark at 23°C. All

manipulations of pure fungal cultures prior to inoculation were performed in a Class II safety cabinet (Airtech, Jiangsu, China).

DNA analyses of fungal and insect material

Total DNA was extracted from dried vouchers (20–30 mg) and pure mycelial cultures (50–150 mg) using a modified CTAB procedure of Gardes and Bruns (1993). The internal transcribed spacer (ITS) rDNA region was amplified by PCR using the primers ITS1F and ITS4 previously described by Gardes and Bruns (1993) and White et al. (1990), respectively. PCR analyses were carried out on a LifeECO thermocycler (Life Bioer Technology, China) in a final volume of 25 μ L containing 16.2 μ L of H₂O, 2.5 μ L of 10X buffer (Takara Bio Inc., Japan), 1.5 μ L of 1 mM dNTP mix (Takara), 1 μ L of 1% BSA (Genview), 0.5 μ L of 25 mM MgCl₂ (Takara), 0.3 μ L of Taq DNA polymerase (5 U/ μ L, Takara), 1 μ L of each primer (5 μ M, Sangon Biotech, China), and 1 μ L of DNA template. PCR conditions consisted of preheating at 95°C for 5 min, 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min.

During the acclimation stage of seedling growth, insects were surveyed with sticky traps, and captured insects were identified by both morphological characters and molecular approaches (see also 'Mycorrhiza, rhizomorphs and insect analyses' section). DNA was extracted from one adult fly of each different insect morphotype using the HiPure™ Insect DNA Kit (Magen, China). The mitochondrial cytochrome c oxidase subunit I was amplified by PCR using the primers LCO1490 and HCO2198 (Folmer et al. 1994). PCR reactions with Takara reagents were performed in a final volume of 15 μ L containing 8.775 μ L of H₂O, 1.5 μ L of 10X reaction buffer, 1.5 μ L of 0.2 mM dNTP mix, 1.5 μ L of 25 mM MgCl₂, 0.125 μ L of Taq, 0.3 μ L of each primer (10 μ M, Sangon Biotech), and 1 μ L of DNA extract. PCR conditions consisted of 96°C for 3 min, followed by 40 cycles of 95°C for 15 s, 56°C for 15 s, 60°C for 2.5 min, and 60°C for 10 min as a final extension step. Sequences of fungal and insect PCR products were generated in one direction by Tsingke Biology (Kunming, China) and edited

manually using Sequencher™ 4.1.4 (Gene Codes, USA). Sequences were queried against the NCBI public database GenBank with the BLASTn algorithm for identification. All fungal sequences generated in this study have been deposited in GenBank (Table 1).

Plant material

Seeds were obtained from the following sources: *Pinus yunnanensis* Franch. (Mei Di, Kunming), *P. massoniana* Lamb., and *P. tabuliformis* Carr. (Blue Sky Seed Company, Jiangsu, China), *P. armandii* Franch. (Ciba market, Kunming), and *P. radiata* D. Don (Proseed, New Zealand). The seeds were surface sterilised by immersing them in 30% hydrogen peroxide for 5 min (*P. yunnanensis*, *P. massoniana*) or 10 min (*P. radiata*, *P. armandii* and *P. tabuliformis*), and then rinsed thoroughly in distilled water. They were germinated on a mixture of perlite (Jing-rui, Kunming) and peat (Jiffy, The Netherlands) (1:1, V:V), which had been previously sterilised by autoclaving (1 h at 121°C). Given the time required to prepare seedlings and inocula, the age of seedlings at the time of inoculation varied between 6 and 18 weeks (Table 2). All seedlings were grown under natural light in a glasshouse (e.g. 169 $\mu\text{mol}^{-2}\cdot\text{s}^{-1}$ inside, in June) on the KIB campus, which was fitted with openable roof panels and an extractor fan cooling system. Unfortunately, the cooling system was not able to maintain the temperature below 27°C. The glasshouse was newly built but surrounded by abundant vegetation on two sides, which could have been a source of insects. Roof panels were opened to lower the temperature during sunny periods. Seedlings were watered with tap water three times a week, until inoculation.

Mycorrhizal syntheses in nursery containers using vegetative inoculum

A mixture of coarse vermiculite:perlite:peat:pine bark (4:2:1:1, V:V) impregnated with distilled water (14%, w/v dry substrate) was autoclaved for 90 min at 121°C. Vermiculite (3–8 cm particles) and pine

bark were sourced from Niu-niu Gardening (Hebei province) and Mu-mu Biology (Zhejiang province), respectively. Two hundred and fifty mL of mixture was distributed into 300-mL autoclavable plastic vessels (Mu-mu Biology) whose lids were fitted with aeration holes covered with sterilised PTFE filter membranes (pore size < 0.2 µm). Seventy-five mL of liquid m+p were added to each vessel, which was then autoclaved for 30 min at 121°C. Agar fungal cultures were made on 90-mm Petri dishes (x3 c. 100 mm² agar plugs per plate) and grown for 8 weeks. The resulting mycelia were cut into c. 100 mm² pieces and inserted homogeneously within the mixture of each vessel (about eight pieces per vessel, i.e. the mycelia covering half a Petri dish). The vessels were incubated in darkness at 23°C 8–10 weeks, allowing the *Lactarius* mycelium to colonise the mixture. Prior to use, the content of all vessels was observed under the dissecting microscope to assess *Lactarius* mycelial growth (rhizomorph-like hyphae are easily detectable) and check for possible contamination. For each fungal isolate tested, a subsample (c. 5 mL) of the inoculated mixture collected from one random vessel was plated onto m+p agar medium to check the inoculum viability and purity. The inoculation of seedlings took place in 142 mm high, 67 mm square tubes ‘olive pots’ (Daltons Ltd, New Zealand). About a fifth of the inoculated mixture in each vessel was then carefully taken (minimising breaking) and applied directly to the top third part of the root systems of 6- to 18-week-old seedlings. Inoculated seedlings were potted and grown in the same mixture (without addition of liquid m+p), which had been previously autoclaved (1 h at 121°C) in 5-L bags twice at 48 h intervals. Non-inoculated control seedlings were generated by adding the same amount of mixture grown in vessels in exactly the same way as the inoculated treatments, but the mixture had not been inoculated with *Lactarius* agar cultures. All seedlings used for inoculation were devoid of mycorrhization. Three seedling series were produced at three inoculation dates, i.e. 3 December 2015, 26 January, and 22 March 2016 (Table 2). Replicate treatment and control seedlings were made for each of the combinations tested (see Table 2 for replicate numbers). Seedlings in each series (i.e. inoculation date) were arranged according to a randomised block design on grid tables. The grid tables supporting the seedlings were located in a mesh-covered (0.5 x 0.7 mm) metal frame (c. 3 x 4 x 2 m,

width x depth x height) set up within the glasshouse. Temperature and humidity inside the glasshouse were monitored with an electronic thermometer (Wan Yunshan, Fujian) in June and July 2016. Seedlings were watered with tap water three times a week. Three months after inoculation, 2.5 mL of slow release Osmocote® fertiliser (No5, LILY'S GARDENING, Shanghai, N:P:K / 14:13:13) was added per container. Needle height, i.e. the distance between the collar (point of attachment of the first needles on the stem) to the apex of the longest needle, was measured for all inoculation treatments¹.

Mycorrhizal syntheses in pouch or square dish using mycelium grown in liquid medium

The pouch method was used as described by Guerin-Laguet et al. (2000a). Pouches were filled with the W solution (reverse osmosis water with MES biological buffer, pH 5.7) used by these authors. Square dishes (10 x 10 cm, Zhen Xin, Jiangsu) were filled with the previously described sterile mycorrhization mixture. The root system of a germinated seedling was laid over the substrate while the stem and shoots protruded from the pouch or dish. Six-week-old agar cultures were cut in fine pieces (c. 10–20 mm³) which were transferred to a thin layer of liquid m+p medium in 90-mm Petri dishes and grown for 2 months. About a quarter of the mycelium covering the plate was used to inoculate the seedlings: the mycelium was cut into c. 0.5 to 2 cm² pieces, which were applied directly onto short lateral roots of the root systems. Liquid grown mycelia provide a better adherence to the roots than rigid mycelial agar pieces. Pouches and plates were assembled under a laminar flow hood. Plates were sealed with plastic tape and covered with aluminium foil. Pouches and plates were incubated in a growth cabinet (Yi Heng Technical) under 18/6 h light/dark cycle at 23°C, and a photosynthetic active radiation (PAR) of 100-μmol.m⁻².s⁻¹ (400–700 nm). Plant/fungal isolate combinations tested are listed and shown².

¹ Supplementary material Fig. S1

² Supplementary material Fig. S2

Mycorrhizal synthesis in olive pots using spore slurries

In parallel with the mycelial inoculations, we attempted, using the same potting mixture, mycorrhizal synthesis from spores using six combinations of plant/fungus species (eight plant/isolate combinations) involving two mushroom and five pine species: *P. armandii* x *L. vividus* LV-115; *P. massoniana* x *L. vividus* LV-107; *P. radiata* x *L. vividus* LV-115; *P. tabuliformis* x *L. hatsudake* LH-122; *P. yunnanensis* x *L. hatsudake* LH-108 or LH-122 and *P. yunnanensis* x *L. vividus* LV-115 or LV-118. Spore suspensions were made from full caps (without the stipe) air-dried at room temperature and stored at 4°C. The pilei from dry basidiomata were finely ground to powder and suspended in distilled water. A haemocytometer (Qiujiang, China) was used to determine the number of spores per g of dry fungal tissues for each basidiomata (a drop of Tween® 80 was added to suspensions to enable spore dispersion). A spore suspension was prepared for each of the *Lactarius* species and stored overnight at 4°C before use. The spore inoculations were attempted only with *L. vividus* and *L. hatsudake* since we could not source basidiomata of the other species at the time. When seedlings were 8 weeks old, spore suspensions were inserted into a hole (c. 3 cm deep) next to the stem, using a pipette fitted with a blunt 5-mL tip. Each seedling received 5 mL x 2 injections or about 10 M spores. A second inoculation of 50 M spores per seedling was repeated 3 months later using freshly ground pilei stored at 4°C. Control, non-inoculated seedlings were injected with sterile distilled water. Five replicates were made for each of the combinations tested.

Mycorrhiza, rhizomorph and insect analyses

The root systems of seedlings grown in containers were assessed for mycorrhiza formation and insect activity at three times following inoculation, approx. 5–6, 8–9, and 11–14 months after inoculation (Table 2). The dates of the assessments differed since they depended on the inoculation date for each series. However, the third and last assessment was performed in early February 2017 for all series, i.e. from 10 to 14 months after inoculation. Seedlings inoculated in pouches and square

dishes were assessed weekly for mycorrhiza formation between 2 to 8 weeks after inoculation. Successfully colonised seedlings were transferred to containers. Mycorrhiza assessments were non-destructive. A simple quantification of the colonisation was made as follows: 'High' when mycorrhizae were abundant and easily detected on the root systems, 'Low' when their detection required up to several minutes inspecting the root system, including looking for mycorrhizae deep within the root ball. Furthermore, when replicate syntheses were made for a given host/fungus association, the number of successfully mycorrhized seedlings indicated the consistency of the mycorrhization process. All observations were performed under a dissecting microscope (Leica, Germany) fitted with a DFC450 C digital camera. Mycorrhizae of *Lactarius* spp. were identified following morphological characteristics (Guerin-Laguet 1998; Guerin-Laguet et al. 2000a; Wang et al. 2002). Orange to green rhizomorphs were also identified visually and their presence/absence was recorded for each seedling over the three assessments.

Insects outside the root system (mostly flying insects) were captured on 10 sticky fly traps (25 x 15 cm Jida, Shanghai) placed amongst the containers between July and September 2016. A preliminary morphological analysis sorted the caught insects into groups. One insect representative of each group was further analysed through DNA sequence analysis. Following the discovery (first mycorrhiza assessment) and the worsening (second assessment) of the insect damage, each seedling received 30 mL of Shi-qi insecticide solution (active substance 50% acetamiprid, Guoguang, Sichuan province; 1 g per 2.5 L as per the manufacturer's instructions) applied three times as a liquid over the potting mix, on 22 November, 30 December 2016 and on 30 January 2017.

Results

Fungal identification and pure mycelial cultures

All fresh basidiomata used for propagation were identified to species by both morphological and molecular analyses. ITS sequences of all pure cultures (see GenBank accession numbers in Table 1) were aligned and compared with reference sequences BLAST and confirmed to be target species. When basidiomata were available, sequences of pure cultures were matched to those of the corresponding specimens. Mycelia were successfully grown in liquid, agar and vermiculite-based mixture and no contaminants were observed. All subsamples of the inoculated mixture in vessels generated characteristic, contaminant-free, *Lactarius*-like colonies on m+p agar medium.

Mycorrhiza formation and seedling growth in nursery containers

Using vegetative inoculum

From the first assessment, *Lactarius* sect. *Deliciosi* mycorrhizae were obtained for nine distinct combinations of plant and fungal species: *P. armandii* x *L. vividus* (Figure 1j); *P. radiata* x *L. deliciosus* (Figure 1a-e); *P. radiata* x *L. vividus* (Figure 1i); *P. tabuliformis* x *L. akahatsu* (Figure 1g); *P. tabuliformis* x *L. hatsudake* (Figure 1f); *P. yunnanensis* x *L. deliciosus* (Figure 2 a-f); *P. yunnanensis* x *L. hatsudake* (Figure 1h); *P. yunnanensis* x *L. vividus* (Figure 2h) and *P. massoniana* x *L. vividus* (Figure 2i-k), corresponding to 13 distinct combinations of plants and fungal isolates (Table 2). All combinations tested successfully produced the expected *Lactarius*-like mycorrhizae, except *P. massoniana*/L. *akahatsu* LA-JPN (Table 2). Synthesised mycorrhizae appeared similar for all species tested, i.e. a thick vivid orange mantle when young (Figures 1 and 2) to occasionally slightly green for *L. hatsudake* (Figure 1f) or with a more pronounced vivid orange colour for *L. vividus* (Figure 1j), with a surface of frequent fine cystidia observable at high magnifications (Figures 1i-j and 2f, i), and with

laticiferous hyphae on the mantle surface (Figure 2e). Mycorrhizae branched frequently and formed clusters as they developed (Figures 1 and 2).

The colonisation rates were 'High' for all combinations tested, as illustrated in Figures 1 and 2, but 'Low' for *P. armandii* x *L. vividus* LV-115 and for one isolate of *L. hatsudake* (LH-23d) with *P. tabuliformis*. *Lactarius hatsudake* LH-122 produced 'High' mycorrhization rates on *P. tabuliformis* (Figure 1f). Mycorrhizae were successfully produced in all replicate seedlings of eight host/isolate combinations out of the thirteen tested (Table 2). The following five combinations had some replicate seedlings that failed to form mycorrhizae: *P. tabuliformis* x *L. akahatsu* LA-QP and *P. yunnanensis* x *L. vividus* LV-115 (four out of five replicates were successful); *P. tabuliformis* x *L. hatsudake* LH-122 and LH-23d (8/10 and 1/5 successful, respectively), and for *P. armandii* x *L. vividus* LV-115 (1/4 successful) (Table 2). Overall, the inoculation process was very efficient, with 81 seedlings successfully mycorrhized out of 97 inoculated, i.e. a success rate of 84% (Table 2). In terms of 'mycorrhization power' of the *Lactarius* sect. *Deliciosi* species tested, regardless of the host pine species, *L. deliciosus* was the most efficient (43 mycorrhized out of 43 inoculated seedlings, i.e. a success rate of 100%) followed by *L. vividus* (20 out of 24, i.e. 83%), *L. hatsudake* (14 out of 20, i.e. 70%), and *L. akahatsu* (four out of 10, i.e. 40%) (Table 2). Characteristic orange to green mycelial rhizomorphs (Figure 1a, e, f, g, h and Figure 2h, j, k) were observed for all *Lactarius* species from the first assessment. As expected, none of the control, non-inoculated, seedlings produced *Lactarius* sect. *Deliciosi*-like mycorrhizae or rhizomorphs. Six months following inoculation, we detected a slight positive effect of *Lactarius* sp. mycorrhization on the back-transformed mean heights of needles, i.e. for *P. radiata* x *L. deliciosus* LD-74 (19.60 cm), *P. yunnanensis* x *L. hatsudake* LH-122 (14.36 cm), and *P. yunnanensis* old x *L. deliciosus* LD-74 (17.34 cm) in comparison with the same non-inoculated plants (16.40, 11.40, and 14.80 cm, respectively)³.

³ Supplementary materials Table S1 and Fig. S1

The number of seedlings harbouring fresh *Lactarius* mycorrhizae dropped considerably in the second assessment (i.e. 9 months following inoculation), with only 17 of the 81 seedlings still having such mycorrhizae (Table 2). At the last assessment, 2.5 months after the start of the insecticide treatment, only 14 seedlings had fresh *Lactarius* mycorrhizae (Table 2). All of these were inoculated in late January or late March 2016.

No mycorrhizal contaminants (i.e. non-*Lactarius* species) were detected on any of the seedlings over the course of the experiment, except for one seedling (*P. massoniana* x *L. vividus* LV-141) in which a few unidentified *Suillus*-like whitish mycorrhizae were observed at the last assessment, along with *L. vividus* mycorrhizae (data not shown). Secondary colonisation by unidentified saprobic fungi was common in the root system of seedlings, but did not appear to affect mycorrhizal synthesis.

Using spore inoculum

Six months following spore inoculation, i.e. when insect damage had not yet killed plants and had only partially damaged mycorrhizae obtained from vegetative inoculum, no mycorrhizae could be detected despite all the six host/species combinations having successfully produced mycorrhizae using mycelial inoculum (this study). In particular, two isolates involved in five of these successful combinations, i.e. *P. armandii*, *P. radiata*, *P. yunnanensis* x LV-115 and *P. tabuliformis*, *P. yunnanensis* x LH-122, failed to produce mycorrhizae from spores on the same plants, despite using exactly similar growing conditions and the fact that vegetative and spore inocula were obtained from the same fruiting-bodies.

Additional mycorrhizal syntheses in growth pouches, square dishes and containers

Successful mycorrhiza formation was obtained between a further four fungus/pine species combinations: *P. radiata* x *L. hatsudake*, *P. tabuliformis* x *L. deliciosus*, *P. tabuliformis* x *L. vividus*, and

P. tabuliformis x *L. hengduanensis* sp. nov. All combinations produced 'High' mycorrhization rates except *P. tabuliformis* x *L. hengduanensis*⁴.

Insect feeding on roots and mycorrhizae

At the first assessment, 5 to 6 months following inoculation, along with the fresh, active mycorrhizae described above, insect damage was observed on both mycorrhizae and roots (Figure 3 – i.e. 'eaten' mycorrhizae; Figure 3b-g – 'eaten' roots) (Figure 3h, i, j, l). Root/mycorrhizal damage was recorded for 37 out of 134 seedlings (28 %), including two P_{Yo} control seedlings (Table 2, Figure 3h). However, mycorrhizal seedlings affected by insect grazing still showed numerous, undamaged, fresh *Lactarius* mycorrhizae (Figures 1 and 2).

By the second assessment, 8–9 months following inoculation, insect damage was recorded in 90 out of 134 seedlings (67%), including 17 control seedlings across all pine species except *P. massoniana* (Table 2). Insect feeding eliminated all fresh *Lactarius* mycorrhizae from the first series of seedlings that were inoculated in early December 2015 (Table 2). Furthermore, symptoms such as yellow needles⁵ were apparent across treatments, with *P. yunnanensis* appearing to be the most affected. Of the 38 seedlings (28%) that died by the second assessment, 28 were of *P. yunnanensis*, five of *P. radiata*, three of *P. armandii* and two of *P. tabuliformis*. Regrowth from dead mycorrhizae was frequent, and there were abundant root hairs without mycorrhizae (Figure 3m, n), suggesting that the fungal partner did not survive the insect damage.

At the final assessment, the seedlings that had conserved their mycorrhizae at the second assessment and that received the insecticide, showed fresh *Lactarius* mycorrhizae without signs of feeding damage (Table 2). However, seedlings that had lost their mycorrhizae by the second assessment did not recover their mycorrhization despite the insecticide treatment.

⁴ Supplementary material Fig. S2

⁵ Supplementary material Fig. S3

Insect identification

Adult insects found on the root systems were not identified to species level but *Collembola* spp. were easily recognised by morphology and were commonly encountered (data not shown). Live fungus gnat larvae were detected on four mycorrhizal seedlings, two *P. radiata* x LD-74, one *P. radiata* x LV-115, one *P. yunnanensis* x LH-122 (Figure 3i), and one *P. radiata* control (Figure 3l). Larva morphology was characteristic of *Bradysia impatiens* (Figure 3k; Ye et al. 2017). The vast majority of insects caught on the sticky traps (1688 of 1976 catches, i.e. 85%) were a dark-winged fungus gnat: the sequence obtained (KY853754) had a 99% similarity match with *B. impatiens* (Johannsen, 1912)[=*Bradysia difformis* Frey 1948](JX418064.1), Sciaridae, Diptera (Ye et al. 2017). Other insects caught on the traps were in decreasing abundance and according to sequence similarities: *Hymenoptera* sp.1, Diapriidae, Hymenoptera (186 catches, i.e. 9%), *Hymenoptera* sp.2 Ichneumonidae, Hymenoptera (37 catches, i.e. 1.9%), *Culex pipiens*, Culicidae, Diptera (19 catches, i.e. 1%), and *Hemiptera* sp.1 (16 catches, i.e. 0.8%). The 11 other insect taxa identified represented minor fractions of the catches (0.3 to 0.05%)⁶.

⁶ Supplementary material Table S2

Discussion

This research provides detailed methodology and foundational data on controlled mycorrhizal synthesis using vegetative inoculum for 13 combinations of four species of *Lactarius* sect. *Deliciosi* with five species of *Pinus* and for 17 distinct combinations of plants and fungal isolates (Table 2)⁷. To the best of our knowledge, except *P. radiata* x *L. deliciosus*, 12 syntheses involving different plant/fungus species combinations are unprecedented. The mycorrhiza formation between *P. radiata* and *L. vividus* is new to science. However, its putative survival and development following the transplantation of seedlings into natural conditions is unknown and needs to be tested. Similarly, the ‘Low’ mycorrhization rate obtained here between *P. tabuliformis* and *L. hengduanensis* sp. nov. may reflect the natural association of this species with *Picea* spp. at high elevation (Wang 2016).

In nursery containers using vegetative inoculum, *L. deliciosus* had the highest incidence of mycorrhiza formation of any of the species tested (100% of inoculated seedlings), followed by *L. vividus* (83%), *L. hatsudake* (70%) and *L. akahatsu* (40%). More work is required to determine whether these differences could be attributed to the *Lactarius* species used. Our latest results using modified potting mixes and growing conditions tend not to support this hypothesis. Indeed, they showed that the nutrient medium used in the present study, originally designed for *L. deliciosus* (Guerin-Laguette et al. 2000), generated efficient and comparable mycorrhization rates on large number of seedlings regardless of the fungal species used, i.e. *L. deliciosus* on *P. radiata*, *L. hatsudake* on *P. yunnanensis*, and *L. vividus* on *P. massoniana* (Wang et al. unpublished). Most replicate seedlings produced ‘High’ rates of mycorrhization, except *Pinus armandii* and *L. vividus* LV-115, which produced a ‘Low’ mycorrhization rate. Since no other isolates or species of *Lactarius* were tested in this study with *P. armandii*, more work is required to understand the potential of this pine species in controlled mycorrhization programmes. *Pinus tabuliformis* showed contrasted results depending on the *L. hatsudake* isolate used, i.e. ‘High’ mycorrhization with LH-122 but ‘Low’ with LH-

⁷ Supplementary material Fig. S2

23d. These results suggest that the screening of a high number of isolates could contribute to select highly performing combinations. Among the trialled combinations, *P. massoniana* x *L. akahatsu* was the only association not to produce any mycorrhizae (Table 2) despite the viability of the inoculum produced. We cannot rule out the possible incompatibility between these two species or, at least, between the fungal isolates and pine varieties used. This is the first comprehensive attempt to synthesize mycorrhizae using several Chinese species of *Lactarius* sect. *Deliciosi* and *Pinus*. Additional research is required to test the stability, over time, of some of these associations in nursery and field conditions. Previous success was reported in China for one association that was not attempted here: *P. massoniana* x *L. hatsudake* (Tang et al. 2008).

Mycorrhizae of the *Lactarius* species obtained in these experiments had morphological features characteristic of the species in this group (Riffle 1973; Guerin-Laguette 1998; Diaz et al. 2007), including being yellowish orange when young to cinnamon, browning when ageing, and short cystidia (Figures 1 and 2). Mature *L. hatsudake* mycorrhizae appeared more frequently less vivid orange than mycorrhizae of the other three species, and slightly greenish (Figure 1f). Occasional differences were also seen, e.g. green rhizomorphs for *L. hatsudake* (Figure 1k), but no other conspicuous morphological differences between species could be found. A high rhizomorph density was observed in several cases (Figure 1g and 2h), indicative of an exploration type of extra-matrical mycelium (Weigt et al. 2012).

Our attempt at synthesising mycorrhizae from spores failed in this study. We aimed to monitor the outcome of spore inoculation over a longer time, but from 8 to 9 months following inoculation the infestation of fungus gnat larvae gradually killed spore-inoculated and mycelial-inoculated seedlings. Despite repeated attempts, we never obtained mycorrhizae of *Lactarius* sect. *Deliciosi* following spore inoculation of nursery seedlings (Guerin-Laguette and Wang, unpublished) and failed to reproduce the results presented by Gonzales-Ochoa et al. (2003). However, we are aware of natural successful spore inoculation events in New Zealand, i.e. the spreads of *L. deliciosus* basidioma to

mature radiata pine trees up to several km distant from a saffron milk cap pine orchard near Nelson, New Zealand (Hannes Krummenacher, pers. comm.). We hypothesise that spores may become dormant under the conditions that we have used so far. Hutchison (1999) highlighted that, in previous studies, spore germination in *Lactarius* was nil or extremely low and that spores were probably stimulated to germinate immediately after release and only in the presence of root exudates from older host trees. Furthermore, mycorrhizae of basidiomycetes are mainly formed by dikaryotic mycelia, which may require additional time, specific conditions or compatibility mechanisms to successfully develop from spore-derived homokaryotic mycelia (Billiard et al. 2012). Although widespread in ascomycete truffle mycorrhizal seedling production, the successful use of spore inoculation for edible basidiomycete species is still rarely reported (Wang et al. 2002; Fangfuk et al. 2010; Carrasco et al. 2015).

The use of similar growing conditions allowed us to compare, at least over 6–8 months after inoculation, the efficacy of mycelium with that of spores, clearly showing that only the vegetative inoculum approach was successful. Within the scope of this study, it was not intended, nor possible, to compare the efficacy of the various mycelium techniques used. However, the high success rate obtained with vegetative inoculum in containers suggests this could be the recommended method for the production of *Lactarius* sect. *Deliciosi* mycorrhizal seedlings for cultivation applications.

The stimulating effect of *L. deliciosus* and *L. hatsudake* mycorrhization on pine growth presented here⁸ is congruent with the previously reported effect of *L. deliciosus* on *Pinus sylvestris* (Guerin-Laguette et al. 2003) and *P. radiata* (Guerin-Laguette et al. 2014). However, the pine seeds used were not screened for homogeneous size; therefore, the growth variability between replicate plants prevented detection of further significant growth stimulation effects due to mycorrhiza formation. Furthermore, beyond 8 months of growth, the damage caused by the insect larvae on the

⁸ Supplementary material Fig. S1

mycorrhizae prevented continued monitoring of the effects of mycorrhiza formation on pine growth. We intend to measure these effects in subsequent experiments.

The acclimation stage in tree nurseries is often a critical step in large-scale production of seedlings colonised with edible *Lactarius* species (Parladé et al. 2004). From 5 to 9 months following inoculation, the insect feeding on *Lactarius* sect. *Deliciosi* mycorrhizae worsened and became widespread across all combinations in the first series of seedlings. This resulted in the loss of all mycorrhizal seedlings by the third mycorrhiza assessment (Table 2). Feeding damage was less intense for the other two seedlings series, and by the third mycorrhiza assessment, more than half the seedlings (14 out of 24) still showed fresh *Lactarius* mycorrhizae (Table 2). Impacts of fungus gnats on plant and mushroom production are well known (Harris et al. 1996). The soft substrate, the lack of predators, and the moist and warm conditions in the glasshouse permeable to its surrounding environment may have favoured the exceptional development of larvae. In June and July 2016, temperatures in the glasshouse (within the meshed frame) frequently reached $\approx 30^{\circ}\text{C}$ for about 2 h in the early afternoon, while ambient humidity exceeded 90% for several hours a day. The harm caused by sciarid larvae, including *B. impatiens* (Johannsen, 1912) on *Pinus montezumae* roots is also well established (Marin-Cruz et al. 2015).

To the best of our knowledge, this is the first report of the extensive physical damage caused by the feeding on ectomycorrhizae by insects. Despite their putative negative impacts on plant yield and health, there is little work detailing the below-ground herbivory by insects (Hunter 2001). In this study, we observed that grazing insects can feed on ectomycorrhizae and roots of pines. We attribute the damage mostly to *Bradysia impatiens*, because this was the dominant species caught on sticky traps, and corresponding larvae were seen alive foraging through roots (Figure 3). It is, however, unclear whether, or to what extent, the other micro-fauna commonly detected under the dissecting microscope (*Collembola* spp., spiders) have contributed to the damage. Experiments involving the inoculation of mycorrhizal plants with specific micro-faunal species in contained

environments could help to answer this question. Since all material were sterilised prior to use, we suggest that the micro-fauna originated from the surrounding environment and developed rapidly.

In summary, this study demonstrated the efficiency of mycelial inoculation methods for production of *Lactarius* spp. mycorrhizal seedlings for establishing trial plantations in China. From the present study, it appears that *L. deliciosus* x [*P. yunnanensis* or *P. radiata*], *L. hatsudake* x [*P. yunnanensis* or *P. tabuliformis*], and *L. vividus* x [*P. massoniana* or *P. radiata*] are particularly promising symbionts for mushroom cultivation. Identifying environmental conditions that could provide appropriate control of the grazing of *Lactarius* sect. *Deliciosi* mycorrhizae by insects appears as a priority for future work: time of inoculation, lower temperature and moisture (i.e. lower watering regime), type of substrate (Olson et al. 2002), and possibly the use of additional control methods, i.e. insecticide (e.g. *Bacillus thuringiensis* protein) or biological control agents such as predators (Ydergaard et al. 1997; Jandricic et al. 2006, Cloyd 2015). Once this issue is solved, the present work will enable the production of large numbers of mycorrhizal milk cap seedlings suitable for establishing trial mushroom orchards in southwest China and other regions of the world.

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Table 1 *Lactarius* section *Deliciosi* pure culture isolates used in this study including the source of basidiomata and GenBank accession numbers (a. n.) of ITS rDNA sequences from pure cultures. All isolates were made in 2015 except JPN and QP, which was isolated in 2014. KIB = Kunming Institute of Botany, China.

<i>Lactarius</i> species	Isolate no.	KIB herbarium voucher	Source of basidioma ^a	Host tree	ITS a. n.
<i>L. akahatsu</i>	LA-QP	na	Shandong (m)	<i>Pinus thunbergii</i>	KY661915
	LA-JPN	na	Japan	<i>P. densiflora</i>	KY687509
<i>L. deliciosus</i>	LD-74	HKAS_90711	Ciba, Kunming (m)	<i>Pinus</i> sp.	KY661913
	LD-98	HKAS_90696	Mu Shui Hua, Kunming (m)	<i>Pinus</i> sp.	KY661914
	LD-NZ	na	New Zealand (h ^b)	<i>P. radiata</i>	KY687508
<i>L. hatsudake</i>	LH-122	HKAS_90695	Ciba, Kunming (m)	<i>Pinus</i> sp.	KY661921
	LH-23d	na	Ciba, Kunming (m)	<i>Pinus</i> sp.	KY661922
<i>L. vividus</i>	LV-115	HKAS_90068	Ciba, Kunming (m)	<i>Pinus</i> sp.	KY661924
	LV-118	HKAS_90069	Mu Shui Hua, Kunming (m)	<i>Pinus</i> sp.	KY661925
	LV-141	HKAS_89733	Yan Zi Bian, Shǎnxī (h)	<i>Pinus</i> sp.	KY661926

^afrom market (m) or harvested (h) by the authors; na, not available; ^boriginal culture from Anglesey, Wales, under *P. sylvestris* (Wang et al. 2012).

Table 2 Distinct combinations of pine and fungal species/isolate tested for mycorrhizal formation in container mycelial inoculations, with records of insect feeding. Entries indicate number of seedlings with fresh *Lactarius mycorrhizae* (Myc), or with severe mycorrhizal and/or root grazing (Graz) per the number of replicate seedlings set up in each combination category, at three assessments (months) following inoculation.

Combinations by species (isolate)	Combination code	Inoculation date	Seedling age (week)	First assessment		Second assessment		Third assessment
				Myc	Graz	Myc	Graz	Myc
		3/12/15		+ 6 months		+ 9 months		+ 14 months
1 (1)	PA x LV-115		9	1/4	1/4	0/4	4/4	0/4
	PA Control		9	0/3	0/3	0/3	1/3	0/3
2 (2)	PR x LD-74		6	18/18	9/18	0/18	17/18	0/18
2 (3)	PR x LD-98		6	5/5	0/5	0/5	4/5	0/5
3 (4)	PR x LV-115		6	5/5	2/5	0/5	5/5	0/5
	PR Control		6	0/5	0/5	0/5	1/5	0/5
4 (5)	PT x LA-QP		6	4/5	1/5	0/5	5/5	0/5
5 (6)	PT x LH-122		6	5/5	2/5	0/5	5/5	0/5
	PT Control		6	0/5	0/5	0/5	4/5	0/5
6 (7)	PY x LD-74		6	5/5	5/5	0/5	5/5	0/5
7 (8)	PY x LH-122		6	5/5	4/5	0/5	5/5	0/5
8 (9)	PY x LV-115		6	4/5	3/5	0/5	5/5	0/5
	PY Control		6	0/5	0/5	0/5	5/5	0/5
8 (9)	PYo x LD-74		18	5/5	4/5	0/5	5/5	0/5
	PYo Control		18	0/5	2/5	0/5	5/5	0/5
		26/1/16		+ 5 months		+ 8 months		+ 13 months
8 (10)	PR x LD-NZ		13	10/10	2/10	6/10	10/10	4/10
	PR Control		13	0/4	0/4	0/4	1/4	0/4
8 (10)	PT x LH-122		10	3/5	2/5	1/5	1/5	0/5
8 (11)	PT x LH-23d		10	1/5	0/5	0/5	1/5	0/5
	PT Control		10	0/5	0/5	0/5	0/5	0/5
		22/3/16		+ 6 months		+ 8 months		+ 10 months
	PM x LA-JPN		14	0/5	0/5	0/5	0/5	0/5
9 (12)	PM x LV-118		14	5/5	0/5	5/5	0/5	5/5
9 (13)	PM x LV-141		14	5/5	0/5	5/5	1/5	5/5
	PM Control		14	0/5	0/5	0/5	0/5	0/5
	All seedlings ^a			81/97	37/134	17/97	90/134	14/97

PA, *Pinus armandii*; PM, *P. massoniana*; PR, *P. radiata*; PT, *P. tabuliformis*; PY, *P. yunnanensis*, PYo, older *P. yunnanensis* seedlings. LA,

Lactarius akahatsu; LD, *L. deliciosus*; LH, *L. hatsudake*; LV, *L. vividus*.

^aAll seedlings means inoculated seedlings only (Myc) or inoculated plus control seedlings (Graz).

Figure 1 Dissecting microscope views of ectomycorrhizae and rhizomorphs of *Lactarius* section *Deliciosi* **a**, *Pinus radiata* x *L. deliciosus* LD-74, note the yellow/orange rhizomorph in the foreground, bar 0.9 mm **b**, *P. radiata* x *L. deliciosus* LD-98, note the cluster stage, bar 1 mm **c**, Cluster of *P. radiata* x *L. deliciosus* NZ, bar 1 mm **d-e**, *P. radiata* x *L. deliciosus* LD-98, in the close-up (e) note the vivid orange rhizomorph emanating from the brown/old mantle surface, bars 0.9 mm and 240 µm, respectively **f**, *Pinus tabuliformis* x *L. hatsudake* LH-122, note the greening of the mantle and the green rhizomorph (arrow), bar 350 µm **g**, Cluster of *P. tabuliformis* x *L. akahatsu* LA-QP covered in abundant light green rhizomorphs, bar 1 mm **h**, *Pinus yunnanensis* x *L. hatsudake* LH-122, bar 1.2 mm **i**, *P. radiata* x *L. vividus* LV-115, note the cistidia (arrow), bar 375 µm **j**, *Pinus armandii* x *L. vividus* LV-115, note the cistidia (arrow), bar 300 µm **k**, Green, branched rhizomorph of *L. hatsudake* LH-122, bar 240 µm.

Figure 2 Dissecting microscopy photographs showing ectomycorrhizae and rhizomorphs of *Lactarius* section *Deliciosi* **a-c**, *Pinus yunnanensis* x *L. deliciosus* LD-74, branched mycorrhizae, bars 0.5 mm (a, b), 430 µm (c) **d-f**, *Pinus yunnanensis* x *L. deliciosus* LD-NZ branched mycorrhizae, note the orange-coloured laticifers (e, arrow), and ‘spiky’ cistidia (f, arrow) visible on the mantle surface, bars 430 µm (d), 200 µm (e), 230 µm (f) **g**, *P. massoniana* x *L. vividus* LV-107, proliferation of branched mycorrhizae, bar 1.2 mm **h**, *P. yunnanensis* x *L. vividus* LV-118 mycorrhizae covered in dense rhizomorphs, right, bar 1.7 mm **i**, *P. massoniana* x *L. vividus* LV-141 with ‘spiky’ cistidia visible (arrow), bar 430 µm **j**, *L. vividus* LV-118 highly branched rhizomorphs, bar 300 µm **k**, *P. massoniana* x *L. vividus* LV-118 mycorrhizae and running thick rhizomorph (arrows), bar 1 mm.

Figure 3 a, Wilting and yellowing pine shoots 10 months after inoculation **b-g**, Dissecting microscope views of damaged ectomycorrhizae. The mantle damage is obvious, exposing the internal tissues (vascular cylinder): *Pinus yunnanensis* x *Lactarius deliciosus* LD-74, bar 0.75 mm (b), *P. radiata* x *L.*

vividus LV-115, bar 375 μm (c), *P. yunnanensis* x *L. vividus* LV-115, bar 0.67 mm (d), *P. radiata* x *L. vividus* LV-115, note the border with the remaining mantle (arrow), bar 0.5 mm (e), *P. yunnanensis* x *L. vividus* LV-115, damaged mycorrhiza clusters, bar 0.75 mm (f), *P. yunnanensis* x *L. deliciosus* LD-74, eaten mantle (arrow), bar 430 μm (g) **h**, Dissecting microscopy photographs of a damaged long root of non-inoculated *P. yunnanensis*, bar 1.5 mm **i-l**, Dissecting microscopy views of *Bradysia impatiens* and damage: larva coming out (arrow) from a *P. yunnanensis* root, bar 400 μm (i), cortex damage of a *P. radiata* root, bar 300 μm (j), larva of *B. impatiens* with characteristic black head and semi-transparent skin, bar 220 μm (k), larva (arrow) foraging through a *P. radiata* root, bar 330 μm (l) **m-n**, Dissecting microscopy views on non-mycorrhizal regrowth of insect-damaged mycorrhizae: remains of a branched *P. radiata* x *L. deliciosus* LD-74 mycorrhiza showing old rhizomorph (arrowhead), remaining mantle (asterisk) and non-mycorrhizal re-growth of the tips, including root hairs (arrow), bar 0.5 mm (m), Growing tips of an ex-mycorrhizal cluster of *P. tabuliformis* x *L. deliciosus* LD-74 covered in root hairs, bar 1.5 mm (n).