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SHORT NOTE

Are fungi from adult orchid roots the best symbionts at germination? A case study

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Abstract

We studied mycobionts from advanced seedlings and adult mycorrhizal roots of the terrestrial orchid *Arundina graminifolia*. Fungi were isolated, identified by ITS sequencing, and tested for their impact on seed germination, protocorm formation, and development of advanced seedlings (emergence of first leaf) in vitro. Among the six fungal species isolated, four were not standard orchid mycorrhizal fungi (*Fusarium solani*, *Cylindrocarpon* sp., *Acremonium* sp., and *Phlebiopsis flavidoalba*) and did not support germination beyond imbibition and greening of the seeds during a span of 35 days. Over the same time, one *Tulasnella* species isolated from adult mycorrhiza allowed protocorm formation but not further development. However, another *Tulasnella* species isolated from advanced seedlings facilitated development to the advanced seedling stage. Our results support (i) the inability of occasional orchid root colonizers to support late seed germination, and (ii) the growing literature showing that fungal associates can change over orchid development. Functionally, we show that mycorrhizal taxa isolated from advanced seedlings can be more efficient than those from adults in supporting germination in some species, leading to recommendations for ex situ orchid conservation.

Keywords *Arundina graminifolia* · Endophytes · Mycorrhizal specificity · Protocorm · Orchid seed germination · *Tulasnella*

Introduction

Mycorrhizal fungi play a vital role in the life history of orchids, from early germination to mycorrhizal association in adults (Rasmussen et al. 2015; Dearnaley et al. 2016). The dust-like seeds of orchids (Arditti and Ghani 2000) cannot germinate unless compatible fungi colonize them and support their nutrition (Dearnaley et al. 2016; Smith and Read 2008). Most green orchids associate with rhizoctonias, a polyphyletic aggregate of species belonging to Serendipitaceae (Sebacinales), Tulasnellaceae and Ceratobasidiaceae (Cantharellales; Dearnaley et al. 2012), which are soil saprobes and/or endophytes in roots of non-orchid plants (Selosse and Martos 2014; Weiß et al. 2016; by endophyte, we mean a fungus growing in living plant tissues that does not cause obvious symptoms or morphological modifications; Selosse et al. 2018). A given orchid species may associate with species from one or more of these rhizoctonia families (Dearnaley et al. 2012). Traditionally, orchid mycorrhizal fungi have been isolated from roots of mature plants to screen their ability to enhance germination and authors retain only the isolates belonging to rhizoctonias (e.g., Zettler and Hofer 1998; Stewart and Zettler 2002; Massey and Zettler 2007; Zettler and Corey 2018). Here, in a pilot experiment, we question (i) the effectiveness of using fungi isolated from adult roots compared with those isolated from seedlings, and (ii) the relevance of discarding non-rhizoctonia fungi in germination attempts.

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Considering the first point, the high diversity of fungi associated with adult roots may not only encompass fungi associated with protocorms and/or advanced seedlings (Zelmer et al. 1996; Salman et al. 2002; McCormick et al. 2004). Even more, protocorm symbionts may have been outcompeted at adult stages, as in *Tipularia discolor* where fungi cultured from adults do not support seed germination ex situ (McCormick et al. 2004), or in *Cynorkis purpurea*, which may switch fungus during development (Rafter et al. 2016). For this reason, researchers have advocated targeting young seedling stages as a source of fungal isolates (e.g., Rasmussen et al. 2015; Yokoya et al. 2015; Rafter et al. 2016). Considering the early development, there is some evidence that orchids have different symbionts at different stages, and only a subset of the fungi allowing the first germination steps may support later developmental stages in situ (Bidartondo and Read 2008; Jacquemyn et al. 2011; Long et al. 2013) and ex situ, where more diverse fungi stimulate very early seed germination (Wang et al. 2011; Zi et al. 2014; Rasmussen et al. 2015). In all, there is published evidence for an hourglass (= a bottleneck) in fungal symbiont diversity during orchid development, with maximum specificity at the advanced seedling stage. However, this trend is mainly reported for temperate, European species, especially for species that are partly heterotrophic (mixotrophic) and do not associate with rhizoctonias (Bidartondo and Read 2008; Těšitelová et al. 2012), and some species (e.g., *Epipactis atrorubens* in the two latter studies) do not show any bottleneck. Thus, more evidence is needed, as are case studies, especially those investigating the developmental stages on a finer scale.

Another aspect concerns the many fungi isolated from adult roots and discarded as "non-mycorrhizal." Although many endophytes, saprobes, and parasites can be isolated, they may act positively on germination, such as described for *Fusarium* (Vujanovic et al. 2000) or *Mycena* (Guo et al. 1997) species. The use of non-rhizoctonia fungi for their potential as mycorrhizal associates has been advocated (Yokoya et al. 2015). The question of a potential role of such non-mycorrhizal fungi is indeed crucial when considering that some taxa (e.g., saprobic fungi) are mycorrhizal in some achlorophyllous (Selosse et al. 2010; Dearnaley et al. 2012) or even chlorophyllous (such as *Mycena* sp. in some *Dendrobium*; Zhang et al. 2012) orchid species.

The current case study uses one population of *Arundina graminifolia*, a widely distributed terrestrial orchid, to test how seed germination is impacted by (i) fungi isolated from advanced seedlings versus from adults, and (ii) by mycorrhizal rhizoctonias versus other non-mycorrhizal endophytes. To reveal a potential hourglass in fungal symbiont diversity during advanced seedling development, we analyzed on a fine scale the successive developmental stages, which may require more or less specific partners.

Materials and methods

Study species and sampling site

Arundina graminifolia (D. Don) Hochreutine is a terrestrial orchid (Fig. 1a) widely distributed in subtropical and tropical Southeast Asia at elevations from 400 to 2800 m on sunny grassy slopes, stream banks, roadsides, and forest edges (Chen et al. 2009). Although used in Dai medicine in China and naturalized as an ornamental plant in the Pacific islands and in the Neotropics, the mycorrhizal symbiosis of *A. graminifolia* remains largely unknown. All samples were collected from a grassy slope near the Mengbang reservoir in Menghai (Yunnan province, China; 21° 54′ N, 100° 16′ E; elevation 1396 m) on April 28, 2016, i.e., during the fruiting period. One root was collected from eight adult *A. graminifolia*, placed in an icebox, and transferred to the laboratory for fungal isolation. A mature, close-to-dehiscence capsule of *A. graminifolia* was harvested at the same time from one of the previous individuals, and seeds were stored in the orchid seed bank of Xishuangbanna Tropical Botanical Garden following Gao et al. (2014). After surface-sterilization with 75% ethanol, the capsules were opened to release the seeds with a scalpel under sterile conditions. Seeds were air-dried over $CaCl_2$ for 4 days at 25 ± 2 °C, and then mixed together and stored in the orchid seed bank in airtight glass containers at 4 °C for short-term preservation and at − 20 °C for long-term preservation, respectively.

Ex situ seed baiting experiments

To bait fungi by advanced seedling formation following our previous methods (Sheng et al. 2012), we collected top soil around 10 adult plants (8 cm deep; on an area of 30 cm) including the eight former ones. Soil samples were then mixed together and distributed in 20 cylindrical glass bottles for fungal baiting. Soil was watered to saturation with sterile water and covered by a layer of autoclaved nylon mesh (4 × 4 cm). Before baiting, seeds were tested to ensure high viability by TTC method (Fig. 1b; Vujanovic et al. 2000), sterilized with 1% (*w*/*v*) NaClO for 5 min, and washed with sterile distilled water 3–4 times. Approximately 150 seeds suspended in 150 μL agar solution were spread on the nylon mesh. After incubation in a high-humidity (75 ± 5%) chamber at 25 ± 2 °C and 12/12-h light/dark cycle, developing advanced seedlings (stage 4, see below) were taken out for fungi isolation.

Fungal isolation and identification

Fungi were isolated from (i) 14 advanced seedlings at emergence of the first leaf (stage 4, see below; Fig. 1c) following our previous methods (Sheng et al. 2012) and (ii) the eight

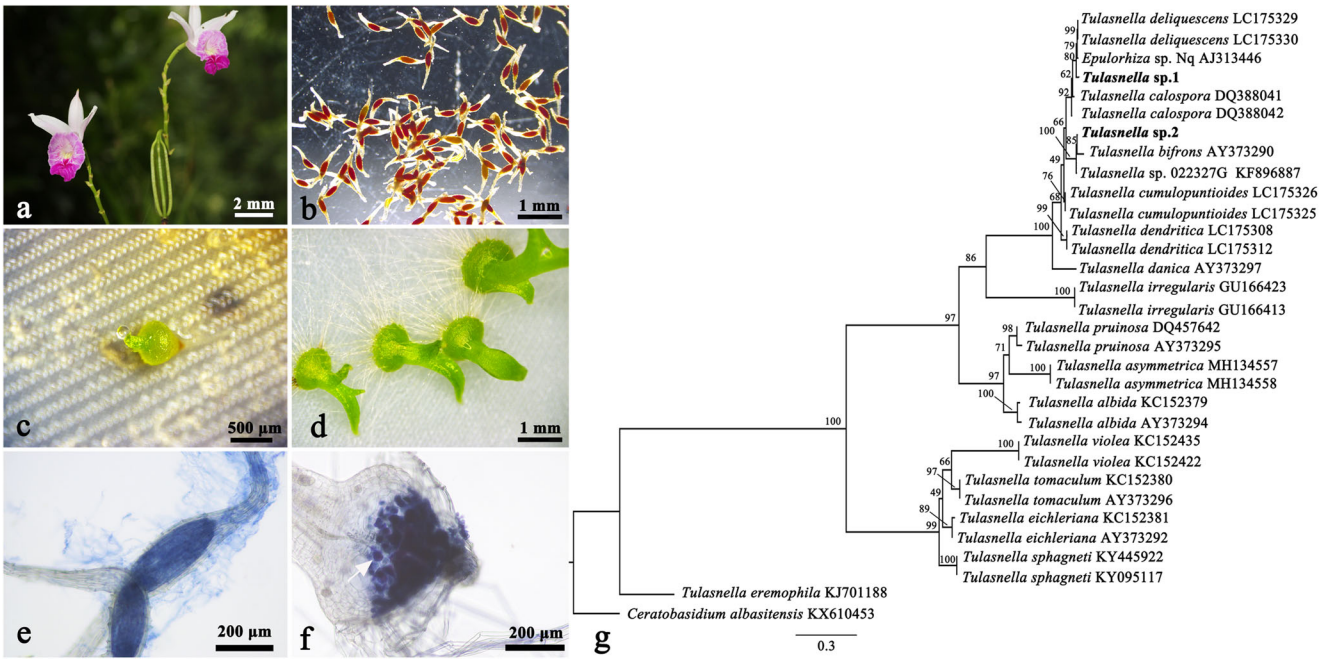

Fig. 1 *Arundina graminifolia* and its *Tulasnella* symbionts. **a** Flowers and fruits of *Arundina graminifolia*; **b** seeds of *A. graminifolia* tested by the TTC method, showing high viability; **c** advanced seedlings at stage 4 obtained during ex situ seed baiting experiments; **d** advanced seedlings with 2 or 3 leaves (stage 5) 35 days after incubation with *Tulasnella* sp.2; **e** trypan blue staining showing fungi colonizing whole embryo incubation with *Acremonium* sp.1; **f** trypan blue staining showing *Tulasnella* sp.2 forming pelotons in cells of advanced seedlings; **g** Maximum-likelihood tree constructed using ITS sequences of *Tulasnella* sp.1 and sp.2 (in bold) found on *Arundina graminifolia* in this study and of other Tulasnellaceae species. Numbers above branches are bootstrap probabilities (whenever ≥ 50%) out of 1000 replicates

sampled roots of *A. graminifolia* following Bayman et al. (2016). For the roots, after checking for colonization and surface-sterilization of a section, we removed the superficial uncolonized tissues (data not shown) using a thin sterile blade and forceps under a dissecting microscope. For roots and advanced seedlings, nine fragments per sample were used for fungal isolation on Petri dishes. In all procedures, we tentatively used three media (3 inoculated fragments per medium), namely water agar, oat agar medium (OMA; 4 g/L ground oat and 8 g/L agar), and potato dextrose agar (PDA; 200 g/L potato, 20 g/L dextrose and 20 g/L agar).

All fungal strains were characterized morphologically (culture characteristics such as color, surface shape, and tissue texture; hyphal characteristics; Prakash and Bhargava 2016) and molecularly by internal transcribed spacer (ITS) sequencing. DNA extraction, ITS amplification by PCR using the ITS1 and ITS4 primers (White et al. 1990), and amplicon sequencing were as in Selosse et al. (2002). ITS sequences were blasted against the GenBank database (National Center for Biotechnology Information) for identification, and later one sequence per species (from the isolate used below; Appendix Table S2) was deposited in this databank (MK651834-39). One isolate per species was conserved in the Guangdong Microbial Culture Collection Center (GDMCC, https://www.gdmcc.net/) under the accession number 3.668-3.673. We are also maintaining live cultures in storage at 4 °C in our fungus bank.

To visualize the phylogenetic position of the *Tulasnella* species from *Arundina graminifolia* among other known *Tulasnella* species associated with orchids, a phylogenetic tree was generated using one ITS sequence of each of these species and closely related taxa obtained by blast searches, as well as sequences of known *Tulasnella* species. The ITS is known to provide good phylogenetic signal in Tulasneallaceae (Linde et al. 2014). Sequences were first aligned using the MAFFT tool implemented in Unipro UGENE v1.26.2 software and then manually adjusted in this software (Okonechnikov et al. 2012). The substitution model selection based on the Bayesian information criterion (BIC) and the "TNe+G4" (freqA = 0.250, freqC = 0.250, freqG = 0.250, freqT = 0.250, R(a)[AC] = 1.00000, R(b)[AG] = 2.06722, R(c)[AT] = 1.00000, R(d)[CG] = 1.00000, R(e)[CT] = 4.38516, R(f)[GT] = 1.00000, gamma shape alpha = 0.764) was selected as the best model according to Modelfinder implemented in IQ-TREE 1.6.10 program (Nguyen et al. 2015). Then, a maximum-likelihood phylogenetic tree was constructed using this model in this program. All parameters in the ML analysis were kept at their default levels, and statistical support was obtained using ultrafast bootstrap with 1000 replicates. The ITS sequence of another Cantharellales species, *Ceratobasidum albasitensis* (KX610453), served as an outgroup.

Testing the fungal promotion of seed germination

The ability to promote germination of one isolate per isolated species was tested on surface-sterilized (as above) seeds of *A. graminifolia*. Approximately 120 seeds per Petri dish were sown, either with fungus or axenically. Axenic growth was used for comparison on two media: OMA (nutrient-poor medium) and Murashige and Skoog (MS; nutrient-rich medium; Murashige and Skoog 1962). Fungal inoculation was performed following our previous methods (Zi et al. 2014; Huang et al. 2018). Each treatment was replicated in 10 Petri dishes placed in germination chambers at 25 ± 2 °C and 12/12-h light/dark cycle. For each Petri dish, the number of seeds and the status of seed germination were assessed 35 days after incubation according to the stages of Arditti (1967): 0, no germination; 1, embryo swells and turns green (an unusual feature observed in lightened protocorm in this species), and testa is propped up (germination); 2, continued embryo enlargement forms a spherule, seed coat broken (protocorm formation); 3, appearance of protomeristem (protocorm differentiation); 4, advanced seedling with emergence of the first leaf; 5, emergence of the second leaf and further development. The percentage of seeds at each developmental stage was calculated. To determine if mycorrhizal symbiosis actually became established, protocorms were randomly selected and examined for the presence of pelotons. They were cleared using 10% KOH solution, washed with 1% HCl solution, stained in 0.05% (*w*/*v*) trypan blue in lactic acid glycerol solution overnight (adapted from Phillips and Hayman (1970), and then de-stained in acetic glycerol solution before observation under the microscope (DM2000, Leica Microsystems GmbH, Wetzlar, Germany).

Data collection and statistical analysis

We used stages 0, 1, (2 + 3), and (4 + 5) respectively to determine no germination, seed germination, protocorm formation, and advanced seedling development. Total seeds (*t*), germinated seeds (*g*), protocorms (*p*), and advanced seedlings (*s*) were counted after 35 days of incubation. The percentages of germinated seeds (*G*), protocorms (*P*), and advanced seedlings (*S*) respectively were calculated as follows: $G = 100 \times (g + p + s)/t$, $P = 100 \times p/t$, and $S = 100 \times s/t$. Generalized linear models were used to test for effects of different fungal inoculations on seed germination, protocorm formation, and advanced seedling development. To account for multiple comparisons, we used Tukey tests for differences among different treatments. All statistical analyses were performed in R (version 3.3.3). The alpha-type I error was fixed at 5% (thus, all non-significant differences have $P > 0.05$).

Results

A total of 50 fungal isolates (out of 198 attempts, i.e., 25.2% success; Appendix Table S1) were obtained from *A. graminifolia*, including 21 fungal strains isolated from advanced seedlings and 29 from roots. Morphological and molecular identification showed that they belong to six species (Appendix Fig. S1; Tables S1 and S2). The species exclusive to roots were *Fusarium solani*, *Acremonium* sp.1, *Phlebiopsis flavidoalba*, and *Tulasnella* sp.1 (Appendix Table S2) isolated 4, 4, 3, and 11 times, respectively (Appendix Table S1). The species exclusive to advanced seedlings is *Tulasnella* sp.2, which was isolated 18 times (Appendix Tables S1 and S2). The species isolated both from roots and advanced seedlings is *Cylindrocarpon* sp.1, which was isolated 7 times from roots and 3 times from advanced seedlings (Appendix Tables S1 and S2). The *Tulasnella* spp. 1 and 2 (GenBank accession numbers MK651837 and MK651838) differ by 9% in ITS sequence and thus likely belong to different species: this is supported by a phylogenetic analysis based on ITS (Fig. 1g).

Fungal capacity in promoting seed germination

After 35 days of incubation, significant differences among treatments were recorded for the percentages of seed germination ($chi^2 = 78.27$, $P < 0.0001$), protocorm formation ($chi^2 = 31.44$, $P < 0.0001$), and advanced seedling development ($chi^2 = 42.45$, $P < 0.0001$; Fig. 2). Nutrient-rich control treatment (MS) allowed more than 95% seed germination, leading to large proportions of protocorms (55.02%) and advanced seedlings (33.64%; Fig. 2). In nutrient-poor control treatment (OMA), most seeds (88.89%) remained at stage 1 (Fig. 2).

From the 6 fungal inoculation treatments, no germinated seeds were found with *Fusarium solani*, *Cylindrocarpon* sp.1, and only a few seeds reached stage 1 with *Acremonium* sp.1 (9.71 ± 3.63%) or *Phlebiopsis flavidoalba* (13.32 ± 4.52%; Fig. 2a). Alternatively, percentages of seed germination were high with *Tulasnella* sp.1 (93.20%) and *Tulasnella* sp.2 (96.97%). Percentages of protocorm were lower with *Tulasnella* sp.2 (10.17 ± 3.81%) than with *Tulasnella* sp.1 (50.43 ± 6.47%). Considering later stages, *Tulasnella* sp.2 allowed development to advanced seedling (79.75 ± 3.84%) stages, while no advanced seedlings occurred with *Tulasnella* sp.1 in the same time (Fig. 2b, c). Although the percentages of seed germination with *Tulasnella* sp.2 did not differ from MS treatment ($P = 0.11$), and protocorm formation was lower than MS treatment, the percentage of advanced seedlings was significantly higher with *Tulasnella* sp.2 than in any other treatments (all $P < 0.05$; Fig. 2c), suggesting that this fungus efficiently promotes seed germination and advanced seedling development. These trends did not differ after 2 months (data not shown).

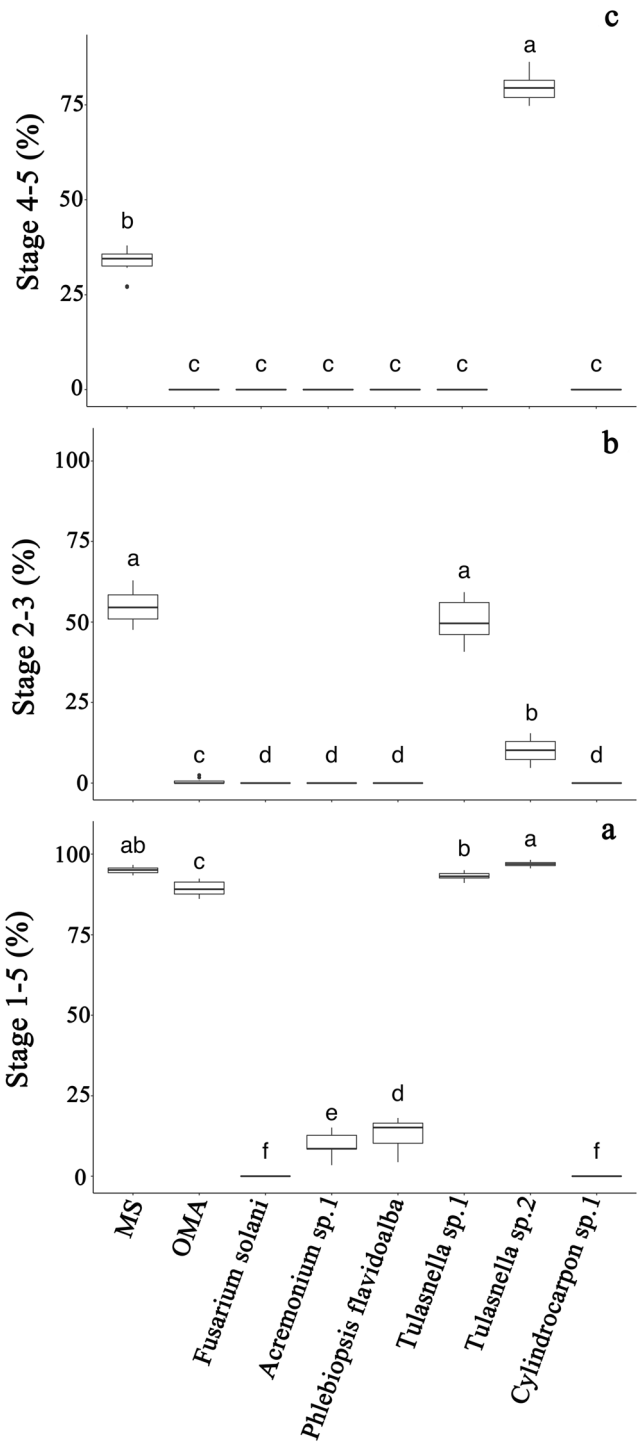

Fig. 2 Effects of different treatments (control on medium MS and OMA, or with addition of one of the six inoculated strains) on the successive stages of the germination process after 35 days. **a** Seed germination, i.e., stages 1 to 5 included, according to Arditti (1967); **b** protocorm formation (stages 2 to 3); **c** advanced seedlings (stages 4 and 5). In each panel, different letters indicate significant differences based on Tukey tests of variance

Contrasting fungal colonizations were observed in inoculated treatments at 35 days. In treatments, *Fusarium solani*, *Acremonium* sp.1, *Phlebiopsis flavidoalba*, and *Cylindrocarpon* sp.1, fungi colonized part or all of embryo tissues in seeds (Fig. 1e), resulting in failed germination. On the other hand, *Tulasnella* sp.1 and sp.2 penetrated the seeds from suspensor cells and colonized the basal part of the protocorms. They formed pelotons in basal cells of protocorms and in advanced seedlings (Fig. 1f).

Discussion

We successfully isolated fungi from adult roots and advanced seedlings (stage 4, emergence of first leaf) of an *A. graminifolia* population to test their ability to support germination ex situ. A limited diversity was recovered from the roots (5 species) and from the soil after baiting by seed germination (2 species). These fungi may form pelotons or any other looser endophytic, non-mycorrhizal colonization. Of the rhizoctonia species isolated, two *Tulasnella* spp. were sampled more often (11 and 18 times) than the non-rhizoctonias (3–10 times), suggesting that they may densely colonize the tissues and likely form pelotons. The six species were all isolated repeatedly, so the limited number of species found may characterize the samples studied rather than a limitation in the number of cultivation attempts.

Interestingly, although soil samples were sampled in the vicinity of the adult from which roots were harvested, the diversity from root and baiting overlapped for one *Cylindrocarpon* species. Three of the non-rhizoctonia species isolated are from genera encompassing plant pathogens that can also behave as symptomless endophytes (*Fusarium*, *Acremonium*, and *Cylindrocarpon*; Rodriguez et al. 2009; Selosse et al. 2018) while *Phlebiopsis flavidoalba* is a saprobe (Hori et al. 2014). We removed the external layers of root cells devoid of peloton to limit the surface contamination by saprobes, so we cannot exclude that the latter species had endophytic abilities as described in some saprobes including *Phlebiopsis gigantea* (Vasiliauskas et al. 2007).

The five non-rhizoctonia species isolated in this study were unable to support germination development beyond stage 1 within 35 days (or 2 months, not shown). This stage is not really evidence of germination since even dead embryos imbibe, yet the greening of the embryos (Appendix Fig. S2) in these seedlings demonstrates some physiological development. This result is reminiscent of the germination of orchids that fail to go beyond early stages with *Fusarium* (Vujanovic et al. 2000) or *Mycena* (Guo et al. 1997) species. We note that the presence of many fungi in living protocorms, even if unable to support the germination, may have an evolutionary relevance: it may be the niche from which some of them evolve, in certain lineages, true symbiotic, mycorrhizal partnerships (Selosse et al. 2010).

Most importantly, we found that, in *A. graminifolia*, the *Tulasnella* mycorrhizal fungi isolated from adults are less efficient at supporting germination than those isolated from advanced seedlings. Being more than 97% divergent (the threshold usually retained for species delineation in Tulasnellaceae; Linde et al. 2014) and with contrasted places in the Tulasnellaceae phylogeny (Fig. 1g), the two isolated strains likely belong to two different species. Thus, their contrasted effects on *A. graminifolia* represent an interspecific, not an intraspecific polymorphism. The finding that *Tulasnella* sp.2 isolated from advanced seedlings is more efficient than *Tulasnella* sp.1 isolated from mycorrhizas agrees with several previous studies on epiphytic orchids, where fungi obtained from protocorms and advanced seedlings effectively promoted seed germination up to advanced seedling development, e.g., for *Cymbidium mannii* (Sheng et al. 2012), *Papilionanthe teres* (Zhou and Gao 2016), *Dendrobium aphyllum* (Zi et al. 2014), or *D. devonianum* (Huang et al. 2018). Although in some orchid species a single *Tulasnella* symbiont is present from protocorm to adult plant (e.g., Phillips et al. 2011), in many other species, developmental change in symbionts may occur: *Tipularia discolor* switches mycobionts at the transition to the adult plant (McCormick et al. 2004); *Cynorkis purpurea* switches partners during development (Rafter et al. 2016), and *Gastrodia elata* shifts from *Mycena osmundicola* at germination to *Armillaria mellea* during adult vegetative growth (Xu and Mu 1990). In the investigated *A. graminifolia* population, we suspect that a partner replacement occurs during development, although more sampling in natural conditions is pending. Indeed, the soil submitted for advanced seedling baiting was collected in the vicinity of adult plants and thus likely contained *Tulasnella* sp.1 in addition to *Tulasnella* sp.2. Despite this expected dual presence in soil, adults only recovered *Tulasnella* sp.1 while protocorms only baited *Tulasnella* sp.2. Thus, although we cannot fully exclude that seed baiting procedure was fatal to *Tulasnella* sp.1, we favor the idea of a developmental symbiont shift.

The ability to switch fungi may be useful for plants to improve tolerance of environmental fluctuations and disturbances, in the context of a different metabolism at adulthood (increase in autotrophy and mineral needs after leaf expansion; McCormick et al. 2004; Těšitelová et al. 2012). Moreover, a shift in mycobiont limits competition with adults, due to the use of different fungal partners, so that even if lifelong association allows a positive mother-effect on advanced seedling (Jacquemyn et al. 2007; Phillips et al. 2011), avoiding partners sharing with adults may constitute an ecological advantage. Ex situ methods may take advantage of strains isolated from young orchids to improve germination, keeping in mind that, in the case of reintroduction in nature of resulting symbiotic seedlings, such a practice will not normally co-introduce fungi in adults.

Conclusions

By comparing the fungus assemblages in roots and advanced seedlings, and by testing their effects on the germination sequence in *A. graminifolia*, we add support to the growing literature showing that, in some species, fungal diversity decreases with advanced seedling development before increasing again at adulthood (bottleneck in diversity). As a result, best germination can sometimes be achieved in orchid species with a fungal partner specifically isolated from early life stages. Considering this development-dependent specificity, our results suggest the use, whenever possible, of fungi isolated by the seed baiting technique in situ or ex situ, or even from young orchid seedlings (even protocorms) collected in the wild, with a view to ex situ orchid conservation practices.

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Compliance with ethical standards

Disclaimer The authors alone are responsible for the content and functionality of these materials.

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