Symbiosis
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Nutrients and fungal identity affect the outcome of symbiotic germination in *Bipinnula fimbriata* (Orchidaceae)

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Abstract
Orchids produce small seeds with no energy reserves, relying entirely on orchid mycorrhizal fungi (OMF) for germination. This process, known as symbiotic germination, can lead to different outcomes depending on abiotic factors, such as nutrient availability. Previous studies have shown that nutrient addition has a negative effect on the outcome of symbiotic germination. However, if this effect varies across OMF species, and if it is related to a fungal response to nutrients, remains unknown. This paper examines the effect of fungal identity and nutrient addition (nitrogen and phosphorus) on the germination of the orchid *Bipinnula fimbriata* using seven OTUs of mycorrhizal fungi from the families Tulasnellaceae and Ceratobasidiaceae. We also evaluated the effect of nutrient addition on mycorrhizal fungi growth rates. Results showed that the nutrient effect on symbiotic germination varied depending on fungal identity. While there was a strong negative effect on symbiotic germination with all *Tulasnella* OTUs and two *Ceratobasidium* OTUs, less or no effect was observed on the other two *Ceratobasidium* OTUs. Further studies are needed to understand the mechanism underlying this variation and how variable is the effect of nutrient addition on symbiotic germination in Orchidaceae and OMF species.

Keywords *Bipinnula* · Fungal identity · Symbiosis outcome · Nutrient addition · Orchidaceae · Orchid mycorrhiza · Symbiotic germination

1 Introduction

One main question in the study of symbiosis is how the context influences the outcome of the interaction (Johnson et al. 1997; Hoeksema and Bruna 2000). The costs and benefits that determine the net effect of a symbiotic interaction can vary over time and space, causing the interaction outcome to change along a continuum from mutualism to parasitism (Bronstein et al. 2006, 2014; Sachs and Simms 2006). This plasticity is frequently driven by the abiotic and biotic context in which the symbiotic interaction occurs, such as the abundance of key nutrients and the identity of species found in the community (Hoeksema and Bruna 2000; Shantz et al. 2016). For example, nutrient addition in legume-rhizobia symbiosis suppresses growth benefits to partners (Heath and Tiffin 2007; Lau et al. 2012), and may lead to the breakdown of coral-algae mutualism (Mumby and Steneck 2008; Morris et al. 2019) depending on the host identity and the type of nutrient involved (Shantz and Burkepile 2014).

The outcome of mycorrhizal associations (i.e. the symbiotic association between plant roots and soil fungi) is also sensitive to soil nutrient availability (Johnson et al. 1997; Treseder 2004; Hoeksema et al. 2010). In this symbiosis, plants provide fungi with carbon in exchange for nutrients (Smith and Read 2008). However, when soil nutrient availability is high, plants with arbuscular and ectomycorrhizal associations frequently show a reduction in mycorrhizal colonization (Johnson et al. 2003; Liu et al. 2012; Corrales et al. 2017), probably due to the costs of the symbiosis surpass the benefits for plants, in this conditions (Treseder and Allen 2002; Kennedy and Peay 2007; Ven et al. 2019).

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Less is known about the effect of nutrients on the outcome of orchid mycorrhizal symbiosis. Orchid mycorrhizas exhibit a wide mutualism-parasitism continuum (Bronstein et al. 2014) that ranges from mutualism in green orchids (Cameron et al. 2006, 2007, 2008) to parasitism in mycoheterotrophic orchids (i.e. non-photosynthetic orchids that obtain carbohydrates from fungal associates; Leake 1994; Merckx et al. 2010), including partially mycoheterotrophic or mixotrophic orchids (i.e. orchids that obtain carbohydrates both from photosynthesis and from fungal associates; Julou et al. 2005; Gebauer et al. 2016). Nevertheless, all orchids are mycoheterotrophic during the early seedling development phase. Since orchid seeds are small and lack reserves (Arditti and Ghani 2000; Barthlott et al. 2014), they rely entirely on orchid mycorrhizal fungi (OMF) for germination. During this process known as symbiotic germination fungi and seeds form a non-photosynthetic spherical body called protocorm, that completely depends on the fungus for its nutrition (Rasmussen 2002; Kuga et al. 2014). Afterwards, seedlings then develop leaves, become autotrophic (in photosynthetic orchids), and nutrient exchange can occur between the mycorrhizal fungus and the orchid (Cameron et al. 2006, 2008; Perotto et al. 2014; Fochi et al. 2017). The encounter of the seed with the mycorrhizal fungus can lead to the formation of mycorrhizal protocorms (mutualistic outcome) or to the overgrowth of fungus throughout the protocorm, impeding its further development (parasitic outcome) (Beyrle et al. 1995; Smith and Read 2008). The outcome of orchid germination is expected to depend on OMF identity, biotic, and abiotic factors (Rasmussen et al. 2015), but this remains poorly understood.

By doing in vitro experiments, Beyrle et al. (1991) observed that at low nitrogen (N) concentrations, the fungus *Rhizoctonia* DC. sp. colonized seeds of the orchid *Dactylorhiza incarnata* (L.) Soo forming normal protocorms, but at high N concentrations, the fungus spread through orchid seeds acting as a parasite, leading to protocorm destruction. The same negative effects of N addition were observed, but only at high levels of carbon (C) supply, in the germination of the orchid *Orchis morio* L. with a *Rhizoctonia* species (Beyrle et al. 1995). These studies exposed the negative effects of nutrient addition on the outcome of orchid symbiotic germination, but the variability of this effect throughout OMF species is still to be determined.

There are two lines of evidence suggesting that the effect of nutrient addition on orchid symbiotic germination should vary depending on fungal identity. First, the fact that diversity and composition of OMF varies as a function of climatic and edaphic conditions (McCormick et al. 2006; Bunch et al. 2013; Mujica et al. 2016; Reiter et al. 2018; Vogt-Schilb et al. 2020). For example, in their study on ex-arable lands in Central Europe, Vogt-Schilb et al. (2020) found that Serendipitaceae dominated in grasslands with higher organic matter content whereas Ceratobasidiaceae prevailed in phosphorus (P)- rich grasslands. Similarly, Mujica et al. (2016) found a significant relationship between soil nutrients (P and N) and the composition and the diversity of mycorrhizal fungi associated with two species of *Bipinnula* Comm. ex Juss. These observations may be explained by the different capabilities of OMF taxa to support orchids depending on edaphic conditions (McCormick et al. 2012, 2018), possibly related to different germination ability in different habitats (i.e. certain mycorrhizal fungi are more successful in germinating seeds under certain soil conditions), however this has not been evaluated.

The second line of evidence is that under in vitro conditions, different nutritional preferences have been observed among OMF genera (Hadley and Ong 1978; Nurfadilah et al. 2013; Fochi et al. 2017) and even among species of the same genus (Smith 1967; Midgley et al. 2006; Nurfadilah et al. 2013), which could cause different responses to nutrient addition in symbiotic germination. Considering these observations, it is expected that the effect of nutrient addition on the outcome of orchid symbiotic germination will vary depending on OMF identity, and this variation should be related to different nutritional preferences of OMF species, but these hypotheses have not been tested yet.

In this study we addressed the effect of OMF identity, nutrient addition and the interaction between them, on the outcome of orchid symbiotic germination in vitro, and two hypotheses were tested. First, nutrients might affect germination negatively, as it has been previously demonstrated (Beyrle et al. 1995), but also a variation in the effect of nutrients on germination depending on the OMF species involved is expected (hypothesis 1). Secondly, if there is a variation in the effect of nutrients on symbiotic germination among fungal species, this variation is likely to be related to differences in the fungal growth response to nutrient availability (hypothesis 2). To test these hypotheses, we studied the germination of *Bipinnula fimbriata* (Poepp.) I.M.Johnst., a terrestrial and photosynthetic orchid, with four OTUs of Ceratobasidiaceae G.W. Martin and three OTUs of Tulasnellaceae Juel separately, isolated from adult plants of the same species. Additionally, the effect of nutrient addition on the outcome of symbiotic germination and on the growth rates of these mycorrhizal fungi was evaluated.

2 Materials and methods

2.1 Orchid species and seed collection

Bipinnula (subtribe Chloraeinae, Orchidoideae) is a genus of terrestrial, photosynthetic orchids endemic to southern South America. It includes a separate group of five species endemic to Chile (Cisternas et al. 2012; Novoa et al. 2015). *B. fimbriata*

is the most frequent of these five species; it is distributed in lowland (<500 m) coastal areas from 29 to 35°S, preferably on sandy stabilized soils, in open sites exposed to sunlight and marine breezes (Novoa et al. 2015). Mature seeds were collected from three large populations of *B. fimbriata* located in Los Vilos (31°58' S), Zapallar (32°56' S) and Concón (32°33' S). Seed capsules from these populations were mixed, because no local adaptation among these populations was assumed. Then, seeds were dried at room temperature and stored in a glass vessel at 4 °C until sowing.

2.2 Mycorrhizal fungi

Mycorrhizal fungi used in the experiments were isolated in Mujica et al. (2016), where seven populations of *B. fimbriata* were sampled, including its entire range of distribution (Fray Jorge (30°39' S), Los Vilos (31°58' S), Zapallar (32°56' S), Concón (32°33' S), San Antonio (33°33' S), Topocalma (34°07' S) and Constitución (35°09' S)). In each population, 10 individuals were selected, collecting three roots from each which were stored in cold until processed in the laboratory. Fungal associates were isolated from root pieces as described in Mujica et al. (2016). DNA from each fungal isolate was extracted, amplified with the fungal primers ITS1-ITS4 (White et al. 1990), purified and sequenced. After sequencing, fungal OTUs were defined by grouping sequences with more than 97% similarity. Among the isolated OTUs that corresponded to OMF (based on Dearnaley et al. 2012), the most frequent OTUs in the orchid populations were selected to be used in this study: four OTUs of the family Ceratobasidiaceae (*Ceratobasidium* sp.1, *Ceratobasidium* sp.2, *Ceratobasidium* sp.3 and *C. albasitensis*; Genbank accession nos. KP306714 KP306692, KP306722 and KP306721, respectively) and three OTUs of the family Tulasnellaceae (*Tulasnella* sp.1, *T. calospora* and *Tulasnella* sp.2; Genbank accession nos. KP306571, KP306672 and KP306574, respectively).

To illustrate the phylogenetic relationships among the OTUs of mycorrhizal fungi used in this study, the phylogenetic trees of Tulasnellaceae and Ceratobasidiaceae (Fig. 1a and b respectively) that were constructed in Mujica et al. (2016) have been modified to highlight the phylogenetic position of mycorrhizal OTUs used in the experiments. Both trees were inferred using maximum parsimony (MP) and maximum likelihood (ML) approaches implemented in PAUP* version 4.0b10 (Swofford 2002). For MP, a heuristic search was undertaken using TBR branch swapping. Bootstrap support of nodes for MP and ML was computed for 10,000 repetitions. Trees were also constructed using the Bayesian Markov chain Monte Carlo (MCMC) inference (BI) method implemented in MrBayes v 3.1.2. Four simultaneous, independent runs were performed for >10,000,000 generations, starting from random trees. For further details of the phylogenetic inferences, see Mujica et al. (2016).

2.3 Experimental design

To evaluate the effect of fungal identity and nutrients on symbiotic germination, seeds were sown with each fungal OTU under four different nutrient treatments based on OMA medium (oatmeal agar). OMA medium is a solid medium frequently used for orchid symbiotic germination (Janes 2009) and has been successfully utilized in symbiotic germination of *B. fimbriata* (Steinfort et al. 2010; Herrera et al. 2016). We used OMA medium as the control treatment, which contained 3 g/L oatmeal, 0.1 g/L yeast extract and 7 g/L agar. The same components with addition of nitrogen (N) and phosphorus (P) in the forms NH_4NO_3 and KH_2PO_4 were used for the elaboration of enriched nutrient media (N, P and N + P) (Table 1). The concentrations used for N and P were based on Nurfadilah et al. (2013). In each treatment 0.16 mg/L of streptomycin and 0.16 mg/L of penicillin were added, and pH was adjusted to 5.0–5.5 before autoclaving, to reproduce the soil pH of isolation sites of the mycorrhizal fungi.

Seeds were sterilized before sowing by placing them in 2 mL Eppendorf tubes with 1.5 mL 1% sodium hypochlorite solution and five drops of Tween 20. Tubes were shaken for 3 min. Then, the solution was removed with a 5 mL sterile syringe. The seeds were washed three times with autoclaved distilled water, removing the water with a sterilized syringe each time. The sterilized seeds were resuspended in 1.5 mL of sterile distilled water and shaken to obtain a homogeneous suspension. After that, seeds were distributed in Petri dishes (with a mean of 120 ± 54 SD seeds per plate) and then a mycelium plug was transferred to the center of each dish. Each nutritional treatment with each fungal OTU, including a control group without mycorrhizal fungi (hereafter "No fungi" treatment), was replicated ten times, resulting in 480 plates (4 media treatments, 8 fungal treatments, 10 replicates). Petri dishes were stored in a dark room at 18 °C. Germination was evaluated 30 days after sowing, calculating the percentage of seeds in each stage of germination (Mitchell 1989).

The same nutrient treatments (OMA medium, N + P, P and N) were used to evaluate the effect of nutrient concentration on fungal growth. A 0.25 cm^2 plug of mycelium from fungal culture was transferred to the center of Petri dishes containing the media treatments, replicated ten times. Plates were placed in a dark room at 18 °C. Fungal growth was daily measured by marking and photographing the mycelium extension, calculating the area of the mycelium with the ImageJ software (Rueden et al. 2017). Plates were measured until the mycelium covered the entire Petri dish or after 40 days of growth. Growth rate was estimated as the mean difference between daily area measurements during the exponential phase of growth curves.

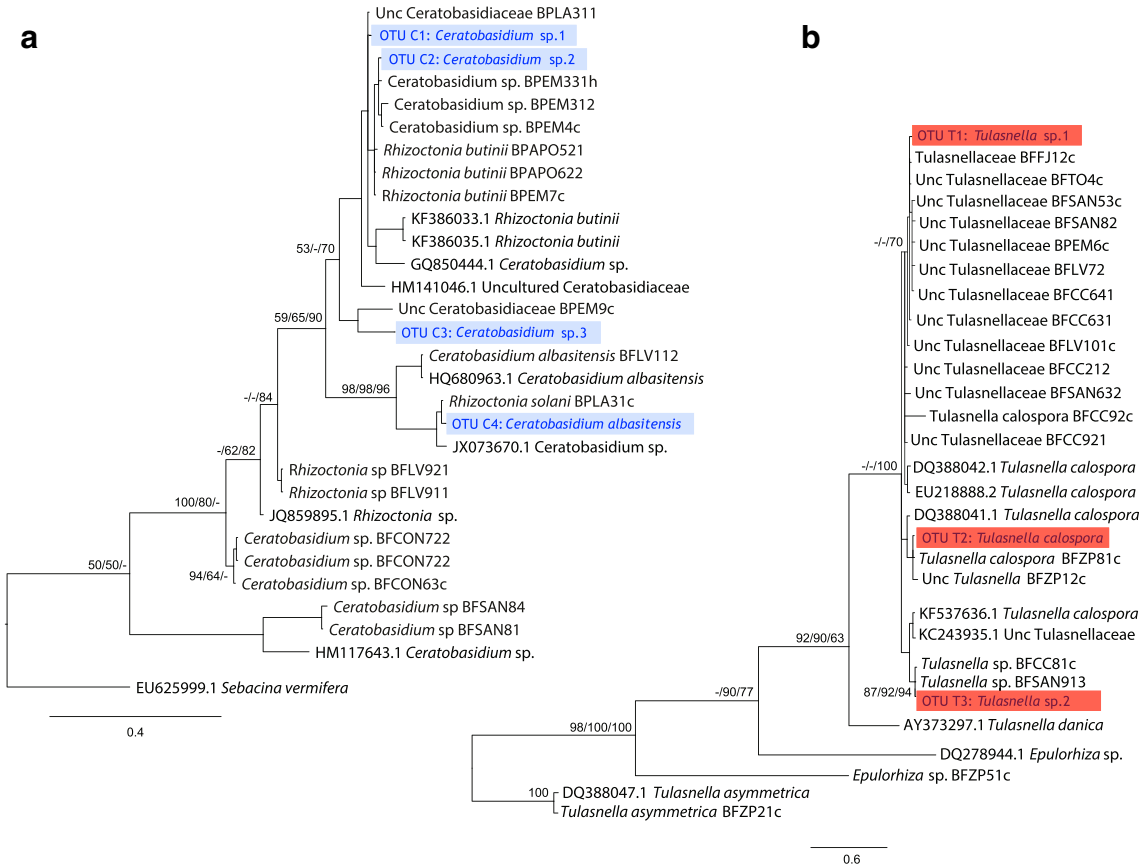

Fig. 1 Phylogenetic relationships among the OTUs of (A) Ceratobasidiaceae and (B) Tulasnellaceae used in this study, modified from Mujica et al. (2016). Trees were constructed based on internal transcribed spacer (ITS) fungal sequences obtained from *Bipinnula fimbriata* and *B. plumosa* roots, and Genbank accessions (see Materials and Methods). Both trees are the Bayesian majority consensus trees, with values on each branch indicating parsimony bootstrap values/maximum likelihood bootstrap values/Bayesian posterior probabilities

2.4 Statistical analyses

We tested the effect of fungal identity and nutrient treatments on the percentage of seeds in stage 1 (coat rupture), on the percentage of seeds in stage 2 (formation of rhizoids), and on fungal growth rates; with fungal identity and treatments as independent factors, including the interaction between them. First, we performed a Shapiro-Wilk normality test to evaluate the normality of the response variables. Stage 1 was the only variable with a normal distribution ($W = 0.99$, p value $= 0.24$), whereas stage 2 and fungal growth rate had distributions significantly different from normal ($W = 0.75$, p value $<2.2e^{-16}$ and $W = 0.93$, p value $= 2.978e^{-09}$, respectively). Then, for stage 1 we used a regular two-ways ANOVAs and a Tukey test for post hoc comparisons. For the other two response variables, we used an Aligned Rank Transform (ART) ANOVA, in which variables are ranked and aligned and then common ANOVA can be used (Wobbrock et al. 2011). For post hoc comparisons we used Wilcoxon signed-rank tests on the original data, and then corrected for multiple comparisons using Holm's sequential Bonferroni procedure (Holm 1978). Analyses were performed in R software (R Core Team 2018).

Table 1 Components of the nutrient treatments used in the germination and fungal growth experiments

Treatment	Component				
	Oatmeal	Yeast extract	Agar	NH4NO3	KH2PO4
OMA medium	3 g	0.1 g	7 g	0 g	0 g
N + P	3 g	0.1 g	7 g	0.3 g (3.7 mM N)	0.3 g (2.3 mM P)
P	3 g	0.1 g	7 g	0.3 g (3.7 mM N)	0.6 g (4.5 mM P)
N	3 g	0.1 g	7 g	0.6 g (7.5 mM N)	0.3 g (2.3 mM P)

3 Results

After 30 days of culture, seeds reached stage 1 (rupture of coat) or stage 2 (formation of rhizoids) of symbiotic germination. The encounter between mycorrhizal fungi and seeds in plates with mycorrhizal fungi had two possible outcomes: a "non-symbiotic" or "parasitic" outcome, in which fungal hyphae grew throughout the seed with no further development of the seeds, observed in intact seeds or seeds that reached stage 1 (Fig. 2a and b); or the mutualistic outcome, where the seedlings would be penetrated by hyphae forming the rhizoids, reaching stage 2 of symbiotic germination (Fig. 2c-f).

3.1 Stage 1: Seed coat rupture

Seed coat rupture was observed under all nutrient treatments, with or without the presence of mycorrhizal fungi. However, the percentage of seed coat rupture was significantly different among fungal OTU (Df = 7, F = 46.09, p value $<2e^{-16}$); higher percentages were observed with the OTUs *Tulasnella* sp.2, *Ceratobasidium* sp.1 and *T. calospora*, whereas lower percentages were observed in *Tulasnella* sp.1, *C. albasitensis*, and in plates without fungi (Fig. 3a). Nutrients and the interaction between them and fungal identity were also significant (Df = 3, F = 27.23, p value = $5.01e^{-15}$ and Df = 21, F = 3.23, p value = $7.18e^{-06}$, respectively). In the fungal species *Ceratobasidium* sp.1, *Tulasnella* sp.1 and also in plates without mycorrhizal fungi, there was a significantly higher percentage of seed rupture in OMA medium than in the other treatments, while in the rest of fungal OTUs no significant differences among nutrient treatments were found (Fig. 3a).

3.2 Stage 2: Rhizoid formation

Similarly to what was observed in stage 1, fungal identity, nutrients and the interaction between them had a significant effect on the percentage of rhizoid formation (fungal identity: Df = 7, F = 145.1, $p < 2e^{-16}$; nutrients: Df = 3, F = 153.7, $p < 2e^{-16}$; interaction: Df = 21, F = 16.26, $p < 2e^{-16}$), showing that the effect of nutrients on symbiotic germination varied among fungal OTUs. Nevertheless, in opposition to stage 1, stage 2 was observed exclusively in plates with the presence of mycorrhizal fungi. All mycorrhizal fungi promoted the development of rhizoids in OMA medium (Fig. 3b), while in under nutrient-enriched media (P, N + P and N), this only occurred in plates with the OTUs *Ceratobasidium* sp.1 and *Ceratobasidium* sp.2 (Fig. 3). The OTU *Ceratobasidium* sp.1 presented no differences in rhizoid formation among the four treatments, whereas *Ceratobasidium* sp.2 showed significantly higher germination in OMA medium, followed by N, without a significant difference between N + P and P treatments. By contrast, rhizoid formation was almost inexistent

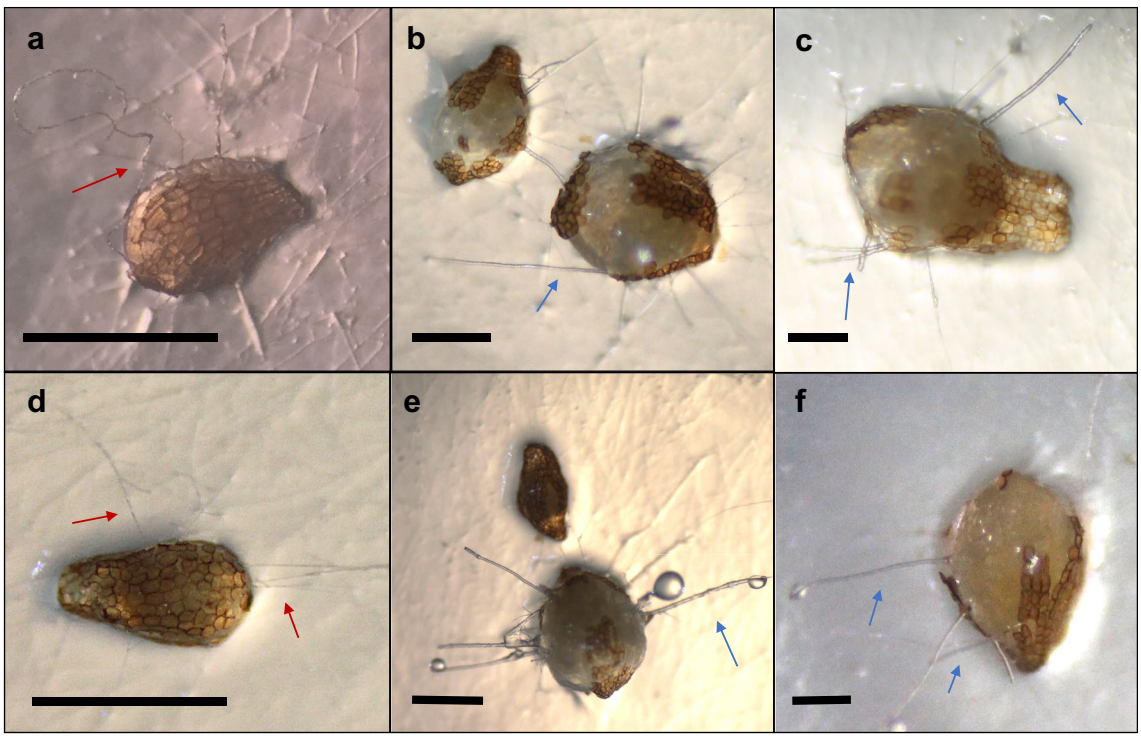

Fig. 2 Different outcomes in the symbiotic germination of *Bipunnula fimbriata*. In the non-mutualistic outcome the fungal mycelium grows covering the seeds and no further development of the seeds is observed (A and B; red arrows indicate fungal hyphae), whereas in the mutualistic outcome, the seedlings are penetrated by hyphae, the embryo increases its size and form rhizoids (D,E,F,G; blue arrows indicate rhizoids). Black scale bar: 500 μm

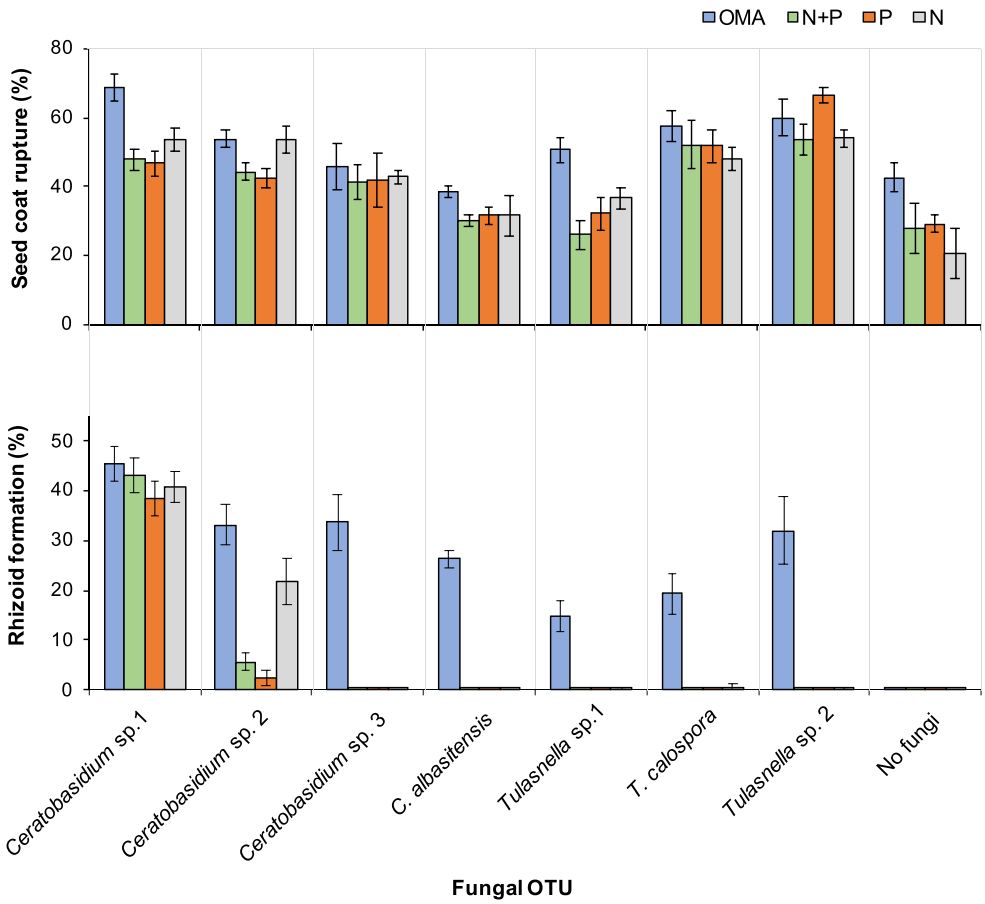

Fig. 3 Percentage of *Bipinnula fimbriata* seeds in the two first stages of symbiotic germination under four nutritional treatments with seven OTUs of orchid mycorrhizal fungi and a control without mycorrhizal fungi. Panel (A) shows the seed coat rupture (stage 1) and panel (B) the formation of rhizoids (stage 2). Treatments are: Oatmeal Agar (OMA), OMA with additional nitrogen and phosphorus (N + P), OMA with additional phosphorus (P), and OMA with additional nitrogen (N). Significant differences among nutrient treatments are shown in lowercase letters or with asterics (*) when only one treatment is significantly different from the rest. n.s. = non-significant

under nutrient addition treatments for *Ceratobasidium* sp. 3, *C. albasitensis*, *Tulasnella* sp.1, *T. calospora* and *Tulasnella* sp.2, with no significant differences among treatments (N, P, N + P). In those treatments, the aforementioned fungal OTUs grew through the seeds and germination did not continue further.

3.3 Fungal growth

There was a significant effect of fungal identity, nutrient treatment and the interaction between them on fungal growth (fungal identity: Df = 6, F = 235.4, $p < 2e^{-16}$; nutrient: Df = 3, F = 163,2, $p < 2e^{-16}$; interaction: Df = 18, F = 29.3, $p < 2e^{-16}$). Nutrient treatment significantly affected growth of all fungal OTUs except for *Ceratobasidium* sp.1 and *Ceratobasidium* sp.2, which showed no significant differences among treatments (Fig. 4). The OTUs *Ceratobasidium* sp. 3, *C. albasitensis*, *Tulasnella* sp.1 and *Tulasnella* sp.2 presented the same pattern of growth under the nutrient treatments. In these OTUs the highest growth rate was observed in OMA medium (N addition = 0 g/L), followed by a similar growth rate in N + P and P (N addition = 0.3 g/L), with the lowest growth rate under N treatment (N addition = 0.6 g/L), suggesting a negative effect of N concentration on growth rate (Fig. 4). Lastly, *T. calospora* presented a different pattern, with the lowest growth rate in OMA medium and no significant differences among N, P and N + P (Fig. 4).

4 Discussion

In this study we found that under controlled conditions, fungal identity and nutrient concentration are determinant for the outcome of symbiotic germination in *Bipinnula fimbriata*. As was expected in hypothesis 1, the effect of nutrient addition on symbiotic germination varied depending on the identity of the fungal partner involved. Also, as expected in hypothesis 2, the variation in the effect of nutrients on symbiotic germination was related to different fungal responses to nutrient availability. However, further research is needed to understand the underlying mechanism.

4.1 Outcomes of symbiotic germination in *B. fimbriata*

In orchid symbiotic germination, hyphal penetration may occur through rhizoids or through the seed suspensor, depending on the orchid species (Rasmussen et al. 2015). In the former

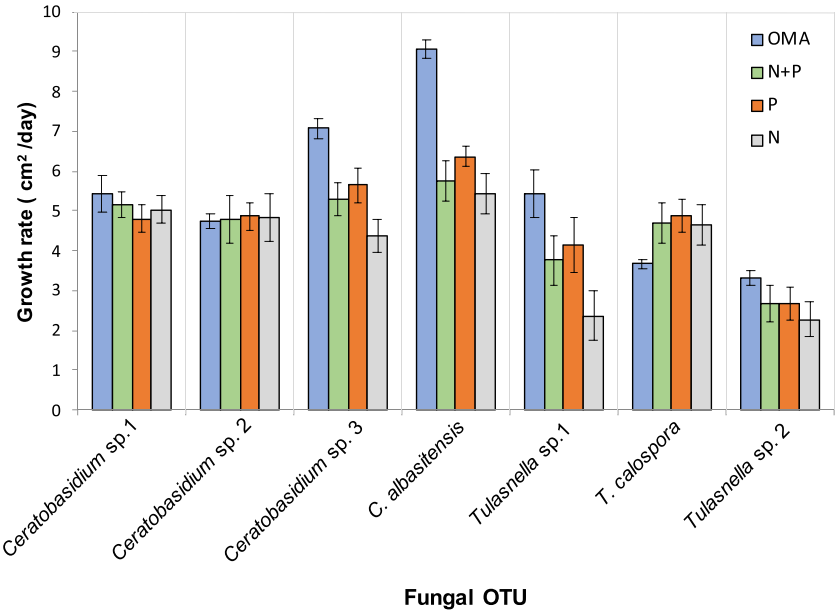

Fig. 4 Growth rate of the seven mycorrhizal OTUs isolated from *Bipinnula fimbriata* roots under four nutritional treatments. Treatments are: Oatmeal Agar (OMA), OMA with additional nitrogen and phosphorus (N + P), OMA with additional phosphorus (P), and OMA with additional nitrogen (N). Significant differences are shown in lowercase letters. ns = not significant

case, seeds can produce rhizoids before the fungal invasion occurs, then seedlings can survive while the nutrient storage last or until a fungus penetrates through rhizoids subsequently assisting development. In the latter, seeds will only develop rhizoids after invasion through the suspensor (Rasmussen and Rasmussen 2014). This study and previous reports observed an absence of rhizoid formation in plates without mycorrhizal fungi in *B. fimbriata* (Steinfort et al. 2010), suggesting that hyphal penetration occurs through the seed suspensor in this orchid species. Swelling of the embryo and rupture of the seed coat were observed in all plates, regardless of nutrient treatment, fungal presence or identity, probably related to seed water uptake (Smith 1967). After this initial stage, the interaction between *B. fimbriata* seeds and mycorrhizal fungi had two possible outcomes the mutualistic or the parasitic outcome (according to Beyrle et al. 1991). In the mutualistic outcome seedlings were penetrated by hyphae and then formed rhizoids. Although rhizoid formation is a signal of compatible interaction, further microscopical observation of pelotons on basal cells of protocorms are needed to support the evidence of a symbiotic association. On the other hand, in the parasitic or non- mutualistic outcome, fungal hyphae grew through the seed without rhizoid formation, with no further development of the seeds. Nevertheless, since we did not observe a notorious overgrowth of the fungus through the entire protocorm (Fig. 2a and b), as was observed in Beyrle et al. (1991), we refer to this outcome as "non-mutualistic" rather than "parasitic".

Contrasting outcomes of symbiotic germination have also been observed in other orchid species associated with rhizoctonia (Smith and Read 2008; Adamo et al. 2020), and it has been suggested that it could be related to changes in fungal gene expression. Recently, Adamo et al. (2020) tested if different outcomes observed in the orchids *Serapias vomeracea* (Burm.f.) Briq. and *Cattleya purpurata* (Lindl. & Paxton) Van den Berg with the fungus *Tulasnella calospora* (Boud.) Juel were related to a switch of the fungus from a mycorrhizal to a parasitic behavior, which would be demonstrated by an increase in the expression of plant cell wall (PCW) degrading enzymes in unsuccessful protocorms. However, no significant differences in fungal enzyme expression were observed between symbiotic and rotting protocorms, discarding that the switch from mutualistic to parasitic outcome was related to a switch in fungal behavior.

4.2 Effect of fungal identity

B. fimbriata was able to form rhizoids (stage 2 of germination) with the seven fungal OTUs tested in this study, suggesting a low mycorrhizal specificity in this orchid species, at least under low nutrient availability. This agrees with other studies on *B. fimbriata* germination that showed lack of specificity (Steinfort et al. 2010; Herrera et al. 2016). Nevertheless, fungal identity had a significant effect on germination; the OTU *Ceratobasidium* sp.1 presented the highest percentages of germination and the OTU *Tulasnella* sp.1 the lowest. This differs from the results obtained by Herrera et al. (2016) in which a higher germination of *B. fimbriata* seeds with *Tulasnella* species was observed. This difference could be due to local adaptation of seeds to different mycorrhizal fungi, since the seeds used in this study were obtained from northern populations of *B. fimbriata*, and in Herrera et al. (2016) seeds were obtained from southern populations. Further studies evaluating the effect of local adaptation would contribute to understand these differences. Nevertheless, both results

suggest that fungal identity affects the result of the symbiosis between *B. fimbriata* and mycorrhizal fungi.

4.3 Effect of nutrient addition

Similar to Beyrle et al.'s (1991) findings, the addition of nutrients in *B. fimbriata* turns the interaction between orchid seeds and mycorrhizal fungi from mutualistic to parasitic in most fungal species, but as expected in hypothesis 1, this effect varies among species. Five out of seven fungal species showed a negative effect of nutrient addition on rhizoid formation. Remarkably, there were no differences among the three enriched treatments (N + P, N and P) on symbiotic germination with these five fungi (Fig. 3). This suggests that the concentration of nutrients in the N + P treatment was high enough to inhibit the germination of *B. fimbriata*, and thus, there was no effect of an additional N or P increase.

The effect of nutrients on symbiotic germination could be related to a direct effect of nutrients on seeds. Accordingly, at stage 1 we observed a negative effect of nutrients in the plates without mycorrhizal fungi, which would support this hypothesis. Studies on non-symbiotic germination have shown similar results. Ponert et al. (2013) demonstrated that nitrate had a negative effect on non-symbiotic germination of *Pseudorchis albida* (L.) Á.Löve & D.Löve, even at extremely low concentration. Likewise, Figura et al. (2020) studied the effect of nitrate on non-symbiotic germination in seven orchid species from meadows in Europe, finding that orchids from sites with natural low concentrations of nitrate were more sensitive than orchids from eutrophic habitats. These results suggest that the observed effect of nutrient addition on symbiotic germination could be mediated by a direct effect of nitrate on seeds. In our case, the three enriched treatments used nitrate at inhibitory levels (according to Ponert et al. 2013; Figura et al. 2020; see Table 1), which could explain the negative effect of nutrients on germination observed in plates without mycorrhizal fungi.

4.4 Variation among fungal species

Although there was a negative effect of nutrient addition on symbiotic germination in most fungal species, we observed no effect of nutrient addition in *Ceratobasidium* sp.1 and a weaker effect in *Ceratobasidium* sp. 2. A similar pattern was observed in the orchids *Gymnadenia conopsea* (L.) R.Br. and *Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh. (Figura and Ponert 2017) in which the effect of nitrate addition on symbiotic germination varied depending on fungal identity. In a similar manner to our observations, a negative effect of nitrate was observed in their study, for *Tulasnella* sp. and *Sebacina* sp., but not for *Ceratobasidium* sp. These authors proposed that an inhibitory effect of nitrate on germination would be counteracted by some fungal species such as *Ceratobasidium* sp. species (Figura and Ponert 2017), which would be supported by our findings.

The variation in the ability to counteract the effect of nutrients could be related to the differences in nutritional tolerances among OMF (Midgley et al. 2006; Nurfadilah et al. 2013; Fochi et al. 2017; Mehra et al. 2017). Accordingly, we observed that nutrient addition did not affect the growth of the OTUs *Ceratobasidium* sp.1 and *Ceratobasidium* sp.2, but it strongly affected the growth rates of the rest of fungal OTUs (Fig. 4), which coincides with their differences in the response to nutrients in symbiotic germination. Further research is required, however, to understand the relationship between nutrient tolerances and response to nutrients in symbiotic germination. Remarkably, *Ceratobasidium* sp.1 and *Ceratobasidium* sp.2, which had a similar response to nutrients in growth and symbiotic germination, are also phylogenetically closer than the other *Ceratobasidium* OTUs (Fig. 1), suggesting that these responses might be phylogenetically conserved.

4.5 Ecological implications

Soil nutrients have been suggested to be an important driver of orchid ecology (Dijk et al. 1997) and recent studies have demonstrated their key role in orchid mycorrhizal associations (Bell et al. 2020; Mujica et al. 2020; Vogt-Schilb et al. 2020), however there is still poor understanding of the underlying mechanisms. The variation of the effect of nutrients on symbiotic germination among fungal species could contribute to understand the role of nutrients in orchid mycorrhizas and may have important ecological implications for orchids. For example, a mycorrhizal fungus that is capable of germinating orchid seeds in a broader range of nutrient availability should be a suitable partner in a wider range of habitats. This was observed for *Ceratobasidium* sp. 1, able to germinate seeds equally under low and high nutrient concentrations (Fig. 3), and present in a wider range of habitats associated with *Bipinnula* (Mujica et al. 2016).

4.6 Methodological limitations

In vitro studies are far from reproducing the complexity of the natural conditions were orchid symbiotic germination occurs. In this study we measured the effect of nutrients in a system with one orchid and one fungal species, whereas in situ symbiotic germination is expected to be affected by the presence of other mycorrhizal species or by other fungal groups such as saprotrophs or pathotrophs. An artificial medium also does not reflect the complex soil structure in which this interaction occurs in nature, where nutrients are not equally distributed in the substrate. Finally, we measured symbiotic germination only until the second stage of seed development (rhizoid formation), in this way, the results might be different if

germination is assessed until further stages. Then, although our study suggests a key role of fungal identity and nutrient availability, further research including in situ and non-symbiotic germination experiments is needed to fully understand the role of nutrients in orchid symbiotic germination.

5 Conclusions

This study demonstrates that nutrients influence symbiotic germination and that its effect varies depending on mycorrhizal fungal identity under in vitro conditions. More studies are needed to understand the underlying mechanism of this variation, and also to outweigh how widespread this response to nutrients is across orchid species and OMF, especially considering its ecological implications.

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