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Ceratobasidium orchid mycorrhizal fungi reveal intraspecific variation and interaction with different nutrient media in symbiotic germination of *Prasophyllum* (Orchidaceae)

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Abstract
Understanding how nutrient requirements of orchid mycorrhizal fungi (OMF) affect symbiotic germination is essential for the ex situ conservation of threatened orchids and their mycorrhizal symbioses. Yet the influence of isolate-level variation in OMF nutrient preferences on orchid germination is unknown. We tested germination of *Prasophyllum frenchii* (Orchidaceae) on 15 different media of varying carbon and macronutrient compositions with three *Ceratobasidium* isolates of the same operational taxonomic unit (OTU) as determined with internal transcribed spacer locus sequencing. There was a significant interaction between media and fungal isolate on percentage germination, with each isolate recording its highest percentage germination on different nutrient media (Isolate 9.3: 5.2 ± 1.4% on MOM–S; Isolate 8.2: 5.4 ± 1.1% on MOM + S; Isolate 4.3: 2.2 ± 0.5% on 1.25 g/L wheat bran agar). Across all isolates, germination (percentage germination > 0) occurred more frequently on wheat bran agar media (39.7% of plates) than on oatmeal agar media (6.0% of plates). There was also an effect of media type on aerial hyphal growth behaviour of the OMF isolate. All isolates supported growth through to adult flowering plants. We demonstrated that symbiotic germination of *Prasophyllum* is affected by media composition. Further, percentage germination and aerial hyphal growth behaviour differed significantly among OMF isolates of the same OTU. This illustrates that a diversity of functionally significant fungal strains occurs within a single OTU, a previously unknown aspect of OMF research with important ecological and conservation implications.

Keywords Orchid mycorrhizal fungi · Symbiotic germination · Orchid · *Prasophyllum* · *Ceratobasidium* · Propagation

1 Introduction

Mycorrhizal symbioses with fungi are an essential component of the ecology of most plants (Balestrini and Lumini 2018; Brundrett and Tederoo 2018). This is accentuated in the Orchidaceae, the world's second largest plant family (WCSP 2022), which are completely dependent on orchid mycorrhizal fungi (OMF) for seed germination in the wild (Bernard 1899, 1902; Smith and Read 2008). As well as their obligate requirement for mycorrhiza, the Orchidaceae is also well known for containing a high proportion of species threatened with extinction (Fay 2018; IUCN 2022), caused by a multitude of anthropogenic factors (reviewed in Reiter et al. 2016; Wraith and Pickering 2019). For some threatened orchids, translocation of plants grown ex situ into areas of suitable habitat represents one of the few options for recovery (Swarts and Dixon 2009; Reiter et al. 2016). Translocations of threatened orchids for conservation purposes are likely to be more successful when using orchid plants grown with their symbiotic OMF, compared to plants grown without their symbiotic OMF using asymbiotic methods (Reiter et al. 2016; Phillips et al. 2020). Therefore, the identification of factors affecting symbiotic orchid germination is a priority for effective conservation of orchids and their mycorrhizal symbioses (Rasmussen et al. 2015; Phillips et al. 2020).

Orchid mycorrhizal fungi require sources of carbon and macronutrients, including nitrogen and phosphorous, both

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for their own growth and to pass to their orchid hosts (Cameron et al. 2006, 2007; Smith and Read 2008). The form and concentration of carbon, nitrogen and phosphorus can affect symbiotic germination of orchids, although the nature of this effect is highly variable across different OMF and orchids (Table 1). While it is possible these differences reflect direct nutrient preferences among seeds of different orchid species (Figura et al. 2020) or other factors influencing germination (Rasmussen et al. 1990; Rasmussen 1992), hyphal growth studies illustrate that OMF genera can have preferences for differing forms of carbon and nitrogen (Hadley and Ong 1978; Nurfadilah et al. 2013; Mehra et al. 2017). Recent studies have begun to explore the influence of genus-level OMF nutrient preferences on symbiotic germination of orchids. Work by Fochi et al. (2017) showed that *Tulasnella calospora* was unable to use nitrate but could access other forms of organic nitrogen including ammonium. Nitrate has also been found to suppress symbiotic germination with *Tulasnella* and *Serendipita* OMF relative to other forms of nitrogen, but not with *Ceratobasidium* OMF (Figura et al. 2021). The effect of nutrient media has recently been demonstrated to vary among OMF taxa within the same genus, with different germination responses observed among different *Ceratobasidium* OTUs by Mujica et al. (2021).

Results from symbiotic orchid germination trials frequently report differences in germination ability among isolates of the same OTU (Rasmussen 1995; Raleigh 2005; Huynh et al. 2009; Tan et al. 2014; Oktalira et al. 2019; Fuji et al. 2020; Freestone et al. 2021). Whether this is due to differences in nutrient requirements among isolates of OMF within an OTU is unknown. If so, it could represent a mechanism for variation in the mycorrhizal 'niche' of sub-OTU

Table 1 Review of studies testing the composition of nutrient media on symbiotic orchid germination

Nutrient	Study	Orchid mycorrhizal fungus	Orchid genus	Additive	C (%)	G	S	Result	Basal medium
Carbon	Perkins et al. 1995	*Ceratobasidium*	*Microtis*	Sucrose	0.5		✓	Increase[1]	1/6 strength NDY agar
	McQualter 2012	*Ceratobasidium*	*Prasophyllum*	Sucrose	0.5	✓		Decrease[1]	OMA
	Tomita and Tsutsui 1988	*Tulasnella*	*Liparis*	Oatmeal	1		✓	Increase[2]	OMA
	Tomita and Tsutsui 1988	*Ceratobasidium*	*Spiranthes*	Oatmeal	2		✓	Increase[2]	OMA
	Mala et al. 2017	*Tulasnella*	*Dendrobium*	Oatmeal	1	✓		Increase[3]	OMA
	Yamamoto et al. 2017	*Tulasnella*	*Bletilla*	Oatmeal	0.25– 1	✓		No effect	OMA
Macro-nutrients	Figura et al. 2021	*Tulasnella, Serendipita*	*Dactylorrhiza*	Nitrate	0–100 (mg/L)	✓		Decrease[1]	OMA
		Ceratobasidium						No effect in one isolate, decrease in another[1]	
	Zettler et al. 2007	*Tulasnella*	*Epidendrum*	$Ca(NO_3)_2$, KH_2PO_4, KCl, $MgSO_4$, yeast extract, sucrose	As per MOM + S[A]	✓		Decrease[1] (compared to OMA)	OMA
	Mujica et al. 2021	*Tulasnella*	*Bipinnula*	Nitrate, phosphate	0.03 or 0.06	✓		Decrease[1]	OMA
		Ceratobasidium	*Bipinnula*	Nitrate, phosphate	0.03 or 0.06	✓		Decrease or no effect[1]	OMA

C concentration of the additive, *G* the effect was tested on germination, *S* the effect was tested on seedling growth, *NDY* nutrient dextrose yeast agar, *OMA* oatmeal agar

[1] compared to absence of added compound(s)

[2] compared to lower and higher concentrations

[3] compared to lower concentrations

[A] refer to Supplementary Table 1

level 'strains' of OMF in the wild (Selosse et al. 2018), as different OMF strains are able to access different nutrient resources (Pellegrino et al. 2014).

Macronutrient concentrations can also affect the stability of the orchid-OMF relationship in culture, with high nitrogen (Beyrle et al. 1991; Beyrle 1995) and low carbon concentrations (Beyrle 1995) leading to a change from a mycorrhizal to a parasitic interaction. Additionally, this effect can be dependent on the family or genus of OMF involved, with pronounced increased parasitism of orchid protocorms with *Ceratobasidium* OMF on high nitrogen media, but not with *Tulasnella* OMF (Dijk and Eck 1995). Symbiotic germination studies with *Ceratobasidium* OMF have often observed parasitism or smothering of germinating protocorms by aggressive hyphal growth with negative effects on germination (Williamson and Hadley 1970; Beyrle et al. 1991; Zettler 1997; Hajong et al. 2013). Aggressive fungal growth of *Ceratobasidium* OMF in vitro is influenced by both the nitrogen (Beyrle et al. 1991; Dijk and Eck 1995) and carbon (Beyrle 1995; Hajong et al. 2013) content of symbiotic germination media, with more stable associations between orchid and fungi observed on media containing insoluble forms of carbon (e.g. cellulose) compared to those containing a soluble carbon source (oatmeal) (Hajong et al. 2013). Optimising the nutrient composition of symbiotic germination media to the family or genus of OMF may help improve germination outcomes (Phillips et al. 2020), particularly with genera of OMF prone to aggressive or parasitic behaviour in vitro.

Prasophyllum R.Br. is a large genus of terrestrial orchids containing over 140 species from southeast and southwest Australia and New Zealand (Jones 2021). The genus contains 38 Australian endemic species currently listed as nationally threatened on the Australian Government's *Environment Protection and Biodiversity Conservation Act 1999* (DCCEEW 2022). *Prasophyllum* form mycorrhizal associations with at least 11 OTUs of *Ceratobasidium* (Basidiomycota) (Freestone et al. 2021). *Prasophyllum* seed are difficult to germinate symbiotically, with promising germination results for some species (*P. diversiflorum* and *P.* sp. aff. *validum*; McQualter 2012) but not others (*P. correctum*; Huynh and Coates 1999). Unreliable symbiotic germination methods is a major issue hampering ex situ conservation efforts for this genus.

We use *Prasophyllum frenchii* F.Muell, an endangered orchid from south eastern Australia (DCCEEW 2022), as a model *Prasophyllum* species. The studied adult population of *P. frenchii* have a specific mycorrhizal association with a single OTU (Freestone et al. 2021; Freestone 2022). We hypothesize that the nutrient media composition will affect the ability of individual isolates of *Ceratobasidium* to germinate seed. Specifically, we investigated if, (i) isolates of *Ceratobasidium* vary in optimal media requirements for germination of *Prasophyllum*; (ii) the nutrient media composition influences the growth habit (presence of aerial hyphae) of the *Ceratobasidium* mycorrhizal fungal isolates; and (iii) there is an effect of isolate or nutrient media composition on post-germination growth and development of seedlings through to flowering.

2 Methods

2.1 Study species and site

Prasophyllum frenchii is a spring–summer flowering (October-December), summer- autumn dormant, terrestrial orchid endemic to lowland native grasslands and swamps across southern Victoria and southeast South Australia (Mueller, 1889). It produces a single, terete leaf to 60 cm height, followed by a single inflorescence containing 10–40 small, nectar-producing flowers (Mueller, 1889) and is listed as endangered in Australia under the *Environment Protection Biodiversity and Conservation Act 1999*. The species is currently known from seven populations containing around 5000 plants (DCCEEW 2022). The site chosen for this study was a large population of ca. 1000 plants growing in a 4 ha remnant lowland native grassland at Yarram, Victoria, Australia. The population of *Prasophyllum frenchii* at Yarram has been intensively studied and adult plants are known to associate with a single *Ceratobasidium* OMF (OTU I in Freestone et al. 2021; Freestone 2022).

2.2 Seed collection

Naturally pollinated seed from 30 plants (inflorescences) was collected in November 2017. Mature fruiting inflorescences with seed pods that were close to or just starting to dehisce were cut at ground level and placed in water for up to one week to finish ripening. When seed pods began to dehisce, the inflorescence was removed from the water and dried to 15% relative humidity for two weeks. The seed was then cleaned by removing all inflorescence material, and seed was pooled together and stored in airtight glass vials over silica gel (Sigma–Aldrich, St. Louis, United States of America) at 4 °C for 16 months prior to sowing. Results from previous asymbiotic germination trials on the Yarram population indicated that naturally pollinated *P. frenchii* seed has low viability ($1.6 \pm 0.5\%$ and $10.3 \pm 1.8\%$ for two separate asymbiotic germination trials; M. Freestone unpublished data), although these data were recorded from different seed batches to that used in this study.

2.3 Fungal isolation

Root samples from three plants were collected from the Yarram population in September 2017. Roots were washed in tap water to remove soil, the epidermis was removed aseptically with a scalpel under a dissecting microscope in a drop of sterile water and the root was then cut open to liberate pelotons. Pelotons were rinsed three times in sterile water with a sterile pipette and plated onto Fungal Isolation Media (FIM) (Clements et al. 1986) containing 0.05 g/L streptomycin sulfate and incubated for 1 week at 20 °C. Hyphal tips from one actively growing peloton per plant were excised (i.e. three isolates, one from each plant), plated onto individual plates of FIM and stored at 20 °C until use.

2.4 Identity of fungi

Although detailed morphological analyses were not undertaken, all three isolates appeared visually identical. Therefore, molecular analysis was used to determine the identity of the three fungal isolates. A single actively growing culture of each isolate was transferred to liquid low CN Melin-Norkrans Medium broth (Marx and Bryan 1975) in preparation for DNA sequencing. Once adequate growth had been attained (about 12 weeks), fungal hyphae were removed from the media, blotted dry on sterile paper towel, immediately frozen and stored at -80 °C until further processing. Frozen tissue from the mycorrhizal cultures was freeze-dried and then ground with a bead and mechanical bead beater before DNA was extracted using a DNeasy Plant Mini Kit (Qiagen).

The internal transcribed spacer (ITS) primer pair ITS5 (White et al. 1990) and ITS4 (White et al. 1990) was used to PCR amplify the ITS1-5.8S-ITS2 region of the nuclear genome, as previously described by (Swarts et al. 2010). Briefly, each 30 µl PCR reaction consisted of 0.12 µl of MyTaq DNA polymerase, 5 µl of 5 × MyTaq Polymerase Buffer (Meridian Bioscience, Cincinnati, U.S.A.), 1 µl of each 10 µM primer, 2 µl template DNA and 20.88 µl sterile dH_20. PCR cycling conditions were 10 min at 6 °C, 3 min at 95 °C; 34 cycles of 15 s at 94 °C, 15 s at 60 °C, 1 min at 72 °C; followed by 10 min at 72 °C. Amplification success was assessed by electrophoresis on an agarose gel. PCR products were purified (ExoCIP) and bi-directionally Sanger sequenced using BigDye 3.1 on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). All sequences were edited and checked manually using Geneious v.10.1 (www.geneious.com).

Sequences of the three fungal isolates were initially aligned with sequences of *Ceratobasidium* OMF from Australia (alignment from Freestone et al. 2021) using MUSCLE in Geneious v.10.1. We used p-distance to calculate pairwise sequence dissimilarity distances as determined by MEGA v.10.2.6 (www.megasoftware.net) with default settings. In this initial alignment, the three fungal isolates in this study appeared most similar to OTU I (0.9% pairwise dissimilarity). To confirm this result, all unique sequences from the group containing OTU G, OTU H and OTU I in Freestone et al. (2021) were aligned with the three sequences from this study using a MUSCLE alignment in Geneious v.10.1. The alignment was trimmed to between 549–559 base pairs (ungapped) and manually edited.

Phylogenetic analysis was undertaken using the MrBayes plugin in Geneious v.10.1. Two parallel runs of four chains each were run for 1 million generations and trees sampled every 1000 generations after a 10% burn-in. To verify that the burn-in was sufficient, likelihood profiles were examined. Convergence of runs was confirmed when the average standard deviation was < 0.01 and effective sample sizes > 200. The analysis was performed using the GTR + G model of nucleotide substitution. The Bayesian tree was visualised in Figtree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) with *T. fusisporus* as the root. Molecular Operational Taxonomic Units (OTUs) were delimited based on posterior probability branch support and using the 5% pairwise dissimilarity threshold for the ITS region from Freestone et al. (2021).

2.5 Effect of media on symbiotic germination

Fifteen different symbiotic media were used: oatmeal agar (OMA) with 0.5 g/L, 1.25 g/L, 2.5 g/L and 5 g/L of oatmeal (all with 0.05 g/L yeast extract), 2.5 g/L OMA without yeast extract, wheat bran agar with 0.5 g/L, 1.25 g/L, 2.5 g/L and 5 g/L of wheat bran with yeast extract, 2.5 g/L wheat bran agar without yeast extract, modified oats medium with sucrose (MOM + S) and without sucrose (MOM–S), water agar, and two media (LLMM-sucrose and LLMM-sucrose + 2.5 g/L oatmeal) that were included based on results from a successful asymbiotic medium (Freestone 2022), but modified by removing the sucrose. The composition of media is given in Supplementary Table 1. All media were adjusted to pH 6 prior to autoclaving with 1 M KOH.

Seeds were pre-soaked in a 1% sucrose solution for 24 h at 20 °C to stimulate germination of any fungal contaminant spores. The sucrose solution was then pipetted off and the seed were bleached for 4 min in a 10% solution of Domestos (Unilever, London, England) containing ca. 0.5% m/v NaOCl, vacuum filtered to remove the bleach, and rinsed in sterile water three times. Seed (100–200 seeds per plate) was pipetted onto sterile filter paper using a vacuum filter, and the filter paper then placed on top of the medium in the plate. Finally, a small (~ 0.5 cm^3) cube of one of the three *Ceratobasidium* isolate cultures was placed to one side of the plate and plates sealed with Parafilm (Bemis, Neenah, Wisconsin). Seventeen replicate plates for each of the three

fungal isolates were used for each of the 15 media (Supplementary Table 1). Sowing was undertaken seven months after the isolation of fungal isolates.

Plates were stored in the dark in two black plastic boxes located next to each other on the same shelf in a temperature-controlled growth room on a 24-h rotation of 16 °C for 8 h and 20 °C 16 h for three months. After three months all plates were scored for germination by assigning protocorms to germination stages from Clements et al. (1986). Plates containing protocorms at stage 3 (initiation of leaf primordia) or above were removed from the boxes and incubated under a 24-h rotation of 8 h dark followed by 16 h light under the same temperature regime. From four to six months post-sowing, the number of germinated seedlings that had reached an advanced stage 5 (> 1 cm leaf length) were recorded and subsequently replated into flasks following the methods of Reiter et al. (2016). Stage 5 seedlings were used to define germination due to the observation that protocorms frequently do not progress beyond earlier stages (Huynh and Coates 1999; Dowling and Jusaitis 2012). The plates were re-sealed to allow further seedlings to germinate. All plates were scored for a second time at six months to ensure seedlings which were slower to germinate were included in the data, with the number of seedlings previously removed being added to the total number of stage 5 seedings. After six months, lids of all plates with vigorous hyphal growth were removed to search for protocorms that may have been obscured by the hyphae, which were added to the counts.

Percentage germination among media, among the three *Ceratobasidium* isolates and the interaction between media and isolate, were modelled using data on the proportion of germinated seeds per plate with a quasi-poisson, full factorial general linear model using the glm() function from the tidyverse package in R (R Core Team 2022). Generalised linear models with a quasi-poisson log link distribution were used due to over dispersion and a deviance higher than the degrees of freedom. Media that did not support any germination with one or more of the three *Ceratobasidium* isolates, were removed prior to analyses to remove zero data values. Differences in modelled germination percentages were tested with ANOVA using a chi-squared test at $\alpha = 0.05$. Individual contrasts were tested using post-hoc Tukey tests without adjusting p values (Nakagawa 2004).

The effect of wheat bran versus oatmeal as a preferred carbon source was compared between OMA media and wheat bran agar media. Due to large numbers of zero values (no germination) in the OMA media, we combined all *Ceratobasidium* isolates and all OMA/wheat bran concentrations for this analysis, i.e. testing only the identity of the carbon source (oatmeal versus wheat bran). All media were scored using binary data for germination (presence / absence). The overall difference between oatmeal-based media and wheat bran-based media was modelled using a binomially distributed generalised linear model with the glmmTMB() function in the car package in R. Differences in modelled germination between oatmeal-based media and wheat bran-based media were tested with ANOVA using a chi-squared test at $\alpha = 0.05$.

2.6 Aggressive aerial *Ceratobasidium* hyphal growth

We measured aerial hyphal growth as an indication of the degree of pathogenic-like behaviour of the *Ceratobasidium* isolate. Aggressive aerial hyphal growth is often observed in unstable orchid-fungus relationships when protocorms are parasitised by the fungus (Beyrle et al. 1991; Zettler 1997; Hajong et al. 2013). Aerial hyphal growth was observed as moderate or aggressive. Aggressive *Ceratobasidium* aerial hyphal growth we define here as aerial hyphae covering more than 20% of the surface of the plate, with moderate growth defined as aerial hyphae covering less than 20% of the plate surface. Aerial hyphal growth was recorded on the germination plates at six-months and scored as either aggressive or moderate. The effect of media and *Ceratobasidium* isolate on the proportion of plates with observed aggressive aerial hyphal growth was modelled using a binomial additive general linear model using the glm() function in R (R Core Team 2022). An additive model was used instead of a factorial model because of the large number of zeros in the data. Media that did not support aggressive aerial hyphal growth with any *Ceratobasidium* isolates were excluded from the analysis. Differences in modelled germination percentages were tested with ANOVA using a chi-squared test at $\alpha = 0.05$. Individual contrasts were tested using post-hoc Tukey tests with non-adjusted *p* values. The relationship between aggressive aerial hyphal growth and germination (summarised to a binary variable) across all isolates and media was tested with a binary binomial general linear model using the glm() function in R (R Core Team 2022).

2.7 Post-germination seedling growth and flowering

Surviving seedlings were transferred into plastic flasks (five seedlings per flask) following the methods of Reiter et al. (2016), with flasks containing a 3 cm layer of vermiculite (mixed with 120 mL/L of water) over a 3 cm layer of 1.25 g/L wheat bran agar, 5 g/L wheat bran agar, OMA, MOM–S or MOM–S with wheat bran instead of oatmeal. There was at least one flask of each of these five flasking media per *Ceratobasidium* isolate. The total number of flasked seedlings was 186 (with isolate 9.3), 90 (with 8.2) and 40 (with 4.3). The increase in length of each seedling's leaf was measured by recording the height of the seedling on the flask wall when flasked and after one month in

the flask, any decrease in media thickness (due to evaporation or consumption by the fungus), was taken into account. Survival of each seedling was also recorded (alive/dead).

The effect of flasking media on leaf length increase of seedlings in the flasks was modelled using a linear mixed model, with the flask number set as a random effect, using the lmer() function in the lmerTest package in R (R Core Team 2022). Differences in the leaf length increase were tested with the summary() function in the tidyverse package in R. Due to small number of flasks for *Ceratobasidium* isolate 4.3 and 8.2, the effect of flasking media was combined across all fungi. The effect of flasking media on survival of seedlings in the flasks was modelled using a binomially distributed generalised linear model with the glmmTMB() function in the car package in R. Differences in modelled leaf length increase and survival among flasking media were tested with ANOVA using a chi-squared test at $\alpha = 0.05$.

Seedlings aged seven months were then transferred to 15 cm pots in the nursery containing Royal Botanic Gardens Victoria terrestrial orchid BioGro potting mix (BioGro, Dandenong, Australia) with 10–20 seedlings per pot and watered as required. *Ceratobasidium* that supported surviving plants to flowering (two years after potting) in the nursery were deemed to have supported plants through to adulthood. The number of surviving seedlings was counted in July 2021, three years post-transferral.

3 Results

3.1 Effect of media on symbiotic germination

Five media did not support any germination (water agar, 1.25 g/L OMA, 2.5 g/L OMA, LLMM-sucrose, LLMM-sucrose + 2.5 g/L oatmeal), two media only supported germination with one isolate (0.5 g/L OMA with *Ceratobasidium* isolate 4.3 and 0.5 g/L bran with *Ceratobasidium* isolate 9.3, both less than 1% germination) and two media supported germination with only two of the three isolates (2.5 g/L OMA-yeast and 5 g/L OMA, both with *Ceratobasidium* isolates 8.2 and 9.3, all less than 1% germination) (Table 2; Figs. 1, 2).

Across the remaining six media treatments, there was a significant interaction between media type and *Ceratobasidium* isolate ($p < 0.001$) meaning the highest percentage germination for each isolate occurred on different media. For isolate 4.3, the highest percentage germination occurred on 1.25 g/L bran (2.2 ± 0.5%), which recorded significantly higher germination than MOM–S (z-ratio = 2.192, $p = 0.028$) and 5 g/L bran (z-ratio = 2.161, $p = 0.031$), but was not significantly higher than 2.5 g/L bran, 2.5 g/L bran-yeast or MOM + S (Table 2; Fig. 1). The highest percentage germination for isolate 8.2 occurred on MOM–S (5.4 ± 1.1%), which was significantly higher than all other media (Table 2; Fig. 1). The highest percentage germination for isolate 9.3

Table 2 Percentage germination across fungi and media in the symbiotic media trial. Germination was defined as Stage 5 (leaf-bearing) seedlings. Significantly different percentage germination (non-adjusted post-hoc Tukey test results) within each medium and overall among fungi, are denoted by superscript ^{A,B,C}; and within each fungus and overall among media, are denoted by subscript $_{i,ii,iii,iv}$

Medium	Mean germination (%) ± SE			Average germination (%) per medium across all fungi ± SE
	Fungus			
	4.3	8.2	9.3	
WATER AGAR	0.0	0.0	0.0	0.0
1.25 g/L OMA	0.0	0.0	0.0	0.0
2.5 g/L OMA	0.0	0.0	0.0	0.0
LLMM–SUC + 2.5 OAT	0.0	0.0	0.0	0.0
LLMM–SUC + 2.5 OAT	0.0	0.0	0.0	0.0
0.5 g/L OMA	0.5 ± 0.5	0.0	0.0	0.2 ± 0.2
0.5 g/L BRAN	0.0	0.0	0.8 ± 0.6	0.3 ± 0.2
2.5 g OMA—yeast	0.0	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.1
5 g/L OMA	0.0	0.4 ± 0.2	0.5 ± 0.3	0.3 ± 0.2
2.5 g BRAN—yeast	$1.0 \pm 0.4^{B,C}{}_{i,ii}$	$0.3 \pm 0.2^{C}{}_{ii}$	$2.4 \pm 0.6^{A,B}{}_{iii}$	$1.3 \pm 0.4_{i}$
2.5 g BRAN	$1.2 \pm 0.4^{A}{}_{i,ii}$	$1.2 \pm 0.5^{A}{}_{ii}$	$2.2 \pm 0.7^{A}{}_{iii}$	$1.5 \pm 0.5_{i}$
1.25 g/L BRAN	$2.2 \pm 0.5^{A}{}_{i}$	$0.5 \pm 0.3^{B}{}_{ii}$	$2.8 \pm 0.7^{A}{}_{iii}$	$1.9 \pm 0.5_{i}$
5 g/L BRAN	$0.4 \pm 0.2^{B}{}_{ii}$	$1.1 \pm 0.3^{B}{}_{ii}$	$3.2 \pm 0.9^{A}{}_{ii,iii}$	$1.6 \pm 0.5_{i}$
MOM + S	$0.7 \pm 0.6^{B}{}_{i,ii}$	$0.7 \pm 0.4^{B}{}_{ii}$	$5.2 \pm 1.4^{A}{}_{i,ii}$	$2.2 \pm 0.8_{i}$
MOM–S	$0.1 \pm 0.1^{B}{}_{ii}$	$5.4 \pm 1.1^{A}{}_{i}$	$3.3 \pm 0.8^{A}{}_{ii,iii}$	$2.9 \pm 0.7_{i}$
Average germination (%) per fungus across all media ± SE	0.9 ± 0.4^{B}	1.5 ± 0.5^{B}	3.2 ± 0.9^{A}	

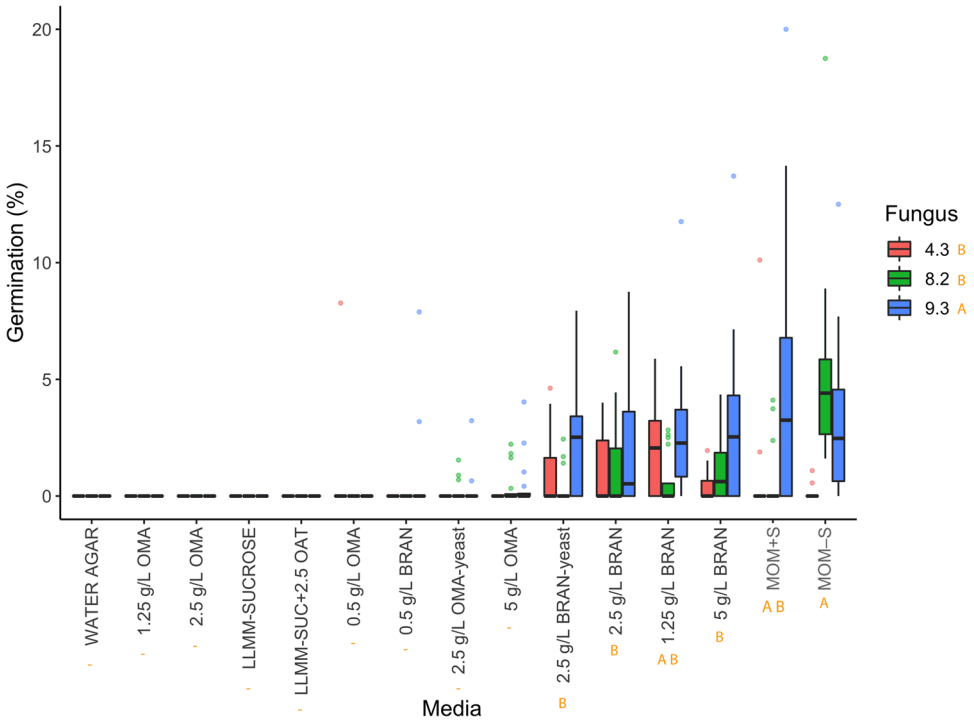

Fig. 1 Boxplots of percentage germination of *Prasophyllum frenchii* seed using three *Ceratobasidium* isolates belonging to OTU C in Freestone et al. (in press) on 15 nutrient media. Dots represent outliers. Results from ANOVA using post-hoc Tukey tests with unadjusted *p* values are in orange; A denotes significantly higher germination than B (groups denoted by – were excluded prior to statistical analysis)

occurred on MOM + S (5.2 ± 1.4%) and was significantly higher than all media except 5 g/L bran (z-ratio = 1.553, $p = 0.121$) and MOM–S (z-ratio = 1.473, $p = 0.141$) (Table 2; Fig. 1).

There was a significant effect of *Ceratobasidium* isolate on germination percentage averaged across all six modelled media ($p < 0.001$) (Table 2; Fig. 1). Germination with isolate 9.3 (3.2 ± 0.9%) was significantly higher than with either isolate 8.2 (1.5 ± 0.5%; z-ratio = 4.820, $p < 0.001$) or 4.3 (0.9 ± 0.4%; z-ratio = 4.897, $p < 0.001$) averaged across all modelled media (Table 2; Fig. 1). There was no significant difference between 8.2 and 4.3 (z-ratio = 1.184, $p = 0.236$).

Across all isolates, germination occurred significantly more often on wheat bran-based media (39.7% of plates) than on oatmeal agar (6.0% of plates; z-value = -7.650, $p < 0.001$).

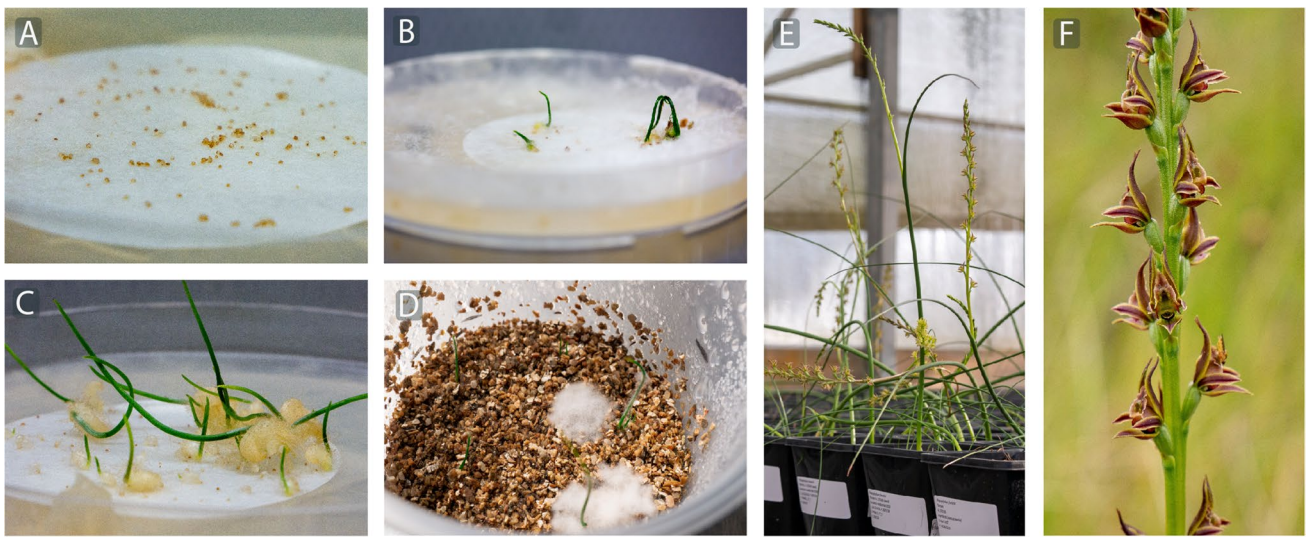

Fig. 2 Example photos of germinating *Prasophyllum frenchii* seed on symbiotic media: **A**) no germination (Stage 2 only) on 2.5 g/L OMA with isolate 9.3; **B**) germination and aggressive fungal behaviour on MOM with isolate 8.2; **C**) germination and non-aggressive fungal behaviour on MOM-sucrose with isolate 9.3; **D**) seedlings in flasks with visible fungal growth; E) mature plants grown with all three isolates flowering 2.5 years after sowing; and, F) *P. frenchii* flowering in the wild

3.2 Aerial hyphal growth

There was a significant effect of fungal isolate on aerial hyphal growth across all isolates ($p < 0.001$; Figs. 2 and 3). Isolate 8.2 recorded 26.6% plates with aggressive aerial growth, significantly more than both 4.3 (4.2% of plates; z-ratio = -6.411, $p < 0.001$) and 9.3 (2.9% of plates; z-ratio = 6.627, $p < 0.001$) (Fig. 3). Isolates 4.3 and 9.2 were not significantly different (z-ratio = 0.831, $p = 0.406$). Despite aerial growth being frequently recorded in this study, we did not observe degraded protocorms that might indicate obvious parasitism.

There was also a significant effect of media on aerial growth across all media ($p < 0.001$; Fig. 3). MOM + S recorded 35.4% of plates with aggressive aerial growth, significantly higher than 0.5 g/L OMA (2.0% of plates; z-ratio = 3.570, $p = 0.004$), 1.25 g/L bran (4.0% of plates; z-ratio = 3.825, $p < 0.001$), 2.5 g/L OMA (7.3% of plates; z-ratio = 3.401, $p = 0.001$), 2.5 g/L OMA-yeast (10.4% of plates; z-ratio = 3.311, $p = 0.001$), LLMM-sucrose (2.0% of plates; z-ratio = 3.614, $p < 0.001$) and 2.5 g/L bran-yeast (10.9% of plates; z-ratio = 3.191, $p = 0.001$). Four media recorded no aerial hyphal growth (0.5 g/L bran, 1.25 g/L OMA, LLMM-sucrose + 2.5 g/L oatmeal and water agar) (Fig. 3).

Germination was significantly more likely to occur in plates that displayed aggressive aerial hyphal growth, with 35.8% of plates displaying aggressive aerial hyphal growth supporting germination, compared to 19.7% of plates with moderate aerial hyphal growth (z-value = 3.26, $p = 0.001$).

3.3 Molecular identity of fungal isolates

We confirm the three *Ceratobasidium* isolates used in this study belong to a single unnamed OTU, related to Thanatephorus fusisporus (OTU I in Freestone et al. 2021; Fig. 4). Sequences of the three isolates were identical according to p-distance, with the only differences being several ambiguities (Prfre4.3_Y_17 = 5 ambiguities, Prfre8.2_Y_17 = 3

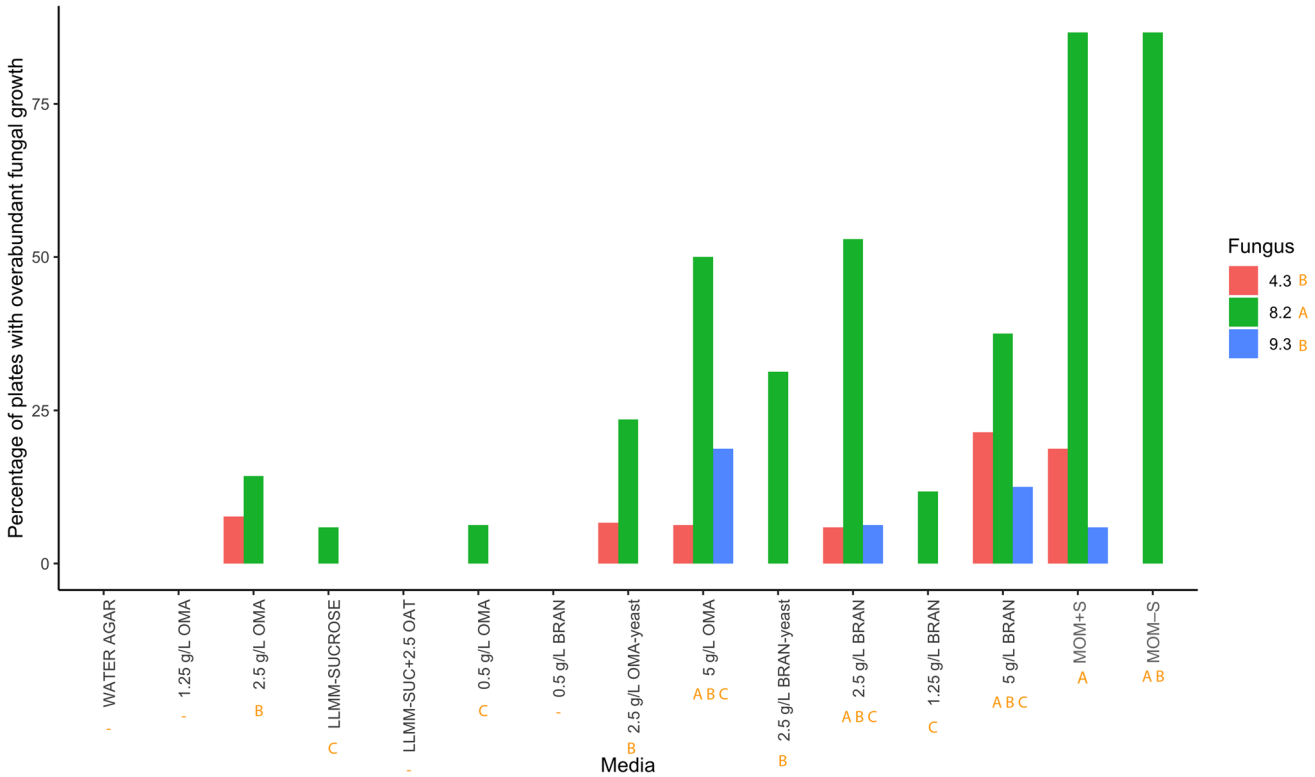

Fig. 3 Percentage of plates displaying aggressive aerial fungal growth across all media and fungal isolates. Results from ANOVA comparing media across all fungal isolates are in orange, using post-hoc Tukey tests with non-adjusted p values. A denotes significantly more frequent aggressive aerial fungal growth than B and B is more frequent than C (groups denoted by – were excluded prior to statistical analysis)

ambiguities; Prfre9.3_Y_17 = 2 ambiguities) due to variation among ITS copies within isolates.

3.4 Post-germination seedling growth and flowering

There was no significant effect of media on leaf length increase across all isolates ($F = 2.084$; $p = 0.094$) with leaf length increase ranging from 3.1 ± 0.9 mm in 5 g/L bran to 7.9 ± 1.5 mm in MOM–S + bran (Table 3). The variance of the random effect (flask) was 3.40 compared to a residual variance of 77.31, indicating that there was much more variance in leaf length increase among flasks than within flasks. There was no effect of media on survival of seedlings in the flasks across all fungi (p = 0.187), with percent survival ranging from 33 ± 7% in 5 g/L bran to 55 ± 4% in MOM–S + bran (Table 3).

All *Ceratobasidium* isolates supported seedlings through to adulthood (Fig. 2). Percentage survival three years post-transferral of seedlings to pots in the nursery was very high at 87.7% overall (85.7% for isolate 4.3, 82.4% for isolate 9.3 and 100% for isolate 8.2).

4 Discussion

We have demonstrated that carbon and nutrient media composition affect the ability of near-identical isolates of the same *Ceratobasidium* OTU to germinate seed, suggestive of functional diversity among strains of an OMF OTU.

4.1 Effect of media on symbiotic germination

The composition of nutrient media has previously been shown to influence the germination of orchid seed symbiotically (Table 1), and here we demonstrate its influence on isolate level germination efficacy. The media composition treatments we applied in this study influenced the ability of isolates of the same *Ceratobasidium* OTU to germinate seed, suggestive of functional diversity among strains of an OMF OTU. There was a significant interaction between *Ceratobasidium* isolate and media in this study. The three isolates were all from a single *Ceratobasidium* OTU (OTU I in Freestone et al. 2021) and had ITS sequences with identical p-distances, only varying in several ambiguities among ITS copies. Yet, despite their high level of ITS sequence similarity, they displayed variable germination efficacy on the media tested. Isolate 4.3 recorded its highest germination percentages on 1.25 g/L bran, isolate 8.2 with MOM–S and isolate 9.3 with MOM + S. The interaction between media and isolate shows that the nutrient composition of the media was affecting the ability of these fungi to germinate the seeds, rather than acting directly on the nutrient requirements of the seeds themselves. Previous studies have observed differing percentage germination among isolates within the same molecular OTU of *Ceratobasidium* (Freestone et al. 2021), *Tulasnella* (Fuji et al. 2020) and *Serendipita* (Oktalira et al. 2019). However, this is the first study to show that isolates within the same OTU support different germination responses on different nutrient media, suggestive of considerable functional diversity among isolates within a single OTU.

The differences observed among isolates within the same OTU in ex situ culture, may reflect variation in the mycorrhizal 'niche' of these isolates in the wild (Selosse et al. 2018), as different OMF strains are able nutrient resources (Pellegrino et al. 2014). Evidence from this study suggests that different nutrient preferences among OMF strains could affect the germination niche of their host orchids, if differential soil nutrient profiles in the wild affect the ability of individual strains to germinate. It is also possible that associations with a diversity of within-OTU fungal strains could assist host orchids in surviving changeable environmental conditions (McCormick et al. 2004) or across large geographic areas (Davis et al. 2015). In addition, the functional significance of within-OTU fungal strains also raises the prospect that the level of OTU may not be the most appropriate taxonomic level to draw conclusions on OMF ecology and interactions with orchids.

Media with insoluble wheat bran as the carbon source recorded higher germination percentages than the oatmeal-based OMA media. Relatively high percentage seed germination on wheat bran media likely reflects a preference for cellulose as a food source by *Ceratobasidium*, perhaps because it more closely resembles organic matter found naturally in soils (Hadley 1969; Smith 1966). Other OMF genera seem to be less specific about their carbon source, with *Serendipita* OMF displaying high biomass when grown on several different carbon sources (most hexoses and disaccharides) (Mehra et al. 2017). There was no obvious effect of the concentration of carbon source on germination of *P. frenchii* with *Ceratobasidium* in this study, suggesting that the amount of the carbon source is not limiting germination in this system.

Media with added macronutrients (N, P, K, Mg; Supplementary Table 1) recorded the highest percentage germination for two of the three *Ceratobasidium* isolates in this study (8.2 and 9.3 on MOM–S and MOM + S respectively). This supports findings from other studies that demonstrated symbiotic germination with *Ceratobasidium* OMF of four species of *Dactylorhiza* on identical media (MOM + S in Clements et al. 1986), and with *Goodyera repens* Br. (Alexander and Hadley 1983) and *Dactylorhiza purpurella* T. Stephenson & T.A.Stephenson (Hadley 1969) on very similar media (identical apart from the latter two studies using 0.08 g/L Ca $(NO_3)_2$ instead of 0.02 g/L in MOM + S and MOM–S in this study). It is possible that the presence of nitrate is important for symbiotic germination with some *Ceratobasidium* OMF (Figura et al. 2021), and *Ceratobasidium* OMF have been shown to prefer nitrate (along with asparagine, glutamine and glutamic acid) to other forms of


Ceratobasidium cornigerum
Ceratobasidium ramicola
OTU A
Rhizoctonia solani AG-6
Thanatephorus cucumeris
OTU B
OTU C
Ceratobasidium bicorne
OTU D
Ceratobasidium papillatum
OTU E
OTU F
OTU G
T. fusisporus
OTU H
OTU I
OTU J
OTU K
OTU L
OTU M
Ceratobasidium anceps
OTU N
OTU O
Ceratobasidium noxium
OTU P
Rhizoctonia butinii
OTU Q complex
OTU R
Ceratobasidium sp. AG-I
OTU S
OTU T
OTU U
OTU V
Thanatephorus_fusisporus_HQ441575.1
Prasophyllum_incorrectum_T25
Prasophyllum_suaveolens_PsuavR1
Prasophyllum_petilum_ppet1
Prasophyllum_validium_PvalB9
Prasophyllum_murfettii_2.4
Prasophyllum_tadgellianum_3.4R
Prasophyllum_tadgellianum_3.1R
Prasophyllum_murfettii_2.3
Prfre4.3_Y_17
Prfre8.2_Y_17
Prfre9.3_Y_17
Prasophyllum_anticum_Panticum
Prasophyllum_viretrum_PVIT2
Prasophyllum_frenchii_3.11_16
Prasophyllum_niphopedium_PNIPH
Prasophyllum_frenchii_1.4
Prasophyllum_tadgellianum_11.8R
Prasophyllum_tadgellianum_12.7R
Prasophyllum_frenchii_5.2_16
Prasophyllum_frenchii_4.2_16
Prasophyllum_frenchii_5.1_16
Prasophyllum_frenchii_4.7_16
Prasophyllum_frenchii_4.10_16
Prasophyllum_regium_JX138542
Prasophyllum_pulchellum
1
1
1
OTU G
OTU I
OTU H

◂**Fig. 4** Simplified MrBayes phylogeny of ITS sequences from the *Thanatephorus fusisporus* clade, adapted from Freestone et al. (2021). Inset box: OTU G, OTU H and OTU I and the three fungi isolates used in this study in black (Prfre4.3_Y_17, Prfre8.2_Y_17 and Prfre9.3_Y_17,). Branch labels are bayesian posterior probabilities (> 0.80). Phylogeny adapted from: Freestone MW, Swarts N, Reiter N, Tomlinson S, Sussmilch FS, Wright MW, Holmes GD, Phillips RD, Linde CC (2021) Continental scale distribution and diversity of Ceratobasidium orchid mycorrhizal fungi in Australia. Ann Bot 128:329–343

nitrogen in hyphal biomass trials (Nurfadilah et al. 2013). However, isolate 4.3 supported almost no germination on the relatively high macronutrient (and nitrate) MOM + S and MOM–S media, instead recording highest percentage germination on 1.25 g/L bran medium with no added macronutrients. A preference for media without added macronutrients was also observed by Dijk and Eck (1995) with European *Ceratobasidium*-associating orchids and in other studies using *Ceratobasidium* OMF on OMA media (Clements and Ellyard 1979; Batty et al. 2001; Bonnardeaux et al. 2007; Fracchia et al. 2016; Decruse et al. 2018). Clearly there is considerable variation in nutrient preferences among *Ceratobasidium* OTUs, and sometimes among isolates within a single OTU as illustrated in our study, and generalised genus-level conclusions about nutrient requirements of OMF are probably not possible. The majority of studies on symbiotic orchid germination use OMA media (Table 1). Evidence from this study indicates that future studies would benefit from testing a wider range of nutrient media composition both within and between OMF OTUs.

4.2 *Ceratobasidium* aerial hyphal growth

We showed that fungi displaying aerial hyphal growth were more likely to support germination than those with less aerial hyphae. Parasitism of orchid seeds in symbiotic germination trials with *Ceratobasidium* OMF has been frequently reported (Dijk and Eck 1995; Beyrle et al. 1991; Beyrle 1995; Hajong et al. 2013; Gowland 2008) likely due to the aggressive growth nature of these fungi, and because some *Ceratobasidium* OMF are closely related to pathogenic taxa (Veldre et al. 2013; Freestone et al. 2021). However, in our study, aggressive aerial hyphal growth was not associated with parasitism of the orchid seed but instead, reflected vigorous growth on high macronutrient media. Different isolates exhibited different aerial fungal growth characteristics, supporting our hypothesis that there are substantial differences among OMF strains of the same OTU, not just in their ability to germinate orchid seed but in their growth in ex situ culture.

4.3 Post-germination seedling growth and flowering

Despite the strong effect of nutrient composition of germination media on percentage germination, there was no difference in seedling growth or survival across five different flasking media. It is likely that once seedlings start to photosynthesise, they become less dependent on their fungal partner (Cameron et al. 2008) and, therefore, the nutrient

Table 3 The effect of flasking media on growth of symbiotically germinated seedlings. Media are as per Supplementary Table 1, excepting for MOM–S + bran, which is MOM–S but with 2.5 g/L wheat bran instead of oatmeal

Medium	Fungus	No. of flasks	Average % survival ± SE	Average % survival per medium ± SE	Average increase in seedling leaf length (mm) ± SE	Average increase in seedling leaf length (mm) per medium ± SE
OMA	4.3	2	30 ± 10	48 ± 5	1.2 ± 0.0	3.8 ± 0.8
	8.2	4	60 ± 8		7.2 ± 3.9	
	9.3	7	46 ± 7		2.7 ± 1.1	
1.25 g/L bran	4.3	1	60	43 ± 8	3.6 ± 1.5	5.7 ± 1.2
	8.2	3	27 ± 18		3.5 ± 2.9	
	9.3	9	51 ± 9		6.6 ± 3.3	
5 g/L bran	4.3	2	30 ± 10	33 ± 7	1.0 ± 0.6	3.1 ± 0.9
	8.2	2	60 ± 0		9.0 ± 4.0	
	9.3	8	28 ± 8		2.2 ± 0.8	
MOM–S + bran	4.3	1	40	55 ± 4	2.4 ± 2.5	7.9 ± 1.5
	8.2	3	47 ± 7		4.5 ± 1.6	
	9.3	7	60 ± 4		10.2 ± 4.6	
MOM–S	4.3	2	30 ± 10	37 ± 4	3.7 ± 1.9	5.2 ± 1.2
	8.2	6	33 ± 4		5.3 ± 4.7	
	9.3	7	43 ± 8		5.6 ± 1.9	

media in which the fungus is growing has a negligible effect on seedling growth. The causes of high variation in seedling growth among replicate flasks of the same treatment are unknown, but could possibly be due to differences in surface moisture on the agar, humidity, temperature, genetic differences among seed or differences in plant hormones (Phillips et al. 2011).

The flasking process resulted in the death of 50% or more of *P. frenchii* seedlings, which was unexpectedly high. It is unclear why *Prasophyllum* in this study were less likely to survive the flasking step than other genera (e.g. 1–4% mortality rate for *Caladenia*, *Diuris* and *Thelymitra*; Batty et al. 2006) and this step needs to be further optimised for this genus. In contrast, survival three years post-deflasking was very high (87.7%). Despite different germination media preferences among *Ceratobasidium* isolates, once germinated all isolates supported seedlings through to reproductive maturity. This was important to clarify as some orchids are known to associate with a larger number of OMF in the germination phase than are able to support ongoing seedling development (Bidartondo and Read 2008; Těšitelová et al. 2012). A high survival rate following deflasking to the nursery, combined with rapid development to reproductive maturity, shows symbiotic propagation to be an effective method for ex situ propagation of *Prasophyllum.*

5 Conclusions

This study further reveals the complexity of the OMF-orchid relationship. Near-identical isolates of the same *Ceratobasidium* OTU required different nutrient media to achieve the highest percentage germination of *Prasophyllum* and displayed different levels of aggressive aerial hyphal growth in ex situ culture. This illustrates that a diversity of functionally significant fungal strains occurs within a single OTU, a previously unknown aspect of OMF research, which may underpin the ability of these symbioses to span heterogenous edaphic conditions across large geographic areas. Our study illustrates the importance to the orchid-OMF relationship of diversity among fungal strains within an OMF OTU, and we suggest that this theme should be a focus for future research.

In this study, we achieved symbiotic germination of the endangered *P. frenchii,* with potted seedlings displaying high percentage survival and vigorous growth to reproductive maturity (Fig. 2). This represents a major advance in the potential for ex situ conservation for *Prasophyllum* and potentially other orchid genera for which symbiotic germination has not yet been achieved.

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Author contribution All authors contributed to project inception and preparation of manuscript. Data collection and analysis were undertaken by MF.

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