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ORIGINAL ARTICLE

Non-specific symbiotic germination of *Cynorkis purpurea* (Thouars) Kraezl., a habitat-specific terrestrial orchid from the Central Highlands of Madagascar

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Abstract Orchids, particularly terrestrial taxa, rely mostly on basidiomycete fungi in the *Cantharellales* and *Sebacinales* that trigger the process of seed germination and/or initiate the full development of the seedling. During the course of development, orchids may associate with the same fungus, or they may enlist other types of fungi for their developmental needs leading to resilience in a natural setting. This study examined in vitro seed germination and seedling developmental behavior of *Cynorkis purpurea*, a terrestrial orchid from the Central Highlands of Madagascar. This species is mostly restricted to gallery forests in the Itremo Massif, in moist substrate between rocks bordering streams. The main objective was to understand the influence of diverse mycorrhizal fungi on seed germination and further development of *C. purpurea*. The study aims to compare symbiotic versus asymbiotic germination and seedling development with seeds and fungi collected from a 13-km^2 area in the Itremo region. Seeds collected from the wild were sown with diverse orchid mycorrhizal fungi (OMF) spanning 12 operational taxonomic units (OTUs) in three genera (*Tulasnella*, *Ceratobasidium*, and *Sebacina*) acquired from different habitats. Treatments were assessed in terms of the percentage of germinated seeds and fully developed seedlings against those in asymbiotic control media treatments. Overall, OMF significantly improved seedling development within the 12-week experiment period. *Sebacina* as a genus was the most effective at promoting seedling development of *C. purpurea*, as well as having the ability to enter into successful symbiotic relationships with orchids of different life forms; this new knowledge may be especially useful for orchid conservation practiced in tropical areas like Madagascar. A *Sebacina* isolate from an epiphytic seedling of *Polystachya concreta* was the most effective at inducing rapid seedling development and was among the five that outperformed fungi isolated from roots of *C. purpurea*. *C. purpurea* was found to be a mycorrhizal generalist, despite its specific habitat preference, highlighting the complex interaction between the plant, fungi, and the environment. The potential impact on conservation strategies of understanding the requirements for orchid seed germination and development by identifying and using OMF from diverse sources is discussed in detail.

Keywords *Sebacina* · *Ceratobasidium* · *Tulasnella* · Mycorrhizal fungi · Orchidaceae · Seedlings · Conservation · In vitro

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Introduction

Orchidaceae is widely regarded as the largest and the most diverse angiosperm family with ~17,000–35,000 species (Dressler 1993) encompassing 8 % of the vascular plant total (Stokstad 2015). All orchids are uniquely tied to higher fungi for their seed germination needs, and most also for subsequent growth and existence (Rasmussen 2002). Given this obligate requirement—at least in the early growth stages—understanding the patterns of orchid-mycorrhizal relationships is important to integrated orchid conservation (Swarts and Dixon 2009). The current study investigates *Cynorkis purpurea* (Thouars) Kraezl., a terrestrial orchid typically found along wet rocks bordering river margins and streams in the Central Highlands of Madagascar (Cribb and Hermans 2007), often in

gallery forest remnants (Fig. 1). There are about 135 species of *Cynorkis* in Madagascar alone (Cribb and Hermans 2007) with an additional 25 species found in Africa and Mauritius nearby. The considerable diversity within this biodiversity hotspot provides a unique opportunity to study symbiotic relationships between orchids and orchid mycorrhizal fungi (OMF) in different habitat settings. Furthermore, the information gathered has the potential to enhance conservation efforts in the region and worldwide, as the Orchidaceae is the plant family considered most at risk from extinction (Merritt et al. 2014). Among the Orchidaceae, over two thirds fall under the category of epiphytes and lithophytes, with terrestrial species comprising the remainder (Zotz 2013). According to the International Union for Conservation of Nature (IUCN), almost half of the extinct species are terrestrial herbaceous perennials, and the remaining extant taxa are at an elevated risk imposed by anthropogenic factors and climatic change (Swarts and Dixon 2009). In Madagascar, where 90 % of the 1000 orchid species are endemic (Tyson 2000) like *Cynorkis purpurea*, mass deforestation and the prevalence of slash and burn agriculture have led to small, fragmented orchid populations where natural regeneration is low (Whitman et al. 2011). Recovery of many orchids in this region is constrained by their need for specialized pollinators necessary for reproduction (fruit set), and the lack of viable seeds available for studies and/or ex situ conservation. Terrestrial orchid seeds are also often difficult to store; thus, there is a need for improvements to cryopreservation protocols (Hirano et al. 2009).

Noticeable OMF diversity has been reported in different habitats, with the presence of an assortment of fungi in both seedlings and mature phase plants (Hadley 1970; Rasmussen 1995; Currah et al. 1997; Brundrett 2004). Almost all known orchid mycorrhizal symbionts are restricted to two orders of basidiomycetes in the *Cantharellales* and *Sebacinales* (Bidartondo et al. 2004; Selosse et al. 2004), the majority of which fall under the broad category of "*Rhizoctonia*-like fungi" (Arditti 1992; Otero et al. 2002; Rasmussen 2002; Dearnaley 2007) whose members span three separate families (*Ceratobasidiaceae*, *Sebacinaceae*, and *Tulasnellaceae*; Wells 1994). While some orchids apparently target a narrow range of fungi for their physiological needs, especially those with restricted distributions (Swarts and Dixon 2009), photosynthetic (green) orchids of open habitats commonly associate with all three major groups of *Rhizoctonia*-like fungi (Dearnaley et al. 2012). Most of this knowledge stems from studies involving adult orchid growth stages (Shefferson et al. 2007; Roy et al. 2009). Much less is known about the associations in earlier life stages, from initial seed germination leading to maturity, and this is especially true for tropical orchids. To further explore the latter, we investigated the fungal diversity needs of *Cynorkis purpurea* spanning all three families of *Rhizoctonia*-like fungi that we recovered from Madagascar in a previous study (Yokoya et al. 2015). We anticipate that these findings will provide a framework for other species of *Cynorkis* that are endemic to the island (Cribb and Hermans 2009), and many threatened with extinction.

Materials and methods

Plant material

Seeds of *Cynorkis purpurea* were collected from mature ripening capsules on at least 10 different individual plants within the Itremo Massif region of Madagascar in May, 2013, and processed for transport and storage using standard protocols (e.g., Zettler 1997). The seeds were desiccated and stored at 4 °C.

Culture media for seed germination

Cynorkis purpurea seeds and protocorms were sown and cultured on three agar-solidified media. Oatmeal medium (3.5 g powdered oatmeal and 0.1 g yeast extract per liter) of the same or similar formulation has been used by other researchers for orchid symbiotic germination tests and was therefore used for all germination trials with fungi; seeds were also sown on this medium in the absence of fungi (control 1). Oatmeal medium,

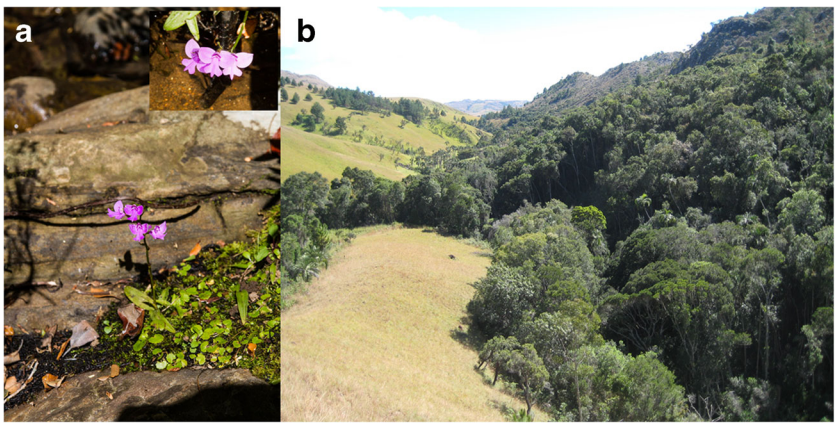

Fig. 1 *Cynorkis purpurea* in the Itremo region of Madagascar's Central Highlands. **a** One flowering and several near-mature size plants of *C. purpurea* growing in accumulated substrate between rocks along a stream in a gallery forest. (*Inset*) Close-up of *C. purpurea* flowers (~15 mm across). **b** Gallery forest in Itremo where *C. purpurea* was found

as above, was supplemented with 10 g sucrose per liter, to test whether the orchid had a requirement for simple carbohydrates (that symbiotically germinating seeds might receive, via fungal associations, from the complex carbohydrates in the standard Oatmeal medium) during germination (control 2). Phytamax™ P6668 (Sigma-Aldrich, UK), a nutrient-rich medium commonly used for asymbiotic germination and propagation of orchids (control 3), was used with the expectation that it would provide all necessary nutrients to support orchid germination and growth. All media were supplemented with 0.6 % (*w*/*v*) agar (A-7002; Sigma-Aldrich, UK) and the pH was adjusted to 5.7 before sterilization by autoclaving. Following sterilization, media were poured into 5-cm-diameter petri plates.

Seed viability staining

Cynorkis purpurea seed viability was assessed using 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; VWR, UK) staining test based on the procedure developed by Hosomi et al. (2012). The seeds were transferred to a layer of filter paper and observed under a stereo microscope (Leica Microsystems MZ16, Germany). The number of full embryos and fully stained embryos was counted to determine the percentage viability of *Cynorkis purpurea* seeds. No significant differences were detected between three different incubation protocols tested. The mean estimated viability of all three treatments was 69.9 %.

Selection of mycorrhizal fungi for germination study

Orchid roots were collected in April/May, 2013, from the Itremo Massif region of Madagascar, immediately after the rainy season, as described in Yokoya et al. (2015). Fungal pelotons were isolated from root cortical cells and identified to genus level (*Ceratobasidium*, *Sebacina*, or *Tulasnella*) from their ribosomal DNA internal transcribed spacer (ITS) sequences. Isolates were grouped into operational taxonomic units (OTUs) based on a conservative similarity threshold of 95 % of the ITS sequence as described previously (Yokoya et al. 2015). Fungi were maintained on fungal isolation medium (FIM; Mitchell 1989).

Fourteen fungal isolates from 12 OTUs were selected for the germination study. Isolates were chosen to represent a selection from each fungal genus and to represent collections from a range of orchid species (including the test species *Cynorkis purpurea*) of different types and habitats. The fungal isolates used in the study are described in Table 1. All fungal strains utilized in this study have been safeguarded at Kew in cryopreservation.

Seed sterilization and sowing

For sterilization, several hundred seeds of *Cynorkis purpurea* were secured within a filter paper packet made of a folded and stapled 54-mm-diameter circular hardened filter paper (Whatman, UK). Packets were sterilized in 100 ml of 0.5 % (*w*/*v*) sodium dichloroisocyanurate (Sigma-Aldrich, UK) solution and a drop of Tween 20 (Sigma-Aldrich, UK) with gentle agitation on an orbital shaker for 30 min (Mitchell 1989). The packets were washed in sterile water three times. Under aseptic conditions, the packets were opened and ~100–200 *Cynorkis purpurea* seeds were added per 5-cm petri plate containing culture media.

Oatmeal medium inoculation for symbiotic culture

Following seed sowing, plates containing oatmeal medium were inoculated with a fungal isolate identified in Table 1, by transferring a 1-cm^3 cube of fungal culture on FIM onto the oatmeal medium. Control treatments were not inoculated with fungi. The plates were sealed with Parafilm (Sigma-Aldrich, UK) and maintained at 18 °C in darkness for 1 week before being moved to light (Cool White, Osram, Germany; PPFD, 10 μmol m^{-2} s^{-1}) under 16/8-h light/dark photoperiod. Each fungal isolate was used to inoculate two plates.

Assessment of germination

Using a stereo microscope (Leica-microsystems MZ16, Germany), every seed in each treatment was assessed according to six developmental stages defined by Yamazaki and Miyoshi (2006): stage 0, no embryo growth; stage 1, embryo is swollen, filling the seed coat; stage 2, embryo begins to emerge from the seed coat; stage 3, embryo is released from the seed coat, forming a protocorm; stage 4, protocorm begins to form rhizoids on its surface; and stage 5, protocorm forms a differentiated shoot and becomes a seedling (Fig. 2).

The seeds were assessed at the end of the first and second week and then every fortnight, until the end of the 12-week trial. Seeds were recorded as germinated once they reached stage 3 germination to rule out the possibility that embryo swelling was attributed to water imbibition alone. The effectiveness of each treatment was assessed by the percentage of stage 5 seedlings produced by the end of the 12-week trial, to help identify fungal isolates that induced both germination and seedling development.

Statistical analysis

Statistical analysis was performed in R 3.0.1 using the survival package, to test whether there was a significant difference in the rates of stage 5 seedling development with different

Table 1 Details of the different *Ceratobasidium*, *Sebacina*, and *Tulasnella* OTU number, isolate number, orchid source, life forms of orchids, growth stage of orchid, and habitat from where plant material was collected

Genus (OTU)	Fungal isolate	Orchid source	Orchid type	Root sample	Description of habitat
Ceratobasidium (cer1)	E1	*Aerangis* sp. 1	Epiphytic	Mature?	Seasonally dry gallery forest
Ceratobasidium (cer2)	L1	*Aerangis ellisii*	Lithophytic	Seedling	Montane grassland
Ceratobasidium (cer3)	E2	*Aerangis* sp. 2	Epiphytic	Seedling	Dry gallery forest
Ceratobasidium (cer4) *Sebacina* (seb1)	C1 C2	*Cynorkis purpurea*	Terrestrial	Seedling Mature	Gallery forest alongside stream
Sebacina (seb2) *Sebacina* (seb3)	E3 E4	*Polystachya concreta* seedling	Epiphytic	Very small seedling	Seasonally dry, exposed granite, and grassland
Tulasnella (tul1)	L2	*Angraecum magdalenae*	Lithophytic	Seedling	Montane grassland in a crack in granite
Tulasnella (tul3)	L3	*Angraecum protensum*	Lithophytic/Terrestrial	Seedling	Montane grassland on an exposed granite slope facing east
Tulasnella (tul3) *Tulasnella* (tul3)	C3 C4	*Cynorkis purpurea*	Terrestrial	Mature Seedling	Gallery forest alongside stream
Tulasnella (tul4)	C5			Seedling	
Tulasnella (tul5)	T1	*Tylostigma* sp.	Terrestrial	Mature?	Montane moorland, among grass, dark acidic soil
Tulasnella (tul6)	T2	*Tylostigma nigrescens*	Terrestrial	Mature	Montane grassland

Isolate numbers were assigned with a prefix to indicate the life form of the original host orchid: E1–E4 for epiphyte, L1–L3 for lithophyte, and T1–T2 for terrestrial, except for C1–C5 for isolates isolated from *Cynorkis purpurea*

isolates. Fungal isolates that did not induce any stage 5 development were omitted from the test.

Standard statistical analysis could not be used on the data due to the method of data collection. Each fortnight, the same seeds sown with each isolate were classified in terms of their germination stage; this violates the assumption of independent measures, which is used in most statistical tests. Therefore, a Kaplan–Meier graph and the more specialized comparison of

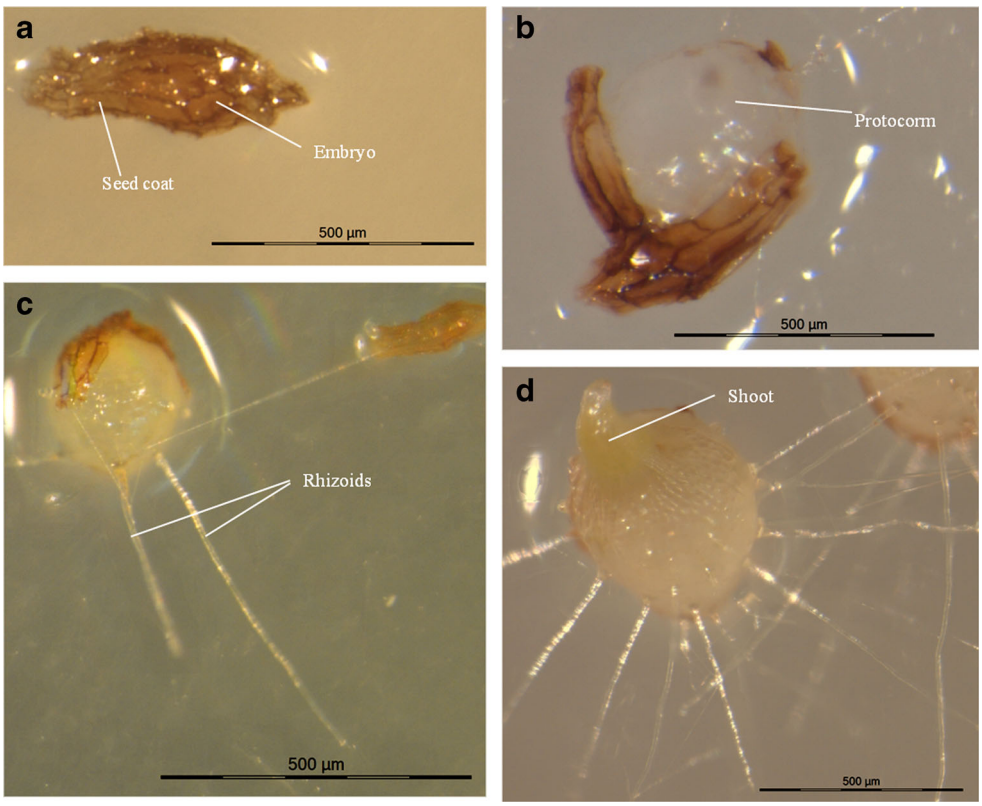

Fig. 2 The successive germination stages of *Cynorkis purpurea* from seed embryo imbibition to seedling formation: **a** stage 0, no embryo growth; **b** stage 3, embryo is released from the seed coat; **c** protocorm begins to form rhizoids on its surface; **d** stage 5, protocorm forms a differentiated shoot and becomes a seedling

survivor functions test were used to analyze whether there were statistically significant differences between the cumulative percentages of stage 5 seedlings produced (McNair et al. 2012). A Kaplan–Meier estimator of survivor function was plotted to model the probability of seeds not developing into stage 5 seedlings. An analysis of survivor function was then performed to test whether there was a significant difference between the curves.

Orchid–fungus interaction

Confocal microscopy was used to investigate colonization of fungi at stage 4 (protocorm stage). Four fungal isolates, T1 (OTUtul5) and E2 (OTUcer3) that facilitated germination to stage 5, and C1 (OTUcer4) and C5 (OTUtul4) that failed to support conversion to stage 5, were studied. A protocorm germinated on oatmeal medium alone was also included for comparison with symbiotically germinated protocorms. Four weeks after sowing, protocorms were removed from the medium and stored in 70 % (*v*/*v*) ethanol at 4 °C. Protocorms were fixed in 4 % formaldehyde as described (Deshmukh et al. 2006) and mounted in Vectashield containing DAPI (Vector Laboratories, Peterborough, UK). The natural autofluorescence of the fungus and plant cell walls was observed using a Nikon A1 Plus confocal microscope (Nikon Instruments Europe B.V., Kingston upon Thames, UK) in oil immersion. The samples were excited and fluorescence detected at 403.4, 487.8, 561.5, and 638.9 nm. DAPI was excited at 360 nm and emitted at a wavelength of 460 nm when bound to DNA. The images were viewed and 3D composite images assembled using NIS Elements Confocal Microscope Imaging Software (Nikon Instruments Europe B.V., Kingston upon Thames, UK).

Results

Assessing fungal isolates for their ability to induce germination and development

Cynorkis purpurea seeds were germinated in combination with potentially mycorrhizal fungi isolated from Madagascan orchid roots, as well as on several different media without the inoculation of fungi. In all treatments using oatmeal medium, with or without sucrose and in the presence or absence of fungi, germination was first recorded 2 to 4 weeks after the seeds were sown, whereas germination was not seen on the asymbiotic medium P6668 (control 3) until week 6. After 12 weeks, all treatments yielded germination although, at 7 %, considerably less on the asymbiotic medium P6668 compared to other treatments (Table 2).

Although TTC staining predicted that the seed batch had a viability, and therefore a maximum possible germination rate, of 70 % (represented by the thick black line in Fig. 3), control treatments on oatmeal medium and oatmeal plus sucrose medium yielded germination rates of 76 and 96 %, respectively. The seeds sown with OTUseb3, OTUtul1, and one of the three isolates of OTUtul3 also exceeded this estimate, producing 74, 74, and 70 % germination, respectively (Table 2). A chi-square test comparing seed viability and the germination rate with E4 (OTUseb3) and L2 (OTUtul1) showed $p < 0.001$, demonstrating a significantly greater than expected rate of germination in the presence of these isolates.

After 12 weeks, none of the seeds developed to stage 5 on oatmeal medium, whereas 39 % of the seeds sown on oatmeal plus sucrose medium had converted to stage 5 seedlings after the same period of time. The asymbiotic P6668 control 3 medium did not support the conversion to stage 5 seedlings in 12 weeks, but a small number of seedlings eventually developed on this medium subsequent to the 12-week trial period (data not shown). However, even after a similar extended period, no stage 5 seedlings were seen on oatmeal control 1 medium (data not shown).

There does not appear to be a correlation between the germination percentage and the conversion to stage 5 seedlings (Fig. 3). For example, E4 (OTUseb3), which induced the highest percentage of germination (74 %), also induced the highest proportion of stage 5 seedlings (69 %), whereas L2 (OTUtul1) which induced the second highest percentage of germination (74 %) did not induce the development of any stage 5 seedlings (Table 2). Interestingly, the conversion rates display stark contrasts between isolates (Table 2); seeds sown with *Sebacina* tended to facilitate the development of the most seedlings, followed by *Ceratobasidium* and then *Tulasnella*.

Overall, fungal isolates that were not collected from *Cynorkis purpurea* roots induced a greater percentage of seedlings compared to fungi isolated from *Cynorkis purpurea* (Fig. 3). Only two out of the five *Cynorkis*-derived isolates produced any seedlings, in comparison to seven out of nine non-*Cynorkis* isolates. *Cynorkis purpurea* isolates also had a far lower yield of stage 5 seedlings (between 1 and 4 %) in comparison to non-*Cynorkis* isolates (2–69 %; Table 2).

The four isolates that induced the most development to stage 5 [E4 (OTUseb3), E3 (OTUseb2), T1 (OTUtul5), and E2 (OTUcer3)] had already produced more seedlings by week 6 than did the other isolates by week 12. In particular, E4 (OTUseb3) induced the most rapid development of *Cynorkis purpurea*, with 50 % of the seeds already reaching stage 5 by the sixth week. By contrast, the second best performing isolate, E3 (OTUseb2), only managed to induce 49 % of seeds to develop to stage 5 by week 12 (Table 2).

A Kaplan–Meier estimator of survivor function was carried out to test whether there was a significant difference in the rates of stage 5 development between different isolates. Only fungal isolates that induced seedlings were considered for this analysis. The analysis showed that the probabilities of seeds

Table 2 Fungal isolates tested for symbiotic germination of *Cynorkis purpurea*, ranked by percentage of stage 5 seedlings (fully developed seedling) after 12 weeks

Genus	OTU (isolate name)	Germination by week 12 (%±SE)	Stage 5 by week 12 (%±SE)	Conversion rate (%±SE)
Sebacina	seb3 (E4)	74±13	69±1	92±4
Sebacina	seb2 (E3)	61±3	49±1	80±4
Tulasnella	tul5 (T1)	51±3	33±5	65±6
Ceratobasidium	cer3 (E2)	56±0	29±14	47±25
Ceratobasidium	cer1 (E1)	38±5	8±1	20±2
Sebacina	seb1 (C2)	38±2	4±1	11±2
Tulasnella	tul6 (T2)	61±8	3±3	4±4
Ceratobasidium	cer2 (L1)	34±0	2±2	7±7
Tulasnella	tul3 (C4)	39±28	1±1	1±1
Tulasnella	tul1 (L2)	74±1	0±0	0±0
Tulasnella	tul3 (C3)	70±6	0±0	0±0
Ceratobasidium	cer4 (C1)	59±7	0±0	0±0
Tulasnella	tul3 (L3)	57±4	0±0	0±0
Tulasnella	tul4 (C5)	46±3	0±0	0±0
Control 1 (oatmeal medium, no fungus)	N/A	76±5	0±0	0±0
Control 2 (oatmeal medium plus 1 % sucrose, no fungus)	N/A	96±2	39±5	40±5
Control 3 (asymbiotic medium P6668, no fungus)	N/A	7±1	0±0	0±0

Isolates that did not produce stage 5 seedlings are ranked by percentage germination after 12 weeks. Conversion rate represents the percentage of germinated seedlings that reached stage 5 after 12 weeks. Isolates with a "C" prefix were isolated from *C. purpurea* roots

not developing into seedlings with isolates E4 (OTUseb3), E3 (OTUseb2), T1 (OTUtul5), and E2 (OTUcer3) were significantly different as there is little overlap between their 95 % confidence limits. The remaining isolates were not considered to be significantly different. The analysis of survivor function gave a chi-square $p<0.001$, indicating a statistically significant difference between E4, E3, T1, and E2 when compared to each other and the other five isolates used in the test. These four isolates not only produced the highest stage 5 yield but also the largest stage 5 seedlings that were clearly visible even without the use of a stereomicroscope.

Orchid–fungus interaction

UV autofluorescence was used to study 4-week-old protocorms by confocal microscopy. The seeds on control 1 medium germinated to stage 4, a protocorm with rhizoids (Fig. 4a), with an average cell diameter of ~50 μm. Rhizoid formation can be seen where the epidermal cells at the basal end have elongated, and the developing shoot primordium is characterized by smaller, compact cells in the highly nucleated area of the distal end of the protocorm. Some stage 4 protocorms and stage 5 seedlings from symbiotic cultures

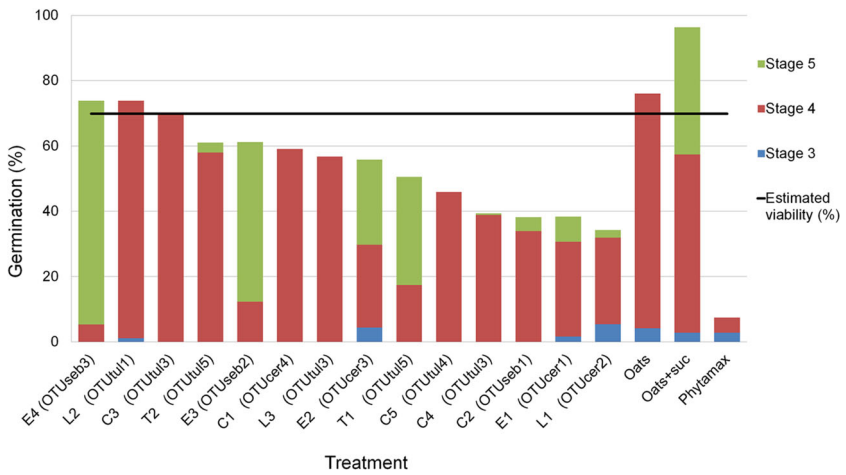

Fig. 3 Percentage of *Cynorkis purpurea* seeds that reached stage 3, 4, or 5 with each fungal isolate by week 12. Germination was defined as seeds reaching stage 3 (a loose protocorm) or further. Predicted seed viability (69.9 %) is represented with the *horizontal black line*. Isolates prefixed with "C" denote fungi isolated from *C. purpurea* roots

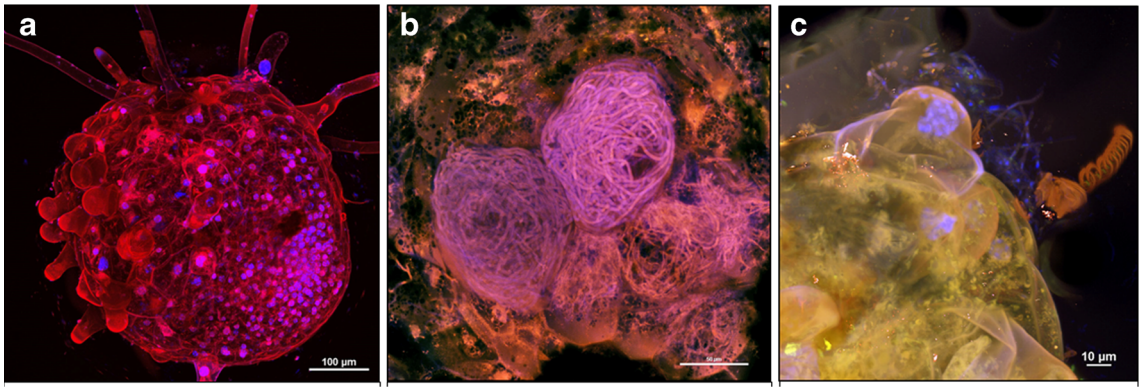

Fig. 4 *Cynorkis purpurea* protocorms showing asymbiotic, symbiotic, and unsuccessful symbiotic systems. **a** Composite stacked image of a stage 4 protocorm after asymbiotic germination. Cell walls show red autofluorescence and nuclei stained blue by DAPI. Cells at the basal end elongate to form rhizoids and a region of cell division can be seen in the *bottom right*. **b** Single transverse optical section of the center of a 4-week-old symbiotically germinated stage 4 protocorm colonized by the fungal isolate T1 (OTUtul5). Well-developed pelotons can be seen (in *purple*) filling the enlarged colonized cells. **c** Composite stacked image of the surface of a stage 4 protocorm germinated with isolate C1 (OTUcer4). No fungal colonization of the protocorm (*yellow*) is visible, but fungal hyphae with DAPI-stained nuclei can be seen near the emerging rhizoid (Color figure online)

had evidence of fungal entry via the rhizoids, as well as colonization and peloton formation in the cortical cells. In a 4-week-old protocorm grown with T1 (OTUtul5), the cortical cells in the central part of the protocorm were colonized, and several fully formed pelotons were seen (Fig. 4b). There was also evidence of fungal colonization within the central cells of the protocorm after 4 weeks when E2 (OTUcer3) was used during germination. Cells containing pelotons were enlarged in relation to the surrounding cells, approximately twice the diameter of uncolonized cells.

Protocorms germinated with a fungus that did not develop the seeds to stage 5 did not have evidence of fungal entry into the protocorm or colonization. *Cynorkis purpurea* seeds germinated with C1 (OTUcer4) reached stage 4 but had no evidence of fungal colonization within the protocorm. Fungal hyphae were observed massing close to an elongating rhizoid but did not enter inside the protocorms through the rhizoids (Fig. 4c). A similar effect was observed with C5 (OTUtul4) where no colonization of the stage 4 protocorm had taken place. In addition, unlike the protocorm germinated with C1 (OTUcer4), no massing of fungal hyphae was seen around the elongating epidermal cells.

Reintroduction of *Cynorkis purpurea*

Over the course of this study, over 300 stage 5 *Cynorkis purpurea* seedlings were produced and, after another 13 weeks, there were ~450 seedlings. These seedlings have been transferred to larger culture vessels to prepare them for reintroduction trials in the gallery forest from where the seeds were collected.

Discussion

Asymbiotic germination tests of *Cynorkis purpurea*

In this trial, *Cynorkis purpurea* seeds were able to germinate and develop to stage 4 protocorms on oatmeal medium (control 1), the standard medium used for symbiotic germination, as well as on water agar (data not shown), demonstrating that this orchid species does not need a fungus to initiate germination. It seems that, for *Cynorkis purpurea*, the availability of water is sufficient for the initiation of germination. Germination rates exceeded the estimated viability, indicating that TTC staining underestimated possible germination rate and most of the seeds were actually viable. Other researchers have found TTC-based viability estimates to be lower than eventual germination rates (Johnson et al. 2007; Dowling and Jusaitis 2012).

Cynorkis purpurea were typically found growing among rocks at the edge of streams in a gallery forest and, with the seeds being dispersed at the start of the dry season, their ecological strategy may be to grow into established seedlings quickly, making use of the available water. In most other habitats in this region of Madagascar, orchid seeds dispersed during the dry season would not have such access to water and would probably need to undergo a dormant period and wait for the subsequent wet season for germination. The poor germination on the asymbiotic medium P6668 (control 3), compared to the viability estimate and germination performance on other treatments, suggests that this medium is somehow inhibitory to germination, possibly due to an unfavorable osmotic balance caused by the rich mineral and organic content. This is in contrast to the favorable germination responses seen with this commercially available asymbiotic orchid germination medium

from many other tropical orchid seeds, particularly of epiphytes and lithophytes (data not shown). Unfavorable asymbiotic germination outcomes relative to viability estimates is common in many species of terrestrial orchids (Lee 2011).

Germination with OMF was less than the germination recorded on the same medium in the absence of OMF. Our results support the concept suggested by Johnson et al. (1997), who described the plant–fungus interaction in a mycorrhizal system as a continuum ranging from parasitism to mutualism. At least some of the isolates that were tested appear to have inhibited the germination of the seeds, and even fungi that support seedling development may be exerting a level of antagonism during germination.

Symbiotic germination and seedling development

Seeds of *Cynorkis purpurea* inoculated with some mycorrhizal fungi showed dramatically improved seedling development when compared to seeds incubated in the absence of fungi (control 1). Furthermore, the presence of seedling development in the control 2 medium, which was supplemented with sucrose, compared to the lack of seedlings in the sucrose-free control 1 medium, suggests that the fungus provides the orchid seedling with a carbon source. The oatmeal in the control 1 medium would contain polysaccharides that are inaccessible to the developing orchid but are being made available via the fungus. The relatively poor development that was seen on control 2 medium subsequent to the conversion to stage 5 suggests that symbiotic fungi are able to provide more than just the necessary carbon source and are probably also providing other necessary nutrients to the orchid. Studies using isotope-labeled nutrient sources have demonstrated the fungus-dependent uptake of C and N (Cameron et al. 2006), and P (Cameron et al. 2007) by orchids. Phosphate uptake was shown to be up to 100 times greater in orchid *Goodyera repens* infected with mycorrhiza compared to that in non-mycorrhizal plants (Alexander et al. 1984). In addition to supplying carbon and minerals directly to the orchid, mycorrhizal fungi facilitate the absorption of free water (Yoder et al. 2000). These reports can explain why fungus-infected *Cynorkis purpurea* seedlings are able to develop rapidly in vitro.

Rhizoctonia-like fungi have previously been shown to benefit seed germination (Quay et al. 1995; Shimura and Koda 2005; Nikabadi et al. 2014; Zi et al. 2014), protocorm growth (Zettler and Piskin 2011), and seedling development (Johnson et al. 2007; Mahendran et al. 2013; Nikabadi et al. 2014). This study has also shown that the right symbionts can indeed increase the rate of seedling development relative to other strains in the *Rhizoctonia* complex, exemplified by E4 (OTUseb3), which induced the greatest percentage of germination and showed the fastest rate of seedling development. The conversion rate of protocorms to seedlings of more than 90 % is a very successful outcome, as is the rapid pace of conversion; within 6 weeks, 50 % of seeds sown with E4 (OTUseb3) had developed into stage 5 seedlings, which was greater than what the second best symbiont, E3 (OTUseb2), could achieve by week 12. The symbiotic seed germination and seedling development of *Cynorkis purpurea* appeared to mirror the observations of Stewart and Kane (2007) with symbiotic germination tests of *Spiranthes brevilabris*; seeds readily germinated to become enlarged embryos with rhizoids (equivalent to stage 4 in this study), even in the absence of fungi, but not all fungi tested were able to support the subsequent development of the protocorm into a seedling within 12 weeks. Bonnardeaux et al. (2007) also found that four out of six terrestrial orchid species tested produced protocorms with or without fungal inoculation, with a specific subset of fungi that stimulate further seedling development.

By confocal microscopy, intracellular colonization of a 4-week-old protocorm was confirmed for two of the fungal isolates that supported development to stage 5 seedlings, while two fungal isolates that did not stimulate development beyond stage 4 were not seen in the plant tissue in week 4 protocorms. In the colonized protocorms, many rhizoids had fungal hyphae within them, suggesting the rhizoid cells as points of entry of fungi into the protocorm. It has been reported that OMF enter protocorms via rhizoids and suspensor cells (Rasmussen 1995), but the latter was not seen in this study. This colonization occurs rapidly, being visible at 4 weeks in the two isolates observed, and explains the appearance of stage 5 seedlings within 6 weeks with the best-performing fungi. The failure of intracellular colonization in week 4 protocorms by fungi that did not support seedling development indicates an incompatibility preventing colonization and, therefore, successful mycorrhizal symbiosis between these fungi and *Cynorkis purpurea*. The fungal isolates that failed to enter the cells were congregating around the rhizoids suggesting that there is a mechanism preventing entry and successful colonization. Rasmussen and Rasmussen (2014) speculated that there are signaling mechanisms between plant and fungus at different stages during protocorm mycorrhization, including response to fungal entry, suggesting that a compatible infection elicits little defense reaction.

It has been reported that fungi isolated from a different orchid species can support orchid seed germination and subsequent growth (Bonnardeaux et al. 2007; Stewart and Kane 2007; Zettler et al. 2007; Yokoya et al. 2015). However, unlike the rapid symbiotic seedling development seen after inoculation with fungal isolates from roots of the same species observed by Bonnardeaux et al. (2007) and Stewart and Kane (2007), *Cynorkis purpurea* seedlings performed relatively poorly with *Cynorkis purpurea*-derived fungi. *Cynorkis purpurea* seedlings instead showed better development with fungi from other orchid species, some with different life forms. Reports of orchid specificity with regards to mycosymbionts (Otero et al. 2007; Rasmussen 2002) would suggest that

isolates from orchids of a similar growth form or habitat would perform better, as they would be more likely to fulfill germination and development requirements. In the present study, however, fungal isolates acquired from the same species (*Cynorkis purpurea*) underperformed in supporting seedling development when compared to fungal isolates acquired from other taxa—a surprising outcome, although this phenomenon has been reported previously (e.g., Warcup 1973; Hajong et al. 2013). Although the five *Cynorkis purpurea* isolates showed germination levels ranging from 38 to 70 %, only two isolates induced the development of stage 5 seedlings. These results may be due to the accidental selection of suboptimal *Cynorkis purpurea* isolates; Huynh et al. (2004) found that only certain developmental stages of a terrestrial orchid *Caladenia formosa* yielded fungi that were suitable for symbiotic germination. It could be that, in nature, some of the non-*Cynorkis* isolates such as OTUseb3 are widely abundant and already associate with *Cynorkis purpurea*. Further sampling of orchid roots from across the Itremo Massif region is needed to assess and map the abundance of fungal isolates and the species they have been found to associate with.

It is also possible that the fungus that associates with roots of *Cynorkis purpurea* is not the same fungus that has entered the protocorm to assist that plant during seedling development. Although we were able to isolate and test fungi from *Cynorkis purpurea* seedlings (C4, C5, C1), we did not acquire protocorms during our field work due to their very small size. The use of seed packets for seed baiting (Rasmussen and Whigham 1993) would be one technique that could be used for acquiring in situ protocorms of *Cynorkis purpurea* on future trips to determine if protocorms do indeed harbor different kinds of fungi than those found in seedlings and/or mature plants. Different orchid species have been found to have variable patterns of specificity at protocorm and adult stages (reviewed by Rasmussen et al. 2015). With this in mind, it appears that *Cynorkis purpurea* protocorms may undergo fungal succession, switching preference for the fungus to associate with once the seedling has developed roots. With a distinct annual cycle of wet and dry seasons, the dominant members of the fungal population in the soil may also be cyclical and transient. It would be very informative if a series of collections could be made at different times of the year in the same location.

The four best fungi for seedling development are represented by all three genera investigated, while *Cynorkis purpurea* roots yielded a different set of four OTUs, again represented by the three genera (Yokoya et al. 2015). *Cynorkis purpurea* would thus be described as a generalist, as both protocorm and adult stages are able to associate with a wide range of fungal partners.

It is interesting to note that the two best performing isolates, E4 (OTUseb3) and E3 (OTUseb2), were both identified as *Sebacina* strains. Of the five isolates derived from *Cynorkis purpurea*, the best seedling conversion rate of 11 % was achieved with OTUseb1, another *Sebacina*. This may be a further indication that the *Sebacina* group of fungi are more suitable partners for *Cynorkis purpurea* seedling development than either *Ceratobasidium* or *Tulasnella*.

As to why the *Sebacina* OTUseb3 was so effective remains to be resolved but could be attributed to a combination of factors. As a group, fungi in the *Sebacinales* are commonly present as endophytes of various plant taxa and in a variety of habitats (Weiss et al. 2011) and may play potentially important roles in both natural and human-altered ecosystems. *Sebacinales* fungi were identified as orchid endophytes in achlorophyllous orchid taxa *Neottia* and *Epipactis* (Weiss et al. 2011), and phylogenetic analysis of the large ribosomal subunit distinguished these as group A, also containing fungi found as tree ectomycorrhizae. Group B *Sebacinales* sensu (Weiss et al. 2004) includes culturable orchid mycorrhizal fungi as well as ericoid mycorrhiza, jungermannoid mycorrhiza, and the model endophyte *Piriformospora indica* (Verma et al. 1998). All three *Sebacina* isolates used in this study were of group B according to their sequence (data not shown) and their culturable nature. *Pyrola asarifolia*, an Ericaceous species with dust seeds, were found to germinate with a *Sebacinales* clade B fungus revealing an intriguing evolutionary convergence with orchids (Hashimoto et al. 2012). These findings are both relevant for applied research as well as for basic research on the role of *Sebacinales* in ecosystem functioning and possible shaping of plant communities (Weiss et al. 2011; Oberwinkler et al. 2013). Our results show that *Sebacina* as a genus has the ability to enter into successful symbiotic relationships with orchids of different life forms, and this new knowledge may be especially useful for orchid conservation practiced in tropical areas like Madagascar.

The two fungi that showed the best conversion rate to seedlings, E4 (OTUseb3) and E3 (OTUseb2), were isolated from roots of very small epiphytic seedlings that were tentatively identified as *Polystachya concreta* located ~2 km from the gallery forest where the *Cynorkis purpurea* was found. The fungi that showed the fourth and fifth best conversion rates [E2 (OTUcer3) and E1 (OTUcer1), respectively] were also from epiphytic orchids. Thus, it is slightly surprising that these isolates would be most effective at germinating seeds of the terrestrial *Cynorkis purpurea*, given that the donor orchids originated from a very different habitat. Few studies have attempted to germinate seeds of terrestrial orchids with mycorrhizal fungi from epiphytes and vice versa (e.g., Zettler et al. 2007), but our results suggest that this practice could have merit for further investigation, helping to understand orchid–fungus–environment interactions in nature.

Given that *Cynorkis purpurea* colonizes wet rocks bordering streams and river margins (Cribb and Hermans 2009), it is conceivable that branches and woody debris harboring epiphyte seedlings (e.g., *Polystachya concreta*) and their associated mycorrhizal fungi (e.g., E4) would fall to the forest floor.

These branches, and the fungal inoculum load they carry, could enter water runoff into nearby streams favorable to colonization by *Cynorkis purpurea*, creating fungal "hotspots" along stream margins that might trigger germination if orchid seeds land in the vicinity. This scenario also makes sense from the standpoint of a generalist orchid such as *Cynorkis purpurea*. In other words, one might expect orchids that inhabit stream margins to be fungal generalists—or opportunists—given that water runoff could be a source of high fungal diversity, at least seasonally. Despite its generalist nature, the distribution of *Cynorkis purpurea* is confined to a specific niche that is apparently defined by water availability; this species was not found anywhere else in the gallery forest except beside streams, nor at any of the other sites in the Itremo region visited during two expeditions in the last 2 years. It may be that, for *Cynorkis purpurea*, it is the specific environment—one that suits its life cycle—that restricts its distribution, rather than a dependence on a single, niche-specialized fungal symbiont. There have been reports of high mycorrhizal specificity in rare orchids (Shefferson et al. 2005; Swarts et al. 2010), which logically explained the rarity of those orchid species by their dependence on a fungus that is possibly restricted in habitat or distribution. However, more recently, Pandey et al. (2013) have expressed surprise in finding broad mycorrhizal associations in *Piperia yadonii*, an orchid species that is rare and narrowly endemic. Furthermore, Shefferson et al. (2005) found that, out of seven *Cypripedium* species, *Cypripedium californicum* was found to have the broadest range of fungal associates despite having the narrowest distribution; similarly to *Cynorkis purpurea*, it associated with fungi of *Ceratobasidiaceae*, *Tulasnellaceae*, and *Sebacinaceae*. In considering the high mycorrhizal specificity of orchids of southern Australia, Phillips et al. (2011) hypothesized that the relatively old, stable landscape of the region offered an environment that allowed greater specialization compared to terrestrial orchids in temperate Eurasia and North America. On the other hand, Shefferson et al. (2005) suggested that, because *Cypripedium californicum* was generally restricted to wet, serpentine sites, the nutritionally limited, potentially toxic environment resulted in a need for the orchid to be able to associate with a broader range of fungi. It is possible that similar interactions are taking place on the rocky outcrops along the streams in the Itremo gallery forest.

Symbiotically grown orchids are anticipated to be more suitable propagules for restoration, by being likely to survive reintroduction in situ (Aggarwal and Zettler 2010), as well as already harboring fungi that can help the orchid to complete its life cycle. Seedlings grown in vitro with mycorrhizal fungi have been shown to have higher survival rates compared to asymbiotically grown seedlings (e.g., Anderson 1991).

Conclusions

In summary, we found that

- Unlike some other orchid species that have been studied, *Cynorkis purpurea* appears not to be dependent on symbiotic fungi for germination in vitro up to protocorm formation while they were essential for further seedling development.
- Successful symbiotic seedling development was underpinned by intracellular colonization of the fungal isolates while unsuccessful development was associated with hyphae that failed to enter the cells.
- Although *Cynorkis purpurea* is seemingly a generalist, with seedling development supported by all three fungal genera tested, the two isolates that supported the best conversion rate to seedlings in this terrestrial orchid were epiphyte-derived *Sebacina* cultures.

Symbiotic protocols remain underutilized due to a limited knowledge of the most appropriate symbionts for different orchid species, and further study to understand mycorrhizal associations in terrestrial orchids is urgently needed to improve in vitro orchid seed germination for recovery, restoration, and storage programs (Reed et al. 2011). The superior seedling development with *Sebacina* isolates compared to the *Cantharellales* fungi is particularly intriguing. Furthermore, the high seedling conversion rates achieved by epiphyte-derived *Sebacina* isolates with the terrestrial *Cynorkis purpurea* suggest that the *Sebacina* group of fungi are generalist symbionts, and this feature deserves further investigation. Our study shows that *Cynorkis purpurea* is a generalist with regards to fungal symbiont-mediated seedling development. However, even though the right symbionts are apparently available in nearby areas and buffer zones, this species is habitat-specific, being restricted to riverbanks and wet rocks. Further detailed research of the *Cynorkis* genus in Madagascar, many species of which are also found in moist or wet substrate (Cribb and Hermans 2009), would be very interesting and will help to understand whether the findings from one orchid taxon could apply to conservation efforts in other taxa.

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