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Conservation Potentials of Mycorrhizal Fungi Root Cultures of 23 North American Orchids from Herbarium Specimens

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Research Article

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ABSTRACT

Root samples from 23 species of preserved orchid specimens dating back to 1884, and housed in the Jacobs Herbarium at Wittenberg University (Ohio, USA), were sampled for culturable mycorrhizal fungi to determine if herbarium specimens could be enlisted as a source of fungi for conservation purposes (e.g., symbiotic seed germination). Root segments were detached from the specimens, soaked one hour in water, macerated, and immersed in three different types of standard culture media. To verify that this sampling technique was effective, a positive control was established that involved living roots of a greenhouse specimen. After >one week incubation at ambient temperature, seven of 33 orchid herbarium specimens yielded fungal colonies. None of the 800+ Petri plates prepared in this manner yielded living fungus cultures assignable to typical orchid mycorrhizal genera. We conclude that herbarium specimens are generally void of living *Rhizoctonia*-like fungi, thereby placing a heavier burden on fresh material as a source of these fungi for orchid conservation.

INTRODUCTION

Most orchids form a symbiotic relationship with *Rhizoctonia*-like fungi that include those assignable to *Ceratobasidium* and *Tulasnella* [1-6]. Tulasnelloid fungi have been isolated with regularity in North America [7]. These strains are typically extracted from masses of fungal hyphae (pelotons) present in the cortical region of lateral (branch) roots in living specimens. Pure cultures acquired from pelotons are used as a tool for generating large numbers of seedlings *in vitro* [8].

According to Rasmussen, orchid mycorrhizal fungi are not guaranteed that they are associated with an orchid unless isolated from pelotons [6]. As orchid populations continue to decline, securing material (roots) will become increasingly difficult [6], raising concerns that potentially useful fungi will be lost before they are recovered and safeguarded for use in conservation. This study determines if orchid mycorrhizal fungi could be cultured from herbarium specimens. We are testing the hypothesis that these specimens may represent an untapped resource of orchid mycorrhizal fungi. The Jacobs Herbarium at Wittenberg University (Springfield, OH) was chosen for this study. Methods for preservation and storage used in this herbarium are common for small liberal arts colleges throughout North America. The Jacobs herbarium is a model facility because it contains a mixture of newer (1986) and older (1884) orchid specimens (**Figures 1A and 1B**). Specimens were preserved and stored on acid-free paper without high heat or drying treatments. To verify the effectiveness of our fungus isolation technique, roots of a live orchid (*Phalaenopsis* sp.) maintained in the Wittenberg University greenhouse for many years **(Figure 1C)**, was examined to serve as a control.

MATERIALS AND METHODS

Sections of roots (1 cm portions) were excised from the orchid, and they were soaked in 20 ml of sterile deionized double-distilled water (DI water) in Syracuse dishes for one hour (fresh water, twice for 30 min). The root portion was then surface sterilized following the method outlined in Zettler [9]. The root portions were then cut into smaller pieces in a 100 × 15 mm sterile Petri dish and covered with 20 ml molten agar: modified Melin-Norkrans agar (MMN), potato dextrose agar (PDA), and modified potato dextrose agar (MPDA, pH lowered to 5.5. with lactic acid) (Fisher Scientific, Pittsburgh, PA). Three different agar media were used in order to obtain as many different fungi as possible. Petri plates were incubated at 20 ± 1°C and total darkness once the agar solidified.

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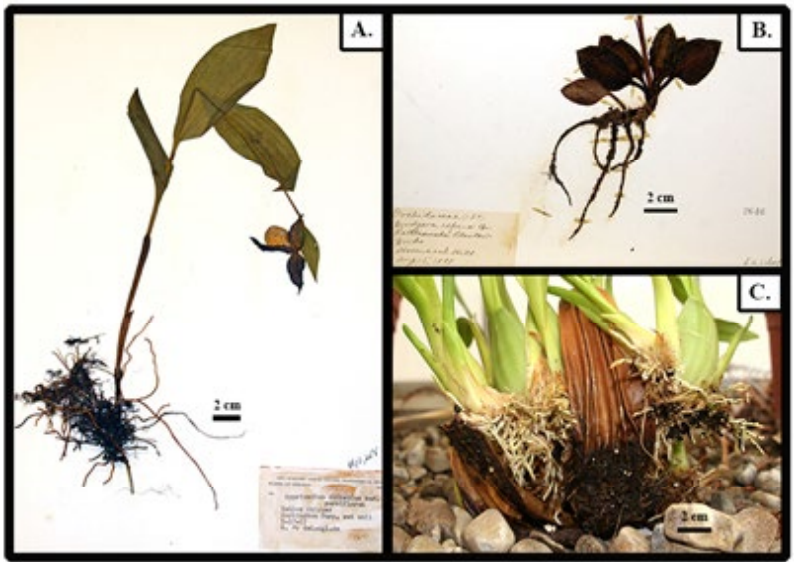

Figure 1. (A) Herbarium specimen of an orchid (*Cypripedium calceolus*; 1957) showing intact root system taken from the Jacobs Herbarium, (B) magnified view of root system from a century old orchid herbarium specimen (*Goodyera repens*; 1898), and (C) live *Phalaenopsis* sp. from the University greenhouse showing living root system. Photo credit: M. P. Dameron, Department of Biology, Wittenberg University, Springfield, OH (Canon EOS 20D Digital SLR camera; Melville, NY).

Plates were examined daily by light microscopy (40/100x) for emerging fungal hyphae (each one was an isolate). Hyphae that could not be traced back to the internal content of the root portion were not used. Subcultures for fungal identification were conducted by plating excised hyphal tips as a 1 cm^3 block that was placed on to fresh solidified agar media. Fungal identification was based on macroscopic (colony) and microscopic (conidia) characteristics at 1000x under oil. Fungi were identified according to Currah and Barnett and Hunter and by pure culture comparison once spore characteristics appeared [10,11].

Each experiment was replicated three times. Nine 1 cm root portions (main and lateral roots) from each orchid specimen were cut into thirds and plated on each type of agar media. Root samples from a live orchid (*Phalaenopsis* sp.; n=5 plants in different pots) acted as a control. The numbers of Tulasnelloid isolates from the herbarium specimens to the number of isolates in the control were compared using an analysis of covariance (ANCOVA, P=0.05; SPSS 14.0 for Windows; IBM, Armonk, NY) [12].

RESULTS AND DISCUSSION

Seven of the 33 herbarium specimens spanning 23 orchid taxa (n>800 plates) yielded fungi: *Corrallorhiza maculata* (Rafinesque) Rafinesque (1927, PDA only), *Cypripedium acaule* Aiton (1965, 1970, PDA only), *Cypripedium passerinum* Richardson (1973, MPDA only), *Goodyera pubescens* (Willdenow) R. Brown (1936, MMN only), *Liparis liliifolia* (L.) Richard *ex* Lindley (1982, PDA only) *Listera cordata* (L.) R. Brown (1969, PDA only), *Platanthera hyperborean* (L.) Lindley (1973, MMN only), and *Spiranthes ovalis* Lindley (MPDA only) **(Table 1)**. These fungi were identified as common saprophytic molds verified by microscopic examination of asexual propagules and cultural morphology on PDA, consisting of: *Mucor* (two isolates), *Cunninghamella* (one isolate), *Aspergillus* (one isolate), *Penicillium* (one isolate), *Trichoderma* (two isolates), and *Mycelia sterilia* (four isolates). Fungal isolates from *Cypripedium passerinum* and *Spiranthes ovalis* appear to require a lower pH, because these isolates are restricted to growth on MPDA that contained lactic acid, pH 5.5. The majority (5/8) of the isolates were acquired from thinner lateral root sections immersed and cultured on MMN **(Table 1)**. Common saprophytic molds were also evident in roots of the live orchid, and these were identified as: *Aspergillus niger* (one isolate), *Penicillium glabrum* (two isolates), and *Cladosporium cladosporioides* (one isolate). In addition, more fungal isolates were acquired from the live orchid than any of the herbarium specimens (P<0.05 in each pairwise comparison), the majority of which were obtained using MMN **(Table 1)**. In contrast, most of the fungi from the herbarium specimens were cultured on PDA **(Table 1)**.

Fungi recovered from root cortical cells in the live orchid control (*Phalaenopsis* sp.) matched published descriptions for ubiquitous orchid mycorrhizae, in particular Tulasnelloid fungi (with many examples provided in a review by Currah [13]. Despite inspection of >800 plates, we did not isolate any *Rhizoctonia*-like fungi from the herbarium specimens in dated (1884) and more recently collected (1986) orchid material. The presence of Tulasnelloid fungi in the live orchid control rules out that our isolation protocol was ineffective. These findings support an earlier report by Zettler et al. [9]. which attempted to isolate mycorrhizal fungi from four orchid specimens housed in the Illinois College Herbarium (Jacksonville, IL) spanning four species: *Epidendrum conopseum*– syn. *E. magnolia* Mühlenberg (1995), *Goodyera repens* (L.) R. Brown (1895), *Orchid spectabilis*– syn. *Galearis spectabilis* (L.) Rafinesque (1890), and *Platanthera integrilabia* (Cornell) Luer (1995). In that study, fungi from cortical pelotons failed to initiate growth on MMN, even in recently preserved specimens collected approximately five years earlier. As to why common molds were successfully cultured whereas orchid mycorrhizal fungi were not remains to be determined, but it is conceivable that

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Table 1. Isolation attempts of endomycorrhizal fungi using three different agar media from orchid herbarium specimens taken from the Jacobs Herbarium (Wittenberg University, Springfield, OH) and live orchid (Phalaenopsis sp.) from the Wittenberg University greenhouse. MMN, modified Melin-Norkrans agar; PDA, potato dextrose agar; MPDA, modified potato dextrose agar (PDA, pH lowered to 5.5 with lactic acid). Data are the number of fungal isolates that were obtained from nine–1 cm portions of roots, three portions on MMN, three portions on PDA and three portions on MPDA.

Orchid species (from collection label)	Year collected	No. fungal isolates on agar media			Total
		MMN	PDA	MPDA	
Live specimen	2012	5	0	2	7
Phalaenopsis sp.	2008	3	1	1	5
Herbarium specimens Aplectrum hyemale	1894	0	0	0	0
Corallorhiza maculata	1927	0	2	0	2
Corallorhiza multiflora	1884	0	0	0	0
Corallorhiza odontorhiza	1937	0	0	0	0
Cypripedium acaule	1979	0	0	0	0
	1970	0	1	0	1
	1968	0	0	0	0
	1965	0	1	0	1
	1899	0	0	0	0
Cypripedium calceolus	1957	0	0	0	0
Cypripedium passerinum	1973	0	0	1	1
Goodyera oblongifolia	1927	0	0	0	0
Goodyera pubescens	1979	0	0	0	0
	1974	0	0	0	0
	1973	0	0	0	0
	1936	1	0	0	1
Goodyera repens	1898	0	0	0	0
Habenaria clavellata	1912	0	0	0	0
Habenaria hyperborea	1969	0	0	0	0
Habenaria obtusata	1969	0	0	0	0
Habenaria orbiculata	1885	0	0	0	0
Habenaria repens	1968	0	0	0	0
Liparis liliifolia	1982	0	2	0	2
	1912	0	0	0	0
	1899	0	0	0	0
Listera cordata	1969	0	1	0	1
Orchis spectabilis	1974	0	0	0	0
	1894	0	0	0	0
Platanthera hyperborea	1973	1	0	0	1
Spiranthes cernua	1960	0	0	0	0
Spiranthes gracilis	1968	0	0	0	0
Spiranthes ovalis	1966	0	0	1	1
Spiranthes romanzoffiana	1951	0	0	0	0

mold spores (conidia) might be more resistant to prolonged desiccation on specimens in storage. Although many strains of orchid mycorrhizal fungi do produce spore-like structures they do not typically produce asexual spores common to many of the molds recovered in the present study (conidia, sporangiospores) [13,14].

We emphasize that a living orchid must have a fungal symbiont to allow for proper growth, reproduction and development. Therefore, the orchid specimens we sampled possessed a fungal symbiont, where the specificity of both fungus and host orchid are codependent [15-19]. Although pelotons may be present in the cortical cells of our preserved specimens, they did not produce viable fungal isolates as evidenced by our study. For conservation purposes, targeting herbarium specimens as a source of living orchid mycorrhizal fungi does not appear to be a viable alternative to collecting live material. Thus, conservation programs that enlist mycorrhizal fungi for orchid species recovery projects and symbiotic seed germination will likely require the persistence of extant orchid sites as a source of fresh root material. As natural populations continue to experience environmental degradation accelerated by human activities, securing orchid material for this purpose will become increasingly more difficult in the coming decades [20-22].

CONCLUSION

We recommend that professional botanists consider sending fresh root samples to specialists (mycologists) from orchid

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specimens destined for herbaria as one possible mechanism for safeguarding mycorrhizal fungi for conservation purposes [23,24].

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REFERENCES

1. Otero JT, et al. Diversity and host specificity of endophytic *Rhizoctonia*-like fungi from tropical orchids. Am J Bot. 2002;89:1852-1858.
2. Bidartondo MI, et al. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proceedings of the Royal Society B: Biological Sciences. 2004;271:1799-1806.
3. Selosse MA, et al. 2004. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microb Ecol. 47:416-426.
4. Dearnaley JDW. Further advances in orchid mycorrhizal research. Mycorrhiza. 2007;17:475-486.
5. Atala C, et al. Orchidioid fungi of the form-genus *Rhizoctonia* associated with the roots of *Chloraea cuneate* Lindl. from Araucanía, Chile. Gayana Botánica. 2015;72:145-148.
6. Rasmussen HN, et al. Germination and seedling establishment in orchids: a complex of requirements. Ann Bot. 2015;116:391-402.
7. Stewart SL and Kane ME. Symbiotic seed germination of *Habenaria macroceratitis*(Orchidaceae), a rare Florida terrestrial orchid. Plant Cell Tissue Organ Cult. 2006;86:159-167.
8. Zhu GS, et al. A novel technique for isolating orchid mycorrhizal fungi. Fungal Diversity. 2008;33:123-137.
9. Zettler LW, et al. Do herbarium specimens harbor viable orchid seeds and mycorrhizal fungi for conservation? Southeastern Biol. 2001;48:149.
10. Currah RS, et al. *Epulorhiza inquilina* sp. Nov. from *Platanthera* (Orchidaceae) and key to *Epulorhiza* species. Mycotaxon. 1997b;61:335-342.
11. Barnett HL and Hunter BB. Illustrated genera of imperfect fungi. American Phytopathological Society Press, St. Paul, MN. USA. 1998;218.
12. Sokal RR and Rohlf FJ. Biometry: The principles and practice of statistics in biological research. W.H. Freeman and Co., San Francisco, CA. USA. 1995;887.
13. Currah RS, et al. Fungi from orchid mycorrhizas. In: J. Arditti and A.M. Pridgeon (eds.) Orchid Biology: Reviews and Perspectives, VII. Kluwer Academic, Dordrecht, Netherlands. 1997a;394.
14. Chagnon PL, et al. A trait-based framework to understand life history of mycorrhizal fungi. Trends Plant Sci. 2013;18:484-49.
15. Pereira G, et al. Essential mycorrhizal partners of the endemic Chilean orchids *Chloraea collicensis* and *C. gavilu*. Flora. 2014;200:95-99.
16. Lee YL, et al. The importance of associations with saprotrophic non-*Rhizoctonia* fungi among fully mycoheterotrophic orchids is currently under-estimated: novel evidence from sub-tropical Asia. Ann Bot. 2015;116:423-435.
17. Pereira MC, et al. Characterization of seed germination and protocorm development of *Crytopodium glutiniferum* (Orchidaceae) promoted by mycorrhizal fungi *Epulorhiza* spp. Acta Botanica Brasilica. 2015;29:569-576.
18. Klironomos JN, et al. Inter-specific differences in the response of arbuscular mycorrhizal fungi to *Artemisia tridentata* grown under elevated atmospheric CO_2. New Phytol. 1998;138:599-605.
19. Koch AM, et al. Diversity effects on productivity are stronger within than between trophic groups in the arbuscular mycorrhizal symbiosis. PLoS One. 2012;7:e36950.
20. Chagnon M. Causes and effects of the worldwide decline in pollinators and corrective measures. Canadian Wildlife Federation, Quebec Regional Office, QC, Canada. 2008;67.
21. Meisel JE, et al. Orchids of tropical America an introduction and guide. Cornell University Press, Ithaca, NY. 2014. pp. 276.
22. Fay MF, et al. Orchid conservation: Making the links. Ann Bot. 2015;116:337-379.

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p-ISSN:2347-2308

23. Hoeksema J, et al. A meta-analysis of context-dependency in plant response to mycorrhizal fungi. Ecol Lett. 2010;13:394-407.

24. Van Der Heijden MGA, et al. Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytol. 2015;205:1406-1423.