THE FUNGAL ENDOPHYTES OF DIPODIUM VARIEGATUM (ORCHIDACEAE)

J.J. Bougoure [1] and J.D.W. Dearnaley [2, 3]

[1] School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, Crawley 6009, Australia.
[2] Department of Biological and Physical Sciences, The University of Southern Queensland, Toowoomba 4350, Australia.
[3] Author for correspondence: Fax +617 4631 1530; Email: dearns@usq.edu.au

Abstract

We have attempted to grow fungi from pelotons isolated from roots of the myco-heterotrophic orchid species *Dipodium variegatum* on 3 different growth media. As well, we have analysed the fungal DNA within roots of the orchid using ITS-PCR analysis, cloning and molecular sequencing.

Fungi failed to grow out from pelotons isolated from plant roots. ITS rDNA sequences were successfully amplified and cloned from roots of three orchid plants. Comparison of these sequences with ITS rDNA in GenBank revealed that the fungal community of *D. variegatum* roots consists of *Russula* spp. and non-mycorrhizal soil Deuteromycetes.

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Introduction

Myco-heterotrophs are plant species that obtain a carbon source from an associated fungus. Myco-heterotrophic plants exist as both monocots and dicots, with most being monocotyledonous. The largest group of such plants is the Orchidales, 280 species of which are entirely myco-heterotrophic (Leake 1994). Although myco-heterotrophy is generally linked with achlorophyllous plants, a number of species exist that obtain carbon both autotrophically and heterotrophically. For example, most members of the Family Orchidaceae, which are generally autotrophic when mature, pass through a heterotrophic phase during their development, often associated with the limited food reserves of the generally minute seeds (Smith & Read 1997).

Fungi are generally quite easy to isolate from pelotons (hyphal coils) in orchid roots and are amenable to growth in pure culture. Although a diversity of orchid species have been sampled across different geographical areas of the world, the fungal endophytes are largely similar (Rasmussen 2002, Smith & Read 1997). The fungi are all basidiomycetes with the majority belonging to the form genus *Rhizoctonia*, within the heterobasidiomycetes (Table 1). A few orchid mycorrhizal fungi, associated with myco-heterotrophic species, are representatives of the homobasidiomycetes (Table 1).

Research into the fungal endophytes of Australian terrestrial orchids has mostly focussed on the autotrophic orchid species (e.g. Huynh *et al.* 2004, Milligan & Williams 1988, Perkins & McGee 1995, Perkins *et al.* 1995, Pope & Carter 2001, Warcup 1971, 1973, 1981) while the myco-heterotrophic species have largely escaped attention. Warcup (1985, 1991) successfully isolated *Rhizoctonia*-like fungal endophytes from two species of the rare subterranean orchid *Rhizanthella*. He named the endophyte of *R. gardneri*, *Thanatephorus gardneri* sp. nov. after succeeding in producing its teleomorph (Warcup 1988).

The orchid genus *Dipodium* or 'hyacinth orchids', contains a number of leafless myco-heterotrophic species that appear to rely heavily on carbon provided by endophytic fungi (Warcup 1990). The identity of these fungal endophytes is unknown. Warcup (1981, 1991) succeeded in isolating a fine, white, unclamped and slow growing endophyte from roots of *D. punctatum* J.E. Sm. but was unable to

Table 1. A list of fungal genera capable of forming orchid mycorrhizas. Anamorphic and teleomorphic genera are included for the Heterobasidiomycetes. Adapted from Rasmussen (2002) and the recent systematic overview of Basidiomycetes according to Jens H. Petersen, http://www.mycokey.com/systematics.html.

Heterobasidiomycetes			**Homobasidiomycetes**	
Order	**Genera**		**Order**	**Genera**
	Anamorph	**Teleomorph**		
Ceratobasidiales	*Ceratorhiza*	*Ceratobasidium*	Gloeocystidiales	*Russula*
	Moniliopsis	*Oliveonia* *Thanatephorus*	Hymenochaetales	*Erythromyces*
Tulasnellales	*Epulorhiza*	*Tulasnella*	Thelephorales	*Thelephora*, *Tomentella*
Auriculariales		*Sebacina*	Agaricales	*Armillaria*, *Mycena*

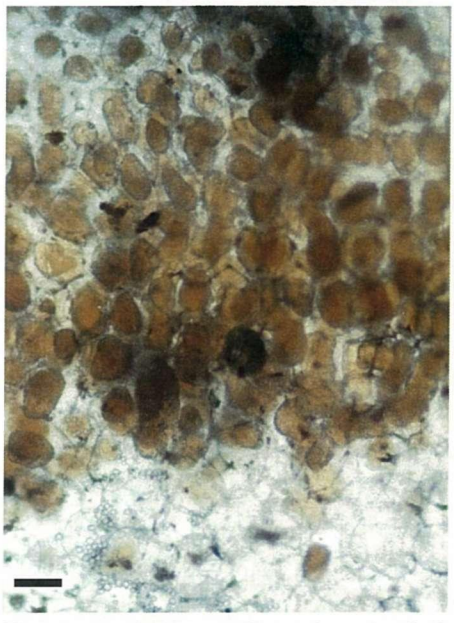

Figure 1. Cross section of root of *D. variegatum* showing large numbers of brown-coloured fungal structures. Scale bar = 100 µm.

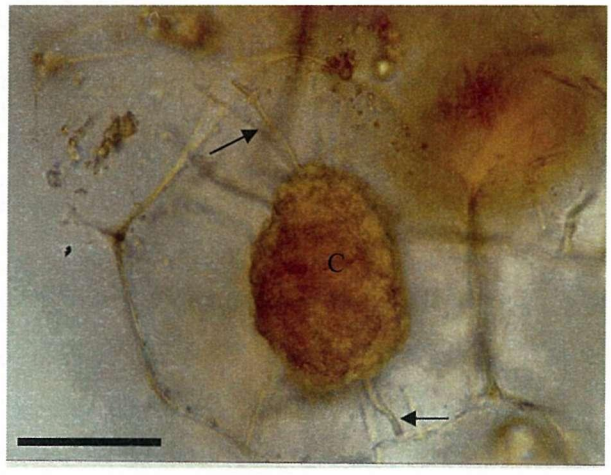

Figure 2. Higher magnification view of a fungal structure showing both collapsed fungal material (c) and intact hyphae (arrows). Scale bar = 70 µm.

assign the fungus a taxonomic identity other than 'not a rhizoctonia'. Warcup (1981) isolated a *Tulasnella*-like fungal endophyte from the epiphytic *D. pandanum* F.M. Bail. In this study we have used two approaches to identify the fungal endophyte of the myco-heterotrophic orchid *Dipodium variegatum* R. Br., a widespread woodland species in South-east Queensland (Stanley & Ross 1989). We have isolated single pelotons from roots of the orchid using microtechniques and attempted to grow these on a number of different media. As well, we have used PCR-amplification of rDNA ITS regions, cloning and sequence comparison of fungi from colonised *D. variegatum* roots. The results of these investigations are presented here.

Materials and Methods

Acquisition of orchid and fungal material

Roots were collected from two *Dipodium variegatum* plants at Toohey State Forest, Brisbane, QLD, Australia (27°33'S, 153°02'E) and two plants from Duggan Park, Toowoomba, QLD, Australia (27°35'S, 151°59'E). One cm long root portions were cut and rinsed under tap water. These portions were then soaked in 100% bleach for 30 seconds and rinsed with sterile dH_2O three times. Thin, transverse sections were then cut from the root portions and these were then crushed to release individual pelotons from cortical cells (some uncrushed sections were kept and photographed using a Nikon E600 upright photomicroscope (Nikon Corporation, Tokyo, Japan)). While viewing under a compound microscope, healthy (non-collapsed) individual pelotons were removed with a 2.5 µl volume micropipette to malt extract agar (MEA) or one sixth strength neutral dox yeast (NDY6) agar plates containing 15 mg l^{-1} streptomycin and 15 mg l^{-1} tetracycline or potato dextrose (PD) agar plates containing 100 mg l^{-1} streptomycin and 50 mg l^{-1}

tetracycline. At least six individual pelotons were removed from each plant. Culture plates were sealed and incubated in the dark at 20°C for at least three weeks.

Molecular analysis of *Dipodium* endophytes

Roots were collected from three flowering *Dipodium variegatum* plants at Duggan Park, Toowoomba. DNA was extracted from these roots using a DNeasy Plant mini kit (Qiagen, Doncaster, Vic., Australia) following the manufacturer's instructions.

The fungal ITS region of each sample was amplified in 50 µl reaction volumes, each containing 38 µl sterile distilled H_2O, 5 µl 10X buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100; Invitrogen Australia, Mt Waverley, Vic., Australia), 2.5 µl 50 mM $MgCl_2$ (Invitrogen Australia), 1 µl 10 mM dNTP (Invitrogen Australia), 1 µl of each of the fungal specific ITS1F primer (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990), 0.5 µl of *Taq* DNA polymerase (Invitrogen Australia) and 1 µl of extracted genomic DNA. PCR amplifications were performed in a Thermo Hybaid PCR Express thermocycler (Integrated Sciences, Willoughby, NSW, Australia) with 35 cycles of 95°C for 1 min., 50°C for 1 min. and 72°C for 1 min., with a final incubation at 72°C for 10 min. These reactions were performed in duplicate and a negative control was included without DNA. The resulting amplification products were electrophoresed in 2% (w/v) agarose gels with ethidium bromide, and visualized under UV light.

ITS-PCR products were purified using a DNA purification kit (Roche Applied Science, Castle Hill, NSW, Australia) prior to cloning with the pGEM-T Easy vector system (Promega, Annandale, NSW, Australia), both conducted as per the manufacturer's instructions. Sequencing reactions were performed in 10 µl volumes containing 400 ng of purified plasmid DNA, 6.4 pmoles of T7 promotor primer at the Brisbane laboratory of the Australian Genome Research Facility (AGRF). ITS sequences were analysed using BLAST searches (http://www.ncbi.nlm.nih.gov/).

Results

Fungal isolation

Fungal colonisation was obvious within the roots of the *Dipodium* plants with the cortex containing large numbers of brown-coloured structures (Fig. 1). At higher magnification these structures contained both collapsed fungal material and intact hyphae (Fig. 2). Although single fungal pelotons were successfully removed from root cortical cells these failed to grow on ME, NDY-6 and PD agar.

Molecular analysis of *Dipodium* endophytes

ITS-PCR amplification produced a number of bands between 600 and 800 bp for each DNA sample (data not shown). Full length ITS sequences were obtained for two clones from each of the three sampled *D. variegatum* plants and these have been deposited in GenBank with the accession codes listed in Table 2. Both clones from Plant 1 showed high identities (90% over 393 bp) to *Russula occidentalis* Singer (Table 2). The first clone from Plant 2 had a high identity to *Trichoderma hamatum* (Bonorden) Bainier (99% over 607-611 bp) while the second clone had 89–88% identity (over 337-372 bp) to two species of *Russula* (Table 2). The first clone from Plant 3 had high identity to a *Verticillium* sp. (95% over 585 bp) and an uncultured fungus (97% over 367 bp), while the second clone had affinity (96 & 95% over 166-168 bp) to two homobasidiomycete fungi, *Halocyphina villosa* Kohlm. et Kohlm and *Merisimodes fasciculata* (Schwein.) Earle (Table 2).

Discussion

Sequence comparison of the cloned fungal ITS rDNA samples with GenBank revealed that the fungal community of *D. variegatum* roots consists of a number of species. The non-mycorrhizal Deuteromycetes, *Trichoderma hamatum* and *Verticillium* sp. are likely to be contaminants on the orchid roots as they are common soil inhabiting fungi (Alexopoulous, Mims & Blackwell 1996). As *Halocyphina villosa* is a marine fungus (Hibbert & Binder 2001) and *Merismodes* spp. have never been recorded as forming orchid mycorrhizas (see Rasmussen 2002) the results strongly suggest that the primary peloton forming fungi in *D. variegatum* roots are members of the homobasidiomycete genus *Russula*. This has good correlation with molecular studies of species of North American myco-heterotrophic orchids which have also shown to be colonised by *Russula* spp. (Taylor & Bruns 1999). While the sample size here is small, these findings also compare favourably with other studies of myco-heterotrophic orchids which have shown quite specific fungal associations (Selosse *et al.* 2002, Taylor & Bruns 1997, 1999). Indeed, as more molecular analyses of autotrophic orchid endophytes are being conducted it appears that the lack of specificity usually attributed to these orchid types (e.g. Warcup 1981, Zelmer *et al.* 1986) may not be the case (see Bougoure *et al.* 2005, McCormick *et al.* 2004).

Table 2. Closest two matches from BLAST searches of fungal sequences amplified from the three *D. variegatum* plants. Included are the two closest GenBank matches and accession codes, sequence identity and overlap of each match.

Plant & clone no.	GenBank Accession Code	Closest species match & accession code		Sequence identity (%)	Sequence overlap (bp)
Plant 1 clone 1	AY702070	*Russula occidentalis*	AY228349.1	90	393
		Russula occidentalis	AY534206.1	90	393
Plant 1 clone 2	AY702071	*Russula occidentalis*	AY228349.1	90	393
		Russula occidentalis	AY534206.1	90	393
Plant 2 clone 1	AY702072	*Trichoderma hamatum*	AY154937.1	99	607
		Trichoderma hamatum	Z48816.1	99	611
Plant 2 clone 2	AY702073	*Russula solaris*	AF418627.1	89	337
		Russula lepida	AY061686.1	88	372
Plant 3 clone 1	AY702074	*Verticillium* sp.	AY172097.1	95	585
		Uncultured fungus	AF504849.1	97	367
Plant 3 clone 2	AY702075	*Halocyphina villosa*	AY571042.1	96	166
		Merismodes fasciculata	AY571052.1	95	168

The endophytes of a number of European and North American myco-heterotrophic orchids have been recently identified via molecular biology techniques. Taylor & Bruns (1997, 1999) and McKendrick *et al.* (2000a) found that *Cephalanthera austinae* and *Corallorhiza trifida* were colonised by members of the Thelephoraceae, whereas *Corallorhiza maculata* and *C. mertensiana* were colonised by the Russulaceae. Selosse *et al.* (2002) recently showed that *Neottia nidus-avis* was colonised by sebacinoid fungi, while Taylor *et al.* (2003) showed that the primary fungal partner of *Hexalectris spicata* was also a member of the Sebacinaceae. Together with the present study, these analyses show that both homobasidiomycetes and heterobasidiomycetes can be endophytes of myco-heterotrophic orchids.

Research into myco-heterotrophic orchids and their fungal partners suggests that they are often involved in tripartite interactions with surrounding tree and shrub species, thus providing the orchid with a greater and more reliable nutrient source (McKendrick *et al.* 2000a, b, Selosse *et al.* 2002, Warcup 1985). The orchids used in this study all occurred close to species of *Eucalyptus* and it is possible that the orchids are linked to these tree species via ectomycorrhizal associations. Support for this comes from the fact that *Russula* species, which are well known ectomycorrhizal fungi in Australia (Brundrett *et al.* 1996), have been implicated in tripartite relationships between trees and myco-heterotrophic orchids (Taylor & Bruns 1999).

The lack of success in growing extracted pelotons may be owing to the absence of specific cultural requirements in the media used or that there were problems with the extraction methodology. Taylor & Bruns (1997) and Zelmer *et al.* (1996) have also encountered difficulties in isolating fungi from various species of terrestrial orchids (including members of the Russulaceae in the former study). The latter authors suggest that some orchid endophytes may have antibiotic sensitivity or have an obligate dependency on their plant hosts.

In conclusion, in the absence of pure culture synthesis of an association under experimental conditions, we have identified the primary peloton-forming fungal endophytes of *D. variegatum* as members of the Russulaceae using molecular-based techniques. It would be interesting to examine the fungal endophytes of other *Dipodium* species to determine if these were similarly colonised by these homobasidiomycetes.

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