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REVIEW

Further advances in orchid mycorrhizal research

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Abstract Orchid mycorrhizas are mutualistic interactions between fungi and members of the Orchidaceae, the world's largest plant family. The majority of the world's orchids are photosynthetic, a small number of species are myco-heterotrophic throughout their lifetime, and recent research indicates a third mode (mixotrophy) whereby green orchids supplement their photosynthetically fixed carbon with carbon derived from their mycorrhizal fungus. Molecular identification studies of orchid-associated fungi indicate a wide range of fungi might be orchid mycobionts, show common fungal taxa across the globe and support the view that some orchids have specific fungal interactions. Confirmation of mycorrhizal status requires isolation of the fungi and restoration of functional mycorrhizas. New methods may now be used to store orchid-associated fungi and store and germinate seed, leading to more efficient culture of orchid species. However, many orchid mycorrhizas must be synthesised before conservation of these associations can be attempted in the field. Further gene expression studies of orchid mycorrhizas are needed to better understand the establishment and maintenance of the interaction. These data will add to efforts to conserve this diverse and valuable association.

Keywords Orchid mycorrhizas · Mixotrophy · Myco-heterotrophy · *Rhizoctonia* · Russulaceae

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Introduction

The Orchidaceae is the world's largest plant family with estimates of more than 25,000 species (Jones 2006). Orchids have three main growth habits: soil dwelling (terrestrial), on other plants (epiphytic) and on rock surfaces (lithophytic). As the seeds of orchids are minute and contain few stored food reserves, colonisation by a compatible fungus is essential for germination and/or early seedling development in or on the substrate (Smith and Read 1997). In the interaction, fungal hyphae grow into orchid tissues and form elaborate coiled structures known as pelotons within cortical cells. The majority of orchids are photosynthetic at maturity. However, more than 100 species of orchid are completely achlorophyllous (Leake 2005) and are nutritionally dependent on their fungal partners throughout their lifetime. These latter orchids have previously been termed saprophytic, but a more accurate designation is myco-heterotrophic (MH; Leake 1994, 2005; Bidartondo 2005).

Orchids are economically important. Vanilla is used to flavour food and drink, the tissues of *Gastrodia* are an important natural medicine, and orchids are a huge horticultural market worth 100 million dollars annually in the US alone (Griesbach 2002). Thus, it is surprising that research of orchids lags well behind that of other important mycorrhizas. Many problems remain. While epiphytic species are easy to grow asymbiotically in complex nutrient media, many terrestrial orchids, including both photosynthetic and MH species, have not yet been cultivated. Largely because of human-induced habitat loss and theft of attractive individuals, many orchid species are in danger of extinction across the planet. Conservation measures require a full understanding of the biology of each species in question.

A review by Rasmussen (2002) elegantly summarised the then current state of orchid mycorrhizal research. In her work, she described the latest cytological, ecological and physiological aspects of this mycorrhizal field. Rasmussen reported some of the early studies on orchid mycobiont identification using molecular techniques (e.g. Taylor and Bruns 1997, 1999) and highlighted new evidence that some MH orchids could derive their carbon from tree species via an ectomycorrhizal (ECM) connection (McKendrick et al. 2000). In the past 5 years, there has been a steady flow of new research published on orchid mycorrhizas, with a predominance of molecular mycobiont identification studies which have clarified some major issues in orchid mycorrhizal biology. Recently, Cameron et al. (2006) published results of a study showing, for the first time, carbon transfer from orchid to fungus, which has important implications for all subsequent research into photosynthetic orchid mycorrhizas.

New discoveries in orchid-mycorrhizal physiology

A landmark new paper demonstrating orchid mycorrhizas are a true mutualism

Orchid mycorrhizas have historically been depicted as anomalous mycorrhizal associations in that nutrient flow was plant focussed, and the fungal partner received little in return for its services (Smith and Read 1997). In two prominent papers, Hadley and Purves (1974) and Alexander and Hadley (1985) reported that when mycorrhizal *Goodyera repens* (L.) R.Br. was exposed to $^{14}CO_2$, they were unable to detect any passage of carbon to the fungal partner. In a recent repeat of these experiments, Cameron et al. (2006) have clearly shown that $^{14}CO_2$ passes from adult *G. repens* to the mycobiont (Fig. 1a). These authors also showed that mycorrhizal fungi continued to provide some carbon to adult photosynthetic plants, a result again in contrast to Alexander and Hadley (1985). Differences in results have been attributed to the higher physiological activity of both partners (i.e. sink sizes) in the later study created by more naturally equivalent experimental conditions such as moderate temperature, humidity and lighting.

Orchids receive compounds other than carbon from their fungal partners. Alexander et al. (1984) found that mycorrhizal *G. repens* acquired 100 times more P than non-mycorrhizal controls. P and N (as glycine) transfer from fungus to plant was confirmed in radiolabelling experiments (Cameron et al. 2006, 2007). Mycorrhizal fungi may also be a key source of water for orchids. In both the terrestrial *Platanthera integrilabia* (Correll) Luer and the epiphytic *Epidendrum conopseum* R.Br., water content was higher for mycorrhizal seedlings than uncolonised controls (Yoder et al. 2000). Thus, the overall picture of nutrient exchange in at least photosynthetic orchids appears more complete. All orchids need fungi to provide inorganic and organic nutrients for seed germination and/or early protocorm development. In adult photosynthetic orchids, N, P and water continue to flow from the fungal partner, but carbon exchange is essentially reversed with photosynthate providing incentive for continued fungal colonisation. The reward for fungi at the seed/protocorm stage is still a matter for conjecture.

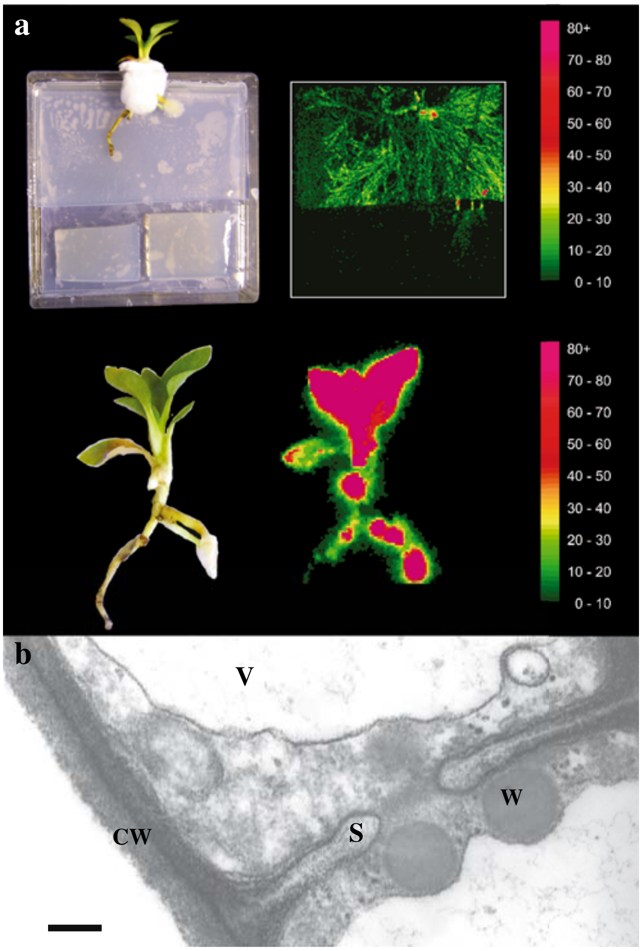

Fig. 1 Important recent discoveries in orchid mycorrhizal physiology and ecology. **a**. False colour digital autoradiographs showing movement of ^{14}C from *G. repens* (upper and lower images) to intact colonising fungal hyphae (RHS block of top image). The colour scale is indicative of the number of counts detected in pixel areas of 0.25 mm^2 over 60 min (Fig. 5 from Cameron et al. 2006) reproduced with kind permission of Blackwell Publishing). **b**. Transmission electron micrograph of non-dolipore ascomycete peloton forming hyphae in roots of *E. microphylla*. *W* Woronin bodies, *S* septum, *CW* fungal cell wall, *V* vacuole. *Scale bar* is 0.2 μm (Fig. 1c from Selosse et al. 2004, reproduced with kind permission of Springer Science and Business Media)

More evidence of transfer of carbon from neighbouring trees to orchids

More evidence has accumulated indicating that photosynthetic and MH orchids indirectly derive carbon from neighbouring trees since the study of McKendrick et al. (2000). This evidence has taken two forms. Identical fungal internal transcribed spacer (ITS) sequences in orchid roots and ECM of surrounding trees indicate epiparasitic interactions, although fulfilment of Koch's postulates remain (Taylor and Bruns 1997; Selosse et al. 2002a, 2004; Bidartondo et al. 2004; Girlanda et al. 2006; Abadie et al. 2006). In the second form of experiment, stable isotope ratios of carbon and nitrogen within orchids match those of local ECM fungi (Gebauer and Meyer 2003; Trudell et al. 2003; Bidartondo et al. 2004; Whitridge and Southworth 2005; Julou et al. 2005; Abadie et al. 2006), indicating common pools of nutrients. The common mycelium linking orchids and trees (Selosse et al. 2006) has major conservation implications (Girlanda et al. 2006). Protection of populations of threatened MH and other ECM-dependent orchids will require complementary preservation of suitable associated host tree species (Whitridge and Southworth 2005) in undisturbed habitats.

Mixotrophic orchids

The majority of orchids are photosynthetic in the adult stage with a small number being MH throughout their lifetime (Leake 2005). Recent evidence shows that a third orchid nutritional mode exists—mixotrophy (Julou et al. 2005). Such orchids are photosynthetic at the adult stage but augment their carbon requirements via mycorrhizal fungi (Gebauer and Meyer 2003; Bidartondo et al. 2004; Selosse et al. 2004; Julou et al. 2005). Mixotrophic orchids may be an evolutionary step between photosynthetic and MH orchids (Julou et al. 2005). Furthermore, the presence of ECM fungi in green orchids (Bidartondo et al. 2004; Irwin et al. 2007) and the recent discovery that the mycorrhizal partner of *Goodyera* continues to supply small amounts of carbon to its adult plant host (Cameron et al. 2006) suggests that this mode of nutrition may be more common in the Orchidaceae than first thought. Interestingly some members of the Tulasnellaceae and Ceratobasidiaceae have been demonstrated as ECM fungi (Bidartondo et al. 2003; Warcup 1985, 1991; Bougoure, personal communication), so further study of carbon flow to many photosynthetic orchids is warranted.

Gene expression studies in orchid mycorrhizas

In comparison to other mycorrhizal types [for recent reviews of arbuscular mycorrhizal (AM) interactions, see Hause and Fester 2005; Balestrini and Lanfranco 2006; ECM associations, see Duplessis et al. 2005; Frettinger et al. 2007], the molecular physiology of orchid mycorrhizas has been little studied. Gene expression was analysed in mycorrhizal and non-mycorrhizal *Cypripedium parviflorum* var *pubescens* (Willd.) Knight (Watkinson and Welbaum 2003). mRNA was extracted from non-mycorrhiza and plants in the early stages of mycorrhizal establishment and differentially expressed bands identified through amplified fragment length polymorphism (AFLP) complementary DNA differential display. Two genes showed differential expression, and these were mycorrhizal specific as both were unaffected by infection by a pathogenic fungus. A trehalose-6-phosphate synthase phosphatase decreased in expression during mycorrhizal establishment suggesting changes to orchid carbohydrate transport. A nucleotide-binding protein was upregulated in the interaction possibly because of enhanced cytokinesis in preparation for the entry of the fungus into the orchid tissues.

Recent advances in identification of orchid mycobionts

Ascomycetes as orchid mycobionts

Since the review by Rasmussen (2002), a large number of additional orchid mycobionts have been identified globally mainly through molecular biology approaches (Table 1). In agreement with Rasmussen (2002) the majority of orchid mycobionts are basidiomycetes, but a striking exception has been the fungal partners of *Epipactis*. Selosse et al. (2004) analysed the fungal ITS regions of colonised roots of chlorophyllous and achlorophyllous individuals of *Epipactis microphylla* (Ehrh.) Swartz over three French sites. Seventy-eight percent of root pieces analysed contained *Tuber* spp., with the remainder containing other ascomycete fungi and a few basidiomycete fungi. Electron microscopy confirmed the presence of non-dolipore ascomycete hyphae forming pelotons within roots of the species (Fig. 1b). Bidartondo et al. (2004) have also found *Tuber* in other *Epipactis* spp. and indicated that *Wilcoxina* and *Phialophora* are other potential mycorrhizal ascomycetes in orchids. The simple presence of ascomycete fungi in orchid roots does not necessarily indicate a functional association. These fungi will need to be isolated and grown in orchid seedlings before they can be designated as mycorrhizal partners.

Green orchids with specific fungal associations

Rasmussen (2002) suggested that photosynthetic orchids associated with a wider range of mycobionts than MH species. Subsequent studies indicate a more complex situation. Some photosynthetic orchids, even when sampled over a wide range, have a single dominant mycorrhizal fungus (McCormick et al. 2004, 2006; Shefferson et al. 2005 and Irwin et al. 2007; Fig. 2a,b). A fairly specific association for single fungi, particularly members of the Tulasnellaceae and Ceratobasidiaceae, occurs in (photosynthetic) epiphytic orchids (Otero et al. 2002; Ma et al. 2003;

Table 1 Summary of mycobionts identified in orchids since Rasmussen (2002)

Author and year of publication	Country of study	Orchid species, nutritional mode and habit[a]	Dominant mycobiont taxa present
Kristiansen et al. (2001)	Denmark	*Dactylorhiza majalis* (Rchb. F.) Hunt & Summerh. (P)	Tulasnellaceae Hydnangiaceae
McKendrick et al. (2002)	Britain	*Neottia nidus-avis* (L.) Rich. (MH)	Sebacinaceae
	Germany	*N. nidus-avis*	Sebacinaceae
Otero et al. (2002)	Puerto Rico	*Campylocentrum fasciola* (Lindl.) Cogn. (P+)	Ceratobasidiaceae
		C. filiforme (Sw.) Cogniaux (P+)	Ceratobasidiaceae
		Erythrodes plantaginea (L.) Fawcett & Randle (P)	Ceratobasidiaceae
		Ionopsis satyrioides (Sw.) Reichenbach f. (P+)	Ceratobasidiaceae
		I. utricularioides (Sw.) Lindl. (P+)	Ceratobasidiaceae
		Oeceoclades maculata (Lindl.) Lindl. (P)	Ceratobasidiaceae
		Oncidium altissimum (Jacq.) SW. (P)	Ceratobasidiaceae
		Tolumnia variegata (Sw.) Braem (P+)	Ceratobasidiaceae
Selosse et al. (2002a)	France	*Neottia nidus-avis*	Sebacinaceae
Shan et al. (2002)	China	*Eulophia flava* (Lindl.) Hook f. (P)	Tulasnellaceae
		Goodyera procera (Ker Gawl.) Hook. (P)	Tulasnellaceae
		Spiranthes hongkongensis S.Y.Hu & Barretto (P)	Tulasnellaceae
Ma et al. (2003)	Malaysia	*Oncidium nona X O. varimyre* (P+)	Tulasnellaceae
		Vanda "Miss Joaquim" (P+*)*	Tulasnellaceae
		Arachnis "Maggie Oei" (P+)	Tulasnellaceae
		Dendrobium crumenatum Swartz (P+)	Tulasnellaceae
		Arundina graminifolia (D.Don) Hochr. (P)	Tulasnellaceae
		Diplocaulobium sp. (P+*)*	Tulasnellaceae
		Spathoglottis plicata Bl. (P)	Tulasnellaceae
Pereira et al. (2003)	Brazil	*Epidendrum rigidum* Jacq. (P+)	Tulasnellaceae
		Polystachya concreta (Jacq.) Garay and Sweet (P+)	Tulasnellaceae
Sharma et al. (2003)	USA	*Platanthera praeclara* Sheviak and Bowles (P)	Ceratobasidiaceae, Tulasnellaceae
Taylor et al. (2003)	USA	*Hexalectris spicata* (Walt.) Barnh. (MH)	Sebacinaceae
		H. spicata var. arizonica (S.Watson) Catling & V.S.Engel (MH)	Sebacinaceae
		H. revoluta Correll (MH)	Sebacinaceae
Taylor et al. (2004)	USA	*Corallorhiza maculata* (Rafinesque) Rafinesque (MH)	Russulaceae
Otero et al. (2004)	Puerto Rico	*Ionopsis utricularioides* (P+)	Ceratobasidiaceae
		Tolumnia variegata (P+)	Ceratobasidiaceae
McCormick et al. (2004)	USA	*Goodyera pubescens* (P)	Tulasnellaceae
	USA	*Liparis lilifolia* A. Rich ex Lindl. (P)	Tulasnellaceae
	USA	*Tipularia discolor* Nutt. (P)	Tulasnellaceae et al.
Kristiansen et al. (2004)	Malaysia	*Neuwiedia veratrifolia* Bl. (P)	Tulasnellaceae, Ceratobasidiaceae
Bidartondo et al. (2004)	Germany	*Cephalanthera damasonium* (Mill.) Druce (MX)	Thelephoraceae, Hymenogasteraceae et al.
	Germany	*C. rubra* (L.) L.C.M Rich (P)	Thelephoraceae, *Phialophora*
	Germany	*Dactylorhiza majalis* (P)	Ceratobasidiaceae, Tulasnellaceae
	Germany	*Epipactis atrorubens* (Hoffm. ex Bernh.) Besser (P)	Pyronemataceae, Tuberaceae et al.
	Germany	*E. distans* Arvet-Touvet (MX)	Pyronemataceae
	Britain	*E. dunensis* (T. & T.A. Stephenson) Godfrey (P)	Tuberaceae, Pezizales, Cortinariaceae
	USA	*E. gigantea* Douglas ex Hooker (P)	Pyronemataceae, Tulasnellaceae et al.
	Britain	*E. helleborine* (L.) Crantz (MX)	Ceratobasidiaceae

Table 1 (continued)

Author and year of publication	Country of study	Orchid species, nutritional mode and habit[a]	Dominant mycobiont taxa present
	Canada	*E. helleborine* (MX)	Tuberaceae
	Germany	*E. helleborine* (MX)	Pyronemataceae, Tuberaceae et al.
	USA	*E. helleborine* (MX)	Pyronemataceae, Tuberaceae et al.
	USA	*E. helleborine* (MH)	Tuberaceae
	Germany	*E. palustris* (L.) Crantz (P)	Ceratobasidiaceae, Sebacinaceae
	Germany	*Plantanthera chlorantha* (Cust.) Rchb. p. (P)	Tulasnellaceae, *Phialophora*, Ceratobasidiaceae
Selosse et al. (2004)	France	*Epipactis microphylla* (MX)	Tuberaceae, Russulaceae et al.
		E. microphylla (MH)	Tuberaceae, Sebacinaceae et al.
Bougoure et al. (2005)	Australia	*Acianthus exsertus* R. Br. (P)	Tulasnellaceae
	Australia	*A. pusillus* D.L. Jones (P)	Tulasnellaceae
	Australia	*Caladenia carnea* R. Br. (P)	Sebacinaceae
	Australia	*Pterostylis nutans* R. Br. (P)	Ceratobasidiaceae
	Australia	*P. obtusa* R. Br. (P)	Ceratobasidiaceae
Bougoure and Dearnaley (2005)	Australia	*Dipodium variegatum* M. Clements & D. Jones (MH)	Russulaceae
Illyes et a. (2005)	Hungary	*Liparis loeselii* (L.) Rich (P)	Tulasnellaceae, Ceratobasidiaceae
Julou et al. (2005)	France	*Cephalanthera damasonium* (MH, MX)	Thelephoraceae, Cortinariaceae et al.
Pereira et al. (2005)	Brazil	*Epidendrum rigidum* (P+)	Tulasnellaceae
	Brazil	*Isochilus linearis* (Jacq.) R.Br. (P+)	Ceratobasidiaceae
	Brazil	*Maxillaria marginata* Fenzl. (P+)	Ceratobasidiaceae
	Brazil	*Oeceoclades maculata* (Lindl.) Lindl. (P)	Tulasnellaceae
	Brazil	*Oncidium flexuosum* (Kunth) Lindl. (P+)	Ceratobasidiaceae
	Brazil	*O. varicosum* Lindl. and Paxton (P+)	Ceratobasidiaceae
	Brazil	*Polystachya concreta* (P+)	Tulasnellaceae
Shefferson et al. (2005)	Estonia	*Cypripedium calceolus* L. (P)	Tulasnellaceae
	USA	*C. californicum* A. Gray (P)	Tulasnellaceae, Ceratobasidiaceae et al.
	USA	*C. candidum* Mühl ex Willd.(P)	Tulasnellaceae, *Phialophora* et al.
	USA	*C. fasciculatum* Kellogg ex S. Watson (P)	Tulasnellaceae, *Phialophora* et al.
	USA	*C. montanum* Douglas ex Lindl (P)	Tulasnellaceae, *Phialophora* et al.
	USA	*C. parviflorum* Salisb. (P)	Tulasnellaceae, *Phialophora* et al.
Whitridge and Southworth (2005)	USA	*Cypripedium fasciculatum* (MX)	Russulaceae, Tulasnellaceae et al.
		Goodyera oblongifolia Raf. (P)	Ceratobasidiaceae
		Piperia sp. (P)	Tulasnellaceae
		Corallorhiza sp. (MH)	Russulaceae
Yamato et al. (2005)	Japan	*Epipogium roseum* (D. Don) Lindl. (MH)	Coprinaceae
Abadie et al. (2006)	Estonia	*Cephalanthera longifolia* (L.) Fritsch (MH, MX)	Thelephoraceae, Pyronemataceae et al.
Dearnaley (2006)	Australia	*Erythrorchis cassythoides* (MH)	Russulaceae, Sebacinaceae, Tricholomataceae et al.
Dearnaley and Le Brocque (2006)	Australia	*Dipodium hamiltonianum* (MH)	Russulaceae
Girlanda et al. (2006)	Italy, France	*Limodorum abortivum* (L.) Swartz (Mix)	Russulaceae, Tuberaceae
	Italy	*L. brulloi* Bartolo & Pulvirenti (MX?)	Russulaceae
	Italy	*L. trabutianum* Battandier (MX?)	Russulaceae
Suarez et al. (2006)	Ecuador	*Stelis concinna* Lindl. (P+)	Tulasnellaceae
	Ecuador	*S. hallii* Lindl. (P+)	Tulasnellaceae
	Ecuador	*S. superbiens* Lindl. (P+)	Tulasnellaceae
	Ecuador	*Pleurothallis lilijae* Foldats (P+)	Tulasnellaceae
Boddington and Dearnaley (2007)	Australia	*Dendrobium speciosum* Smith (P+)	Tulasnellaceae
Irwin et al. (2007)	Australia	*Pterostylis nutans* R.Br. (P)	Ceratobasidiaceae, Russulaceae
Bonnardeaux et al. 2007	Australia	*Pyrorchis nigricans* (R.Br.) D.L. Jones & M.A. Clem. (P)	Tulasnellaceae, Ceratobasidiaceae

Table 1 (continued)

Author and year of publication	Country of study	Orchid species, nutritional mode and habit[a]	Dominant mycobiont taxa present
	Australia	*Disa bracteata* Sw. (P)	Tulasnellaceae
	Australia	*Thelymitra crinita* Lindl. (P)	*Phialophora* sp.
	Australia	*Prasophyllum giganteum* Lindl. (P)	Tulasnellaceae
	Australia	*Diuris magnifica* D.L. Jones (P)	Tulasnellaceae
	Australia	*Caladenia falcata* (Nicholls) M.A.Clem. & Hopper (P)	Sebacinaceae
	Australia	*Microtis media* R.Br. (P)	Sebacinaceae
	Australia	*Pterostylis sanguinea* D.L. Jones & M.A. Clem. (P)	Ceratobasidiaceae
	Australia	*P. recurva* Benth. (P)	Ceratobasidiaceae

Plus sign indicates epiphytic species.

[a] *P* Photosynthetic, *MH* mycoheterotrophic, *MX* mixotrophic

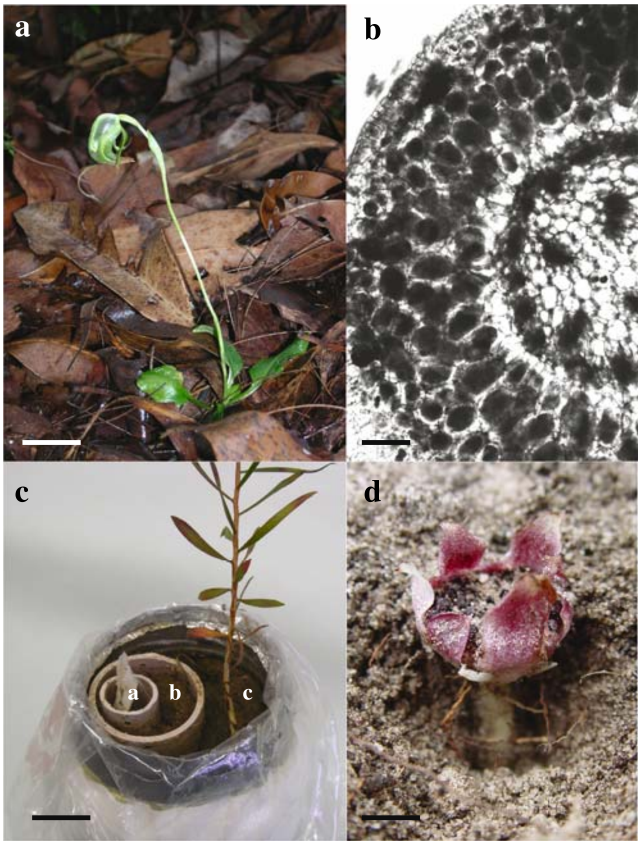

Fig. 2 Recently investigated Australian orchid–fungal relationships. **a** The common and widespread photosynthetic orchid, *Pterostylis nutans* recently investigated by Irwin et al. (2007). The species has a specific relationship with two *Ceratobasidium* fungi across its range in Eastern Australia. **b** Heavy fungal colonisation in the roots of *P. nutans*. *Scale bars* approximately **a**=2 cm, **b**=250 μm. **c** Ex situ growth system for the MH orchid *R. gardneri* (images courtesy of Jeremy Bougoure). *a* inner pot with fungal inoculum and nylon bags containing orchid seedlings, *b* pot with fungal inoculum only, *c* outer pot with *Melaleuca uncinata* R. Br. host and fungal inoculum; 35 μm mesh between pots allow movement of hyphae but not plant roots between compartments. **d** Adult plant of *R. gardneri*. *Scale bars* approximately **c**=2 cm, **d**=1 cm

Suarez et al. 2006). In contrast, some MH orchids contain a range of unrelated mycobiont taxa (Julou et al. 2005; Dearnaley 2006). Although specificity has been a contentious issue for many years (e.g. Warcup 1981; Masuhara and Katsuya 1994; Zelmer et al. 1996) reliable techniques (i.e. fungal ITS sequencing) are now available for identifying orchid mycobionts. Fungal specificity is thus a common phenomenon in many orchids regardless of nutritional mode.

The specific mycorrhizal associations seen in some green orchids warrant further investigation. Specificity possibly leads to high rates of seed germination and a more efficient physiological association when the interaction is fully functional (Bonnardeaux et al. 2007). In photosynthetic orchids with prolonged dormancy periods or species confined to heavily shaded habitats, there may be a higher dependency on fungal carbon than evergreen or annually flowering plants and plants of exposed habits (Girlanda et al. 2006), and thus an efficient and specific association is advantageous. Fungal specificity and orchid rarity may also be linked if the fungal partner is rare or patchily distributed in the landscape (Brundrett et al. 2003; Bonnardeaux et al. 2007). However, Feuerherdt et al. (2005) have shown that a fungus compatible with and likely specific to (Warcup 1971) the threatened *Arachnorchis behrii* Hopper & Brown is found in areas away from orchid populations, so fungal distribution does not appear to be responsible for the rarity of the orchid species. Thus, there is still more to be learnt about the causes of fungal specificity in the Orchidaceae and its impact on the conservation status of individual species.

Mycoheterotrophic orchids with heterobasidiomycete mycobionts

While heterobasidomycete fungi are well known as mycobionts of photosynthetic orchids (Rasmussen 2002), recent molecular analyses have demonstrated the presence of heterobasidiomycete fungi in a number of MH orchid species. Bougoure (personal communication) has recently confirmed through DNA sequence analysis the original observation of Warcup (1991) that an ECM *Thanatephorus* sp. is the main mycobiont of the subterranean MH *Rhizanthella gardneri* R.S. Rogers. McKendrick et al. (2002), Selosse et al. (2002a, b), Taylor et al. (2003), Bidartondo et al. (2004), and Dearnaley (2006) have demonstrated members of the Sebacinaceae in a range of MH orchid species worldwide. The Sebacinaceae are known to be ECM on a diversity of plant families including the Ericaceae, Betulaceae, Fagaceae, Tilliaceae, and Myrtaceae (Berch et al. 2002; Selosse et al. 2002b; Glen et al. 2002). Study by Selosse et al. (2002a) suggests that MH orchids probably exploit these associations by withdrawing carbon from the ECM network.

Investigations of orchid-associated heterobasidiomycete fungi have clarified some taxonomic issues within the group. The anamorphic members of the Sebacinaceae have historically been aligned with members of the *Rhizoctonia* form genus (Warcup 1981, 1988). However, the group is taxonomically distinct from the Tulasnellaceae and Ceratobasidiaceae, and diversity within this group is sufficient to justify a new order, Sebacinales (Weiß et al. (2004). These authors suggest that within the Sebacinales, *Sebacina* spp. that form ECM and associate with MH orchids (subgroup A) are distinct from essentially saprotrophic species and associates of photosynthetic orchids including the probable species complex *Sebacina vermifera* (subgroup B). Recent phylogenetic analyses have cast light on two other important orchid mycorrhizal fungal genera, *Ceratobasidium* and *Thanatephorus* (Binder et al. 2005; Sharon et al. 2006; Gonzalez et al. 2006), but more sequences need to be examined to complete the picture. The common orchid-associating genus *Tulasnella* contains many undescribed species and some phylogenetically problematic taxa, e.g. *T. calospora* (Boudier) Juel, which more extensive sequence analysis should clarify (Suarez et al. 2006). Taxonomic research of orchid-associated heterobasidiomycetes is important from a pure scientific perspective but is crucial for orchid conservation to ensure appropriate mycorrhizal fungi are sustained with their host and potentially pathogenic fungi are excluded from pristine natural systems.

Evidence of partner switching in adult orchid species

Some evidence indicates fungal partners may switch during the life of the orchid. Seed germination often fails with mycobionts extracted from adults (Rasmussen 2002), though failure may be due to isolation of non-mycotrophic fungi from the cortex of the host. However, the fungal partner of *Gastrodia elata* Bl. changed from *Mycena* to *Armillaria* as the plant matured (Xu and Mu 1990), which suggests switching of fungal partner in the transition from juvenile to adult orchid. Partner switching may also occur in adult orchids. Protocorms and adult plants of the photosynthetic *Goodyera pubescens* R. Br. contained the same fungal species, but when environmentally stressed, surviving orchids were able to switch to new fungal partners (McCormick et al. 2006). The MH vine, *Erythrorchis cassythoides* Cunn. (Garay) associates predominately with ECM fungi while it interacts with a living host, but the main mycobiont is a saprotrophic species when the tree host is dead (Dearnaley 2006). Fungal-orchid associations appear sensitive to environmental stimuli and can possibly adjust to favour survival of the plant partner. Orchid species with partner switching need special conservation approaches. If adults and seeds require different mycobionts, it is essential that both of these are isolated and perpetuated during recovery programs (Zettler et al. 2005). It is also essential to determine and perpetuate the range of fungi an adult orchid associates with under different environmental conditions.

The global importance of the Russulaceae in orchid mycorrhizas

Recent studies in Australia and Europe have expanded the range of orchid species colonised by members of the family Russulaceae. Taylor and Bruns (1997, 1999) showed that *Corallorhiza* spp. always associated with members of this important ECM group of fungi across a wide range in the Western US (Taylor and Bruns 1999; Taylor et al. 2003). Girlanda et al. (2006) have recently shown that *Limodorum* spp. associate predominately with *Russula* spp. across sites in France and Italy. The Australian MH orchid *Dipodium hamiltonianum* F.M. Bailey associates primarily with hypogeous members of the Russulaceae (Dearnaley and Le Brocque 2006). This discovery has led the authors to discuss the importance of marsupials in the ecology of the orchid as these fungi are common dietary components of such animals in Australian woodlands (Claridge and May 1994), and roots of the orchid are eaten (unpublished results). Russulaceae spp. are difficult to culture (Taylor and Bruns 1997; Girlanda et al. 2006; Bougoure and Dearnaley 2005) but the recent development of shaking culture techniques (Sangtiean and Schmidt 2002) suggests that ex situ growth of threatened MH orchids that require pure culture inoculation with these fungal partners may be possible.

Molecular studies of the mycobionts of epiphytic orchids

Mycorrhizal fungi of epiphytic orchids have been neglected possibly because early studies indicated low levels of colonisation in such species (Hadley and Williamson 1972). In recent times, a number of authors have used molecular taxonomic techniques to document the fungal partners of epiphytic orchids (Otero et al. 2002, 2004; Ma et al. 2003; Kristiansen et al. 2004; Pereira et al. 2005; Suarez et al. 2006; Boddington and Dearnaley 2007). Overall, the main mycobionts found in these orchids are similar to terrestrial photosynthetic species and include *Ceratobasidium* and *Tulasnella* species (see Table 1). Epiphytic and lithophytic orchids have provided opportunities to investigate aspects of orchid–fungal ecology. Field-grown *Lepanthes rupestris* Stimson were treated with fungicides to test the effect of removal of mycorrhizal fungi on plant growth and survival (Bayman et al. 2002). Although results were difficult to interpret due to the presence of a range of non-mycorrhizal fungi, fungicides clearly reduced the plant population, highlighting the importance of mycorrhizal colonisation for orchid growth and survival. Otero et al. (2004) demonstrated that although *Ionopsis utricularioides* (Swartz) Lindley was more restricted in the *Ceratobasidium* fungi, it could associate with than the related species *Tolumnia variegata* (Swartz) Braem; it had higher seed germination and seedling development rate, suggesting that specificity leads to more efficient mycorrhizal interactions. Thus orchid-fungal specialists tradeoff the risk of not finding a suitable mycorrhizal fungus in nature with a more efficient interaction when a suitable partner is found. Individuals of a population of *T. variegata*, an orchid fungal-generalist, vary in their symbiotic seed germination rate, and certain *Ceratobasidium* are better at inducing germination than others. These results show that fitness varies in members of orchid populations as well as in the mycorrhizal fungi they associate with, and thus natural selection could impact on orchid–fungal relationships (Otero et al. 2005).

Advances in symbiotic orchid conservation techniques

New techniques for symbiotic seed germination

A number of recent studies have determined factors crucial to the germination of orchid seeds under ex situ and in situ conditions. Cold treatment of seeds has been shown to be necessary to break dormancy in seed of *Cypripedium macranthos* var. *rebunense* (Kudo) Miyabe et Kudo, and directly after this is the ideal time for fungal inoculation (Shimura and Koda 2005). Chilling (6°C) and darkness appeared to accelerate symbiotic protocorm growth in the threatened *Platanthera leucophaea* (Nutt.) Lindley and probably mimics natural conditions for this species (Zettler et al. 2005). Optimal symbiotic seed germination conditions in some Australian orchid genera involve seed dessication followed by storage in liquid nitrogen before colonisation with compatible fungi (Batty et al. 2001). Associated mycorrhizal fungi can also be stored in liquid nitrogen for long periods (Batty et al. 2001). Continual darkness inhibited seed germination but stimulated protocorm development in the rare *Habenaria macroceratitis* Willdenow (Stewart and Kane 2006). Pelotons with fine loose hyphae and monilioid cells obtained from leafing to flowering stages appear to be best for ex situ symbiotic seed colonisation in the vulnerable *Caladenia formosa* G.W. Carr (Huynh et al. 2004). Diez (2007) showed that seed of *G. pubescens* should be sown within 1 m of parent plants to enhance germination success or at sites that had higher organic matter and moisture content and lower pH than less suited areas. Brundrett et al. (2003) introduced new in situ and ex situ soil baiting methods for orchid mycorrhizal fungi. The ex situ technique, which involved overlying soil with membranes holding orchid seed, was easy to construct, was not season dependent and made it possible to closely monitor plant development in a range of species under close to natural conditions. The in situ technique allowed simultaneous detection of mycorrhizal fungi of a range of orchid species under field conditions. These studies have given a clearer understanding of the ecology of specific orchids, which may lead to more successful methods for germinating seed and growing orchids generally.

New techniques for introduction of symbiotic seedlings to the wild

Conservation procedures for threatened orchid species involve ex situ growth of plants and release to the wild. This is not a simple procedure, but work from researchers in Australia has provided some recent breakthroughs. An intermediate culture stage in correctly aerated sand-agar-containing vessels can overcome the high rate of mortality often observed when moving symbiotically grown orchid seedlings from the high humidity of the petri dish to the glasshouse (Batty et al. 2006a). Seedling and tuber transfer to the wild is superior to the release of seed to field sites for establishment of orchid populations (Batty et al. 2006b). The factors that affect survival of translocated symbiotically grown seedlings are site aspect, weed cover and orchid species, not presence of individuals of the same species nor compatible soil fungi (Scade et al. 2006), suggesting that site selection and management are key to the survival of translocated populations. Release of symbiotically grown orchid seedlings to areas dominated by ericaceous plants may not be disadvantageous as there does not appear to involve competition for carbon substrates of associated fungi (Midgley et al. 2006).

Future directions in orchid-mycorrhizal research

Orchid mycorrhizal research has benefited from the introduction of molecular biology techniques to mycobiont identification. Orchid mycorrhizas now represent an excellent system to study symbiosis-related gene expression. However, many orchid species are on the verge of extinction and urgently require ecological and physiological examination. I suggest there will be two main foci in this field over the next few years.

Analysis of gene expression in orchid mycorrhizas

The discovery that photosynthetic orchid mycorrhizas are truly mutualistic (Cameron et al. 2006) suggests that the interaction represents a useful model to study the genetics of plant mycorrhizal associations. Unlike ECM and AM symbioses, both partners are easy to culture axenically, and the association can be quickly formed in vitro. Two main areas of gene expression could be dealt with using modern molecular approaches such as quantitative reverse transcription-polymerase chain reaction (RT-PCR), microarray techniques and in situ hybridisation. The first would involve determining the genes that are modified in the initial stages of interaction of orchids with fungi. A target could include a potential diffusible fungus-derived molecule that signals compatibility between partners. Investigation of orchid genes encoding signal transduction and cell wall-modifying proteins that are upregulated by fungal exudates and initial hyphal contact is key to understanding the early stages of the colonisation process. It would be intriguing if plant hyphal branching inducing molecules such as the sesquiterpenes of the *Lotus*-AM interaction (Akiyama et al. 2005) also existed in orchid–fungal interactions.

A second focal point would be the genes involved with the maintenance of the symbiosis. As colonisation involves cell wall modification such as penetration of root cortical cells by fungal hyphae and the formation of the interfacial matrix between plant and fungus (Dearnaley and McGee 1996), it is likely there are related transcriptional changes in wall-loosening genes such as those encoding expansins and xyloglucan degrading enzymes and genes responsible for wall synthesis such as cellulose- and hemicellulose-assembling enzymes. Defence genes are typically downregulated during mycorrhizal associations (for review, see Balestrini and LanFranco 2006). As pelotons are short-lived structures, it would be interesting to monitor the expression of genes of well-known anti-fungal proteins such as chitinases, glucanases and thaumatins during orchid mycorrhizal functioning. As we now have a clearer picture of orchid mycorrhizal nutrition, it is timely to begin studies of nutrient transporters and answer some key questions about the association. Are plant carbon transporters found on the plant cell membrane around intact pelotons analogous to the situation for the AM symbiosis (Harrison 1996)? Where does inorganic nutrient exchange occur in orchid mycorrhizas—solely through collapsing pelotons, or are plant and fungal P and N transporters and aquaporins active around healthy pelotons? Studies of gene expression in orchid mycorrhizas may also provide insights into plant–pathogen interactions, given that recent transcriptome analyses of mycorrhizas have shown conservation of transcriptional pathways between mycorrhizal and pathogenic interactions (Güimil et al. 2005).

Determination of conservation methods for orchids reliant on ECM fungi

A number of MH and mixotrophic orchids are threatened, e.g. *Hexalectris* spp., *Epipactis* spp., *Dipodium* spp. (Taylor et al. 2004; Selosse et al. 2004; Dearnaley and Le Brocque 2006), and further study (e.g. mycobiont identification; stable C and N isotope ratios, CO_2 exchanges) is required of rare chlorophyllous species to confirm physiological status (i.e. dependency on ECM associations). Conservation approaches for these species are closely dependent on determination of appropriate ex situ methods of growth so that more seed and/or seedlings can be used to stabilise natural populations. As these orchid species depend on ECM fungi for their nutrition (Taylor et al. 2004; Selosse et al. 2004; Dearnaley and Le Brocque 2006, but see Yagame et al. 2007 for a recent review of MH orchids that can be cultivated with non-ECM fungi), ex situ growth will require establishing tripartite symbiotic interactions with tree seedlings, ECM fungi and orchids under controlled conditions. Warcup (1985, 1988, 1991), McKendrick et al. (2000) and Bougoure (personal communication, Fig. 2c,d) have successfully grown ECM-dependent orchids within controlled systems, but the majority of these have involved more easy to culture heterobasidiomycete mycobionts and not difficult to grow homobasidiomycete fungi; thus more research on growth techniques is required. Establishment of pure cultures or at least long-term storage methods for ECM fungi is imperative to any conservation effort. Retention of suitable host trees is a vital in situ management approach for these species as is long-term monitoring of appropriate, naturally occurring ECM fungi to ensure continued seedling recruitment (Findlay 2005; Leake 2005).

Concluding remarks

Research into orchid mycorrhizas is set to increase over the next decade. Motivation for increases must come from a desire to learn more about the essential biology of these intriguing associations and critically from a conservation viewpoint. Protection of orchid populations and orchid-associated fungi is important in maintaining global biodiversity, and it also has implications for overall ecosystem health. As photosynthetic orchids pass photosynthate back to their fungal partners (Cameron et al. 2006), orchids and their associated fungi are contributors to the common mycelial network that appears to be key to the integrity of terrestrial ecosystems (Selosse et al. 2006).

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