12 Orchid Mycorrhizas: Molecular Ecology, Physiology, Evolution and Conservation Aspects

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I. Introduction

Since the pioneering works by Noël Bernard (1874–1911; Boullard 1985), whose centenary of death was celebrated in 2011, scientific interest in orchid mycorrhizas has continued to grow. This might seem somewhat surprising as the association concerns a single angiosperm family and is less widespread among land plants than the ectomycorrhizal (ECM) or arbuscular mycorrhizal (AM) symbioses (Smith and Read 2008). Impetus for research into orchid mycorrhizas has been multifaceted. As the earth's largest flowering plant family with 27,135 accepted species (The Plant List 2010), accumulating basic biological knowledge of what represents approximately 10 % of the botanical kingdom diversity is justified. For the mycologist, these plants that shifted from an ancestral AM symbiosis with Glomeromycetes to an original symbiosis with new fungal partners (Yukawa et al. 2009) offer a window on mycorrhizal abilities in numerous, and sometimes unexpected, fungal lineages. Moreover, many species, such as aromatic *Vanilla* spp. or many ornamental species, are of economic value. Illuminating studies of orchid mycorrhizas have shown that some non-chlorophyllous plants live as parasites on ECM interactions or saprotrophic fungi and that some green orchids are partially heterotrophic as adults. Recent research has given insights into the nature of the mycorrhizal association of autotrophic orchids, suggesting that the association may be mutualistic (Cameron et al. 2006, 2008). The mycorrhizas of orchids also offer general perspectives on the evolution of specificity and

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mycorrhizal networks among plants. Finally, as orchids require the presence of suitable fungal partners for seed germination and seedling establishment, a more complete understanding of the mycorrhizal biology of the many threatened orchid species is required for conservation action plans.

Orchids have historically been divided into three main types on the basis of lifestyle, i.e. terrestrial (soil dwelling), epiphytic (plant surface dwelling) and lithophytic (rock surface dwelling) species. Recent literature (e.g. Gebauer and Meyer 2003; Selosse et al. 2004; for reviews, see Merckx et al. 2009; Selosse and Roy 2009) has suggested a division of orchids into three physiological types based on carbon nutrition. Fully autotrophic species (the majority of taxa) are those that are chlorophyllous and, as adults, obtain their carbon compounds via photosynthetic pathways. Fully mycoheterotrophic (MH) species (approximately 200 species worldwide; Leake 1994, 2004) are dependent on fungal carbon throughout their life cycle. A third type, the partial MH species or mixotrophs (Julou et al. 2005; Selosse and Roy 2009) are intermediate, carrying out some photosynthetic carbon fixation as well as receiving fungal carbon.

Regardless of their carbon nutrition at adult stage, all orchids produce minute, endosperm-lacking seeds and are dependent on fungal colonization for germination and growth into an underground heterotrophic, achlorophyllous stage called a protocorm (Rasmussen 1995; Smith and Read 2008). Environmental fungi colonize though embryo suspensor tissues or epidermal hairs and enter the cortical cells. Colonizing fungal hyphae do not breach the cortical cell membrane but ramify in the space between cell wall and membrane, forming elaborate coiled structures known as pelotons (Fig. 12.1) that collapse at later stages as a result of plant digestion. Intact fungal coils and not collapsed pelotons are likely the site of nutrient exchange between plant and fungus, as indicated by the fact that in vitro grown protocorms show a growth response **before** peloton collapse (Hadley and Williamson 1971) and that the nutrient fluxes after labelling pulses occur too rapidly to be accounted for by hyphal digestion (Cameron et al. 2008).

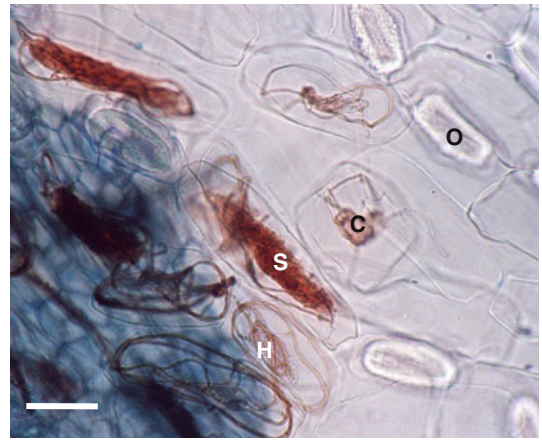

Fig. 12.1. Light microscopy image showing healthy (*H*), slightly degraded (*S*) and collapsed (*C*) fungal pelotons of a *Thelephora* sp. in *Cephalanthera longifolia* roots (Ülle Püttsepp, unpublished micrograph; plant investigated in Abadie et al. (2006)). O Oxalate crystal. *Bar* 50 μm

This chapter will focus on current understanding of the mycorrhizal associations of these three physiological orchid types. For a more comprehensive review of the colonization process and anatomy of orchid mycorrhizas readers are directed to Smith and Read (2008). Here it is intended to update the 10-year-old excellent reviews by Rasmussen (2002) and Taylor et al. (2002), to provide an overview of the contemporary approaches to studying these interactions, to elaborate on what has been gleaned from these studies with regards to the ecology, physiology, evolution and conservation aspects of orchid mycorrhizas and to highlight areas of the association that need further exploration.

II. New Techniques for Studying Orchid Mycorrhizas

A. Molecular Barcoding Approaches

Historically, much knowledge about orchid mycorrhizas has been acquired from **in vitro isolation of fungi**. This has allowed basic fungal identification and simple in vitro seed germination experiments conducted with some root-isolated fungi (e.g. Warcup 1971;

Clements 1988). Indeed, the orchid mycorrhizal association represents possibly one of the easiest symbiotic systems to manipulate under laboratory conditions as both partners can be cultured axenically, at least in the case of the early stages of the fully autotrophic orchids. A hurdle in these types of investigations has been an inability to accurately identify the isolated fungal partners and this has been especially critical to orchid conservation procedures involving restorative work; moreover, isolation often provided mostly contaminants or endophytes (i.e. fungi that for all or part of their life cycle inhabit living plant tissues but do not form pelotons nor cause any obvious disease symptoms; Wilson 1995).

Molecular taxonomy approaches have enhanced fungal taxonomy, especially by isolating fungal DNA and sequencing the nuclear ribosomal DNA (Seiffert 2009). The fungal partners of orchid mycorrhizas can be more accurately and routinely identified from cultured fungi or directly from orchid protocorms, roots, tubers and rhizomes (e.g. Bougoure et al. 2005; Martos et al. 2009; Swarts et al. 2010). For mycobionts recalcitrant to axenic growth, PCR amplification of colonized orchid tissues using fungus-specific primers is commonly used (Dearnaley and Le Brocque 2006; Dearnaley and Bougoure 2010).

Sequencing of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA after PCR amplification using a variety of primer combinations (White et al. 1990; Gardes and Bruns 1993) has been the method of choice for identifying orchid mycobionts over the past decade. One problem is the amplification recalcitrance of Tulasnellaceae, a frequent orchid mycorrhizal taxon (see Sect. III.A), to the 'universal' fungal ITS primers, because they have highly derived nuclear ribosomal DNA sequences. This entailed the need for additional PCR amplifications using Tulasnellaceae-specific PCR primers (Bidartondo et al. 2004; Selosse et al. 2004). Suarez et al. (2006) introduced the Tulasnellaceae-specific primer 5.8S-Tul to amplify the 5' part of 28S rDNA, which was used by Martos et al. (2012). This primer works well on a wide range of clades of Tulasnellaceae and is expected to be frequently used in future studies of orchid mycorrhizal fungi because of the high heterogeneity of the ITS alignment. Recently, some primer pairs specifically devoted to orchid mycorrhizal fungi were described (Taylor and McCormick 2007), but the constantly growing number of fungal taxa (see Sect. III) questions their relevance in the new orchid lineages to be explored. Sequencing of cloned ITS PCR products is often carried out with orchids displaying low fungal specificity (e.g. Selosse et al. 2002; Dearnaley 2006; Liebel et al. 2010; Martos et al. 2012). Sequencing of the large subunit (LSU) of the nuclear ribosomal DNA of the Sebacinales, common orchid mycobionts worldwide, is necessary for higher resolution separation of groups A and B, two major clades in this group (Weiß et al. 2004, 2011; Selosse et al. 2009). Huynh et al. (2009) also recently showed that ITS sequencing may not sufficiently distinguish isolates of the '*Sebacina vermifera*' complex (Sebacinales group B), common mycobionts of spider orchids in Australia. ITS sequencing and cloning may also reveal many endophytes (Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006; Roy et al. 2009a). In Martos et al. (2012), a wide range of ascomycete and basidiomycete endophytes was identified, perhaps more than in any study of orchid associated fungi to date.

Fungal endophytes are very frequently selected during in vitro isolation or PCR amplification from orchid tissues with fungus-specific primers: dissecting single fungal pelotons from roots tissues before in vitro isolations (Zhu et al. 2008) or PCR amplifications (Rasmussen 1995; Kristiansen et al. 2001) is thus strongly recommended in future work to avoid endophytes. The important diversity of endophytic fungi, mainly from the Helotiales (e.g. *Phialophora*, *Leptodontidium* or *Bisporella* spp.) or Xylariales, will not be discussed in detail here (for a review, see Bayman and Otero 2006), while their effect on orchid growth (potentially deleterious in some species; Bayman et al. 2002) and physiology deserves further study. Chaetotyriales are very common orchid endophytes, at least in tropical areas. Capnodiales are also common in epiphytic taxa, but they might be involved in lichenic symbioses. Many epiphytic orchids root in bryophytes or lichens.

Recently, Jacquemyn et al. (2010) and Lievens et al. (2010) introduced **DNA array technologies** for the identification of orchid fungal partners: oligonucleotides were prepared from a preliminary exploration of fungal diversity in a limited number of individuals (Lievens et al. 2010), and the array was successfully used to investigate the fungal partners of three closely related *Orchis* species and their hybrids (Jacquemyn et al. 2011). This method allows

fast and efficient handling of numerous samples, especially compared to the cloning of PCR products. However, some fungal partners may remain overlooked when using this procedure because preliminary exploration overlooks rare fungal taxa that may not be targeted during further investigation (such as taxon 8 and 9 from the Thelephoraceae and Cortinariaceae, respectively, in Lievens et al. 2010; Jacquemyn et al. 2011).

B. Stable and Radioactive Isotopes

A common, but indirect approach to determine the mode of nutrition of individual orchid taxa is **mass spectrometric analysis** of natural C and N isotope abundances (Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006; Zimmer et al. 2007; Ogura-Tsujita et al. 2009; Martos et al. 2009). Fully MH species have been identified to have ^{13}C signatures similar to those of their mycorrhizal partners (Gebauer and Meyer 2003; Trudell et al. 2003) and similar or higher ^{15}N abundance than their mycorrhizal fungi, suggesting a limited trend to ^{15}N accumulation along the food chain (Trudell et al. 2003). As expected, mixotrophs have stable isotope signatures intermediate between fully MH and autotrophic species (Julou et al. 2005; Abadie et al. 2006). Some fully autotrophic species such as *Goodyera* spp. have even lower amounts of these natural isotopes as expected for plants less reliant on nutrient acquisition from fungi (Gebauer and Meyer 2003; Bidartondo et al. 2004). The strength of this method is that abundances oversee the long-term metabolism of the plants, with little interference from the observer.

On the fungal side, Latalova and Balaz (2010) showed that a *Tulasnella* species associated in vitro with the orchid *Serapias strictiflora* was able to mix carbon from the orchid (a C_3 plant) and dead maize roots (a C_4 plant enriched in ^{13}C). The fungus was able to grow with the orchid alone, with ^{13}C abundance close to its host, while addition of dead maize roots resulted in an isotopic shift, so that the latter source furnished ca. 30 % of the fungal biomass.

Experiments tracing **the movement of isotopically labelled compounds** to orchid mycorrhizas have been especially revealing. Although they only provide snapshot views of the metabolism at the time of pulse, they allow tracking of exchanges between symbionts. McKendrick et al. (2000) provided the first clear demonstration of movement of ^{14}C-labelled photosynthates from tree species to the fully MH orchid *Corallorhiza trifida* via ECM fungi. Bougoure et al. (2010) recently demonstrated the flow of ^{13}C-labelled carbon from *Melaleuca scalena* to the fully MH orchid *Rhizanthella gardneri* via an ECM fungal conduit. *R. gardneri* also obtained nitrogen from its fungal partner as indicated by adding $^{13}C+^{15}N$-labelled glycine to hyphae and surrounding soil. Labelling experiments also demonstrated that the fully autotrophic orchid *Goodyera repens* acquires carbon, nitrogen and phosphorous from its fungal partner (Cameron et al. 2006, 2007, 2008). Notably, *G. repens* also transfers significant amounts of photosynthate (likely greater than 3 % of its photosynthetic carbon) back to its *Ceratobasidium* mycobiont – the first direct demonstration of a net carbon flow from orchid to fungi (Cameron et al. 2006, 2008).

C. Other Approaches

In contrast to other mycorrhizal symbioses, such as ECM and AM associations, **gene expression studies** in orchid mycorrhizas have largely been neglected. Watkinson and Welbaum (2003) analysed gene expression in the mycorrhizal association of *Cypripedium parviflorum* var. *pubescens* via differential mRNA display. A trehalose phosphate phosphatase was downregulated in the association, indicating changes to orchid carbohydrate transport. Upregulation of a nucleotide binding protein possibly indicated increased cytokinesis during orchid colonization. As indicated by Dearnaley (2007), modern gene expression techniques such as microarrays, RT-PCR and in situ hybridization may provide additional understanding of the molecular functioning of orchid mycorrhizas. In particular, whole-genome sequencing and transcript profiling of orchid mycorrhizal

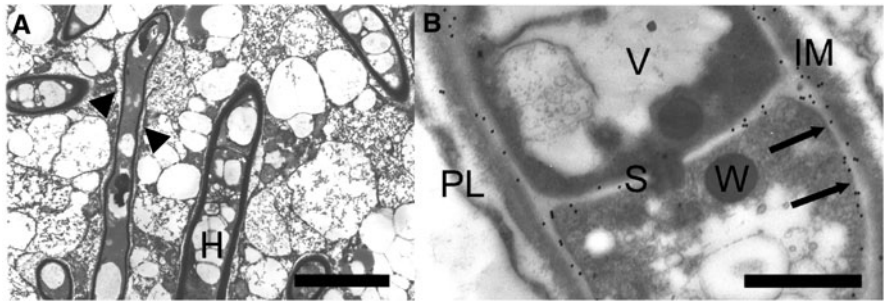

Fig. 12.2. TEM images of *Epipactis microphylla* cells colonized by truffles. (**A**) Truffle septate hyphae (*H*) inside an orchid host cell, where the host plasma membrane (*arrowheads*) tightly surrounds the fungus (*bar* 4 μm). (**B**) Gold granules are regularly distributed (*arrows*) on the longitudinal and septum wall of the fungus after immunogold reaction with the anti-Tbsp1 antibody specific for a truffle phospholipase. *IM* Interfacial material, *PL* plasma membrane of the host cell, *S* septum, *V* vacuole, *W* Woronin bodies. *Bar* 0.6 μm (Modified from Selosse et al. (2004), reproduced with permission of the publisher)

fungi, both free-living and *in planta*, may reveal fungal genes that are upregulated in the symbiosis (Martin et al. 2008).

The use of **electron microscopy** to investigate fungal symbionts in orchid mycorrhizas had somewhat of a rebirth in the past decade (e.g. Pereira et al. 2003; Selosse et al. 2004; Suarez et al. 2008; Martos et al. 2009; Kottke et al. 2010; Schatz et al. 2010; Martos et al. 2012). First, features of the fungal cell wall as well as septal structure, e.g. dolipore and parenthesomes, allow a distinction of the three major mycorrhizal taxa encompassed under the name 'rhizoctonia' (see Sect. III.A; Moore 1987). Moreover, it has been used to confirm how some unexpected taxa do form pelotons and thus are mycorrhizal. Kottke et al. (2010) has given support to molecular data suggesting that Atractiellomycetes, members of the rust lineage (Pucciniomycotina), are mycorrhizal in some neotropical orchids. Selosse et al. (2004) corroborated molecular identification of ascomyceteous *Tuber* spp. as the main mycorrhizal partners in *Epipactis microphylla* by using transmission electron microscopy to check for the presence of Woronin bodies in pelotons and immunogold reactions using antibodies specifically raised against a truffle phospholipase A2 (Fig. 12.2) – interestingly, in this study, basidiomycetes that were found by molecular means were never seen by microscopy. Immunolabelling transmission electron microscopy has been used to demonstrate pectin deposition in the interfacial matrix around *Ceratobasidium* hyphae, but not *Russula* hyphae, in adjacent mycorrhizal root cells of *Limodorum abortivum*, highlighting an orchid's exquisite capability to react distinctly to different fungal symbionts (Paduano et al. 2011). Finally, Huynh et al. (2004) used scanning electron microscopy imaging of stems and protocorms to determine the most effective fungal isolates for conservation of the threatened *Caladenia formosa*.

Other valuable new approaches include: (1) an orchid root peloton isolation and culturing method that maximizes the number of mycorrhizal fungi obtained but minimizes contamination from non-mycorrhizal fungi and bacteria (Zhu et al. 2008) and (2) a modification of the seed packet burial technique originally conceived by Rasmussen and Whigham (1993) which involves removal of site soil and monitoring of symbiotic seed germination under laboratory conditions (Brundrett et al. 2003). (3) Another method of orchid mycorrhizal fungal identification was proposed by Kristiansen et al. (2001), that is, PCR amplification from single pelotons. Now that large-scale environmental detection of fungi is possible through such approaches as t-RFLP (Dickie and FitzJohn 2007), DGGE (Bougoure and Cairney 2005), pyrosequencing (Dumbrell et al. 2011) and DNA microarrays (Lievens et al. 2010), it will be intriguing to see how populations of orchid mycobiota change with time, orchid life stage and environmental conditions.

III. The Diversity of Orchid: Fungus Associations

A. The Diversity of the 'Rhizoctonias'

For many years orchids were considered to interact largely, if not only, with members of the '**rhizoctonia**' complex. This assemblage contains three now taxonomically disparate Agaricomycetes (=Hymenomycetes) taxa: **Sebacinales**, **Ceratobasidiaceae** and **Tulasnellaceae** (Table 12.1). None of them actually fit the exact definition of the asexual genus *Rhizoctonia* by De Candolle (1815), i.e. the absence of sporulation and formation of sclerotia, so that the name 'rhizoctonia' will be used here not in a taxonomic way, but only to conveniently encompass the three above-mentioned taxa, which are common orchid partners. 'Rhizoctonias' have also been divided into two asexual genera, namely *Ceratorhiza* and *Epulorhiza* (Table 12.1), but this approach is uncomfortable to non-mycologists and, given the current trend to abandon asexual classification, we recommend no longer using these names.

Recent research has highlighted the diverse ecology of these three 'rhizoctonia' taxa. While some species are known to be parasitic, such as in the Ceratobasidiaceae, or are suspected to be saprotrophic, e.g. due to their cultivability in vitro on organic substrates, this classical view (Smith and Read 2008) is now challenged at least for some species. Sebacinales encompasses two major groups (Weiβ et al. 2011) that both occur as endophytes in the roots of many plant species (Selosse et al. 2009): group B additionally forms mycorrhizae with green orchids and Ericaceae, while group A forms ECM on trees and is also associated with some MH orchids (see Sect. III.B; group A is usually not encompassed in 'rhizoctonias'). In an interesting example of convergent evolution, group B is involved in symbiotic germination of *Pyrola* spp. (Ericaceae), another taxon with dust-seeds and MH germination (Hashimoto et al. 2012). ECM clades may exist within the Tulasnellaceae (Bidartondo et al. 2003) and Ceratobasidiaceae (Yagame et al. 2008, 2012; Collier and Bidartondo 2009), and noteworthy MH orchids were instrumental in establishing ECM abilities in these taxa (see Sect. III.B). However, it is unlikely that ECM 'rhizoctonias' are mycorrhizal in fully autotrophic orchids. We are far from a complete understanding of the diversity of nutritional strategies (out of orchid roots) for the Tulasnellaceae and Ceratobasidiaceae: at least, we suspect that their main ecological niche exists out of orchids roots.

Molecular taxonomic identification of orchid mycobionts has now revealed that the **diversity of orchid associates** is much more complex and that other basidiomycetes and even ascomycetes can be involved in orchid mycorrhizas (Table 12.1). The recent overall picture (discussed by Motomura et al. 2010) is that autotrophic orchids largely associate with 'rhizoctonias' worldwide. However, in tropical regions, Atractiellomycetes (Pucciniomycotina) may be common mycorrhizal partners of some epiphytic and terrestrial autotrophic orchids, as shown in the neotropics (Kottke et al. 2010) and in the paleotropics (Martos et al. 2012). The study of South African Diseae (*Pterygodium* and *Corycium* spp.) revealed ECM Ascomycetes such as *Tricharina* and *Peziza* (Waterman et al. 2011), although no direct visualization was obtained. One may expect this list of mycobionts to enlarge in the future. Nevertheless, the study of the earliest-diverging orchid lineages and distribution of fungal associates across orchid phylogeny support that the ancestral state is an association to the three 'rhizoctonia' lineages (Yukawa et al. 2009). Interestingly, Tulasnellaceae turn out to be the most frequently found 'rhizoctonias', in both temperate and tropical regions (Rasmussen 1995; Yuan et al. 2010): in a survey of 77 orchid species from La Réunion island (Indian Ocean), Martos et al. (2012) found them in 88 % of the investigated species (versus 42 % for Sebacinales and 18 % for the Ceratobasidiaceae). By contrast, mixotrophic or fully MH orchids revealed associations with more diverse fungal lineages.

Table 12.1. Summary of the fungal genera forming orchid mycorrhizas. Examples of studies which have identified mycorrhizal genera are given. Taxa in bold are the three groups usually named 'rhizoctonias' in the orchid literature (see text; including the asexual genera *Ceratorhiza*[b] and *Epulorhiza*[a])

Phylum Basidiomycota
- Sub phylum Pucciniomycotina
  - Class Atractiellomycetes (e.g. Kottke et al. 2010)
- Sub phylum Agaricomycotina
  - Class Agaricomycetes
    - Order Agaricales
      - *Armillaria* (e.g. Kikuchi et al. 2008)
      - *Campanella* (e.g. Dearnaley and Bougoure 2010)
      - *Coprinus* (e.g. Yagame et al. 2007)
      - *Gymnopus* (e.g. Dearnaley 2006)
      - *Hymenogaster* (e.g. Julou et al. 2005)
      - *Inocybe* (e.g. Roy et al. 2009b)
      - *Marasmius* (e.g. Burgeff 1959)
      - *Mycena* (e.g. Ogura-Tsujita et al. 2009)
      - *Psathyrella* (e.g. Yamato et al. 2005)
    - *Order Cantharellales*
      - ***Tulasnella***[a] (e.g. Jacquemyn et al. 2010)
      - *Clavulina* (e.g. Selosse, unpublished data)
      - *Ceratobasidium*[b] (e.g. Otero et al. 2002)
      - *Thanatephorus*[b] (e.g. Warcup 1991)
    - *Order Russulales*
      - *Gymnomyces* (e.g. Dearnaley and Le Brocque 2006)
      - *Russula* (e.g. Taylor et al. 2004)
    - *Order Hymenochaetales*
      - *Erythromyces* (e.g. Umata 1995)
      - *Resinicium* (e.g. Martos et al. 2009)
    - *Order Sebacinales*
      - *Sebacina* group A (e.g. McKendrick et al. 2002)
      - ***Sebacina* group B**[a] (e.g. Bougoure et al. 2005)
    - Order Thelephorales
      - *Thelephora/Tomentella* (e.g. Bidartondo et al. 2004)

Phylum Ascomycota
- *Sub phylum Pezizomycotina*
  - Class Pezizomycetes
    - Order Pezizales
      - *Tuber* (e.g. Selosse et al. 2004)
      - *Tricharina* (e.g. Waterman et al. 2011)
      - *Peziza* (e.g. Waterman et al. 2011)

[a]The unrelated sexual genera *Tulasnella* and *Sebacina* encompass species from the asexual genus *Epulorhiza*.
[b]The sexual genera *Ceratobasidium* and *Thanatephorus* encompass species from the asexual genus *Ceratorhiza*.

B. Fully Mycoheterotrophic Orchids and Ectomycorrhizal Fungi

MH orchids are achlorophyllous and receive all their carbon from their mycorrhizal fungi. In this way, they are paedomorphic, i.e. preserving a juvenile trait (heterotrophy, that is a feature of protocorms **only** in other orchid species) during the adult stage. Since the key studies of *Corallorhiza* and *Cephalanthera* species by Taylor and Bruns (1997) and McKendrick et al. (2000), a large number of works indicate that many other fully MH orchids receive carbon from the ECM associations of autotrophic plants in temperate regions (e.g. Selosse et al. 2002; Taylor et al. 2004; Dearnaley and Le Brocque 2006; Roy et al. 2009a) and in some tropical forests (Roy et al. 2009b). Two studies (Taylor and Bruns 1997; Selosse et al. 2002) provided evidence that the same fungal individual was

present on MH orchids and surrounding ectomycorrhizal tree roots in situ: although this relied on the polymorphism of a single genetic marker (nuclear ribosomal DNA), it supports that **hyphal connection** can transfer sufficient carbon from surrounding trees to the MH plants to support their growth, as was more recently supported by ex situ resynthesis experiments (Bougoure et al. 2010). While the association is always specific in temperate regions, a recent study showed that, at least in some tropical areas, some *Aphyllorchis* MH species harboured several different ECM fungi in their roots (Roy et al. 2009b).

In most cases, the Russulaceae, Sebacinales and Thelephoraceae are the most frequently involved taxa (Kennedy et al. 2011); Clavulinaceae may also occur in some *Gastrodia* species (M.-A. Selosse, unpublished data) – interestingly, they also belong to the most frequent taxa in ECM communities (Tedersoo and Nara 2010). For the less specific tropical orchids, lack of specificity may ensure the finding of suitable partners at most sites, and one can speculate that associating to less frequent partners may be evolutionarily risky. Nevertheless, some orchids do associate with rarer taxa, such as *Inocybe* spp. (Roy et al. 2009a; Liebel and Gebauer 2011) or the ECM Ceratobasidiaceae (Yagame et al. 2008, 2012; Bougoure et al. 2009, 2010): the latter are so rare in ECM communities (Collier and Bidartondo 2009) that MH orchids were instrumental in confirming their ECM status (Bougoure et al. 2010; see also Yagame et al. 2012). The common features of ECM fungi supporting MH orchids are unclear, as they are dissimilar in phylogenetic position, ecological preferences and mycelial morphology (shape of mycorrhiza and soil exploration type; R. Agerer, personal communication).

C. Fully Mycoheterotrophic Orchids and Saprotrophic Agaricomycetes

Shifts of fungal partners from non-ECM 'rhizoctonia' to various ECM fungi during MH orchid evolution are considered to give orchids a more continuous carbon supply than that provided by the putatively saprotrophic 'rhizoctonias' (Taylor and Bruns 1997). However, some tropical MH orchids live in forests that are devoid of ECM fungal communities (Smith and Read 2008). Other investigations using molecular fungal identification and stable isotope analyses have now shown **associations to non-'rhizoctonia' saprotrophic partners**.

In the fully MH orchid genus *Gastrodia* the main mycobionts are related to *Marasmius* (Martos et al. 2009; Dearnaley and Bougoure 2010), *Mycena* (Martos et al. 2009; Ogura-Tsujita et al. 2009), *Resinicium* (Martos et al. 2009), or *Armillaria* (Kikuchi et al. 2008), depending on the species. Wood-decaying *Erythromyces* occur in *Galeola* species (Umata 1995) and litter-decaying *Mycena* in *Wullschlaegelia aphylla* (Martos et al. 2009). In both *Epipogium roseum* and *Eulophia zollingeri*, the mycobionts involved are saprotrophic Coprinaceae (Yamato et al. 2005; Yagame et al. 2007; Ogura-Tsujita and Yukawa 2008a). There are also a number of pre-molecular, morphological studies identifying diverse saprobic fungal taxa in fully MH orchids (including *Lycoperdon*; for a review, see Ogura-Tsujita and Yukawa (2008a) that need to be revisited by modern molecular tools.

Although direct data (e.g. isotope tracer studies) that tropical orchids receive carbon from decomposing plant matter via a hyphal conduit is still lacking (Selosse et al. 2010), fungal rhizomorphs linking dead organic matter to the orchid mycorrhizal roots can sometimes be visualized (Kusano 1911; Martos et al. 2009; Fig. 12.3). Moreover, the stable isotope abundance signatures of these orchids are distinctive: they often have slightly higher ^{13}C abundance but substantially lower ^{15}N than ECM-associating plants (Ogura-Tsujita et al. 2009), reflecting the higher ^{13}C and lower ^{15}N abundance of saprotrophic fungi as compared with ECM fungi (Hobbie et al. 2001).

Typically fungal colonization is sparse in fully MH orchids that rely on saprotrophic fungi than on ECM fungi (Dearnaley 2006; Dearnaley and Bougoure 2010), or even not continuous over the year for *Wullschlaegelia aphylla* (Martos et al. 2009), suggesting that the mechanism of obtaining carbon is possibly more efficient than with ECM fungi, but this requires further study. Additionally, hyphae colonize some dead cortical root cells in *Wullschlaegelia aphylla* (Martos et al. 2009), while a complicated pattern of colonization exists in *Gastrodia* roots, with passage cells where hyphae enter the root, in host cells that are permanently colonized and in digestion cells where a carbon flux may occur

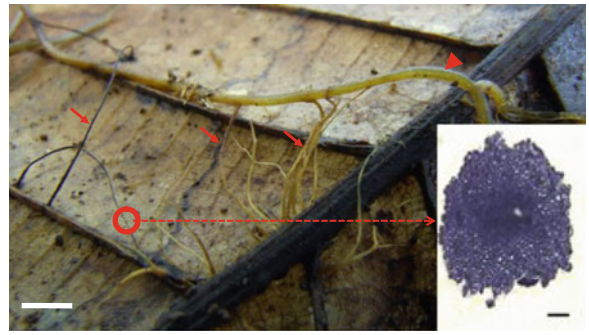

Fig. 12.3. Fungal rhizomorphs (*arrows*) of *Mycena* linking dead leaves to mycorrhizal roots (*arrowhead*) of the fully MH orchid *Wullschlaegelia aphylla*, in which the fungus is mycorrhizal (*bar* 1 cm). *Inset* Transverse section of a rhizomorph, with a central hole, and hyphae with thicker, melanized walls at the external border (*bar* 100 μm). F. Martos, unpublished micrograph

(Kusano 1911; Wang et al. 1997). Although their raison d'être remains unclear, these patterns may be evolutionarily derived, emphasizing the secondary evolution of this kind of mycoheterotrophy. Martos et al. (2009) and Selosse et al. (2010) speculated that the shift of fungal partners to various saprotrophic fungi during MH orchid evolution might have occurred in tropical and wet temperate regions, because these environmental conditions stimulate decomposing activity by fungi and might allow higher carbon gain for the plant. Indeed the need to support the large carbon requirement of plants may explain why non-ECM 'rhizoctonias' are rarely found in MH orchids, although they support MH germination in many orchid species: they may simply be too C-limited to fulfil the plant's needs beyond the protocorm stage.

D. Mixotrophs: Green Orchids that Obtain Carbon from Fungi

Stable isotope investigation of an increasingly large number of green orchids, e.g. in the genera *Cephalanthera*, *Epipactis* or *Cymbidium*, has revealed natural abundances of ^{13}C and ^{15}N higher than surrounding autotrophic plants but less than that of fully MH orchids (Gebauer and Meyer 2003; Julou et al. 2005; Abadie et al. 2006). Such intermediate values suggest that these orchids obtain part of their carbon via photosynthesis and part through their mycorrhizal fungi – that is, these plants are mixotrophic (Julou et al. 2005). **Mixotrophy** is thought to be an intermediate step in the evolution of full mycoheterotrophy (Bidartondo et al. 2004; Selosse et al. 2004; Abadie et al. 2006; Motomura et al. 2010). Identifying photosynthesis inefficiency in many chlorophyll-containing orchids may also uncover cryptic mixotrophic orchids (e.g. Girlanda et al. 2006).

These orchids are rarely specific in their mycorrhizal associations and associate with several ECM fungi, with few exceptions, such as *Platanthera minor* that is specific to an ECM *Ceratobasidium* (Yagame et al. 2012). *Epipactis* spp. associate with truffles and related ECM Pezizales, as one of the rare Ascomycete-associated orchid clades known so far (Fig. 12.2; Selosse et al. 2004; Bidartondo and Read 2008; Ogura-Tsujita and Yukawa 2008b; Shefferson et al. 2008). *Tuber* spp. also occur as rare mycobionts in the closely related mixotrophic *Limodorum abortivum* (Girlanda et al. 2006). Mycorrhizal associations with terrestrial orchid species were documented for 13 *Tuber* species that belong to five of the nine main *Tuber* clades (the Excavatum, Aestivum, Rufum, Maculatum and Puberulum clades; Bonito et al. 2010). In a thought-provoking paper based on field data collection in Hungary, Ouanphanivanh et al. (2008) showed that *Epi-*

pactis spp. co-occurred more often than at random with truffle stands (a similar situation was shown for *Cephalanthera* and *Hymenogaster*) and could indicate truffle habitats. A noteworthy feature is the presence of some 'rhizoctonias' in mixotrophic orchids, together with the dominant ECM fungi (Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006; Motomura et al. 2010; Paduano et al. 2011). This feature also supports that these orchids are an intermediate step in the evolution of full mycoheterotrophy, deriving from autotrophic orchid ancestors associated with 'rhizoctonias'.

Some ECM fungi are found from time to time in autotrophic orchids where 'rhizoctonia' are dominant: Russulaceae occur in some *Cypripedium* spp. (Shefferson et al. 2007) and *Pterostylis nutans* (Irwin et al. 2007), *Thelephora* and *Cortinarius* in *Orchis* spp. (Lievens et al. 2010) and putatively ECM Pezizomycetes and Helotiales in *Gymnadenia conopsea* (Stark et al. 2009). ECM Ascomycetes (Waterman et al. 2011) even dominate in some South African Diseae (*Pterygodium* and *Corycium* spp.). Although the exact interaction with the orchid is unknown, the detection of these fungi is highly unexpected as they are not usually contaminants of soil samples. We speculate that their presence in root tissues may give opportunity for their evolution into mycorrhizal partners, accompanying the emergence of mixotrophy. The same may apply for evolution of mycoheterotrophy based on saprotrophic fungi: *Mycena*-related fungi, that are sometimes associated with MH orchids as mentioned above (Sect. III.C), can sometime be found in roots of some green, 'rhizoctonia'-associated orchids (Fan et al. 1996; Guo et al. 1997).

E. Epiphytic Orchids and '*Rhizoctonias*'

Although epiphytic species represent the largest number of orchids worldwide (Jones 2006) they are surprisingly less well studied with regards to their mycorrhizal associations. Mycorrhizal fungi sparsely colonize **roots of epiphytic orchids** in comparison with terrestrial orchids (Boddington and Dearnaley 2008; Smith and Read 2008; Graham and Dearnaley 2012; Martos et al. 2012) but molecular identification of the mycobionts present reveals them as the typical 'rhizoctonias' of green orchids. This has included members of the Ceratobasidiaceae (Otero et al. 2002, 2004, 2005, 2007; Pereira et al. 2005; Gowland et al. 2007; Graham and Dearnaley 2012; Martos et al. 2012), the Tulasnellaceae (Pereira et al. 2003; Suarez et al. 2006; Kottke et al. 2008; Martos et al. 2012) and the Sebacinales (Suarez et al. 2008; Martos et al. 2012).

In the largest survey of orchid mycorrhizas conducted from 77 orchid species from La Réunion island, Martos et al. (2012) found that communities of 'rhizoctonias' significantly differed between epiphytic and terrestrial orchid communities in terms of OTUs, whereas the three 'rhizoctonia' taxa did not differ in frequency. This may reflect that different fungal species are available in soil and on tree bark, but we lack information about the diversity and ecology of 'rhizoctonias' in soil **versus** bark environments.

The dependency of epiphytic orchids on mycorrhizal fungi throughout the life cycle is not surprising as the mycobionts, with their increased surface area, may improve access to water and minerals for plants which can be especially limiting in the epiphytic state (Zotz and Schmidt 2006; Osorio-Gil et al. 2008). The habitat of many epiphytic species that live in the shade of dense forest canopy is typified by low irradiance and it is possible that species will be soon identified as mixotrophic with a dependence on external supplied carbon as well as photosynthesis.

IV. Nutrient Exchanges Between Orchid and Mycobiont

The minute seeds of orchids lack food reserves and colonization by a suitable fungus is necessary for further development under natural conditions (Smith and Read 2008). Both organic and inorganic nutrients have been shown to be transferred from mycorrhizal fungus to protocorms. Experiments using **split-plate systems** and labelled glucose accessible only to the fungal partner have demonstrated carbon flow to orchid protocorms (Purves and Hadley 1975; Alexander and Hadley 1985). A similar split-plate system indicated that phosphate (labelled by ^{32}P) is passed from fungus to protocorms of *Dactylorhiza purpurella* (Smith 1966).

Adult mycorrhizal orchids continue to receive both organic and inorganic nutrients

from their fungal partners. When the *Ceratobasidium* partner of *Goodyera repens* is supplied with ^{14}C-labelled glycine in the substrate, labelled carbon is passed to the orchid seedlings (Cameron et al. 2006). The addition of ^{13}C/^{15}N-labelled glycine to the fungal compartment also demonstrated a transfer of nitrogen to orchid seedlings. Cameron et al. (2007) have shown that adult *Goodyera repens* also receive phosphate from their fungal partner under experimental conditions. Mycorrhizal fungi may be key to ensuring optimal water uptake from the environment: mycorrhizal *Plantanthera integrilabia* and *Epidendrum conopseum* both had higher water content than uncolonized controls (Yoder et al. 2000).

Orchid mycorrhizas have often been considered to be atypical mycorrhizal associations, with the fungus deriving little benefit from the orchid host (Dearnaley 2007; Smith and Read 2008). Key to this assumption was research conducted by Hadley and Purves (1974) and Alexander and Hadley (1985) on mycorrhizal *Goodyera repens*. In their experiments, the orchid was exposed to $^{14}CO_2$ and no subsequent passage of labelled carbon to the fungal partner was detected. These experiments were more recently repeated by Cameron et al. (2006, 2008) using more naturally equivalent conditions (e.g. moderate temperature, lighting, humidity) with contrasting results. In the latter experiments approximately 0.4–3.0 % of the carbon label originally provided to the orchid was passed to the fungal partner (Cameron et al. 2006, 2008). Adult orchid mycorrhizas thus potentially represent a truly mutualistic interaction similar to ECM and AM associations.

Rasmussen and Rasmussen (2007) and Hynson et al. (2009) rightly note that adult orchid mycorrhizal systems other than *Goodyera repens* should be investigated similarly, as well as under field conditions. The importance of the carbon acquired from the orchids in the whole nutritional budget of the fungus has also been questioned (Rasmussen and Rasmussen 2009). However, using a clever experimental design where the carbon source have different ^{13}C abundances, Latalova and Balaz (2010) showed that a *Tulasnella* species received 70 % of its carbon from its host, *Serapias strictiflora* and 30 % from dead maize roots added to the system, but these experiments were again carried out in vitro.

Most importantly, from an evolutionary point of view, the reciprocation in a mutualism cannot only be quantified in terms of nutrient flow, but should result in a **fitness improvement**. Fitness is notoriously difficult to measure in fungi (Pringle and Taylor 2002), and there is currently no evidence that fungi reproduce better with the orchid than without.

Intriguing indirect evidence for mutualism arose recently from the analysis of the **architecture of interaction networks** between autotrophic orchids and their 'rhizoctonias'. These may vary according to the nature of the interaction, especially when comparing mutualistic and trophic interactions (Thébault and Fontaine 2010). Recent analyses (Jacquemyn et al. 2010 and unpublished data) concluded that orchid mycorrhizal interaction networks displayed a significantly nested structure, i.e. specialized (fungus-specific) orchid species tended to associate with 'rhizoctonia' species that themselves associated with more generalized (not fungus-specific) orchid species, and vice versa. Conversely, Martos et al. (2012) found that orchid–fungus networks displayed a highly modular structure in a tropical context, which could be interpreted as an ecological divergence between epiphytic and terrestrial guilds of plant and fungal partners in tropical communities, although the authors also confirmed the presence of some level of nestedness in the epiphytic and terrestrial sub-networks (Fig. 12.4). The trend of nestedness is a specific feature of mutualistic networks, as opposed to parasitic or trophic networks that are more compartmentalized, and is viewed as a consequence of the reciprocation process itself (Thébault and Fontaine 2010). In other words, this may be an indirect indication that some reciprocation occurs with most 'rhizoctonias'; thus, the investment in protocorm development may be viewed as a transient cost to develop a host that will be beneficial for the fungus later (Leake et al. 2008).

Should we conclude that, conversely, mixotrophic and MH orchids are fungal parasites? This is the tacit idea when calling these plants epi-parasites, or cheaters on ECM symbioses (Merckx et al. 2009), but we still lack rigorous

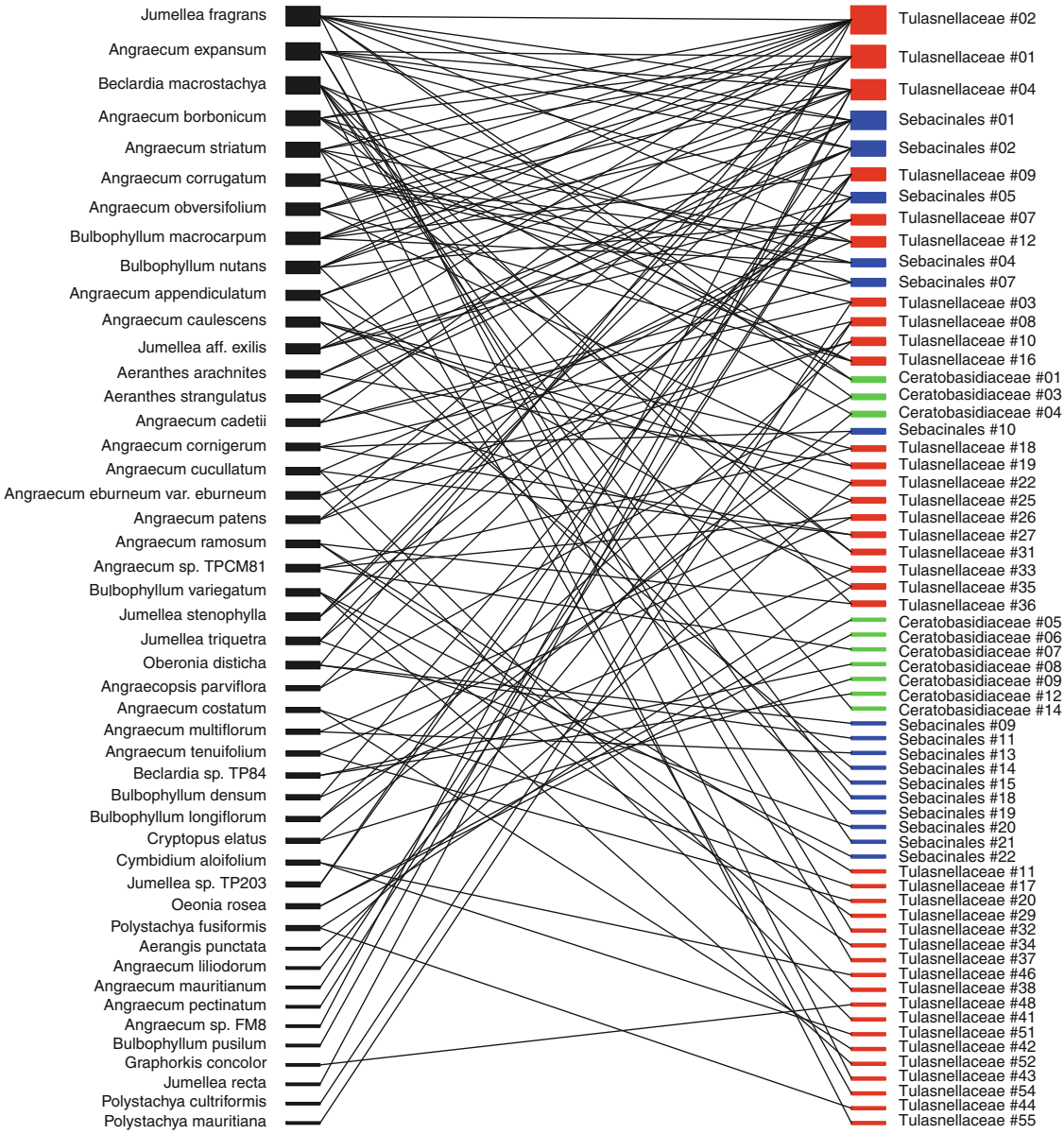

Fig. 12.4. Nested architecture of an orchid–rhizoctonia network as formed by epiphytic orchids on La Réunion island (from Martos et al. 2012). The *left* column shows orchid species with lines linking to various 'rhizoctonia' taxa (*right* column). A nested structure (i.e. less compartmentalized) is a specific feature of mutualistic networks

evidence for this. Indeed, some vitamins, or some protection at some time of the year may enhance fungal fitness. Obviously, we need more studies on the fungal side before any conclusion can be made. Hopefully, the development of models tractable in vitro for autotrophic (Cameron et al. 2006, 2008) and MH orchids (Yagame et al. 2007; Bougoure et al. 2010) will help in investigating these questions.

V. Fungal Specificity in Orchids

A. Patterns and Evolutionary Significance

Fungal specificity is the association of an orchid species with a small number of fungal partners (Irwin et al. 2007), and can be quantified as the phylogenetic breadth (= antiquity of the last common ancestor) of its range of associates (Thompson 1994; Shefferson et al. 2010). This can take the form of narrow specificity whereby an orchid associates exclusively with a single mycobiont across its range, such as the rare underground orchid *Rhizanthella gardneri* (Bougoure et al. 2009). Typically, **specificity** is expressed as an orchid species associating with a limited number of related fungal taxa, e.g. *Corallorhiza maculata* associating with Russulaceae species in the western United States (Taylor et al. 2004), or *Pterostylis nutans* associating with two *Ceratobasidium* species in eastern Australia (Irwin et al. 2007). Both fully MH and autotrophic species can display fungal specificity (e.g. McCormick et al. 2004; Yamato et al. 2005) although the phenomenon is more common to the former orchid physiological type. A small number of the investigated orchid species display little fungal specificity. For example, the widespread Australian grassland species *Microtis intermedia* associates with members of both the Sebacinales and the Ceratobasidiaceae (Bonnardeaux et al. 2007), while two fully MH *Aphyllorchis* species from Thailand associate with an array of unrelated ECM fungi, including members of the Thelephoraceae, Russulaceae and Sebacinales (Roy et al. 2009b).

The **evolution of fungal specificity** has been recently evaluated by mapping the phylogenetic breadth of mycorrhizal partners across orchid phylogenies. Shefferson et al. (2007, 2010) analysed fungal specificity across two orchid phylogenies of the genera *Cypripedium* and *Goodyera* and found that both widening and broadening depended on orchid clades, so that the level of fungal specificity was concluded to be an evolvable trait subjected to reversion in orchids. Considering the evolution of fungal partners across orchid diversification, Waterman et al. (2011) showed that fungal partners are conserved between closely related species of South African Coryciinae.

Martos et al. (2012) used both orchid and fungal phylogenies to assess phylogenetic signal in the interaction network of tropical angraecoid orchids on the island of La Réunion and found a stronger signal on the orchid side than on the fungal side: fungal partners that belong to the Tulasnellaceae, Sebacinales and Ceratobasidiaceae are statistically more conserved between closely related angraecoids than orchid partners of closely related fungi are. Such an asymmetry of phylogenetic signal may reveal different constraints for the partners in orchid mycorrhiza, especially the lower dependence of fungal partners on the symbiosis.

Sudden **partner shifts** have also occurred during the evolution of the MH genus *Hexalectris* (Kennedy et al. 2011), or in the genus *Epipogium*, where *E. aphyllum* associates with ECM *Inocybe* spp. (Roy et al. 2009b) while *E. roseum* associates with saprotrophic *Psathyrella*-related partners (Yamato et al. 2005). Partner shift can thus rapidly evolve, although how the shift occurs remains unclear. As mentioned below (Sect. V.B), the observation that some unexpected fungi are sometimes detected in roots, in addition to the major mycorrhizal fungi, may be relevant as a starting point in the transition – this led to the suggestion that 'molecular scraps' (unexpected fungi considered as contaminant or marginal in the mycobiont spectrum) obtained in symbiont typing should always be reported (Selosse et al. 2010). A particularly interesting stage for the transition may be the germination step: in some orchid species at least, the fungi enhancing the first stage of germination are more diverse than the fungi allowing further development (Vujanovic et al. 2000; Bidartondo and Read 2008). From this situation, where early embryos contact diverse fungi, a mutant for specificity may survive.

B. Adaptive Significance

The adaptive significance of fungal specificity in orchid mycorrhizas is a source of some conjecture. Specific fungal partners may lead to

enhanced seed germination rates (Otero et al. 2004; Bonnardeaux et al. 2007) and thus increased fitness. The **efficiency of nutrient exchange** between partners may be heightened with specific plant–fungus combinations (Bonnardeaux et al. 2007) and this may be critical for carbon uptake for mixotrophic and fully MH orchids in low-light habitats where there is a higher dependency on fungal carbon (e.g. Girlanda et al. 2006). More efficient nutrient exchange as a driver for fungal specificity in orchids can be suggested by examples of partner switching in adult orchids. For example *Goodyera pubescens* switched from one *Tulasnella* species to another when plants were drought-stressed (McCormick et al. 2006). Several studies have suggested that autotrophic orchids associate with different clades of 'rhizoctonias' depending on the environment, e.g. when comparing terrestrial and epiphytic orchid communities in tropical forests (Martos et al. 2012), or European terrestrial orchids in dry and wet habitats (where different Tulasnellaceae subclades dominate; Illyés et al. 2009). However, it remains unknown whether this results from choosing optimal fungal partners or simply from different availability of fungal taxa.

C. The Impact of Mycorrhizal Specificity on Orchid Speciation

Fungal specificity was recently linked to speciation in the Orchidaceae by a number of authors (Otero and Flanagan 2006; Shefferson et al. 2007; Waterman and Bidartondo 2008; Waterman et al. 2011). Distribution of fungi in soils is highly heterogeneous (Richard et al. 2004; Pickles et al. 2010) and this, combined with narrow fungal specificity, may determine the small, over-dispersed populations of many orchid species (Otero and Flanagan 2006). The resulting **patchiness of orchid distribution** may limit gene flow between isolated populations and the number of reproducing individuals, leading in turn to genetic drift and allopatric speciation (Tremblay et al. 2005; Waterman and Bidartondo 2008). Support for this process comes from the observation that different populations of the *Hexalectris spicata* complex display distinct mycorrhizal fungi (Taylor et al. 2003). Natural selection may also act on small, isolated populations of orchids, as highlighted in the study of Otero et al. (2005) that showed varying levels of germination rates (or fitness) after reproducing in vitro associations between *Tolumnia variegata* and different 'rhizoctonia' fungi.

In contrast, Roche et al. (2010) showed that multiple species of *Chiloglottis* associated with a narrow group of Tulasnellaceae fungi across eastern Australia. The fact that each of these species associates with a distinct wasp pollinator suggests that pollination systems and not fungal specificity is driving speciation in the orchid genus. A similar interpretation was made by Waterman et al. (2011) when studying shifts of pollination modes and mycorrhizal partners across the phylogeny of South African Coryciinae orchids. Roche et al. (2010) suggested that a common mycorrhizal fungus in *Chiloglottis* spp. has enabled rapid pollination-mediated speciation via co-occurrence of multiple potential species types.

Mycorrhizal associations during species hybridization, a potential source of speciation in the Orchidaceae, have been examined by some researchers. In crosses between *Caladenia* spp., Hollick et al. (2005) showed that hybrids have genetically similar fungi to one of the two parents. The hybrid formed between crosses of *Orchis simia* and *Orchis anthropophora* also had similar Tulasnellaceae fungi to its parents (Schatz et al. 2010). Interestingly, hybrid *Orchis* plants had higher levels of mycorrhizal colonization than the parents but this was possibly related to the inability to attract pollinators and to produce seeds, therefore providing more carbon for the colonizing fungus. Jacquemyn et al. (2010) also investigated the mycorrhizal associations of *Orchis* hybrids and concluded from common mycobionts in protocorms and adults that mycorrhizal fungi play a small role in reproductive isolation. One generalizing speculation that can be derived from these studies is that mycorrhizal symbiosis acts in a permissive way, i.e. that, for successful hybridization to occur, the parent's fungi need to be related or identical.

VI. Orchid Mycorrhizas and Plant Conservation

A dependence on narrowly specific interactions with fungi and pollinators may predispose many orchids to become rare (Bonnardeaux

et al. 2007; Dearnaley 2007; Swarts et al. 2010). However, Phillips et al. (2011) have recently shown that fungal specificity has not led to rarity in West Australian *Drakaea* spp. as the associated *Tulasnella* fungus is widely distributed in the environment. Nevertheless, as humankind continues to have **negative impacts on natural ecosystems** through such perturbations as vegetation clearing, altered fire regimes, weed and feral animal introduction and climate change, populations of many rare orchid taxa are further declining (Brundrett 2007). Conservation approaches for such orchids include on site protection of existing populations, ex situ storage of tissues and restoration procedures (Swarts and Dixon 2009). All of these approaches require an understanding of the mycorrhizal biology of the species in question, since fungi are vital for orchid seed germination and adult vegetative life.

A. Orchid Mycorrhizas and On-Site Management

Molecular identification of the mycobionts of many orchid species has given an insight into the ecological position of fungal species. This has highlighted **management procedures** that are needed to protect existing populations. The conservation of fully MH and mixotrophic orchids dependent on ECM associations such as *Hexalectris*, *Epipactis*, *Dipodium* and *Rhizanthella* (Taylor et al. 2003; Selosse et al. 2004; Bougoure and Dearnaley 2005; Bougoure et al. 2010) clearly need maintenance of stands of suitable host trees. Fully MH species such as *Gastrodia*, *Epipogium* and *Erythrorchis*, which are nutritionally dependent on wood-rotting fungi (Yamato et al. 2005; Dearnaley 2006; Martos et al. 2009; Dearnaley and Bougoure 2010), will need the retention of a suitable decomposable substrate. For the majority of (autotrophic) orchids, preservation of the uppermost organic layer of soils is essential, as this location is the key habitat of their 'rhizoctonias' associates (Brundrett et al. 2003). As this layer is particularly susceptible to frequent burning (Brundrett 2007), careful monitoring of fire regimes is a necessary conservation measure.

For all orchids with partial or full mycoheterotrophy, the fungus cannot be separated from its own carbon source and, if such occurs during relocation, both the fungus and the plant may die. This was shown in an overlooked book by Sadovsky (1965) dealing with the cultivation of 'orchids in your own garden': among other studies, Sadovsky trialled the relocation of a number of orchid species at a time where protection laws were more flexible in Europe, and the resulting list showed that mixotrophic and MH species could not be transplanted. Thus, there may be problems saving populations of such orchids by transferral to another site in the case of major disturbance. However, the effective glasshouse relocation of *Rhizanthella slateri* (with its ECM partner and photosynthetic host) threatened by a major road development in eastern Australia (M. Clements, personal communication) provides an exemplar of success.

Regular monitoring for the continued presence of orchid-associated fungi is a necessary management procedure. This can be done simply by seasonal observations of macrofungal fruiting bodies for some associated orchid species. For microfungi and rarely sporulating fungi, such a most 'rhizoctonias' (e.g. clade B Sebacinales that do not fruit; Weiβ et al. 2004), seed baiting procedures carried out both in situ and ex situ, are cost-effective (Brundrett et al. 2003). Molecular detection of orchid-associated fungal DNA using specific or general fungal primers will also ensure that sites continue to harbour the appropriate mycobionts. The best way to preserve orchids is therefore to preserve their fungi and, from there, given the uncertainties on the ecology of fungi, the whole environment. This is indeed good news for mycologists since orchid protection therefore protects fungi – not only those taxa involved in mycorrhizal associations, but the surrounding ones as well.

Some fully MH orchids rely on ECM fungi that have fruiting bodies that are consumed by native mammals (Bidartondo et al. 2004; Selosse et al. 2004; Dearnaley and Le Brocque 2006). In Australia, members of the Russulaceae are consumed by marsupials such as Bettongs and Potoroos (Claridge and May 1994). To ensure continued cycling of fungal propagules through

ecosystems, protection of these spore-dispersing animals should be a long-term priority.

Two recent works suggest that the presence of fungi may not be the sole limiting factor for orchid settlement: in experimental seed sowing at different sites where the focal orchid species does not grow, there is evidence that early development into a protocorm can occur in *Cephalanthera* spp. (Bidartondo and Read 2008) and *Epipactis* spp. (Těšitelov et al. 2012), with successful access to appropriate fungal partners. Although these plants are mixotrophic and may not represent general models, limitations to orchid development may thus be more than fungal – as the authors discuss, a limitation on seed dispersal or abiotic factors may also be involved. Thus, the fungal symbiosis, although crucial to the orchids, may not be seen as the sole factor explaining why orchids develop and why a given site is suitable for orchid growth.

B. Ex Situ Conservation and Orchid Mycorrhizas

Ex situ symbiotic germination of seed is a common approach in conservation procedures for threatened orchids (Batty et al. 2006b; Stewart and Kane 2007; Zettler et al. 2007). Mycorrhizal fungi can be obtained from adult plants in situ or via buried seed germination packets (Batty et al. 2001; Dearnaley et al. 2009). Damage to adult threatened orchids can be minimized by taking a small sliver of colonized stem (Wright et al. 2009; Smith et al. 2010) and the best plants to isolate fungi from are leafing to flowering stages (Huynh et al. 2004). Surface sterilization of isolated orchid tissues reduces the amount of contamination from bacteria and faster-growing ascomycetes (Huynh et al. 2009). Once pelotons are separated from the host tissue, the best 'rhizoctonias' to choose for symbiotic autotrophic orchid seed germination are those with fine loose hyphae and monilioid cells (Huynh et al. 2004). Pure fungal inoculum and surface-sterilized orchid seed are traditionally co-cultured on oatmeal-based agar medium (Clements et al. 1986). The growth of orchids dependent on ECM fungi and photosynthetic hosts requires special culturing set-ups such as that developed by Bougoure et al. (2010) for *Rhizanthella gardneri* (Fig. 12.5). Fully MH orchids reliant on saprotrophic Agaricomycetes can be grown in seed packets with a medium of sawdust and fungal inoculum (Yagame et al. 2007). Ex vitro approaches whereby seed is sown in pot soil inoculated with the appropriate mycorrhizal fungus has an additional advantage in that seedlings may form associations with other micro-organisms present in the medium (Wright et al. 2009). Procedures for maintaining orchid mycorrhizal fungi in the long term include immersing the inoculum in liquid nitrogen (Batty et al. 2001) or via encapsulation of both seed and fungi in alginate beads, with low-temperature storage (Sommerville et al. 2008). It is important that a range of fungal taxa and isolates are preserved in orchid conservation work as multiple fungi might co-exist in plants (Irwin et al. 2007; Wright et al. 2010) or orchids may switch fungi as they mature (Xu and Guo 2000) or even as adults (McCormick et al. 2004; Dearnaley 2006). Furthermore, the fungal isolate that is best at germinating seed does not necessarily ensure the best long-term survival of orchid species (Wright 2007; Huynh et al. 2009).

C. Use of Mycorrhizal Fungi in Restoration Procedures

Symbiotically grown orchid seedlings can be transferred directly to the natural state but plant persistence is enhanced by growth in potting media for several seasons (Swarts 2007). Moving plants from Petri dish to soil can be a significant hurdle (Wright et al. 2009) but an intermediate deflasking procedure involving carefully aerated sand–agar containers has been shown to effectively prepare agar-grown symbiotic seedlings for transfer to soil in pots (Batty et al. 2006a). There appears to be no benefit to inoculating the pot soil with compatible fungi, with the original colonizing fungus proving sufficient for nutrient uptake for the seedlings (Batty et al. 2006a). For establishing orchid populations in the natural state, seedlings and tubers appear to be better than seed sowing, although the latter is more cost- and time-effective (Batty et al. 2006b; Wright et al.

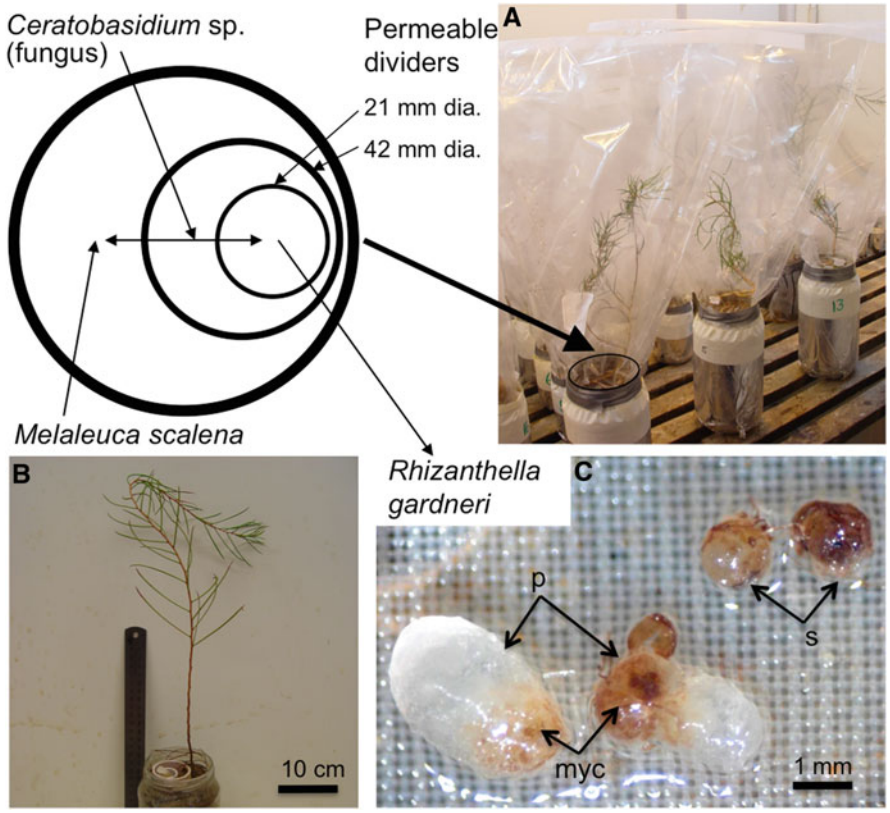

Fig. 12.5. *Rhizanthella gardneri* growth pot arrangement (from Bougoure et al. 2010; used with the permission of author and publisher). The orchid (shown in **C**) is grown in the inner of three pots while the ECM *Ceratobasidium* partner, inoculated into the middle pot, passes photosynthate from the autotrophic *Melaleuca scalena* (outer pot, also seen in **A** and **B**) to the orchid via small holes in each pot

2009). Both in situ (Rasmussen and Whigham 1993) and ex situ (Brundrett et al. 2003) seed baiting can be used to confirm the presence of fungi at introduction sites. Translocation success can be enhanced by a combination of adding fungal inoculum and loosening soil at sites; the latter potentially enhances the activity of the fungi at the location (Smith et al. 2009). Fungal inoculum can be introduced to new sites without orchids and can persist in soils for several seasons in preparation for restoration procedures (Hollick et al. 2007).

VII. Conclusions

Orchid mycorrhizas are predominantly represented by **associations between photosynthetic plants and 'rhizoctonia' fungi**. These associations, which likely represent the plesiomorphic condition for orchids, gave rise through repeated evolutionary shifts to interactions with other diverse fungal lineages and diversification of orchid metabolism. How orchids recruit and allow new fungi (even some 'naïve' fungi from non-mycorrhizal clades) to enter the dual morphogenesis of mycorrhizas remains unclear. However, orchid mycorrhizas are excellent models to reveal the general properties of mycorrhizal systems as well as providing insights into the fungal world via specificity aspects, ecological networks and evolution of the mycorrhizal state.

Although considerable advances have been made in understanding the ecology and evolution of orchid mycorrhizas in recent years, substantial knowledge gaps still exist. In particular, many

aspects of orchid mycorrhizal physiology still require investigation, for example the ubiquity of plant to fungus carbon transfer in green orchids, the metabolism of fungi involved in the process and the expression of genes throughout the symbiosis. Moreover, research is often orchid-focussed, so that a lot of questions remain on the fungal side which is probably less easy to investigate. The exact nutrition, diversity, benefits from the association (if any) and repartition in soil of many mycobionts, such as the Tulasnellaceae, are often ignored and with some exceptions (e.g. Selosse et al. 2002; McCormick et al. 2009), the fungus is rarely investigated out of the orchid roots. It is hoped that these and other areas will continue to contribute to understanding these fascinating mycorrhizal interactions, with more emphasis on the involved fungal taxa.

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