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WHAT DRIVES ORCHIDS TOWARD MYCO-HETEROTROPHY?

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Abstract

Myco-heterotrophy describes the capability of orchids to obtain carbon from fungi. Depending upon varied durations of association with fungi, orchids have been categorized as those that require fungi during germination only as compared to those that are associated with fungi throughout the life span. It has always been the subject of interest to all the scientists to evaluate the mode of nutrition between this mutualistic association for which various modern approaches have been used. There are factors which led to evolution of autotrophy to initial or partial mycoheterotrophy and subsequently, to fully mycoheterotrophy. The present paper presents the overview of mycoheterotrophy, habitat, morphology, reproduction strategies, techniques, threats and conservation of mycoheterotrophy, and major opportunities for the future research.

Introduction

MYCO-HETEROTROPHY IS a potential of a plant to procure carbon from fungi. Leake (1994) coined the term mycoheterotrophy (as *myco-heterotrophy*). To understand mycoheterotrophy, a basic understanding about the mycorrhizal symbiosis is a pre-requisite. The term mycorrhiza is derived from the Greek words, *mykos*= *'fungus'* and *rhiza*= 'root', indicating the mutualistic association of soil fungi with plant roots that benefits each other, mycorrhizal fungi improve the nutrient uptake of the host plants and in return they receive photosynthetically fixed carbon that is essential for growth and reproduction of the fungi. Arbuscular mycorrhizae (AM) involve almost all the members of Glomeromycota and Ectomycorrhizae involve some members of Ascomycota and Basidiomycota. In some cases, saprophytic fungi (SAP) are considered to be the third source of myco-heterotrophy in the plants (Smith and Read, 2008). The mycorrhizae involved in the myco-heterotrophic interactions with Orchidaceae have been termed as orchid mycorrhiza. Two discrete types of orchid mycorrhiza are recognized as tolypophagous and ptyophagous type. The former type is most common, in which hyphae infect the rhizome or root, form pelotons (coil) in cortical cells and are digested. However, the latter one is infrequent in which hyphae that have entered a root, experience lysis at the tips and cell contents are released (Burgeff, 1932).

Orchidaceae is one of the largest families of the flowering plants, with 736 genera distributed throughout the world except polar areas and deserts. Morphologically and functionally, it is considered to be the one of the highly specialized families amongst the monocotyledons (Hajra and De, 2011). The family is divided into five sub-families: Apostasioideae, Vanilloideae, Cypripedioideae, Orchidoideae and Epidendroideae, of which Epidendroideae is the largest subfamily comprising of *ca.* 18,000 spp. and 650 genera (Chase *et al.*, 2015). As far as is known, all orchids depend on a myco-heterotrophic interaction with a symbiotic fungus for a part of their life cycle, especially for germination (Leake, 1994).

There has been a conjecture that orchids rely on symbiotic association with mycorrhiza to obtain complex organic compounds for a part of their life *i.e.*, at the time of germination only, throughout the life cycle due to unavailability of the light and scarcity of nutrients and some plant species show plasticity in trophic strategy in relation to environmental conditions. It has been speculated that the family Orchidaceae includes shifts from autotrophy to initial mycoheterotrophy to partial myco-heterotrophy and from partial mycoheterotrophy to full mycoheterotrophy. Present paper deals with the detailed description about, the strategies adopted by orchids to avail nutrients, habitats and morphology of mycoheterotrophy, tools that will help to determine the nutrient fluxes between fungi and mycoheterotrophic plants, and threats and conservation strategies.

Overview of Myco-heterotrophy

Orchids have evolved in different ways to obtain organic compounds through autotrophy, mycoheterotrophy, and partial mycoheterotrophy. Almost all the plants are autotrophs, convert carbon dioxide into the carbohydrates in the presence of sunlight. Achlorophyllous plants have lost the ability to photosynthesize and rely on symbiotic association

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with fungi to obtain carbon. To understand mycoheterotrophy, the designation of plant species according to their trophic capabilities is needed.

A fully myco-heterotrophic plant exclusively depends on fungal carbon during its life cycle. Thus, orchids that lack visible traces of chlorophyll and do not possess direct relation with autotrophic plants are considered fully mycoheterotrophic plants (Merckx, 2013).

A partial myco-heterotrophic plant combines autotrophy and mycoheterotrophy to procure carbon during at least one stage of its life cycle suggesting thereby that partial MH retain the functional photosynthetic apparatus. Orchids that are green and can survive in the extreme low light conditions are the prime candidates for partial mycoheterotrophy. Gebauer and Meyer (2003) and Preiss *et al*. (2010) reported that the dependency on fungal carbon can greatly differ between partial mycoheterotrophic species and between populations of the same species growing in different illumination conditions (Merckx, 2013; Rasmussen, 1995).

An initially myco-heterotrophic plant is completely dependent on associated fungi for its carbon supply during the early stages of development. Comprehensively, all full myco-heterotrophs are initial myco-heterophs as well, but the term is used for the species that depend on autotrophy or partial myco-heterotrophy, at maturity. Thus, all orchids except fully myco-heterotrophs are initial myco-heterotrophs (Merckx, 2013).

Habitats and Morphology of Myco-heterotrophic Orchids

Habitats

Fully myco-heterotrophic species grow in shaded habitats in closed canopy forests. These habitats are generally characterized by a lack of understory plants, where sufficient sunlight fails to reach till ground level to aid plants to perform photosynthesis. Partial myco-heterotrophs often occur in forest habitats where there is no sufficient sunlight but can also be found in open vegetations, such as bogs and meadows (Girlanda *et al.*, 2011; Matthews *et al.*, 2009). Preiss *et al.,* (2010) demonstrated that light availability is one of the major determinants of the degree of myco-heterotrophy in two partially myco-heterotrophic species of *Cephalanthera*. These observations support a strong correlation between irradiance level and dependency on fungal carbon. Hence, an evolutionary shift from autotrophy to full myco-heterotrophy seems to be accompanied by a switch towards more shaded sites.

Subterranean Morphology

Leake, (1994) observed that in fully myco-heterotrophic plants, root hair are mostly absent, roots are stout and mostly clumped, rhizomes are with a specialized fungal colonization pattern and increase in the width of the root cortex to accommodate mycorrhizal infection and to store carbohydrates and other nutrients, obtained from fungal association.

Shoots

Many myco-heterotrophic orchids have slender and thread like stems, resulting in a hyaline appearance. Vascular tissues are often reduced to a single narrow cylinder of bicollateral bundles or to four or six narrow bundles in the cortex. Most of the species lack secondary thickening and their stems are succulent and brittle (*e.g.*, *Rhizanthella*). Lignification is generally confined to a narrow ring of xylem vessels; phloem is present in very small amounts mainly as parenchyma (Leake, 1994).

Leaves

In fully myco-heterotrophic orchids, the nutrients are obtained solely from fungi, so leaves no longer serve a useful function. Thus, leaves are reduced to widely spaced achlorophyllous scales on the inflorescence axis. Occasionally, leaves are present only on underground rhizomes or tubers or even totally absent. The vascular supply to the leaf scales is mostly reduced to a single trace or may be absent. Stomata are generally absent, but some species retain rudimentary stomata on their leaves and shoots (Leake, 1994).

Seeds

Most species of myco-heterotrophic orchids have extremely small seeds, termed as dust seeds (Arditti and Ghani, 2000). A reduction in seed size and complexity is one of the most significant modifications in mycoheterotrophic orchids. The reduction in seed size is coupled with a reduction of endosperm and a lack of differentiation of the embryo at the maturity, *e.g.*, a single capsule of the mycoheterotrophic orchid *Galeola altissima* contains about 18,000 seeds (Arditti and Ghani, 2000). However, not all orchids produce large number of seeds per capsule, *e.g.*, *Rhizanthella gardneri* produces 20-25 seeds only (George and Cooke, 1981). As seeds are small and reserve less, germination depends on colonization by a mycorrhizal fungus (Eriksson and Kainulainen, 2011).

Reproductive Strategies of Myco-heterotrophs

A very little is known about the reproductive strategies adopted by mycoheterotrophic orchids. Bidartondo (2005) in his review on mycoheterotroph biology hypothesized that mycoheterotrophic plants specialized on fungi, will infrequently be specialized towards pollinators due to the evolutionary instability for specializing on two interactions. Since mycoheterotrophic plants already engage in exclusive specialized symbiotic association with fungi in one aspect of their life history, and it would be an evolutionarily unstable strategy to engage in additional compulsory associations. Hence, mycoheterotrophs are likely to evolve reproductive traits free from highly specialized symbiotic associations, with characteristics including a generalist pollination syndrome, high occurrence of autogamous self- pollination and resource allocation away from metabolically exorbitant reproductive structures, such as large attractive flowers, instead dedicating resources to production of a vast number of seeds. Production of a large number of tiny seeds could be a reproductive strategy aimed at increasing the likelihood of at least a few offsprings locating a suitable host.

Since myco-heterotrophs are found growing under shady forests, it has also been hypothesized that myco-heterotrophs will converge on floral characters to attract pollinators more common in understory forests. This could potentially lead to a myco-heterotroph floral syndrome, consisting of small white flowers, with a scent attractive to fungus gnats, as seen in *Neottia cordata*, and tight synchrony of blooming period among co-occurring population members (Ackerman and Mesler, 1979).

According to Dressler (1981) and Leake (1994), limited carbon supply, patchy distributions, and a restriction to habitats with few pollinators, may lead to a reliance on autogamy among mycoheterotrophs. It has also been shown that variations in abiotic factors may have severe impact upon myco- heterotroph reproduction. Removal of the forest canopy and change of litter com-position can severely affect mycoheterotroph reproductive effort, with observed declines in the reproductive output of mycoheterotrophs possibly corresponding to unfavorable shifts in abiotic factors affecting the composition of mycorrhizal communities and/or the vitality of obligate fungal associates (Luoma, 1987; Moola and Vasseur, 2004).

Techniques to Study Orchid Mycorrhiza

Molecular Approach

Earlier, knowledge about orchid mycorrhiza has been procured from *in vitro* isolation of fungi. This has allowed the identification of basic fungi and conducting *in vitro* seed germination experiments with some root isolated fungi as both can be cultured axenically, at least in the early stages of fully autotrophic orchids (Clements, 1988; Warcup, 1971). However, there has been difficulties in accurately identifying the isolated fungal partner. However, isolation often provides 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).

In the recent years, fungal taxonomy is studied, especially by isolating fungal DNA from host roots and sequencing the nuclear ribosomal DNA (Seiffert, 2009). The fungal partners of orchid mycorrhiza can be more accurately identified directly from orchid protocorms, roots, tubers and rhizomes (Bougoure *et al.,* 2005; Martos *et al.,* 2009; Swarts *et al.,* 2010). PCR amplification of colonized orchid tissues using fungus-specific primers is commonly used (Dearnaley and Bougoure, 2010; Dearnaley and Le Brocque, 2006). Sequencing of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA after PCR amplification using a variety of primer combinations (Gardes and Bruns, 1993; White *et al.,* 1990) is now a popular and reliable method for identifying orchid mycobionts. Jacquemyn *et al.* (2010) and Lievens *et al.* (2010) introduced DNA array technologies for the identification of orchid fungal partners in which 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 swift and efficient handling of numerous samples, especially as compared to the cloning of PCR products.

Stable and Radioactive Isotopes

There is an indirect approach to evaluate the mode of nutrition of an individual orchid is mass spectrometric analysis of natural C and N isotope abundances (Abadie *et al.,* 2006; Bidartondo *et al.,* 2004; Julou *et al.,* 2005; Martos *et al.,* 2009; Ogura-Tsujita *et al.,* 2009; Zimmer *et al.,* 2007). Stable isotopes also provide access to trace elements for which no natural

radioisotopes exist (*e.g.*, nitrogen and oxygen) (Schimel, 1993). Fully mycoheterotrophic orchids have been identified to have ^{13}C signatures similar to those of their mycorrhizal partners (Gebauer and Meyer, 2003; Trudell *et al.,* 2003) whereas similar/higher ^{15}N abundance than their mycorrhizal fungi, suggesting thereby a limited trend to ^{15}N accumulation along the food chain (Trudell *et al.,* 2003). Partial mycoheterotrophs showed stable isotope signatures intermediate between fully mycoheterotroph and autotrophic species (Abadie *et al.,* 2006; Julou *et al.,* 2005). As expected, some fully autotrophic orchids such as *Goodyera* species have even lower amounts of these natural isotopes as autotrophic orchids are least dependent on nutrient acquisition from fungi (Bidartondo *et al.,* 2004; Gebauer and Meyer, 2003).

The movements of isotopically labelled compounds can be traced from fungal partner to orchid mycorrhizas. Although they only provide snapshot views of the metabolism at the time of pulse, they also allow to track the exchanges between symbionts. The first demonstration of movement of ^{14}C labelled photosynthates from tree species to the fully MH orchid *Corallorhiza trifida* via ECM fungi was demonstrated by McKendrick *et al.* (2000). Bougoure *et al.* (2010) provided the flow of ^{13}C-labelled carbon from *Melaleuca scalena* to the fully mycoheterotrophic orchid *Rhizanthella gardneri* via an ECM fungal conduit. *R. gardneri* also obtained nitrogen from its fungal partner, indicated by adding $^{13}C + ^{15}N$-labelled glycine to hyphae and surrounding soil. Labelling experiments by Cameron *et al.* (2006, 2007, 2008) demonstrated that the fully autotrophic orchid *Goodyera repens* obtains carbon, nitrogen, and phosphorous from its fungal partner. Notably, *G. repens* also transfers significant amounts of photosynthates (likely greater than 3 % of its photosynthetic carbon) back to its *Ceratobasidium* mycobiont, which is the first direct demonstration of a net carbon flow from orchid to fungi (Cameron *et al.,* 2006, 2008). Recently, Ercole *et al.* (2015) investigated temporal variations in the mycorrhizal fungi and nitrogen stable isotope natural abundance in adult plants of *Anacamptis morio* (a wintergreen meadow). They observed that irrespective of differences in the seasonal environmental conditions, plant phenological stages, and the associated fungi, the isotopic content in mycorrhizal *A. morio* remains quite constant over time.

Other Approaches

The gene expression studies of ECM and AM associations with mycoheterotrophs have largely been neglected. Watkinson and Welbaum (2003) studied gene expression in the mycorrhizal association of *Cypripedium parviflorum* var. *pubescens* via differential mRNA display. A trehalose phosphate phosphatase was down-regulated in the association, showing changes to orchid carbohydrate transport. Dearnaley (2007) speculated that modern gene expression techniques such as *in situ* hybridization, microarrays and RT-PCR may provide additional understanding of the molecular functioning of orchid mycorrhiza. Specifically, whole genome sequencing and transcript profiling of orchid mycorrhizal fungi, both free-living and in plants, may disclose the fungal genes that are up-regulated in the mutualistic association (Martin *et al.,* 2008).

Study of fungal symbionts in mycorrhiza with the help of electron microscopy provides a fair idea of ultrastructural details (Kottke *et al.,* 2010; Martos *et al.,* 2009, 2012; Pereira *et al.,* 2003; Schatz *et al.,* 2010; Selosse *et al.,* 2004; Suarez *et al.,* 2008). First, characters 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* (Moore, 1987). It has helped to confirm how some unexpected taxa form pelotons and thus are mycorrhizal. Atractiellomycetes, members of the rust lineage (Pucciniomycotina), are mycorrhizal in some neotropical orchids was supported by molecular approaches (Kottke *et al.*, 2010). Selosse *et al.* (2004) verified 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, interestingly, 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 capability to react differently to different fungal partners (Paduano *et al.,* 2011). Eventually, 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 orchid *Caladenia formosa*.

Threats

Myco-heterotrophs prefer regions that have been free from disturbance in recent history (Cheek and Williams, 1999; Taylor and Roberts, 2011). The major threat for the survival of myco-heterotrophic plants is habitat destruction. This is the unavoidable result of the

expansion of human populations and anthropogenic activities. Habitat destruction is the primary cause of the loss of biodiversity in terrestrial ecosystems (Pimm and Raven, 2000). Ecosystems can suffer from anthropogenic activities causing pollution. Pollution that impacts plant and fungal diversity is commonly caused by pesticides, sewage, fertilizers from agricultural fields, industrial chemicals and wastes, emissions from factories and automobiles, and sediment deposits from eroded hillsides (Relyea, 2005). Herbivory may also have a negative impact on reproductive success of orchids (Klooster and Culley, 2009; Taylor and Roberts, 2011). Since, introduction of herbivores into myco-heterotrophic orchid habitats can cause potential harm to local myco-heterotrophs, rare and endangered species of myco-heterotrophs may also suffer from overenthusiastic botanists, who collect materials and trample populations during collection trips (Taylor and Roberts, 2011). The global climate change also has severe impact on the existence of myco-heterotrophs. The emission of greenhouse gases (GHGs) has been constantly increasing over the past 100 years. Scientific evidence says that the increased levels of GHGs produced via anthropogenic activities have already affected the world's climate and ecology and these effects will possibly increase in the future (Primack, 2008) and this may be especially harmful for montane forests and their associated myco-heterotrophs (Foster, 2001; Pounds *et al.,* 1999).

Strategies for Conservation of Myco-heterotrophic Orchids

The best and most straightforward approach to conserve the myco-heterophic plants is to protect the habitats where they grow. Conservation of rare and endangered orchids can be supported by establishment of new populations. Seed germination of fully mycoheterotrophic orchids has been achieved by burying seed packages near ectomycorrhizal trees (Bidartondo, 2005; Bidartondo and Bruns, 2001; McKendrick *et al.,* 2000), showing the possibility of re-introducing myco-heterotrophs into existing suitable habitats. A dependence of myco-heterotrophic orchids on narrowly specific interactions with fungi and pollinators may predispose many orchids to become threatened (Bonnardeaux *et al.,* 2007; Dearnaley, 2007; Swarts *et al.,* 2010). Moreover, the anthropogenic activities such as vegetation clearing, altered fire regimes, herbivores introduction and global climate change have further led to the decline in the populations of many rare orchids (Brundett, 2007) for which suitable measures should be taken to conserve the symbionts. The areas of their natural occurrence should be declared as strictly protected areas and human interventions should be strongly prohibited. For the majority of orchid mycorrhiza, protection of the uppermost organic layer is important, as this location is the key habitat for *Rhizoctonia* associates (Brundrett *et al.*, 2003). Regular monitoring of orchid associated fungi is an essential management procedure. This can be done by seasonal observations of fungal fruiting bodies for some associate orchid species. *Ex situ* conservation by germinating the seeds of threatened mycoheterotrophic orchids is a common approach (Stewart and Kane, 2007; Zettler *et al.,* 2007). *Ex vitro* approach where seed is sown in pot soil inoculated with the appropriate fungal partner has an additional advantage in that seedling may form associations with other microorganism present in the medium (Wright *et al.,* 2009). Batty e*t al.* (2001) reported that by immersing the orchid mycorrhizal fungi inoculum in liquid nitrogen or by encapsulation of both seeds and fungi in alginate beads with low temperature (Sommerville *et al.*, 2008) may assist in conservation strategies.

Conclusion

Myco-heterotrophic orchids do not possess any direct economic importance; they are neither useful for consumption nor for pharmaceutical purposes with only one exception *i.e.*, *Gastrodia elata* used in Chinese traditional medicinal system (Xu and Guo, 2000). However, the presence of mycoheterotrophs in forest ecosystems may offer an indirect economical value through recreational services for mankind. Apart from this value, myco-heterotrophic plants offer a unique model system to study mycorrhizal mutualism and ecological symbioses in general, which is being overlooked. Moreover, considerable advances have been made in understanding the ecology and evolution of orchid mycorrhiza in the recent years, but considerable knowledge gaps still exist which need to be studied.

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