Investigating the ecology of partial and full myco-heterotrophy among Orchidaceae and Ericaceae using the stable isotope natural abundance analysis approach

DISSERTATION

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Zusammenfassung

Zur Gruppe der nicht-photosynthetischen (chlorophylllosen) Pflanzen zählen etwa 4100 Arten, die direkt auf autotrophen Pflanzen parasitieren und mehr als 400 Arten, die organische Nährstoffe von Wurzelpilzen beziehen und daher als mykoheterotroph bezeichnet werden. Etwa ein Viertel aller mykoheterotrophen Arten gehört zur Familie der Orchidaceen. Alle Orchideen sind während ihrer juvenilen, unterirdischen Phase mykoheterotroph; die meisten Arten entwickeln im adulten Stadium jedoch grüne Blätter. Eine ähnliche Situation ist im Tribus Pyroleae (Ericaceae) anzutreffen. Pyroleen sind zumeist photosynthetisch aktive Arten, die, ebenso wie Orchideen, winzige Samen ohne Endosperm produzieren und daher in ihrer frühen Entwicklungsphase auf Nährstoffzufuhr von Pilzpartnern angewiesen sind. Analysen der natürlichen Isotopensignatur einiger grüner und somit scheinbar autotropher Orchideen und Pyroleen zeigten kürzlich, dass diese Pflanzen, zusätzlich zum Kohlenstoffgewinn durch Photosynthese und zur Stickstoffassimilation, organische Kohlenstoff- und Stickstoffverbindungen von Wurzelpilzen beziehen. Pflanzen, die diese Mischform der Ernährung aufweisen, werden als partiell mykoheterotroph bezeichnet.

Die komplexe und faszinierende Ökologie partiell und vollständig mykoheterotropher Pflanzen zu verstehen, ist die Grundlage, um diese zumeist gefährdeten Arten und ihre Habitate erfolgreich schützen zu können. Analysen der natürlichen Isotopenhäufigkeit und die genetische Identifikation von Mykorrhiza-Pilzen sind moderne Techniken, die ohne experimentelle Störung Einblick in pflanzliche Ernährungsweisen unter natürlichen Bedingungen geben können. Basierend auf diesen Methoden, sowie auf der Bestimmung von Chlorophyll-Gehalten, der Messung von Lichtverfügbarkeit und einem *in situ* ^{13}C-Markierungs-Experiment, liefert die vorliegende Arbeit neue, fundamentale Kenntnisse über die Nährstoffaufnahme zahlreicher Orchideen und Ericaceen.

Im ersten Kapitel wird ein verbesserter methodischer Ansatz präsentiert, der Meta-Analysen und eine genauere quantitative Abschätzung der Nährstoffgewinne vom Pilz bei partiell mykoheterotrophen Pflanzen ermöglicht. Unter Anwendung dieser neuen Methode wurde die umstrittene oder zumeist noch völlig unbekannte Ernährungsweise zahlreicher Pflanzenarten untersucht. So konnte unter anderem gezeigt werden, dass innerhalb der Tribus Pyroleae auch eine vollständig mykoheterotrophe Art (*Pyrola aphylla*) vorkommt und dass die auf Ektomykorrhiza-Pilze spezialisierte Orchidee *Corallorhiza trifida* (von der man glaubte, sie sei vollständig vom Pilzpartner abhängig) zur Photosynthese befähigt ist.

Anhand des großen Datensatzes zu Isotopensignaturen und Mykorrhiza-Partnern vieler Orchideen-Arten konnte die Existenz einer bezüglich ihrer Ernährung neuen Gruppe von Orchideen aufgezeigt werden. Daraus lässt sich folgern, dass terrestrische Vertreter der Orchidaceen mindestens vier verschiedene Ernährungsformen aufweisen können: (1) Autotrophie - bei grünen Orchideen, die zumeist mit *Rhizoctonias* vergesellschaftet sind und deren Kohlenstoff-Isotopensignatur sich nicht von derer benachbarter autotropher Pflanzen unterscheidet; (2) partielle Mykoheterotrophie - bei grünen Orchideen, die mit Ektomykorrhiza-Pilzen assoziieren und deren Kohlenstoff-Isotopensignatur zwischen der von autotrophen und vollständig mykoheterotrophen Begleitpflanzen liegt; (3) vollständige Mykoheterotrophie - bei nicht-photosynthetischen Orchideen, die auf Ektomykorrhiza- oder saprotrophe Pilze spezialisiert sind und im ^{13}C ähnlich stark wie ihre Pilzpartner angereichert sind; sowie (4) eine zusätzliche Ernährungsform grüner Orchideen, die vorwiegend mit Pilzen der Gattungen *Ceratobasidium* und *Tulasnella* vergesellschaftet sind und eine relative ^{13}C-Abreicherung im Vergleich zu autotrophen Begleitpflanzen aufweisen. Eine derartige Abreicherung könnte aus einem Pflanze-zu-Pilz-Nettofluss von an ^{13}C angereicherten Kohlenstoffverbindungen resultieren.

Untersuchungen der Ernährungsweise von Orchideen des Mediterranraumes und der Makaronesischen Inseln lassen vermuten, dass das Vorhandensein geeigneter Ektomykorrhiza-Pilze eine Voraussetzung für das Vorkommen partiell und vollständig mykoheterotropher Pflanzenarten ist. Zudem wurde festgestellt, dass Orchideen, die einen großen Anteil ihrer Nährstoffe vom Pilz beziehen, bestimmten taxonomischen Gruppen angehören und an licht-limitierte Waldstandorte gebunden sind. Im Gegensatz dazu scheinen Netto-Kohlenstoff-Flüsse von der Orchidee zum Pilz an offene, licht-gesättigte Standorte gekoppelt zu sein. In einer Studie an grünen *Cephalanthera*-Arten temperater Wälder wurde der Effekt des Mikroklimas auf den Grad der Mykoheterotrophie genauer untersucht. Dabei konnte gezeigt werden, dass bessere Lichtverfügbarkeit die Orchideen sukzessive in Richtung Autotrophie treibt. Partielle Mykoheterotrophie ist folglich keine statische Ernährungsform, sondern ein erstaunlich flexibler Mechanismus, der eine optimal balancierte Nutzung der natürlichen Kohlenstoff-Ressourcen ermöglicht.

Obwohl viele Fragen innerhalb dieses noch jungen und breiten wissenschaftlichen Feldes unbeantwortet bleiben, tragen die Untersuchungen dieser Arbeit erheblich zum Verständnis der mykoheterotrophen Ernährungsweise bei. Die dargestellten Ergebnisse ermöglichen Folgerungen auf die Habitatansprüche mykoheterotropher Pflanzen und liefern somit neue Aspekte für den Artenschutz.

Summary

The group of nonphotosynthetic (chlorophyll-lacking) plants consists of about 4.100 species that directly parasitize on autotrophic plants and more than 400 so-called myco-heterotrophic species that rely upon organic nutrient supplies from associated fungi. Comprising almost a quarter of all known myco-heterotrophic species, the Orchidaceae are the most successful family among myco-heterotrophic plants. All orchids are myco-heterotrophic during their juvenile belowground phases but most species develop green leaves as adults. A similar situation occurs in the tribe Pyroleae (Ericaceae), consisting of mostly photosynthetic species that, as like as orchids, produce 'dust seeds' without endosperm and thus rely upon fungal support during early development. The use of stable isotope natural abundance analyses recently revealed that some adult green and hence putatively autotrophic Orchidaceae and Pyroleae gain organic carbon and nitrogen from their fungal partners in addition to the carbon obtained through photosynthesis and the mineral nitrogen assimilated from the soil. Plants exhibiting this mixed nutritional mode have been referred to as being partially myco-heterotrophic.

Understanding the complex and fascinating ecology of partially and fully myco-heterotrophic plants is the basis for successful conservation to protect these mostly endangered species and to maintain their unique habitats. Analyses of stable isotope natural abundances and molecular identification of mycorrhizal fungi are modern techniques that can give insight into the plants' nutritional modes under natural conditions without experimental disturbance. Based on these two methods, additional analyses of chlorophyll contents, light climate measurements and an *in situ* ^{13}C labeling experiment, this thesis provides some new fundamental knowledge on the intriguing way of nutrient acquisition exhibited by several Orchidaceae and Ericaceae.

A methodological approach that allows meta-analyses and improves the quantitative estimate of nutrient gains by partially myco-heterotrophic plants is presented in the first chapter. Using this enhanced method, the hitherto disputed or in most cases completely unknown nutritional status of a range of species was analyzed. It is evidenced that the ericaceous tribe Pyroleae comprises a fully myco-heterotrophic species (*Pyrola aphylla*) and stated that the leafless, ectomycorrhizal specialist orchid *Corallorhiza trifida* (hitherto considered as completely relying upon mycorrhizal fungi) is capable to photosynthesize.

Based on the broad dataset on isotope signatures and mycorrhizal associates of numerous orchid species, the existence of a nutritionally new group of orchids could be

proved, concluding that at least four nutritional modes can be found among terrestrial members of the Orchidaceae: autotrophy, where green orchids have carbon isotope signatures indistinguishable from those of surrounding autotrophs and mainly associate with *Rhizoctonia* species; partial myco-heterotrophy, where green orchids have carbon isotope signatures intermediate between those of autotrophs and myco-heterotrophs and associate with ectomycorrhizal fungi; full myco-heterotrophy, where orchids have lost the ability to photosynthesize, are specialized on either ectomycorrhizal or saprotrophic fungi and are enriched in ^{13}C similar to their host fungi; and an additional strategy found in green orchids which mainly associate with ceratobasidioid and tulasnelloid fungi and are depleted in ^{13}C compared to surrounding autotrophs - possibly due to a net plant-to-fungus transfer of ^{13}C enriched carbon compounds.

Studies on nutritional modes of orchids from Macaronesia and the Mediterranean region suggested that the availability of suited ectomycorrhizal fungi constrains the occurrence of partially and fully myco-heterotrophic species. Furthermore, we found a general pattern showing that high degrees of myco-heterotrophy in orchids are related to certain taxonomic groups and to the light-limited understory of forest sites while net plant-to-fungus carbon fluxes seem to be coupled to open light-saturated habitats. In a subsequent study on green *Cephalanthera* spp. from temperate forests, the effect of the prevalent micro-scale light climate on the degree of myco-heterotrophy was investigated more explicitly. It could be demonstrated that higher irradiances successively drive the orchids towards full autotrophy and that partial myco-heterotrophy thus is not a static nutritional mode but a surprisingly flexible mechanism allowing a well balanced utilization of carbon resources available in nature.

Although many questions in this broad and novel scientific field remain to be answered, results of this thesis substantially contribute to our knowledge on myco-heterotrophy and the mechanisms behind. The presented findings allow drawing conclusions on habitat requirements and raise new aspects for species conservation.

Introduction

Plants that lack chlorophyll

The green color is a defining feature of the plant kingdom and plants are mostly assumed as autotrophic organisms whose entire resources, i.e., sunlight, water, CO_2 and mineral ions, are acquired from the abiotic environment. However, about 1 % of the angiosperms (estimated 4.500 species) lack chlorophyll and thus are non-photosynthetic (Leake, 1994; Nickrent & Musselman, 2004). Most of them directly invade other plants to acquire nutrients *via* haustoria, i.e., modified roots that form a morphological and physiological link between the parasitizing plant and its host (Kuijt, 1969). Parasitic species occur in ca. 270 genera of higher plants and cover many life forms including annual and perennial herbs, vines, shrubs and trees (Press *et al*., 2005). According to the site of attachment to the host they can be distinguished between stem parasites (e.g., some mistletoes, and Cuscutaceae) and root parasites (e.g., Orobanchaceae). Parasitic plants may also be classified as obligate holoparasites that lack chlorophyll and must rely totally on supply from the host xylem (and phloem), and hemiparasites that contain chlorophyll when mature and mainly obtain water with its dissolved nutrients from the host xylem – the latter also including some facultative parasitic species (Nickrent & Musselman, 2004).

Although it probably is the best-known form, direct parasitism is not the only way to nutritionally exploit autotrophic plants. More than 400 species of vascular plants in 87 genera are achlorophyllous and heterotrophic, but not directly parasitic on autotrophs (Leake, 1994). Members of that group have historically been called ‘saprophytes’ which is misleading since it implies that the plants obtain their nutrients directly from dead organic matter. More properly, these plants have been called ‘cheaters’ or ‘epiparasites’ (Björkman, 1960; Bidartondo *et al*., 2003) because they indirectly obtain photosynthetic products from neighbouring autotrophic plants *via* a mycorrhizal network (with the exception of one known species that directly parasitizes saprotrophic fungi (Ogura-Tsujita *et al*., 2009)). In the following, I will refer to these plants as ‘myco-heterotrophic’, a term that was introduced by Leake (1994) and highlights the fact that such plants rely on organic nutrients from associated fungi.

Mycorrhiza and myco-heterotrophy

More than 80 % of land-plant families are estimated to be mycorrhizal (Trappe, 1987; Wang & Qiu, 2006). Both major types of mycorrhiza - arbuscular mycorrhiza and

ectomycorrhiza - have been invaded by myco-heterotrophic plants. Arbuscular mycorrhizas are the most common mycorrhizal type and characteristically found in species-rich ecosystems (e.g., in the tropics). They are formed between Glomeromycetes and plants of all phyla (Bryophyta, Pteridophyta, all groups of Gymnospermae and the majority of families in the Angiospermae). Arbuscular mycorrhizas are morphologically variable but characterized by fungal structures between and within the cells of the plant root cortex and an extraradical mycelium in the soil (Smith & Read, 2008). Ectomycorrhizas are the dominant nutrient-gathering organs in most temperate and boreal forest ecosystems (Read, 1991) formed by Basidiomycetes and some ascomycetous fungi that associate with woody perennials. The typical structural components are a sheath or mantle of fungal tissue which encloses the root, a labyrinthine inward growth of hyphae between the epidermal and cortical cells called the Hartig net, and an external mycelium which forms essential connections both with the soil and with the sporocarps of fungi forming the ectomycorrhizas (Smith & Read, 2008).

While most mycorrhizal associations are typically generalistic, hitherto studies on myco-heterotrophic plants have shown that they mainly associate with narrow clades of fungi which are simultaneously connected with neighbouring photosynthetic plants, e.g., mycorrhizal trees (Cullings *et al*., 1996; Taylor & Bruns, 1997, 1999; Kretzer *et al*., 2000; Bidartondo & Bruns, 2001, 2002, 2005; Bidartondo *et al*., 2002, 2003; Selosse *et al*., 2002; Taylor *et al*., 2002, 2003, 2004; Young *et al*., 2002; Yokoyama *et al*., 2005; Franke *et al*., 2006; Yagame *et al*., 2008; Yamada *et al*., 2008; Merckx & Bidartonodo, 2008). And quite recently, Ogura-Tsujita *et al*. (2009) demonstrated for the first time that a myco-heterotrophic plant can also form specific mycorrhizas with typically free-living, wood-rotting fungi and exploit these saprotrophs for organic nutrients. The absence of strong mycorrhizal specificity has so far only been found in three tropical species belonging to the Burmannicaeae, Triuridaceae (Franke *et al*., 2006) and Orchidaceae (Dernaley & Le Brocque, 2006), respectively. To explain the extreme fungal specificity seen in most myco-heterotrophs, two hypotheses have advanced (Bruns *et al*., 2002): First, specialization might allow the plants to adapt to particular fungi to enable the most effective capture of fungal carbon; and second, most fungi may develop resistance to exploitation by myco-heterotrophic plants, forcing them to specialize on fungi without this resistance.

Full myco-heterotrophy has evolved independently multiple times, e.g., in the non-vascular liverwort *Cryptothallus mirabilis*, in dicotyledons (Ericaceae, Gentianaceae, Polygalaceae) and tens of times in monocotyledons (e.g., Orchidaceae, Burmanniaceae, Corsiaceae) (Bidartondo, 2005). The wide occurrence of myco-heterotrophic plants challenges the reductionist view that mycorrhizal function is limited to the enhancement of capture of phosphorus, nitrogen or other mineral nutrients (Leake, 1994).

Full myco-heterotrophy in orchids

Besides a comprehensive methodological approach and a study on species of the ericaceous tribe Pyroleae (henceforth referred to as pyroloids), this thesis focuses on members of the world's largest plant family, the Orchidaceae. The roughly 25.000 orchid species (Dressler, 2004) can be classified due to their growth habit as epiphytic, lithophytic or terrestrial (Dearnaley, 2007). One of the most distinctive characteristics of all orchid species is the production of minute seeds that contain only minimal reserves of nutrients (Arditti & Ghani, 2000). Thus, very early colonization by an appropriate fungus is a prerequisite for embryo development in these seeds, meaning that all orchids are fully myco-heterotrophic during juvenile stages. Although the vast majority of orchids develops leaves and is photosynthetic as adults, full myco-heterotrophy in the adult phase may have evolved at least 20 times (Molvray *et al*., 2000). More than 100 terrestrial species, comprising almost a quarter of all known myco-heterotrophs turn the Orchidaceae into the most successful family of myco-heterotrophic plants (Leake, 1994; Taylor *et al*., 2002). With the exception of two genera (*Galeola* and *Gastrodia*) that have been shown to associate with decomposers and soil saprophytes (Bidartondo, 2005; Ogura-Tsujita *et al*., 2009), the parasitized fungi are Basidiomycetes and Ascomycetes of ectomycorrhizal habit that are associated with co-occurring trees or shrubs. In association with orchid roots, these fungi do not form a fungal mantle or a Hartig net but grow into the cortical root cells (Smith & Read, 2008; compare Figure 1 on page 11).

While much is known about the structure of orchid mycorrhizas, the mechanism(s) by which nutrients are transferred from fungus to plant or plant to fungus are unclear. The basic process of orchid mycorrhiza formation consists of fungal hyphae penetrating orchid root cortical cell walls by localized hydrolysis, the formation of a fungal peloton (mass of fungal hyphae) surrounded by plant plasma membrane and an interfacial matrix of unknown origin (Beyrle *et al*., 1995). The fungal peloton is a temporary

structure and eventually lysed allowing the formation of a new peloton in the same cell. It is generally accepted that nutrient transfer (particularly carbohydrate) from fungus to plant occurs after the peloton is lysed, but whether transfer occurs while the fungal peloton is forming or prior to peloton lysis is unknown (Peterson & Masicotte, 2004; Dearnaley, 2007). It is also unclear what the triggers for the lysis of fungal pelotons are, although the involvement of orchid derived fungitoxic phytoalexins seems likely (Beyrle *et al*., 1995).

Stable isotopes shed light on the nutrition of green species

In 2003, a breakthrough in the understanding of plant nutrition was achieved by investigating the nitrogen (N) and carbon (C) stable isotope natural abundances of green orchid species (Gebauer & Meyer, 2003). Most biologically important elements occur as two or more stable isotopes, with one being far more abundant than the other(s). Since fractionation against heavier isotopes is common in physical and metabolic processes, stable isotopes allow tracking of nutrient sources and fluxes in ecosystems (Dawson *et al*., 2002). An important advantage of measuring stable isotope natural abundances is their ability to present a time-integrated picture of functional processes that often are difficult to examine directly.

In the case of investigations on nutritional modes in orchids, the use of nutrient sources over the whole lifespan of the plant can be interpreted without experimental disturbance. This is generally owing to the fact that fungal tissues are enriched in the heavy stable isotopes of nitrogen (^{15}N) and carbon (^{13}C) relative to co-occurring autotrophic plants. Saprotrophic and ectomycorrhizal fungi living on decaying biomass can utilize considerable amounts of the ^{15}N enriched organic nitrogen fraction of the humus which is not directly available to higher plants and it seems as if fungi prefer the organic N for biomass production since it is energetically cheaper than inorganic N compounds (Gebauer & Dietrich, 1993). The ^{13}C enrichment in ectomycorrhizal fungi is generally explained by their major C source which is thought to be current assimilates supplied from the host (Högberg *et al*., 2001) that are enriched in ^{13}C relative to the bulk plant biomass (Gleixner *et al*., 1993; Bowling *et al*., 2008). The enrichment in ^{13}C in saprotrophic fungi is usually attributed to the utilization of organic C compounds and the preferential use of ^{13}C enriched carbohydrates (Boström *et al*., 2008).

Since fully myco-heterotrophic plants completely rely on fungi-derived nutrients, they show isotope signatures similar to those of their fungal associates, fitting the food-

chain model (Trudell *et al*., 2003). Thus, they are enriched in ^{15}N and ^{13}C compared to accompanying autotrophic plants. The first investigation of stable isotope natural abundances in terrestrial green orchids revealed that some of these putatively autotrophic plants are as well enriched in ^{15}N and ^{13}C (Gebauer & Meyer, 2003). The authors concluded that these plants' tissues reflect the incorporation of fungi-derived components and that they thus gain organic C and N from their fungal symbionts in addition to the C obtained through photosynthesis and the mineral N assimilated from the soil. This mixed mode of nutrition which involves the heterotrophic acquisition of C and N *via* associated fungi as well as through autotrophic processes has been referred to as partial myco-heterotrophy (Gebauer & Meyer, 2003) and invalidated the dogma in plant sciences meaning that green plants (with the exception of some hemiparasites) are autotrophic.

Available phylogenies show that partial myco-heterotrophy appeared first and probably facilitated the emergence of myco-heterotrophy (Selosse & Roy, 2009). Achlorophyllous forms of otherwise green species, the so-called albinos, e.g., in *Epipactis helleborine* (Salmia, 1988; Delforge, 1998), *E. microphylla* (Selosse *et al*., 2004), *Cephalanthera damasonium* (Julou *et al*., 2005) and *C. longifolia* (Abadie *et al*., 2006) may also document the evolutionary transition from partially to fully myco-heterotrophic nutrition (Selosse *et al*., 2006).

In addition to stable isotope natural abundance analyses, identification of mycorrhizal fungi of partially myco-heterotrophic orchids plays an important role. Mycologists have developed barcoding methods based on fungal ribosomal DNA, for which reference sequences exist in public databases (Nilsson *et al*., 2008) and the precise identification of the fungal partners is more than a purely descriptive task because the putative ecology of these fungi provides clues on the ultimate C source that is being exploited (Selosse & Roy, 2009). In this context, the study by Bidartondo *et al.* (2004) was the first to show that a switch of mycorrhizal associates enables partially myco-heterotrophic orchids to loot organic nutrients. Green orchids typically associate with Basidiomycetes of the polyphyletic form-group *Rhizoctonia* (Leake, 2004), which encompasses distantly related clades of fungi that are generally assumed to be saprotrophs or plant parasites (Taylor *et al*., 2003; Pope & Carter, 2001). The discovered switch of fungal partners towards ectomycorrhizal associates that are simultaneously connected with trees allows indirect exploitation of C. Interestingly, not only Basidiomycetes but also some ectomycorrhizal Ascomycota like *Tuber* and

Wilcoxina have been found to associate with partially myco-heterotrophic orchids (Bidartondo *et al.*, 2004; Zimmer *et al.*, 2007).

Mechanisms analogue to those seen in partially myco-heterotrophic orchids were recently also found in green pyroloids (Ericaceae) (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). Similar to their fully myco-heterotrophic ericaceous sister tribes Monotropeae and Pterosporeae (Kron *et al.*, 2002) and to the Orchidaceae, pyroloids produce tiny 'dust seeds' that rely on fungal supplies for development. Adult plants form ectendo-type mycorrhizas, referred to as arbutoid (Smith & Read, 2008) with asco- and basidiomycetous fungi that, again, are ectomycorrhizal associates on surrounding trees (Robertson & Robertson, 1985; Bidartondo, 2005; Tedersoo *et al.*, 2007; Smith & Read, 2008). Although fungal hyphae also penetrate the epidermal root cells of pyroloids (Figure 1), no lysis of fungi has been observed in these species (Tedersoo *et al.*, 2007; Vincenot *et al.*, 2008).

It is mostly unknown how nutrients are transferred, but it is clear that carbohydrate transfer does occur in both, orchid mycorrhiza as evidenced by ^{14}C and ^{13}C labeling of fungi and tracing of its movement into orchid tissue (Smith, 1967; Cameron *et al.*, 2006, 2008), and in pyroloids as suggested by Kunishi *et al.* (2004) and Hashimoto *et al.* (2005) who observed C transfer from co-cultivated *Larix kaempferi* to *Pyrola incarnata via* mycorrhizal fungi in ^{13}C labeling pot experiments.

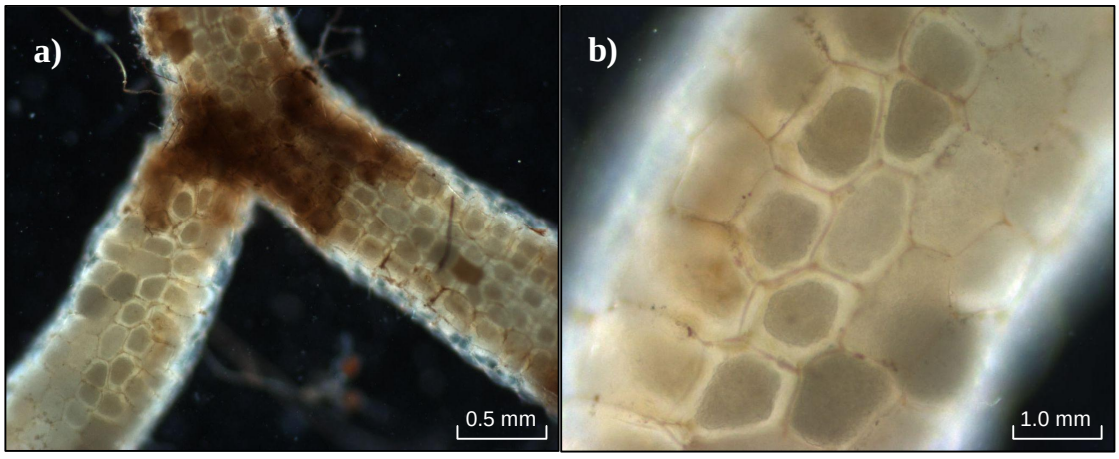

Figure 1. Light micrograph of roots of a partially myco-heterotrophic plant (*Orthilia secunda*). a) mycorrhizal root lacking a fungal mantle; b) epidermal cells densely packed with intracellular hyphal complexes. Photographs by K. Preiss; published in: Mycorrhizal Symbiosis (Smith & Read, 2008).

Research on partial and full myco-heterotrophy is needed

Green pyroloids can be the dominant understorey plants in boreal forests covering large surfaces by vegetative spread (Tedersoo *et al.*, 2007) and thus may play major roles in certain ecosystems. They are known to be particularly susceptible to disturbance from logging or burning (Halpern & Spies, 1995; Haugset *et al.*, 1996; Timoney *et al.*, 1997; Rees & Juday, 2002) and sensitive to anthropogenic nitrogen deposition (Allen *et al.*, 2007). Many orchid species across the planet are also in danger of extinction largely because of human-induced habitat loss (Dearnaley, 2007).

Fungal specificity could be a major reason for the rarity and vulnerability of orchids and pyroloids. A loss of fungal diversity associated with logging (Jones *et al.*, 2003) and N deposition (Wallenda & Kottke, 1998; Taylor *et al.*, 2000) is well documented. In Zimmer *et al.* (2007, not part of this thesis), we mentioned that elimination of key fungal symbionts possibly contribute to the loss of myco-heterotrophic plants at disturbed sites. Furthermore, we suggested that logging may eliminate any competitive advantage that these species might possess from tolerance to shade.

Understanding the intriguing ecology of partial and full myco-heterotrophs is the basis for successful conservation and may help to maintain unique habitats, to protect endangered species and even to preserve important resources like terrestrial orchids harvested for medical purposes (Xu & Guo, 2000).

Objectives of this thesis

The complex ecology of fully myco-heterotrophic plants and the recent discovery of partial myco-heterotrophy raise many questions. Laboratory studies on this issue are limited due to the complicated biological systems behind these fascinating nutritional modes. Analyses of stable isotope abundances and molecular identification of mycorrhizal fungi are modern techniques that can be applied to small samples collected from the field and thus can give insight into the plants' lifestyle under natural conditions. Results of this thesis are mainly based on investigations using these two methods. In addition, chlorophyll extractions, chlorophyll fluorescence measurements, an *in situ* ^{13}C labeling experiment as well as (time-integrated) micro-scale light climate measurements were carried out. As presented in CHAPTER [1-6], the seven publications contributing to this thesis aimed to specifically analyze plants whose nutritional status is disputed [2,3]; to trace indications of a new nutritional mode [4]; and to investigate the ecological constraints to partial and full myco-heterotrophy [5,6] using an enhanced methodological approach [1].

In detail, studies were conducted to achieve the following objectives:

[1] Designing a methodological approach that allows meta-analyses and improves the quantitative estimate of nutrient gains by partially myco-heterotrophic plants

[2] Testing whether the ericaceous tribe Pyroleae includes a fully myco-heterotrophic species in addition to partially myco-heterotrophic members

[3] Investigating the hitherto controversially discussed nutritional mode of the orchid *Corallorhiza trifida* using new analytical methods

[4] Showing that several green orchid species exhibit a new nutritional mode under field conditions - confirming recent indications from a laboratory experiment

[5] Assessing how ecological factors determine the occurrence of nutritional modes found in orchids from the Mediterranean region and the islands of Macaronesia

[6] Unraveling the major determinant for the degree of myco-heterotrophy in green terrestrial forest orchids

Synopsis

- CHAPTER 1 -

Improving estimates of nutrient gains by partial myco-heterotrophs

Since 2003, the stable isotopes of ^{15}N and ^{13}C have been used to investigate the nutritional mode of partially myco-heterotrophic orchids and pyroloids (Gebauer & Meyer). These plants that use the heterotrophic fungal nutrient source in addition to C gained *via* photosynthesis and N gained through assimilation of soil borne nitrogen are characterized by isotope signatures intermediate between autotrophic and fully myco-heterotrophic plants. The application of stable isotope ratio mass spectrometry currently is one of the most informative methods to assess the nutritional mode of adult partially myco-heterotrophic plants and respective studies provided insight into the nature of 23 orchid species and 15 species belonging to the ericaceous subfamily Monotropoideae (Gebauer & Meyer, 2003; Trudell *et al*., 2003; Bidartondo *et al*., 2004; Tedersoo *et al*., 2007; Zimmer *et al*., 2007; Julou *et al*., 2005; Hashimoto *et al*., 2005; Abadie *et al*., 2006; Cameron *et al*., 2006; Ogura-Tsujita *et al*., 2009); plus 29 further species (also belonging to these taxa) that are presented in this thesis (CHAPTER 2-5).

To quantitatively assess the plants' level of myco-heterotrophy, a linear two-source mixing model based on δ values of target and reference plants as introduced by Gebauer & Meyer (2003) is generally applied. This method assumes a linear correlation between nutrient gain from fungi and the enrichment in ^{15}N and ^{13}C. The endpoints of this model are described by mean δ values of autotrophic reference plants from a study site (0 % organic nutrient gain from fungi) and the mean δ values of co-occurring fully myco-heterotrophic plants (100 % nutrient gain from fungi).

Calculations using this method may be biased by variation in irradiance and soil nutrient availability of plots and sites from which material is collected since such variations affect the plants' isotope composition. Misleading results when pooling data from different sites or different plots within an inhomogeneous study site are the consequence. A further limitation of the conventional approach arises if fully myco-heterotrophic species are missing at a study site, since these plants are also influenced by environmental parameters and thus cannot serve as endpoint for calculations *via* the mixing model for target plants from another site.

The approach presented in the first publication of this thesis improves the facility of application and the significance of such model calculations by two ways: First, by introducing normalized (i.e., site- and plot-independent) enrichment factors

$\varepsilon_{Sx} = \delta_{Sx} - \delta_{REFx}$ with S as a single value of a sample from an autotrophic, partially myco-heterotrophic or fully myco-heterotrophic plant, x as a specific sampling plot within the entire study site and REF as the mean value of all autotrophic reference plants; revealing three groups of normalized enrichment factors: $\varepsilon_R = \varepsilon$ of the autotrophic reference plants (whereas the mean ε_R of all reference plants from a site is always 0 ‰), $\varepsilon_{PMH} = \varepsilon$ of the partially myco-heterotrophic plants and $\varepsilon_{MH} = \varepsilon$ of the fully myco-heterotrophic plants. The percentage nutrient gain from fungi ($\%x_{df}$ with x = N or C) can then be calculated from the proportion between ε_{PMH} and ε_{MH} *via* the rule of three, i.e., $\%x_{df} = (\varepsilon_{PMH} / \varepsilon_{MH}) \times 100$. The second improvement offered in this study is the presentation of a universal endpoint for the linear mixing model if fully myco-heterotrophic species are missing at a study site. Means for $\varepsilon_{MH}{}^{15}N = 12.0 \pm 1.7$ ‰ and $\varepsilon_{MH}{}^{13}C = 7.2 \pm 1.6$ ‰ were obtained from 92 values available from the literature.

The normalized approach is advantageous compared to the former method since it provides a possibility for more precisely calculation and statistical validation of the percentage C and N gain of partially myco-heterotrophic plants - even without the occurrence of neighboring fully myco-heterotrophic plants. It furthermore allows meta-analyses between very diverse sites and representation of multiple datasets within one graph. For example, in Figure 1 of CHAPTER 5, the application of the new approach was required to merge data from 20 different sites. In addition, reactions in the degree of myco-heterotrophy to environmental changes (as the effect described in CHAPTER 6) can be assessed more exactly when considering influences of the microclimate on a fine scale, i.e., when regarding normalized, plot-independent ε values instead of mean δ values from a site.

Based on data in Appendix A of the manuscript, the universal ε_{MH} should always be adjusted by new information that arises from subsequent studies. Since the publication of this article, further studies including enrichment factors of myco-heterotrophic plants came up (*Monotropa uniflora* in Ogura-Tsujita e*t al*., 2009; *Pyrola aphylla*, *Corallorhiza maculata*, *Pterospora andromedea* in CHAPTER 2 of this thesis; Neottia *nidus-avis* in CHAPTER 5 of this thesis) and slightly shifted the model endpoints to values of 12.6 ± 2.5 ‰ for $\varepsilon_{MH}{}^{15}N$ and 7.2 ± 1.4 ‰ for $\varepsilon_{MH}{}^{13}C$ (means obtained from meanwhile 147 samples of nine species that completely rely on ectomycorrhizal fungi). Since Ogura-Tsujita *et al*. (2009) have recently shown that the exploitation of saprotrophic fungi by a fully myco-heterotrophic orchid leads to differently enriched plant tissue, it has to be stated, that the universal endpoints presented here should only

be used for calculations on partially myco-heterotrophic species that invade ectomycorrhizas for nutrient acquisition. However, that is the case for all hitherto known partially myco-heterotrophic plants.

- CHAPTER 2 -

Full myco-heterotrophy in the ericaceous tribe Pyroleae

This chapter of the thesis focuses on species of the Ericaceae that are of interest in terms of partial and full myco-heterotrophy. As previously mentioned, pyroloids represent a sister tribe of the two further tribes Pterosporeae and Monotropeae within the ericaceous subfamily Monotropoideae (Kron *et al.*, 2002). Members of the Pyroleae are all myco-heterotrophic in their early stages of development (Leake, 1994) but contain many taxa that develop green leaves and are (at least partially) autotrophic as adults (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). Whereas the Pterosporeae and Monotropeae exclusively consist of fully myco-heterotrophic species, botanists and mycologists have long debated the potential for full myco-heterotrophy within the Pyroleae, especially with regard to the achlorophyllous *Pyrola aphylla*. First indications for its myco-heterotrophic status arose in our previous broad study on nutrient gains from fungi in several pyroloids, monotropoids and orchids using stable isotope abundances (Zimmer *et al.*, 2007). However, that study included only few replicates for *P. aphylla* and the relevance of the findings to the overall distribution of this species thus remained unknown. The publication of CHAPTER 2 confirms previous findings by presenting the C and N stable isotope signatures for three pyroloid species (*P. aphylla*, *P. picta* and *Chimaphila umbellata*) from more intensively sampled populations and sampling over a wider geographic region.

No C gain from fungi could be found for the green species *P. picta* and *C. umbellata*, although they were highly enriched in ^{15}N. We propose two potential possibilities for this pattern. First, although all pyroloid seedlings are myco-heterotrophic, once they develop leaves, they primarily gain C through photosynthesis, but continue to gain N through an unknown uptake mechanism similar to myco-heterotrophic plants. A second possibility is that C gains *via* a myco-heterotrophic strategy are still present, but the analysis of plants' bulk tissue isotope abundances is not sensitive enough to detect these gains, which may only take place during certain seasonal or plant developmental periods (Taylor *et al.*, 2004).

P. aphylla exhibited strong enrichments in ^{15}N and ^{13}C, evidencing its fully myco-heterotrophic status. Haber (1978) assumed that *P. aphylla* was one of many morphological forms of *P. picta* and connected by a rhizome to nearby leafy rosettes. Because of significant differences in the isotope signatures of *P. picta* and *P. aphylla* this study provides no substantiating evidence for rhizomatous connections between the two.

The fact that P. *aphylla* is a nutritionally distinct species is of even greater importance as this species has found to be a mycorrhizal generalist (Hynson & Bruns, in prep). Hence, with exception of albino forms of three orchid species (*Epipactis microphylla,* Selosse *et al*., 2004; *Cephalanthera damasonium,* Julou *et al*., 2005; *C. longifolia,* Abadie *et al*., 2006), *P. aphylla* represents the first known non-tropic plant species that is not specialized on a narrow clade of fungi. This important finding indicates that the loss of photosynthesis in myco-heterotrophs outside the tropics is not contingent upon fungal specialization (Hynson & Bruns, in prep).

Adding the information on isotope signatures of *P. aphylla* to the dataset on the ^{15}N and ^{13}C enrichment of fully myco-heterotrophic plants presented in CHAPTER 1 broadens the significance of the universal model endpoints since they now comprise members of a further plant tribe.

- CHAPTER 3 -

The nutritional mode of Corallorhiza trifida

While the 10 terrestrial species of the orchid genus *Corallorhiza* are in general known to be fully myco-heterotrophic, *C. trifida* might be an exception. Although its 'mycotrophic' nature was already recognized in 1898 (Jennings & Hanna), many questions concerning its nutritional mode have been raised by pigment analyses and assimilation experiments (Montfort & Küsters, 1940) as well as by comparative studies of the plastid DNA (Freudenstein & Doyle, 1994). However, despite contrary evidence, *C. trifida* remained classified as a full myco-heterotroph. This chapter comprises two publications (presented as CHAPTER 3.1 and 3.2) that include methods hitherto not applied in this context, aiming to shed more light on the nutrient source(s) utilized by *C. trifida*.

Molecular identification of the fungi associating with *C. trifida* individuals from the investigated population thriving in a dense broadleaf forest dominated by *Fagus sylvatica* showed mycorrhizal specificity towards ectomycorrhizal fungi of the

basidiomycete genus *Tomentella* which is in accordance with previous findings (Zelmer & Currah, 1995; Taylor, 1998; McKendrick *et al*., 2000). Analyses of the ^{15}N and ^{13}C natural abundance in above-ground tissues of *C. trifida* revealed that autotrophic processes significantly contribute to the nutrient acquisition in adult plants. Calculations *via* the linear mixing-model suggest that this species may supply about half of its N demands and approximately one fourth of its C demands through same processes as autotrophic plants (CHAPTER 3.1). An *in situ* ^{13}C pulse labeling experiment that was conducted one year later on individuals of the same population indicated that photosynthesis may play a considerably less important role than suggested on basis of the mixing-model calculations (CHAPTER 3.2). However, chlorophyll fluorescence measurements and chlorophyll extractions of both studies confirmed the presence of active photosystem II reaction centers and a chlorophyll *a*:*b* ratio similar to that found in other C_3 plants.

A major limitation of calculating the proportion of fungi-derived C using the mixing-model is that the contribution of fungal C to biomass (anabolism) but not to catabolism is established *via* this method. Investigations on respiratory CO_2 will thus be necessary to build a global view of C metabolism in partially myco-heterotrophic species (Selosse & Roy, 2009). The interpretation of data from isotope pulse labeling experiments, however, is restricted as well since assimilation rate is only investigated during a short time and a very small part of the plants' life. Further physiological analyses are required to ascertain whether species with low chlorophyll are quite incapable of photosynthesis in all organs and seasons (Rasmussen & Rasmussen, 2009).

Despite differing evidence on the amount of C that is assimilated in adult *C. trifida*, studies in CHAPTER 3 come to the conclusion that this species is capable of photosynthesis and represents a late stage in the evolutionary development towards complete myco-heterotrophy. *C. trifida* therewith is one of the rare examples of photosynthetic orchids that display specificity towards mycorrhizal fungi. Two further species that were found to exhibit a similar lifestyle in the Mediterranean region are mentioned in CHAPTER 5.

- CHAPTER 4 -

Confirming a new nutritional mode in green orchids

The study of this chapter provides the counterpart to recent findings by Cameron *et al.* (2006, 2008) who studied C fluxes between adult plants of the green orchid species

Goodyera repens and its associated mycorrhizal fungus (*Ceratobasidium cornigerum*). Using ^{14}C-labeled carbon fed either to the mycelia of the orchids' fungal symbiont or to the plant as $^{14}CO_2$, Cameron *et al.* (2008) were able to quantify C transport between the orchid and fungus. They found that the net transfer of C from *G. repens* to *C. cornigerum* was over five times greater than that of C transported from the fungus to the plant. As mentioned in their recent article and the commentary by Johnson (2008), C allocation to fungal biomass within the orchids' root cannot be separated from that to the roots alone; nor can C respiration from the plant *versus* that from the fungus. Furthermore, such labeling experiments give information of C flow within a system for only a relatively short time period and since these measurements were carried out in the laboratory, it is difficult to relate results to field settings.

Analyzing the ^{15}N and ^{13}C natural abundance of two *Goodyera* species collected in the field (*G. repens* and *G. oblongifolia*), we found that both species were significantly enriched in ^{15}N compared to surrounding autotrophic plants (indicating gain of organic N from fungi) but at the same time depleted in ^{13}C. The physiological mechanism leading to this relative depletion is unknown but may be related to the transfer of ^{13}C enriched carbon compounds from these orchids do their associated fungi (Gleixner *et al*., 1993; Bowling *et al*., 2008) which would fit well with Cameron *et al*.'s (2006, 2008) findings of C transfer from orchid to fungus.

Since our results gained from stable isotope natural abundance analysis of field-collected samples give an integrated view of C assimilation throughout the period during which the tissue was synthesized, we can confirm that these two orchid species exhibit a distinct nutritional strategy.

As can be seen in CHAPTER 5, studies from the Mediterranean region and Macaronesia revealed further orchid species that are depleted in ^{13}C compared to autotrophic reference plants. While our findings in CHAPTER 4 represent the link between laboratory investigations by Cameron *et al*. (2006, 2008) and the reaction of *Goodyera* plants under natural conditions, this knowledge enables us to interpret results of CHAPTER 5 concluding that several orchid species exhibit a hitherto unknown nutritional mode. Thus, at least four nutritional modes can be found among terrestrial members of the Orchidaceae, i.e., autotrophy, where green orchids have C isotope signatures indistinguishable from those of surrounding autotrophs and mainly associate with *Rhizoctonia* species; partial myco-heterotrophy, where green orchids have C isotope signatures intermediate between those of autotrophs and myco-heterotrophs and

associate with ectomycorrhizal fungi; full myco-heterotrophy, where orchids have lost the ability to photosynthesize, are specialized on either ectomycorrhizal or saprotrophic fungi and are enriched in ^{13}C similar to their host fungi; and an additional strategy found in green orchids from the tribes Cranichideae and Orchideae which mainly associate with ceratobasidioid and tulasnelloid fungi (CHAPTER 5) and are depleted in ^{13}C compared with surrounding autotrophs.

- CHAPTER 5 -

Constraints to nutritional modes in orchids

The Mediterranean region is a hotspot of orchid diversity, for example, there are 108 orchid species in Italy alone (Ministero dell' Ambiente e della Tutela der Territorio, 2007). In striking contrast, the adjacent climatically similar Macaronesian region is poor in orchid diversity with only 16 species, including not more than eight species on the Canary Islands (Eriksson *et al*., 1979; Hohenester & Welß, 1993). Considering the fact that orchids typically produce 'dust seeds' which are easily transported over large distances by the wind, seed dispersal between the Mediterranean and Macaronesia is to be expected, which raises the question for the factor(s) limiting orchid diversity in Macaronesia.

It has never been investigated whether the occurrence of full and partial myco-heterotrophy is coupled to specific types of habitats and how the diversity of ectomycorrhizal plants and fungi constrain the distribution of these nutritional modes. The study presented in this Chapter is the widest screening for myco-heterotrophy in natural ecosystems carried out to date. We investigated the nutritional mode and fungal associates of Mediterranean (continental Italy and Sardinia) and Macaronesian (Tenerife) orchid species growing in open habitats, shrubland, forest gaps and forests to test whether the occurrence of full and partial myco-heterotrophy is restricted to habitats distinguished by light regime and available fungi.

Based on their isotope signatures, three distinct categories of orchids were obtained from a cluster analysis. One group consisted of orchids collected at forest sites. These species were members of the tribe Neottieae, associated with ectomycorrhizal fungi and turned out to be strongly or fully myco-heterotrophic. Another group included orchids of open habitats and forest gaps belonging to the tribes Orchideae and Cranichideae. In these species, root endophytes were frequently found while associations with (potential) ectomycorrhizal fungi were quite rare. Isotope data of some

Aceras, *Orchis* and *Ophrys* species (tribe Orchideae) within this group showed significant depletion in ^{13}C relative to their autotrophic references. As presented in CHAPTER 4 of this thesis, ^{13}C depletion might be a consequence of a specific net plant-to-fungus C flux. An intermediate group obtained from the cluster analysis was composed of orchids that showed weak C gains through myco-heterotrophy, occurring in all four habitat types.

While orchids from continental Italy and Sardinia cover all three clusters, the group of forest orchids that are highly or fully dependent upon their mycorrhizal fungi (Neottieae) is missing on Tenerife and even though forests are present on the Macaronesian islands, there are no reports for any occurrence of neottioid orchids in the Macaronesian region (Eriksson *et al.*, 1979; Hohenester & Welß, 1993). Our results from vegetation surveys and molecular identification of mycorrhizal fungi raise the hypothesis that this pattern is caused by the reduced number of ectomycorrhizal plants and/or suitable ectomycorrhizal fungi. Based on our wide spectrum of species and habitats investigated, we conclude that a high degree of myco-heterotrophy in orchids is related to certain taxonomic groups (i.e., Neottieae) and to the light-limited understorey of forest sites while we suggest that a net plant-to-fungus C flux is coupled to open light-saturated habitats.

Of particular interest with regard to CHAPTER 3 are the isotope signatures found for *Limodorum* species of this study. These orchids are characterized by reduced leaves, violet color of the stem, specific association with ectomycorrhizal fungi (Girlanda *et al.*, 2006) and have repeatedly been described as nonphotosynthetic or fully myco-heterotrophic, respectively (Fitter *et al.*, 1985; Flora Europaea, 2001; Gebauer & Meyer, 2003). Here we show that *Limodorum abortivum* and *L. trabutianum* are less enriched in ^{13}C than fully myco-heterotrophic plants and conclude that they exemplify a late stage of partial myco-heterotrophy. They thus represent a Mediterranean parallel to the temperate-boreal distributed *Corallorhiza trifida* examined in detail in CHAPTER 3.

- CHAPTER 6 -

The major determinant for the degree of myco-heterotrophy

Although it can be hypothesized that many more green plants than hitherto thought are partially myco-heterotrophic, we know very little on the mechanisms behind this ecologically relevant phenomenon. Previous studies indicate a large range in the proportion of fungi-derived C between and within partially myco-heterotrophic species

(Gebauer & Meyer, 2003; Bidartondo *et al*., 2004; Julou *et al*., 2005; Abadie *et al*., 2006; Tedersoo *et al*., 2007; Zimmer *et al*., 2007; CHAPTER 2-5 of this thesis) but the driving factors for this variation remained mostly unknown. In 2005, Gebauer suggested for the first time that light availability may determine the degree of myco-heterotrophy since the contribution from photosynthesis should be reduced at very dark sites. Furthermore, we could see that *Cephalanthera longifolia* was less dependent upon organic nutrient supply from mycorrhizal fungi when growing at a more exposed site in continental Italy (23 % relative light availability) compared to individuals from a dense *Quercus ilex* forest on Sardinia (2 % relative light availability) (CHAPTER 5).

To test whether the exploitation of mycorrhizal fungi is affected by the prevalent light climate, we combined leaf stable isotope natural abundance analysis with time integrated micro-scale light climate monitoring and investigated two partially myco-heterotrophic orchid species (*Cephalanthera damasonium* and *C. rubra*) together with 12 fully autotrophic and one fully myco-heterotrophic reference species.

$\delta^{13}C$ values in leaves of autotrophic non-orchids and of the fully autotrophic orchid species *Cypripedium calceolus* showed a significant, positive correlation with light availability which is based on the C isotope discrimination during C_3 photosynthesis and on stomatal regulation affecting the intercellular partial pressure of CO_2 (Farquhar *et al*., 1989). Leaf isotope signatures of the achlorophyllous orchid *Neottia nidus-avis* were not correlated with the micro-scale light climate since the C demand of this species is completely covered through organic compounds supplied by mycorrhizal fungi.

A quite interesting pattern was found for the two green *Cephalanthera* species. Relating the isotope data to references of the respective plot, a true relation between enrichment factor and micro-scale light availability becomes obvious. The darker a habitat, the more fungi-derived C is incorporated by the orchids. Under low light conditions, *Cephalanthera* individuals receive about half as much of fungi-derived C as achlorophyllous plants while the proportion of heterotrophic nutrition decreases with increasing irradiance. At sufficiently high irradiances, adult *Cephalanthera* plants completely cover their C demands through assimilation of atmospheric CO_2 as like as fully autotrophic non-orchids and orchids.

This study demonstrates that partial myco-heterotrophy is not a static nutritional mode but a surprisingly flexible mechanism allowing a well balanced utilization of carbon resources available in nature. The fascinating finding that the degree of myco-

heterotopy may successively change – driven by the prevalent micro-scale light climate – further supports our results presented in CHAPTER 5 where we conclude that strong and full myco-heterotrophy in orchids is related to light-limited forest understories while net plant-to-fungus C flux is coupled to open light-saturated habitats. In general, the response to relative light availability could explain several discrepancies between previous studies that investigated the trophic status of numerous green Orchidaceae and Ericaceae.

Outlook

This thesis provides new insights into the intriguing way of nutrient acquisition exhibited by several Orchidaceae and Ericaceae. The hitherto controversial or in most cases completely unknown nutritional status of a range of species was analyzed, revealing the existence of a nutritionally new group of orchids and responding to many further questions on the epiparasitic lifestyle. It is suggested that the availability of suited ectomycorrhizal fungi constrains the occurrence of partially and fully myco-heterotrophic species and the finding that irradiance is the major determinant for the degree of myco-heterotrophy in *Cephalanthera* spp. may represent a milestone in the understanding of these species' ecology. Results of this thesis thus allow drawing conclusions on habitat requirements and raise new aspects for species conservation.

Although some fundamental issues are addressed in the studies presented inhere, a large number of questions remain open. It is, for example, poorly studied whether partial myco-heterotrophy occurs in further plant families. Thus, green plants that are related to fully myco-heterotrophic species should be examined in this context (e.g., among the Burmanniaceae, Gentianaceae and Polygalaceae). The molecular mechanisms behind nutrient transfers between fungi and myco-heterotrophic plants remain to be detected as well. Ongoing studies using stable isotope natural abundance analyses to investigate the plant-fungus exchange of further elements like sulfur, hydrogen and oxygen may increase the understanding of the complex mechanisms and ecological effects of myco-heterotrophy. At the moment, the costs for surrounding autotrophic plants and fungi are unknown and in the case of partial myco-heterotrophs, we even do not know whether we face a parasitism or a somehow balanced exchange. As mentioned by Selosse & Roy (2009), organic nutrient gain could also be 'compensated' by providing vitamins or a shelter (physical or chemical protection) for the fungus. Specific investigations are required to prove such assumptions. With regard to fully myco-heterotrophic plants, it should also be stated that hitherto studies have mainly focused on temperate and mediterranean regions although the majority of myco-heterotrophs occur in the tropics where ectomycorrhizal fungi are less abundant. Isotope signatures of myco-heterotrophic plants associating with arbuscular mycorrhizal fungi are still unknown and the mycorrhizal features of tropical achlorophyllous plants are poorly studied. Thus, mechanisms of nutrient acquisition in these plants could be more diverse than it currently appears.

Exciting times lie ahead for further exploring the molecular, physiological, ecological and evolutionary processes in myco-heterotrophic plants and their mycorrhizal fungi. Stable isotopes will continue to be an informative method to investigate relationships between these plants and their mycobionts. The following table conclusively summarizes the current knowledge on fungi, plants and plant isotope signatures that are of importance in the broad scientific field dealing with full and partial myco-heterotrophy.

Table 1. Overview of the plant families comprising fully myco-heterotrophic or partially myco-heterotrophic species that are either mycorrhizal generalists or specialized to certain ectomycorrhizal, saprotrophic or arbuscular mycorrhizal fungi; including references for information on the plants' carbon and nitrogen stable isotope natural abundances.

FULLY MYCO-HETEROTROPHIC SPECIES		
Mycorrhizal associates	**Plant family**	**Natural isotope signatures**
specific ectomycorrhizal fungi	Orchidaceae, Ericaceae	reviewed in CHAPTER 1; Ogura-Tsujita *et al.* (2009)
specific saprotrophic fungi	Orchidaceae	Ogura-Tsujita *et al.* (2009)
specific arbuscular mycorrhizal fungi	Burmanniaceae, Gentianaceae, Polygalaceae, Aneuraceae, ...	unknown
no strong specificity	Orchidaceae, Burmanniaceae, Ericaceae, Triuridaceae	first data herein (CHAPTER 2) *
CHLOROPHYLL-CONTAINING SPECIES		
Mycorrhizal associates	**Plant family**	**Natural isotope signatures**
specific ectomycorrhizal fungi	Orchidaceae	first data herein (CHAPTER 3, 5)
no strong specificity	Orchidaceae, Ericaceae	e.g., Bidartondo *et al.* (2004) ; Zimmer *et al.* (2007); first explanation for relative ^{13}C depletion herein (CHAPTER 4, 5)

* for data on achlorophyllous forms (albinos) of otherwise green species within the Orchidaceae see Selosse *et al.*, 2004; Julou *et al.*, 2005 and Abadie *et al.*, 2006

Record of my contributions to this thesis

The general introduction, synopsis, outlook and summary of this thesis were written by me. My contributions to each of the seven studies that are part of this thesis are listed below. While the two latest manuscripts have been submitted, five studies are already published in international peer reviewed journals.

- CHAPTER 1 -

Preiss K, Gebauer G. 2008. A methodological approach to improve estimates of nutrient gains by partially myco-heterotrophic plants. *Isotopes in Environmental and Health Studies* 44: 393-401.

Concepts and planning:	50 %	Data analysis:	85 %
Field and laboratory work:	- - -	Manuscript preparation:	75 %

- CHAPTER 2 -

Hynson NA, Preiss K, Gebauer G, Bruns TD. 2009. Isotopic evidence of full and partial myco-heterotrophy in the plant tribe Pyroleae (Ericaceae). *New Phytologist* 182: 719-726.

Concepts and planning:	5 %	Data analysis:	90 %
Field and laboratory work:	0 %	Manuscript preparation:	15 %

- CHAPTER 3.1 -

Zimmer (Preiss) K, Meyer C, Gebauer G. 2008. The ectomycorrhizal specialist orchid *Corallorhiza trifida* is a partial myco-heterotroph. *New Phytologist* 178: 395-400.

Concepts and planning:	60 %	Data analysis:	95 %
Field and laboratory work:	70 %	Manuscript preparation:	85 %

- CHAPTER 3.2 -

Cameron DD, Preiss K, Gebauer G, Read DJ. 2009. The chlorophyll-containing orchid *Corallorhiza trifida* derives little carbon through photosynthesis. *New Phytologist* 183: 358-364.

Concepts and planning:	10 %	Data analysis:	5 %
Field and laboratory work:	25 %	Manuscript preparation:	5 %

- CHAPTER 4 -

Hynson NA, Preiss K, Gebauer G. 2009. Is it better to give than to receive? A stable isotope perspective on orchid-fungal carbon transport in the green orchid species *Goodyera repens* and *Goodyera oblongifolia*. *New Phytologist* 182: 8-11.

Concepts and planning:	25 %	Data analysis:	90 %
Field and laboratory work:	25 %	Manuscript preparation:	15 %

- CHAPTER 5 -

Liebel HT, Bidartondo MI, Preiss K, Segreto R, Stöckel M, Rodda M, Gebauer G. C and N stable isotope signatures reveal constraints to nutritional modes in orchids from the Mediterranean and Macaronesia. Submitted on July 21, 2009 to *Journal of Ecology*.

Concepts and planning:	0 %	Data analysis:	0 %
Field and laboratory work:	10 %	Manuscript preparation:	30 %

- CHAPTER 6 -

Preiss K, Adam IKU, Gebauer G. Irradiance governs exploitation of fungi: Fine-tuning of carbon gain by partially myco-heterotrophic orchids. Submitted on May 25, 2009 to *Proceedings of the Royal Society of London, Series B*.

Concepts and planning:	70 %	Data analysis:	80 %
Field and laboratory work:	50 %	Manuscript preparation:	90 %

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CHAPTER 1

Improving estimates of nutrient gains by partial myco-heterotrophs

A methodological approach to improve estimates of nutrient gains by partially myco-heterotrophic plants

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Key words: partial and full myco-heterotrophy, enrichment factor, linear mixing model, natural isotope abundance, Orchidaceae, Ericaceae

Abstract

The stable isotopes ^{15}N and ^{13}C can be used to investigate the nutritional mode of terrestrial orchids and pyroloids (Monotropoideae, Ericaceae). Some of these plants are putatively autotrophic but meet their nitrogen and carbon demands by gaining organic compounds (e.g., amino acids) from mycorrhizal fungi. This so called partially myco-heterotrophic nutrition is reflected by their isotope signature. The application of a two-source linear mixing model on δ values of such plants allows calculating the percentage of N and C derived from their associated mycorrhizal fungi. Here we present an approach to improve estimates of the plants' degree of myco-heterotrophy. Due to the presented conversion of δ values into enrichment factors (ε), results obtain a better resolution and data from various studies become normalized which facilitates combined representations and meta-analyses.

Introduction

Isotopes are known as excellent tracers for mixing processes and indicate which sources dominate in the mixtures (Fry, 2006). Since 2003, the stable isotopes of nitrogen (^{15}N) and carbon (^{13}C) have been used to investigate the nutritional mode of terrestrial orchids (Gebauer & Meyer, 2003). It has been shown, that some green orchids are not completely autotrophic, gaining C *via* photosynthesis and N *via* assimilation of soil borne nitrogen, but cover significant proportions of their nutrient demands through organic C and N compounds derived from mycorrhizal fungi. Since these fungi are enriched in ^{15}N (Gebauer & Dietrich, 1993) and ^{13}C (Gleixner *et al.*, 1993) compared to accompanying autotrophic vegetation, these orchids' nutritional mode is reflected in their isotope signatures. In addition to the obligate autotrophic and the recently described green orchids that are supplied by fungi, there are more than 100 non-photosynthetic species within the Orchidaceae that entirely rely on heterotrophic nutrient supplies from associated fungi (Leake, 1994). These chlorophyll-lacking plants are referred to as myco-heterotrophic (Leake, 1994) and show $\delta^{15}N$ and $\delta^{13}C$ values similar to those of their ectomycorrhizal fungal partners, fitting the food-chain model (Trudell *et al.*, 2003). The green orchids that may use both the fungal and the autotrophic nutrient source accordingly, are characterized by an isotope signature intermediate between autotrophic and myco-heterotrophic plants. These mixotrophic

plants are more precisely referred to as partially myco-heterotrophic (Gebauer & Meyer, 2003).

Since partially myco-heterotrophic plants become more or less independent of irradiance, they are able to colonize shaded habitats and some of them grow in dense forests under very dark conditions without any other understorey vegetation. Moreover, these green orchids were found to switch their endomycorrhizal associates from the typically associated free living saprotrophic fungi towards fungi that simultaneously form ectomycorrhizas with trees (Bidartondo *et al*., 2004; Selosse *et al*., 2004). Since such ectomycorrhizal fungi are the sole associates of fully myco-heterotrophic plants, it was hypothesized that partially myco-heterotrophic plants provide a missing link in the evolution of myco-heterotrophy (Bidartondo *et al*., 2004). Recently, partial myco-heterotrophy was also described in members of the Ericaceae (Monotropoideae, Pyroleae) (Tedersoo *et al*., 2007; Zimmer *et al*., 2007), raising the question whether there are even more green plant taxa concealing this nutritional mode. The investigation and understanding of the ecological attributes of such mixotrophic and commonly endangered plants is important in terms of nature conservation. The application of stable isotope ratio mass spectrometry currently is one of the most informative methods to assess the nutritional mode of partially myco-heterotrophic plants and already provided insight into the nature of 23 orchid species and 14 species belonging to the ericaceous subfamily Monotropoideae (Gebauer & Meyer, 2003; Trudell *et al*., 2003; Bidartondo *et al*., 2004; Tedersoo *et al*., 2007; Zimmer *et al*., 2007; Julou *et al*., 2005; Hashimoto *et al*., 2005; Abadie *et al*., 2006; Cameron *et al*., 2006; Zimmer *et al*., 2008). To quantitatively assess the plants' level of myco-heterotrophy, a linear mixing model based on δ values of target and reference plants is generally applied (see Gebauer & Meyer, 2003). Although this method is well suited to interpret the information gained from isotope abundance analyses of plant material from a specific site, the following approach provides suggestions to further improve the facility of application and the significance of such model calculations.

Hitherto constraints of the linear two-source mixing model

In the case of partial and full myco-heterotrophy, isotope abundances are mainly presented in the δ notation, as: $\delta^{15}N$ or $\delta^{13}C = (R_{sample}/R_{standard}-1) \times 1000$ [‰] where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the

respective standard. To calculate the relative contribution of fungi-derived nutrients to the plants total N and C gain, a linear two-source mixing model based on these δ values is applied. This method assumes a linear correlation between nutrient gain from fungi and the enrichment in ^{15}N and ^{13}C, respectively. Thus, the endpoints of this model are described by mean δ values of autotrophic reference plants from a study site (0 % nutrient gain from fungi) and the mean δ values of co-occurring fully myco-heterotrophic plants (100 % nutrient gain from fungi). The percentage N and C gain of the individual target plants (%x_{df} with x = N or C) that are assumed to be partially myco-heterotrophic can be calculated according to the following equation (Gebauer & Meyer, 2003):

$$\%x_{df} = (\delta x_{PMH} - \delta x_R) / \varepsilon_{MH} \times 100$$

with δx_{PMH} as the individual δ value of a partial myco-heterotrophic plant, δx_R as the mean δ value of all autotrophic reference plants from the study site and ε_{MH} as the mean enrichment factor of fully myco-heterotrophic plants relative to obligate autotrophic reference plants from the same site.

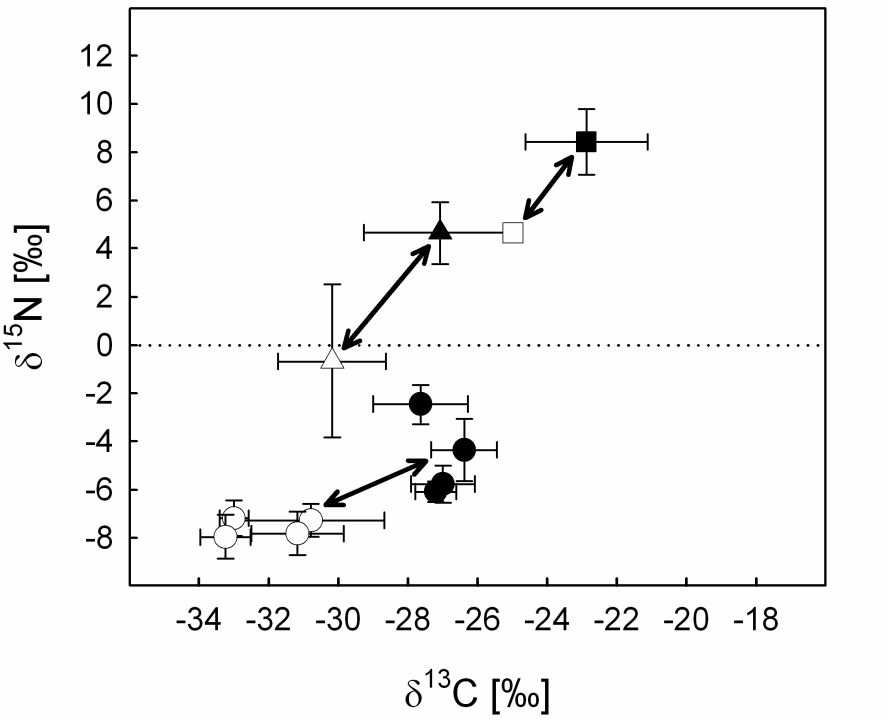

Figure 1. Mean (± 1 SD) values of $\delta^{15}N$ and $\delta^{13}C$ in leaves of two target plant species (triangles), two fully myco-heterotrophic species (squares) and eight fully autotrophic reference species (circles) collected at a beech forest in SE Germany (open symbols) and at a mixed pine forest in S California (closed symbols). Data selected from Zimmer *et al.* (2007). Arrows indicate shifts in $\delta^{15}N$ and $\delta^{13}C$ values of target plants, fully myco-heterotrophic species and autotrophic references between the two study sites.

Calculations using this equation may be biased by the heterogeneity of a study site, e.g., variation in irradiance and soil nutrient availability. These variations, accordingly, may also affect the isotope composition of plant material collected from different plots within such a site. As shown in Figure 1, this effect becomes even more obvious when δ values of plant material from diverse sites, that are located at different continents or climatic regions, are compared. In this example, the Mediterranean climate of S California leads to less negative $\delta^{13}C$ values of plant leaf tissue compared to leaf material of plants sampled in a temperate forest in SE Germany. This shift occurs irrespective of the nutritional mode of the respective plants (autotrophic references, fully myco-heterotrophic species or partially myco-heterotrophic target plants). Moreover, as a result of different soil properties, $\delta^{15}N$ values of plants from the two sites vary significantly as well. These variations in δ values also shift the endpoints of the linear mixing models and it becomes obvious that pooling data from diverse sites to calculate the percentage of fungi-derived C and N in a partial myco-heterotrophic plant will produce misleading results. The same problem applies to an inhomogeneous study site when replicates taken from different plots within that site are pooled.

Another limitation of the conventional approach arises if fully myco-heterotrophic plants are missing at a study site, which frequently is the case. Since δ values of fully myco-heterotrophs are also influenced by environmental parameters, they cannot serve as endpoint for calculations *via* the mixing model for target plants from a different site. Thus, the conventional application of the linear two-source mixing model requires a simultaneous occurrence of plants reflecting both end points of nutrition (i.e., fully autotrophic references and fully myco-heterotrophs) in addition to the target species (i.e., partially myco-heterotrophic plants).

In a recent study, Zimmer *et al*. (2007) showed that the ^{15}N and ^{13}C enrichment of a broad spectrum of fully myco-heterotrophic plants from various sites, relative to their accompanying autotrophic vegetation, is a fairly constant factor and this discovery provides the opportunity to improve the hitherto applied model.

Improved application of the linear two-source mixing model

Conversion of δ values into ε values

Based on common convention, the enrichment factor ε describes the isotope enrichment of a reaction product relative to that of the substrate, as:

$$\varepsilon = (\alpha - 1) \times 1000$$

with α as the isotope fractionation (Högberg, 1997). Thus, ε is also expressed as units per mil (‰) and may be positive or negative. Emmett *et al.* (1998) used ε as the difference between ^{15}N abundance in soil and vegetation to allow for initial differences on soil and plant δ values due to previous land management and soil age. Gebauer & Taylor (1999) calculated ^{15}N enrichment factors for fungal fruit bodies in relation to their substrate to assess if the isotope composition of fungi is also influenced by their mycorrhizal or saprotrophic life form. In the present approach, we define the enrichment factor ε as the relative enrichment in heavy isotopes (^{15}N and ^{13}C) compared to accompanying autotrophic vegetation. This expression of the isotope signature is applied to fully and partially myco-heterotrophic plants and also to autotrophic reference plants.

As already mentioned above and detailed in Gebauer & Meyer (2003), it is important to maintain the spatial resolution of sampling plots as high as possible to minimize impacts caused by variation of environmental parameters. For further applications we therefore suggest the use of normalized enrichment factors based on individual samples from a specific sampling plot as:

$$\varepsilon_{Sx} = \delta_{Sx} - \delta_{REFx}$$

with S as a single value of a sample from an autotrophic, partially myco-heterotrophic or fully myco-heterotrophic plant, x as a specific sampling plot within the entire study site and REF as the mean value of all autotrophic reference plants. Hence, if fully myco-heterotrophic plants are present at the study site, three groups of normalized enrichment factors will result from the conversion: $\varepsilon_R = \varepsilon$ of the autotrophic reference plants (whereas the mean ε_R of all reference plants from a site is always 0 ‰), $\varepsilon_{PMH} = \varepsilon$ of the partially myco-heterotrophic plants and $\varepsilon_{MH} = \varepsilon$ of the fully myco-heterotrophic plants (Figure 2). The percentage nutrient gain from fungi ($\%x_{df}$ with x = N or C) can then be calculated from the proportion between ε_{PMH} and ε_{MH} *via* the rule of three, i.e.,

$$\%x_{df} = (\varepsilon_{PMH} / \varepsilon_{MH}) \times 100.$$

Significant differences from 0 % (no nutrient gain from fungi) and from 100 % (nutrients exclusively derived from mycorrhizal fungi) are verified by statistical tests between ε_{PMH} and ε_{R} or ε_{PMH} and ε_{MH}, respectively, since the enrichment factors already reflect the nutritional mode and the percentage C and N gain is just a more convenient form of data presentation. These site independent enrichment factors can remarkably enhance the application of the linear two-source mixing model.

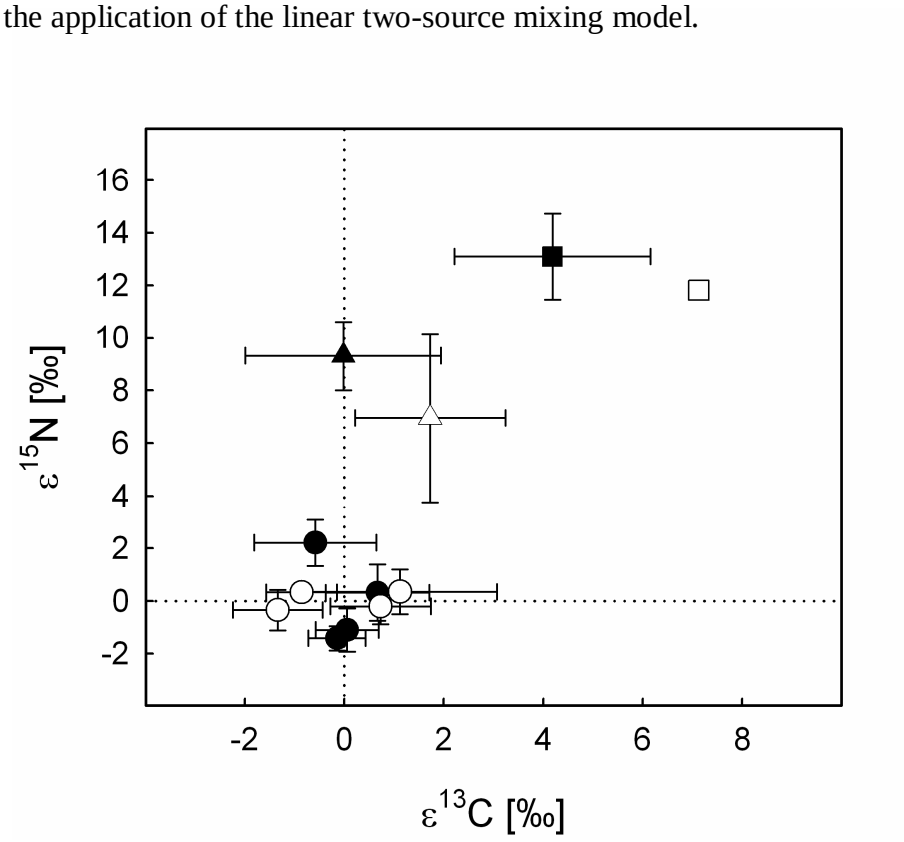

Figure 2. Mean (± 1 SD) enrichment factors (ε) of ^{15}N and ^{13}C in leaves of two target plant species (triangles), two fully myco-heterotrophic species (squares) and eight fully autotrophic reference species (circles) collected at a beech forest in SE Germany (open symbols) and at a mixed pine forest in S California (closed symbols). Data selected and converted from Zimmer *et al*. (2007).

The upper endpoint of the linear two-source mixing model

Based on data from previous studies (see Table 1), enrichment factors ε_{MH} are available for as much as 92 non-photosynthetic individuals from three monotropoid (Ericaceae) and four orchid species. Although the enrichment factors of these single values are normally distributed (Shapiro-Wilk test, $P < 0.05$; Figure 3a,b), there are significant differences in the ^{15}N and ^{13}C enrichment between single species (One-way ANOVA, $P < 0.001$). This effect is not based on systematic differences between species belonging

to the Orchidaceae and Ericaceae (Student's *t*-test, $\varepsilon^{15}N$: $P = 0.991$, $\varepsilon^{13}C$: $P = 0.327$), but reflects a general variation between species. However, due to this species effect and the unequal replications between the different species, an overall mean enrichment factor of fully myco-heterotrophic plants should not be calculated as the mean of the 92 individual values but as mean of the species' particular mean enrichment factors, as shown in Table 1. Although the arithmetic means of the species mean enrichment factors ($\varepsilon_{MH}{}^{15}N = 12.0 \pm 1.7$ ‰ and $\varepsilon_{MH}{}^{13}C = 7.2 \pm 1.6$ ‰) do not account for variances within species, they are close to the weighted means that place greater weight on those values that have less variation ($\varepsilon_{MH}{}^{15}N = 11.9 \pm 0.7$ ‰ and $\varepsilon_{MH}{}^{13}C = 6.9 \pm 0.2$ ‰), and thus still represent a reliable endpoint for the linear mixing model. The advantage of assessing a universal ε_{MH} becomes apparent if fully myco-heterotrophic plants are lacking at a study site or occur in insufficient frequency for statistical analyses.

Table 1. Relative enrichment in ^{15}N and $^{13}C \pm 1$ SD [‰] in leaves of fully myco-heterotrophic Ericaceae (E) and Orchidaceae (O) compared to accompanying autotrophic plants (ε_{MH}). *n* = number of replicates. Data based on literature as indicated by superscript numbers: [1]Zimmer *et al*., 2007; [2]Zimmer *et al*., 2008; [3]Gebauer & Meyer, 2003; [4]Bidartondo *et al*., 2004; [5]Julou *et al*., 2005; [6]Abadie *et al*., 2006. For enrichment factors of all single individuals (*n* = 92) see Appendix A.

Species	$\varepsilon_{MH}{}^{15}N \pm 1$ SD	$\varepsilon_{MH}{}^{13}C \pm 1$ SD	*n*
Monotropa hypopitys (E) [1, 2]	12.1 ± 1.9	8.8 ± 0.9	9
Sarcodes sanguinea (E) [1]	14.6 ± 1.9	5.4 ± 1.4	14
Pterospora andromedea (E) [1]	9.4 ± 1.4	5.3 ± 1.3	9
Corallorhiza maculata (O) [1]	12.8 ± 2.0	7.2 ± 0.7	10
Neottia nidus-avis (O) [1,2,3,4]	10.5 ± 3.0	8.1 ± 0.7	31
Cephalanthera damasonium albino (O) [5]	13.1 ± 1.5	9.2 ± 0.9	10
Cephalanthera longifolia albino (O) [6]	11.8 ± 1.6	6.6 ± 0.2	9
Arithmetic mean	12.0 ± 1.7	7.2 ± 1.6	

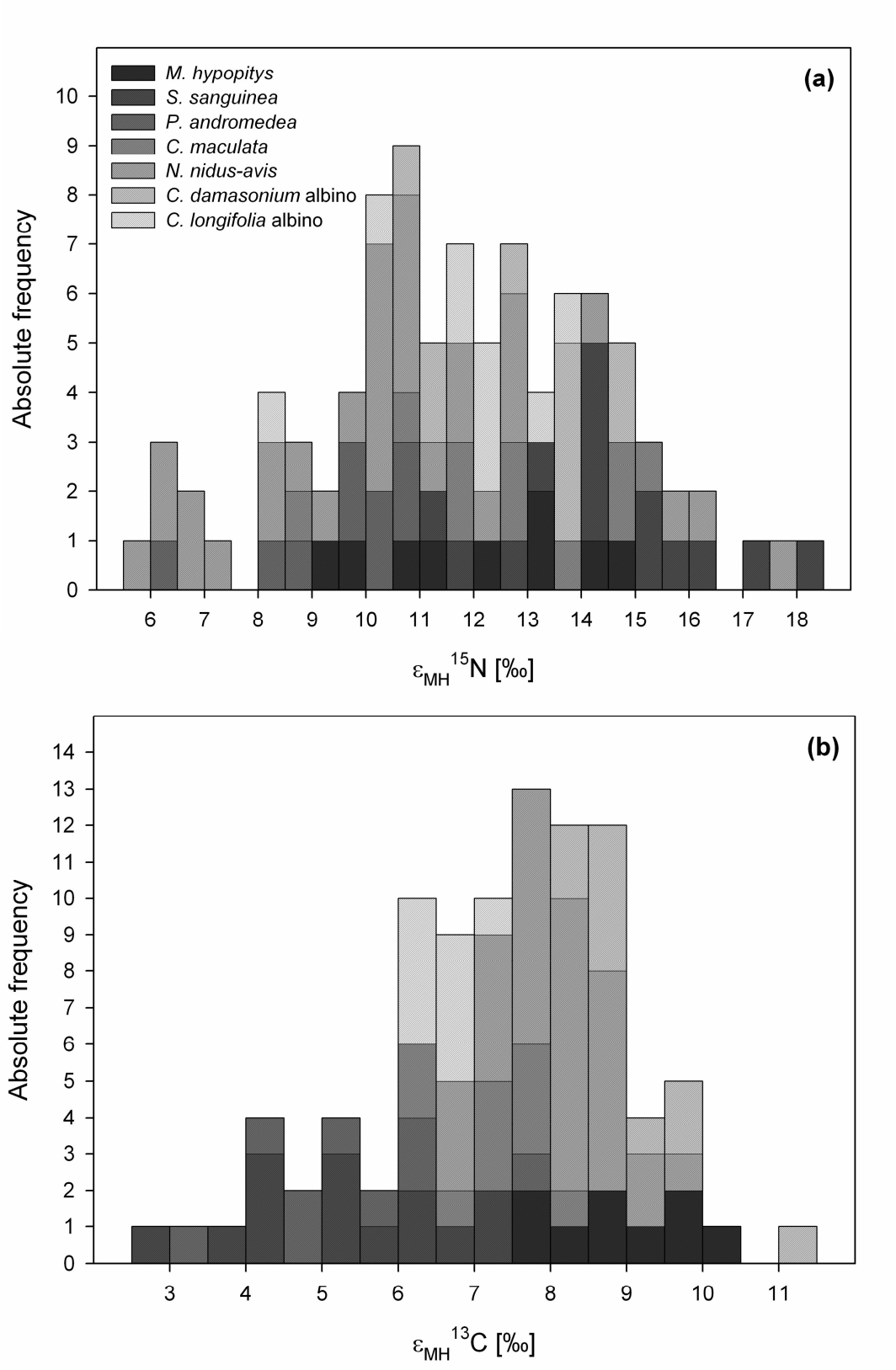

Figure 3. Absolute frequency of ^{15}N (a) and ^{13}C (b) enrichment factors (ε) of seven obligate myco-heterotrophic plant species or albino varieties, respectively, from two plant families (total *n* = 92, see Appendix A). Ericaceae: *M. hypopitys*, *Monotropa hypopitys*; *S. sanguinea*, *Sarcodes sanguinea*; *P. andromedea*, *Pterospora andromedea*. Orchidaceae: *C. maculata*, *Corallorhiza maculata*; *N. nidus-avis*, *Neottia nidus-avis*; *C. damasonium*, *Cephalanthera damasonium*; *C. longifolia*, *Cephalanthera longifolia*.

Advantages of the normalization approach

The hitherto method implied the pooling of autotrophic reference plants from a study site irrespective of their sampling plot within that site. Subsequently, individual values of partially myco-heterotrophic plants from specific sampling plots were compared to the pooled references, leading to a certain information loss due to lower resolution. The approach presented here leads to more precise estimates of the plants' percentage nutrient gain from fungi since ε values are calculated for each individual plant sample relative to references from its respective sampling plot within the study site.

The use of site independent enrichment factors (mean ε_R is always 0 ‰) instead of δ values also allows meta-analyses between very diverse sites and the representation of multiple datasets within one graph. Furthermore, the standardized enrichment factors provide the possibility for calculation and statistical validation of the percentage C and N gain of partially myco-heterotrophic plants, even without occurrence of neighboring fully myco-heterotrophic plants, by referring to ε_{MH} available from the literature (Table 1, Figure 4).

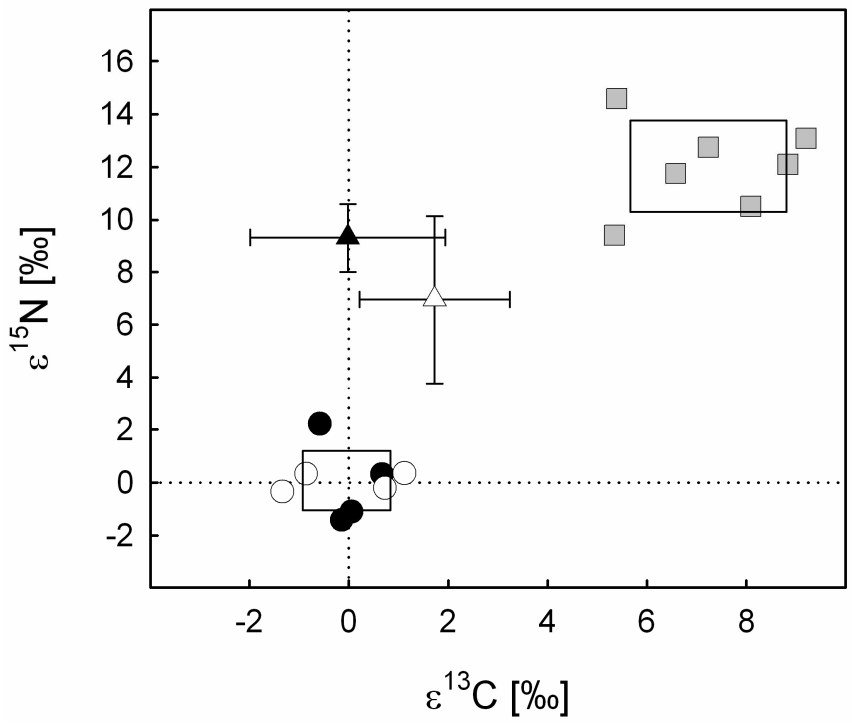

Figure 4. Mean enrichment factors (ε) of ^{15}N and ^{13}C in leaves of two target plant species (triangles) and eight fully autotrophic reference species (circles) collected at a beech forest in SE Germany (open symbols) and at a mixed pine forest in S California (closed symbols); data selected and converted from Zimmer *et al*. (2007). Grey squares represent the mean enrichment factors of all obligate myco-heterotrophic plant species of which data is available from literature (see Table 1, Appendix A). Error bars correspond to 1 SD of the symbol value; boxes represent 1 SD of the mean value from a group of symbols.

Since it is shown that the enrichment in ^{15}N and ^{13}C of fully myco-heterotrophic plants compared to autotrophic vegetation differs significantly between species, one could also argue that the universal ε_{MH} which refers to all hitherto published data is generally the most unbiased and hence best suited upper endpoint for the linear mixing model. However, based on the current data (Appendix A), the universal ε_{MH} should always be adjusted by further information that will arise in the future.

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Appendix

Appendix A. Relative enrichment in ^{15}N and ^{13}C [‰] in leaves of 92 fully myco-heterotrophic individuals from the Ericaceae and Orchidaceae compared to accompanying autotrophic plants (ε_{MH}). Data based on literature.

Species / (Family)	$\varepsilon_{MH}{}^{15}N$	$\varepsilon_{MH}{}^{13}C$	Means published in	Site
Monotropa hypopitys (Ericaceae)	14.3	7.7	Zimmer *et al.*(2007)	*Pinus sylvestris*/ *Quercus robur* forest (SE Germany)
	9.4	8.0		
	14.7	7.8		
	12.2	9.6		
	9.8	8.5		
	11.5	8.9	Zimmer *et al.*(2008)	*Fagus sylvatica* forest (SE Germany)
	13.0	9.1		
	10.9	9.8		
	13.2	10.0		
Sarcodes sanguinea (Ericaceae)	11.9	4.5	Zimmer *et al.*(2007)	*Pinus ponderosa*/ *Quercus kelloggii* forest (S California)
	18.2	4.5		
	15.7	3.8		
	17.2	5.4		
	15.4	5.7		
	14.1	5.2		
	15.4	2.7		
	11.2	6.9		
	12.7	4.2		
	16.2	7.2		
	13.5	7.3		
	14.2	6.5		
	14.4	6.5		
	14.1	5.1		
Pterospora andromedea (Ericaceae)	8.3	4.5	Zimmer *et al.*(2007)	*Pinus ponderosa*/ *Quercus kelloggii* forest (S California)
	10.5	5.0		
	9.9	5.1		
	10.1	3.1		
	10.6	4.5		
	9.6	5.9		
	8.6	6.1		*Abies concolor*/ *Pseudotsuga menziesii* forest (N California)
	10.6	6.3		
	6.3	7.6		
Corallorhiza maculata (Orchidaceae)	11.9	6.3	Zimmer *et al.*(2007)	*Pinus ponderosa*/ *Quercus kelloggii* forest (S California)
	15.3	7.1		
	9.0	7.3		
	12.6	6.7		
	10.7	6.1		
	14.8	7.7		
	13.8	7.3		
	11.9	7.9		
	14.5	7.8		
	13.0	8.1		

(Continued)

Appendix A. Continued

Species / (Family)	$\varepsilon_{MH}{}^{15}N$	$\varepsilon_{MH}{}^{13}C$	Means published in	Site
Neottia nidus-avis (Orchidaceae)	10.6	8.3	Zimmer *et al.*(2007)	*Fagus sylvatica* forest (SE Germany)
	8.4	7.7		
	6.2	7.3		
	5.7	8.7		
	7.1	7.5		
	9.2	7.8		*Fagus sylvatica* forest clearing (SE Germany)
	10.4	7.1		
	10.7	9.1		
	11.3	7.9		
	16.0	8.1	Gebauer & Meyer (2003)	*Fagus sylvatica* forest (SE Germany)
	10.0	8.5		
	14.3	8.1		
	11.7	9.4		
	8.4	7.9		
	10.5	8.7	Bidartondo *et al.* (2004)	open *Pinus sylvestris* forest (SE Germany)
	12.9	8.7		
	10.1	7.9		
	10.0	8.1		
	11.5	7.5	Zimmer *et al.*(2008)	*Fagus sylvatica* forest (SE Germany)
	8.6	6.9		
	6.6	6.6		
	6.4	7.7		
	7.0	6.9		
	9.7	8.4		
	10.9	7.9		
	10.8	9.8		
	12.1	8.4		
	17.9	8.4		
	15.7	8.3		
	12.8	8.6		
	12.7	8.6		
albino *Cephalanthera damasonium* (Orchidaceae)	13.0	9.5	Julou *et al.*(2005)	*Quercus robur*/ *Corylus avellana* forest (N France)
	13.6	8.7		
	10.6	8.3		
	13.9	9.9		
	11.3	8.9		
	13.6	8.4		
	14.8	10.0		
	14.9	11.0		
	13.6	8.5		
	11.3	8.9		
albino *Cephalanthera longifolia* (Orchidaceae)	8.4	6.3	Abadie *et al.*(2006)	*Juniperus communis*/ *Pinus sylvestris* shrubland (Estonia)
	13.4	6.8		
	13.8	6.8		
	12.0	6.5		
	12.1	6.6		
	12.0	6.4		
	10.2	6.6		
	12.0	6.3		
	12.0	7.0		

CHAPTER 2

Full myco-heterotrophy in the ericaceous tribe Pyroleae

Isotopic evidence of full and partial myco-heterotrophy in the plant tribe Pyroleae (Ericaceae)

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Key words: Ericaceae, Pyroleae, *Pyrola*, pyroloids, myco-heterotrophy, mixotrophy, ^{15}N and ^{13}C, mycorrhiza

Abstract

Botanists and mycologists have long debated the potential for full myco-heterotrophy in the achlorophyllous *Pyrola aphylla* (Ericaceae). Here we address the ecophysiology of this putative myco-heterotroph and two other closely related green species in the tribe Pyroleae (*Pyrola picta*, *Chimaphila umbellata*).

The stable isotopes of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) were analyzed from 10 populations of Pyroleae species in California and Oregon. For all populations isotope signatures were tested for significant differences between *P. aphylla*, green pyroloids, surrounding autotrophs and obligate myco-heterotrophs.

Throughout all populations *P. aphylla* was most similar to myco-heterotrophs that associate with ectomycorrhizal fungi in its ^{13}C signature (average enrichment $\varepsilon^{13}C = 6.9 \pm 0.9$ ‰) and even more enriched in ^{15}N than many previously recorded myco-heterotrophic species (average enrichment $\varepsilon^{15}N = 18.0 \pm 2.2$ ‰). The two green Pyroleae species were not enriched in ^{13}C compared to the autotrophic understory (*C. umbellata* average enrichment $\varepsilon^{13}C = -0.5 \pm 1.0$ ‰ and *P. picta* average $\varepsilon^{13}C = 0.3 \pm 1.4$ ‰) and their ^{15}N signatures were similar to myco-heterotrophs that associate with ectomycorrhizal fungi (*C. umbellata* average enrichment $\varepsilon^{15}N = 10.6 \pm 1.6$ ‰ and *P. picta* average $\varepsilon^{15}N = 10.6 \pm 1.9$ ‰).

This is the first study to analyze the isotope signatures of *P. aphylla* from a wide geographic region and our results confirm the variable trophic strategies of adult plants within the Pyroleae and the myco-heterotrophic status of *P. aphylla*.

Introduction

The physiology and taxonomy of pyroloids (species within the tribe Pyroleae, family Ericaceae) has confounded researchers for over 200 years (Jussieu, 1789; Holm, 1898; Henderson, 1919; Camp, 1940; Haber, 1987). The debate over the taxonomy of pyroloids has been partially fueled by the occurrence of leafless forms of plants within the genus *Pyrola* that are potentially myco-heterotrophic. In particular the leafless form of *P. picta* Sm. referred to here as *P. aphylla* Sm. (Figure 1) is thought by some researchers to be an extreme morphological variant of *P. picta* that receives nutrition through parasitizing its mycorrhizal associates (Camp, 1940). Conversely, Haber (1987) considered *P. aphylla* flower stalks to be connected *via* a rhizome to *P. picta* rosettes

that are responsible for photosynthesis for the entire plant, while Smith (1814) considered them discrete individuals and therefore physiologically independent.

Figure 1. Photographs of *Pyrola aphylla*, its rare 'leafy' form, and *P. picta*. From left to right: flowering stalks of *Pyrola aphylla* (inset, close-up of flowers), *P. aphylla* with small leaves (arrow) and a rosette of *P. picta*.

Smith's (1814) determination of *P. picta* and *P. aphylla* as separate species is supported by the existence of *P. aphylla* populations in the absence of *P. picta* plants (Haber, 1987; own pers. obs.). This observation also supports the potential for myco-heterotrophy in *P. aphylla*. Obligate myco-heterotrophy entails a complete dependence on organic nutrient gains *via* a symbiosis with a fungus (Leake, 1994). In many cases these plants are actually 'epiparasites' that receive the majority of their carbon indirectly from surrounding autotrophic plants through a shared mycorrhizal fungus (Taylor *et al.*, 2002), but even in these cases nitrogen is received directly from the fungus (Leake, 1994).

Recently, Freudenstein (1999) and Kron *et al*. (2002) used phylogenetic methods to support the placement of pyroloids in their own tribe: the Pyroleae, which is one of three tribes within the subfamily Monotropoideae. However, the evolutionary relatedness of the tribes in Monotropoideae, and the phylogenetic delimitation of species in the *P. picta*/*P. aphylla* complex has yet to be determined. Despite their unresolved taxonomy pyroloids are also of particular interest to those who study the ecology and evolution of myco-heterotrophy as the tribe contains closely related taxa

that are all myco-heterotrophic in their early stages of development (Leake, 1994), but upon reaching adulthood appear to occupy the full spectrum of trophic habits from autotrophy to mixotrophy (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007) to potentially full myco-heterotrophy in *P. aphylla*. From an evolutionary perspective the variety of trophic abilities in the Pyroleae is intriguing as the tribes' two closest relatives the Monotropeae and the Pterosporeae contain only obligate myco-heterotrophic species (Kron & Johnson, 1997; Freudenstein, 1999). The ecological factor(s) driving the variability in photosynthetic abilities between closely related Pyroleae species remain elusive, but it has been proposed that both limited light availability and the presence of particular mycobionts may be responsible (Bidartondo *et al.*, 2004; Julou *et al.*, 2005).

In this study rather than using a phylogenetic approach to examine evolutionary relationships between pyroloids (this has been done to some extent by Freudenstein, 1999) we chose to address the ecophysiology of these plants through the analysis of the natural abundances of the stable isotopes of carbon (^{13}C:^{12}C) and nitrogen (^{15}N:^{14}N) of pyroloids, surrounding autotrophs and obligate myco-heterotrophs. The analysis of the natural abundance of stable isotopes in plants is a powerful tool to distinguish carbon sources and metabolic pathways (Farquhar *et al.*, 1989; Dawson *et al.*, 2002). Previous work has shown that obligate myco-heterotrophic plants that associate with ectomycorrhizal fungi are significantly enriched in the heavy isotopes of C and N compared to autotrophic understory plants, and have C and N isotope signatures similar to ectomycorrhizal fungi, their sole carbon and nitrogen source (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005). It has also been reported that some green orchids and pyroloids that associate with ectomycorrhizal fungi have carbon isotope values that are intermediate between autotrophs and myco-heterotrophs (Gebauer & Meyer, 2003; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). This finding indicates that these green plants can utilize at least two different trophic pathways and therefore tap into isotopically distinct C and N sources. One trophic pathway available to these plants is C gain through ectomycorrhizal fungi and nitrogen gain through a distinct (but undetermined) pathway compared to autotrophs, while the other pathway available is similar to that of autotrophic mycorrhizal plants. Plants that are capable of gaining nutrition through both of these complementary routes are referred to as mixotrophs or partial myco-heterotrophs (Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). The relative enrichment in ^{13}C of mixotrophic orchids and pyroloids compared to

neighboring autotrophic plants appears to be site specific and possibly influenced by light availability (Bidartondo *et al.*, 2004; McCormick *et al.*, 2004; Julou *et al.*, 2005; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). Mixotrophic plants that associate with ectomycorrhizal fungi are also enriched in ^{15}N compared to surrounding autotrophic plants (Gebauer & Meyer, 2003). The mixotrophic abilities of pyroloids have been at the center of current debate because based on carbon stable isotope abundances the same species from different geographic regions appear to have varying degrees of mixotrophy (Tedersoo *et al*., 2007; Zimmer *et al*., 2007). The potential reasons for this variability among green pyroloids are further addressed here.

The goal of this study was to determine the trophic strategies of the green pyroloid *P. picta* and the achlorophyllous *P. aphylla*. In a previous study (Zimmer *et al*., 2007), both *P. aphylla* and *P. picta* were analyzed for their stable isotope values of C and N from a single site in northern California. The results of this work found *P. aphylla* to have isotope signatures for both elements that were similar to other ericaceous myco-heterotrophs; while *P. picta* had a C isotope signature similar to surrounding autotrophs, but was enriched in ^{15}N similar to myco-heterotrophs that associate with ectomycorrhizal fungi. However, this study was based on a small sampling of the two *Pyrola* species, so the relevance of these findings to the overall distribution of the species is currently unknown. In the present study we sought to confirm these findings by determining the stable isotope signatures of C and N for *P. picta* and *P. aphylla* from more intensively sampled populations as well as sampling over a wider geographic region, and including an additional green pyroloid species (*Chimaphila umbellata*) whose isotope values have only been previously examined from a Bavarian forest. We then compared the isotope signatures of *P. aphylla*, *P. picta*, and *C. umbellata* to each other, and to autotrophic and obligate myco-heterotrophic plants to test for myco-heterotrophy and mixotrophy in the Pyroleae.

Materials and Methods

Study sites

To examine the trophic strategies of pyroloids from a wide geographic area of their natural ranges samples were collected from six National Forests in northern California and southern Oregon including El Dorado, Tahoe, Plumas, Lassen, Shasta and Willamette. The selection of sampling sites (P1-P10) was based on the presence of the target Pyroleae species: *Pyrola aphylla* Sm. and *Pyrola picta* Sm.. All sites are

dominated by second-growth mixed conifer forest at elevations between 700 and 1400 m. Locations and species collected are summarized in Table 1.

Sampling scheme and species investigated

All samples were collected within an eight-day period from June 30 - July 7, 2006. Collection of target species' leaves or flower stalks, autotrophic reference plants' leaves and myco-heterotrophic plants' flower stalks was limited to an area of 2 meters from a target species individual and sampling of autotrophic references was done only from understory saplings. This strategy was used to limit the variability of environmental factors such as atmospheric CO_2 concentrations and isotope signatures that could affect plant carbon isotope values or soil type that could affect nitrogen isotope values (Gebauer & Schulze, 1991). However, variation in the nitrogen isotope values of our collected samples due to possible differences in rooting depths of the plants were not accounted for (Robinson, 2001). Each collection site contained *P. aphylla* or *P. picta,* or both, plus a minimum of five individuals of at least one species that could be used as reference plants representing the autotrophic understory (Table 1). To test for differences in the isotope values between plant organs, whenever possible flowering stalks from *P. picta* were collected and analyzed separately from leaves (Table 1). Four sites (P1, P2, P6 and P7) contained the obligate myco-heterotrophic species *Pterospora andromedea* Nutt. and *Corallorhiza maculata* Raf. and four sites (P1, P5, P6 and P7) contained the green pyroloid *Chimaphila umbellata* (L.) W. Bartram, (Table 1). A total of 37 *P. aphylla*, 42 *P. picta*, 18 *Chimaphila umbellata* individuals along with 17 obligate myco-heterotrophic plants of two different species, and 65 autotrophic reference plants of six species were collected.

Stable isotope analysis

Plant samples were oven-dried at 37ºC and ground to a fine powder. Dried and ground samples were analyzed for nitrogen and carbon stable isotope abundances *via* elemental analyzer/continuous flow isotope ratio mass spectrometry at either the BayCEER - Laboratory of Isotope Biogeochemistry University of Bayreuth, Germany as described by Bidartondo *et al.* (2004) or at the Center for Stable Isotope Biogeochemistry at University of California Berkeley. Both labs used a dual element analysis mode with a continuous flow mass spectrometer coupled to an elemental analyzer (Berkeley: Europa ANCA - SL elemental analyzer coupled to a PDZ Europa Scientific 20/20 Mass Spectrometer, UK; BayCEER: Carlo Erba 1108, Milano, Italy coupled *via* a ConFlo III

interface to a delta S, Finnigan MAT Bremen). Measured abundances are denoted as δ values and are calculated according to the equation: $\delta^{15}N$ or $\delta^{13}C = (R_{sample}/R_{standard}-1) \times 1000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard. At the University of Bayreuth standard gases were calibrated with respect to international standards by using the reference substances N1 and N2 for the nitrogen isotopes and ANU sucrose and NBS 19 for the carbon isotopes (standards from the International Atomic Agency, Vienna, Austria). At the University of California Berkeley standards N2 and NIST 1577 bovine liver, or NIST 1547 peach leaf and corn flour, were used for nitrogen and carbon isotope calibrations, respectively (standards from the National Institute of Standards and Technology, Gaithersberg, MD, USA). In the Bayreuth laboratory reproducibility and accuracy of the isotope abundance measurements were routinely controlled by measures of the test substance acetanilide (Gebauer & Schulze, 1991). At least six test substances with varying sample weight were routinely analyzed within each batch of 50 samples. Maximum variation of $\delta^{13}C$ and $\delta^{15}N$ within as well as between batches was always below 0.2 ‰. In the Berkeley laboratory the long-term precisions for $\delta^{13}C$ and $\delta^{15}N$ based on the laboratory's working standards (NIST 1577 bovine liver and sucrose solution) are: 0.1 ‰ for $\delta\ ^{13}C$ and 0.2 ‰ for $\delta\ ^{15}N$. Differences between the two laboratories are not to be expected because both laboratories refer to internationally accepted standards.

Table 1. Location, species, number of individuals (*n*), plant parts collected for stable isotope analysis at each sampling site (P1-P10), and mean $\delta^{15}N$ [‰] and $\delta^{13}C$ [‰] values ±1 SD. [a], pyroloid, [b]; myco-heterotroph; [c], autotroph, NF, National Forest.

Site	Location	Species (*n*)	Plant part	$\delta^{15}N$	$\delta^{13}C$
P1	El Dorado NF, CA	*Abies concolor*[c] (5)	Leaves	-3.4±0.9	-31.0±0.3
	38˚54'01.70"N	*Chimaphila umbellata*[a] (4)	Leaves	6.7±0.9	-31.2±0.4
	120˚34'26.77"W	*Corallorhiza maculata*[b] (1)	Stalk/Flower	10.9	-20.7
		Pterospora andromedea[b] (1)	Stalk/Flower	5.7	-24.2
		Pyrola aphylla[a] (6)	Stalk/Flower	16.4±2.3	-24.0±0.5
		Pyrola aphylla (1)	Leaves	12.4	-24.7
		Pyrola picta[a] (1)	Leaves	5.6	-31.9
P2	El Dorado NF, CA	*A. concolor*[c] (5)	Leaves	-4.0±0.7	-30.6±0.7
	38˚54'3.47"N	*P. andromedea*[b] (3)	Stalk/Flower	4.8±1.1	-24.9±0.6
	120˚34'28.40"W	*P. aphylla*[a] (1)	Stalk/Flower	17.9	-22.2
		P. picta[a] (4)	Leaves	4.9±1.2	-31.9±0.5
		Ribes roezlii[c] (5)	Leaves	-4.3±1.2	-31.4±0.6

(Continued)

Table 1. Continued

Site	Location	*Species (n)*	Plant Part	$\delta^{15}N$	$\delta^{13}C$
P3	Tahoe NF, CA	*A. concolor*[c] (5)	Leaves	-3.9±0.7	-31.0±0.8
	39˚31'2.84"N	*Lithocarpus densiflora*[c] (5)	Leaves	-4.1±1.5	-30.1±0.9
	120˚59'26.46"W	*P. aphylla*[a] (3)	Stalk/Flower	13.8±0.2	-24.3±0.5
P4	Tahoe NF, CA	*L. densiflora*[c] (5)	Leaves	-3.7±1.0	-30.9±0.4
	39˚31'37.64"N	*P. aphylla*[a] (6)	Stalk/Flower	13.9±3.4	-23.6±0.2
	120˚59'25.47"W	*P. aphylla* (1)	Leaves	9.0	-27.1
		P. picta[a] (3)	Leaves	8.6±1.0	-29.1±2.0
P5	Plumas NF, CA	*A. concolor*[c] (5)	Leaves	-3.8±1.1	-30.4±1.0
	40˚03'36.02"N	*C. umbellata*[a] (4)	Leaves	6.2±1.9	-30.4±1.6
	120˚51'32.99"W	*P. aphylla*[a] (3)	Stalk/Flower	13.7±1.5	-23.0±0.4
P6	Plumas NF, CA	*A. concolor*[c] (5)	Leaves	-5.5±0.9	-30.0±1.0
	40˚03'29.94"N	*C. umbellata*[a] (5)	Leaves	6.3±1.8	-30.5±0.9
	120˚51'28.86"W	*P. andromedea*[b] (3)	Stalk/Flower	5.4±0.9	-28.0±0.1
		P. aphylla[a] (2)	Stalk/Flower	10.4	-24.7
			Stalk/Flower	10.1	-23.9
		P. picta[a] (12)	Leaves	5.0±0.8	-30.3±0.9
		P. picta (2)	Stalk/Flower	5.3	-28.5
			Stalk/Flower	4.5	-30.6
P7	Plumas NF, CA	*A. concolor*[c] (5)	Leaves	-3.7±1.0	-31.6±1.0
	40˚04'00.17"N	*C. maculata*[b] (4)	Stalk/Flower	11.9±1.1	-25.8±0.3
	120˚51'4.17"W	*C. umbellata*[a] (5)	Leaves	6.5±1.5	-32.8±0.6
		P. andromedea[b] (5)	Stalk/Flower	5.5±1.0	-26.6±0.4
		P. aphylla[a] (2)	Stalk/Flower	15.0	-25.2
			Stalk/Flower	14.1	-25.7
		P. picta[a] (6)	Leaves	8.2±1.4	-32.0±1.5
		P. picta (1)	Stalk/Flower	7.6	-31.7
P8	Lassen NF, CA	*A. concolor*[c] (5)	Leaves	-2.9±0.9	-30.9±0.7
	40˚13'39.97"N	*P. aphylla*[a] (5)	Stalk/Flower	13.7±1.1	-23.9±0.7
	121˚11'03.99"W	*P. picta*[a] (10)	Leaves	7.7±3.1	-29.8±1.4
P9	Shasta NF, CA	*P. aphylla*[a] (5)	Stalk/Flower	15.5±0.9	-24.3±1.5
	41º00'44.90"N	*Pseudotsuga menziesii*[c] (5)	Leaves	-5.3±0.8	-30.7±0.7
	121º39'13.35"W	*Quercus kelloggii*[c] (5)	Leaves	-2.5±1.0	-30.8±0.3
P10	Willamette NF, OR	*P. aphylla*[a] (4)	Stalk/Flower	14.6±1.8	-24.6±0.2
	44º18'36.00"N	*P. picta*[a] (5)	Leaves	7.8±1.1	-31.2±1.0
	122º00'36.02"W	*Tsuga heterophylla*[c] (5)	Leaves	-2.2±0.8	-31.9±1.9

Statistics

Once δ values were obtained for all samples (Table 1), for each collection site the $\delta^{15}N$ and $\delta^{13}C$ values of all reference plants were tested for inter-site variation with a one-way ANOVA and Tukey's HSD. Due to significant differences at α 0.05 among $\delta^{15}N$ values of the reference plants between sites (P6-P8 $P = 0.036$, P6-P10 $P = 0.002$) the δ

values could not be pooled to make comparisons across sites. In order to make these comparisons δ values for both elements and all samples were converted into site-independent enrichment factors (ε). The calculation of enrichment factors is a useful method that eliminates the majority of the influence of spatial variation on isotope abundances and therefore allows for comparison among samples from different sites (Emmett *et al.*, 1998; Preiss & Gebauer, 2008) or substrates (Gebauer & Taylor, 1999). First, for each site the $\delta^{15}N$ and $\delta^{13}C$ values of all species of reference plants were averaged. Then, on a per site basis these averages were subtracted from all samples (pyroloids, reference and myco-heterotrophic plants) to create site independent enrichment factors ($\varepsilon = \delta x_S - \delta x_R$) for each sample, where $\delta x_S = \delta^{15}N$ or $\delta^{13}C$ of individual sample per site and δx_R= mean $\delta^{15}N$ or $\delta^{13}C$ of all reference plants per site. Thus, the resulting means of both the $\varepsilon^{13}C$ and $\varepsilon^{15}N$ factors of the reference plants is equal to 0 ‰ and individual samples' ε factors represent their difference from this mean. To appropriately test for differences between trophic groups (pyroloids, references, and myco-heterotrophic plants) the variance around the mean δ values of the autotrophic references used to calculate ε for pyroloids and myco-heterotrophs must be retained. This is done through calculating ε not for only pyroloids and myco-heterotrophs, but as mentioned above, for the reference samples as well. Where the individual $\varepsilon^{15}N$ and $\varepsilon^{13}C$ factors of each autotrophic reference plant sampled represents the variance of these samples' δ values from the mean $\delta^{15}N$ or $\delta^{13}C$ of all references per a site. Furthermore, both the intersite and intrasite standard deviation of the $\varepsilon^{15}N$ and $\varepsilon^{13}C$ factors for all reference species is small (≤ 1 ‰ for both ^{15}N and ^{13}C, Table 1). Statistical comparisons between all ε factors per group (pyroloids, myco-heterotrophic plants and autotrophic references) were made using non-parametric Kruskal-Wallis and sequential Bonferroni-corrected Mann-Whitney U tests for *post hoc* comparisons. To make more robust comparisons between pyroloids and obligate myco-heterotrophs in additin to the two myco-heterotrophic species (*P. andromedea* and *C. maculata*) collected at our sites we included the ε factors of seven fully myco-heterotrophic species *C. maculata* (*n* = 12), *Sarcodes sanguinea* Torr. (*n* = 14), *P. andromedea* (*n* = 13), *Neottia nidus-avis* (L.) Rich. (*n* = 31), *Monotropa hypopitys* L. (*n* = 9), *Cephalanthera damasonium* L. albino (*n* = 10) and *C. longifolia* (L.) Fritsch albino (*n* = 9) from previously published data (Preiss & Gebauer, 2008). For clarity, ε factors of all species collected are reported in the results section and presented in Figure 2 as species

means ± 1 SD. In addition, ε factors of the flowering stalks of *P. picta* were compared to those of their leaves and *P. aphylla* stalks from plots P6 and P7 using independent t-tests.

Results

Comparison of isotope signatures between trophic groups

The δ values of reference plants and myco-heterotrophic plants collected at our sites were within the range of previous records from temperate forests (Trudell *et al.*, 2003; Zimmer *et al.*, 2007; Table 1). The enrichment factors (ε) of individual reference plants clustered around 0 ‰, reflecting the small inter- and intraspecific variations in their isotope signatures that were not significantly different between sites, while enrichment factors for the other groups (pyroloids and myco-heterotrophs) separated out into distinct groups based on the difference of their δ values from the mean of their respective references (Figure 2). Across all sites the two green Pyroleae species were as strongly enriched in ^{15}N as the obligate myco-heterotrophs (*C. umbellata* average $\varepsilon^{15}N$ = 10.6 ± 1.6 ‰ and *P. picta* average $\varepsilon^{15}N$ = 10.6 ± 1.9 ‰, Figure 2). However, these two species were not enriched in ^{13}C compared to autotrophic reference plants (*C. umbellata* average $\varepsilon^{13}C$ = -0.5 ± 1.0 ‰ and *P. picta* average $\varepsilon^{13}C$ = 0.3 ± 1.4 ‰, Figure 2). In contrast, across all sites the achlorophyllous *P. aphylla* had a ^{13}C signature typical for myco-heterotrophic species associated with ectomycorrhizal fungi (average $\varepsilon^{13}C$ = 6.9 ± 0.9 ‰, Figure 2, Appendix A) and was enriched in ^{15}N (average $\varepsilon^{15}N$ = 18.0 ± 2.2 ‰ Figure 2, Appendix A) compared to other pyroloids and surrounding autotrophs, similar to the findings of Zimmer *et al.* (2007).

Interestingly, we did find two *P. aphylla* plants in sites P1 and P4 that had very small basal leaves (Figure 1). These leaves were analyzed separately for their isotope abundances. They were found to be similar to stalks of other *P. aphylla* collections for nitrogen (P1 $\varepsilon^{15}N$ = 15.8 ‰, P4 $\varepsilon^{15}N$ = 12.8 ‰), and similar (P1 $\varepsilon^{13}C$ = 6.3 ‰) or less enriched in ^{13}C (P4 $\varepsilon^{13}C$ = 3.8 ‰) indicating that at least in the latter individual extremely low levels of photosynthesis may still be taking place, similar to the leafless stems of *Corallorhiza trifida* (Zimmer *et al.*, 2008).

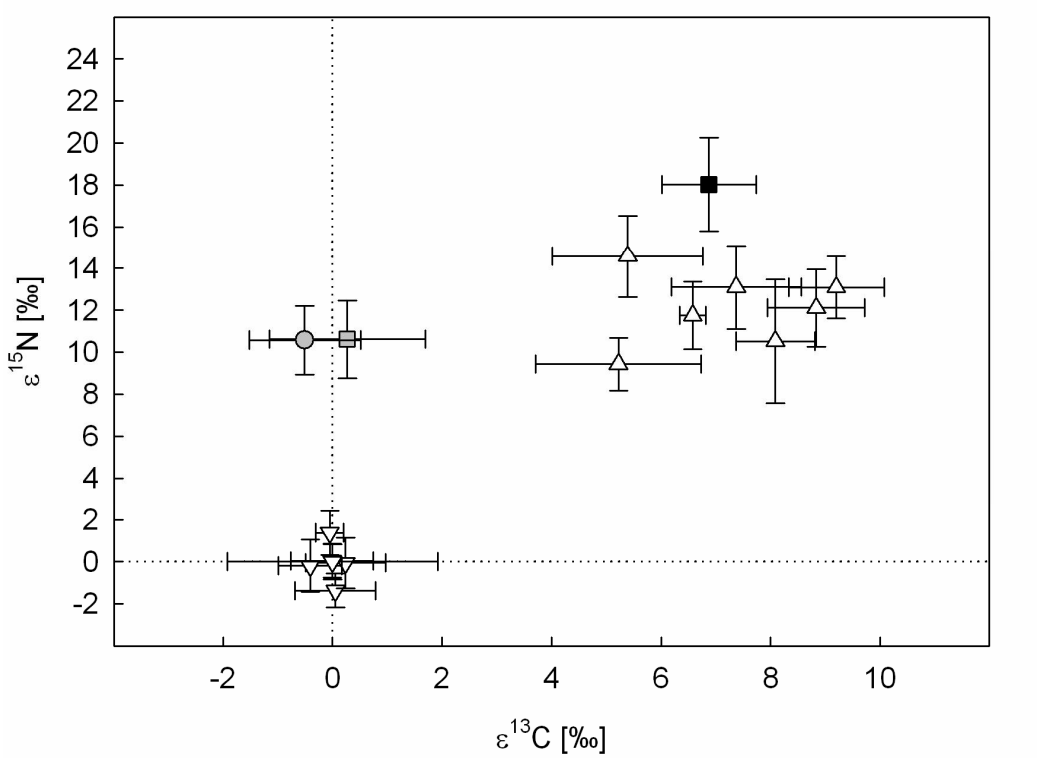

Figure 2. Mean ^{13}C and ^{15}N enrichment factors (ε) of all species analyzed: autotrophic reference plants (open triangles down, two species values overlap), *Chimaphila umbellata* (gray circle), *Pyrola picta* (gray square), *Pyrola aphylla* (black square), myco-heterotrophic plants (open triangles up) including *Pterospora andromedea* and *Corallorhiza maculata* from this and previously published studies (Preiss & Gebauer, 2008) and five additional species (*Sarcodes sanguinea*, *Neottia nidus-avis*, *Monotropa hypopitys*, *Cephalanthera damasonium* albino, and *C. longifolia* albino) from Preiss & Gebauer (2008). Error bars represent 1 SD.

Independent t-tests revealed that comparisons of the enrichment factors of the flowering stalks of *P. picta* (average $\varepsilon^{15}N = 10.7 \pm 0.6$ ‰, $\varepsilon^{13}C = 0.2 \pm 1.1$ ‰) and *P. aphylla* (average $\varepsilon^{15}N = 17.0 \pm 1.5$ ‰, $\varepsilon^{13}C = 5.9 \pm 0.5$ ‰) at α 0.05 were significantly different from each other for both elements ($\varepsilon^{13}C$: $P < 0.001$ and $\varepsilon^{15}N$: $P = 0.008$) and the isotope signatures of the stalks from *P. picta* were not statistically different from the leaves (average $\varepsilon^{15}N = 11.1 \pm 1.3$ ‰, $P = 0.639$; $\varepsilon^{13}C = -0.4 \pm 1.2$ ‰, $P = 0.452$). However, these tests were done with very low sample sizes as flowering stalks of *P. picta* were only collected from three plants in two sites (Table 1).

Discussion

Pyrola aphylla exhibited enrichment in ^{15}N that exceeds that of associated photosynthetic plants, other species in the Pyroleae, and even most other analyzed myco-heterotrophs. While the cause for this enrichment is unclear, it follows both the

pattern of ^{15}N enrichment found in green mixotrophic Pyroleae species (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007; data presented here) and all previously analyzed myco-heterotrophic plants that associate with ectomycorrhizal fungi (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Zimmer *et al.*, 2007). Possible mechanisms that could be driving the high ^{15}N enrichment found in myco-heterotrophs relative to autotrophs include a difference in the physiological processing of nitrogen by mycorrhizal fungi when in association with myco-heterotrophs and differences in N fractionation between fungal species (Gebauer & Taylor, 1999; Taylor *et al.*, 2003; Trudell *et al.*, 2003; Taylor *et al.*, 2004; Nygren *et al.*, 2007). Similar to other ericaceous myco-heterotrophs, the N enrichment seen in *P. aphylla* is coupled with a less dramatic, though significant, enrichment in ^{13}C. Enrichment in ^{13}C is a well established pattern in ectomycorrhizal myco-heterotrophs where carbon is passed from autotrophs to ectomycorrhizal fungi and finally to the myco-heterotroph (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Leake, 2004).

It is interesting that even the green pyroloids from this study have a significant enrichment in ^{15}N compared to surrounding autotrophs as recently there has been debate regarding the mixotrophic abilities of green Pyroleae species (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). Similar to the findings of Zimmer *et al.* (2007) this study found no evidence for C gain *via* mixotrophic means in either *P. picta* or *C. umbellata* adult plants. However, compared to *C. umbellata* individuals from Bavaria the samples from the western U.S.A. were more enriched in ^{15}N relative to autotrophic reference plants and the ^{15}N enrichment of both green pyroloid species from this study were most similar to myco-heterotrophic taxa other than *P. aphylla* (Figure 2). We propose two potential possibilities for this pattern. First, though all pyroloid seedlings are myco-heterotrophic, once they develop leaves carbon gains are primarily through photosynthesis, but they continue to gain nitrogen through an unknown uptake mechanism similar to myco-heterotrophs. A second possibility is that carbon gains *via* a myco-heterotrophic strategy are still present, but the analysis of plants' bulk-tissue carbon isotope abundances is not a sensitive enough method to detect these gains, which may only take place during certain seasonal, or plant developmental periods (Taylor *et al.*, 2004).

In previous studies a linear isotopic mixing model has been used to estimate percent C and N gains *via* fungi in green pyroloids. This model is based on the enrichment factors of pyroloids that are statistically distinct for either element from those of surrounding autotrophs that are then compared to the relative C and N

enrichment of obligate myco-heterotrophs (Gebauer & Meyer, 2003; Preiss & Gebauer, 2008). However, due to the variability in isotope signatures of obligate myco-heterotrophs it is difficult to determine what species accurately represent the isotope signatures of the C and N pools actually accessed by the plants, and the choice of myco-heterotrophic end-members can affect the estimated levels of myco-heterotrophy in the new species being investigated. In the case of *P. aphylla*, if the percent C and N gains *via* fungi were calculated using the mixing model first described by Gebauer & Meyer (2003), and a myco-heterotrophic end-member based on the mean relative enrichment of seven fully myco-heterotrophic plants associated with ectomycorrhizal fungi (Preiss & Gebauer, 2008) the estimated percent C derived from fungal material would be 96 ± 12 %. This indicates that *P. aphylla* essentially gains all of its carbon from a source that is similar to other fully myco-heterotrophic plants associated with ectomycorrhizal fungi. This conclusion fits well with the morphology of *P. aphylla* which lacks photosynthetic organs. In contrast, if the same mixing model is used to calculate percent N gain *via* myco-heterotrophy, *P. aphylla* would gain over 100 % (149 ± 18 %) of its nitrogen from the source(s) utilized by the myco-heterotrophic end-members. Thus the myco-heterotrophic species used as end-members in this scenario obviously do not fully represent the extent of variability in ^{15}N signatures of myco-heterotrophs. Until there is more definitive information on what factors drive the isotopic variability of myco-heterotrophs - especially in the case of ^{15}N enrichment - and the isotope signatures of the nutrient pools accessed by myco-heterotrophs, calculations of percent C and N gains *via* fungi in putative mixotrophs and myco-heterotrophs must be viewed as rough estimates.

Final Remarks

The evidence for myco-heterotrophy in *P. aphylla* is now compelling. Our results based on the isotope signatures of *P. aphylla* and *P. picta* support one of the hypotheses put forth by Camp (1940) and others that *P. aphylla* does indeed behave as a parasite 'deriving their food from the fungous [*sic*] mycelia associated with their roots'. Whereas, Haber (1987) assumed that *P. aphylla* was one of many morphological forms of *P. picta* connected by a rhizome to near-by leafy rosettes. Confirming these connections in the field between *P. aphylla* and surrounding *P. picta* plants is difficult as individual rhizomes can stretch for many meters in the soil. However, because of significant differences in the isotope signatures of *P. picta* and *P. aphylla* (Figure 2) this

study provides no substantiating evidence for rhizomatous connections between the two. Though there are reported differences among $\delta^{13}C$ values of plant organs (Willmer & Roksandic, 1980; Gebauer & Schulze, 1991; Badeck *et al.*, 2005; Bowling *et al.*, 2008) these differences are small compared to those found here between the leaves of *P. picta* and the flowering stalks of *P. aphylla*. Furthermore, when a small number of samples from the same plant organ (flowering stalks) from both plants were analyzed for C and N isotope abundances they were significantly different from each other for both elements.

The confirmation that *P. aphylla* is a myco-heterotroph provides some insights into the order of the evolutionary steps toward obligate myco-heterotrophy, especially because its close relatives exhibit trends towards myco-heterotrophy. These trends include pyroloids' dependency in early stages of development on fungal nutrition (Leake, 1994), an association with ectomycorrhizal fungi shared with overstory trees that could allow for epiparasitism, an enrichment in ^{15}N similar to that of all ectomycorrhizal myco-heterotrophs studied to date, and though not found in this study, some green pyroloids have been found to be enriched in ^{13}C compared to surrounding autotrophs (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). Similar approaches have been used to examine the transition to myco-heterotrophy in the orchids where the loss of photosynthesis is often coupled with an increase in specificity toward particular lineages of mycorrhizal fungi (Bidartondo *et al.*, 2004). Although the identities of the fungi associated with *P. aphylla* are yet to be determined, other closely related green pyroloids, including *P. picta*, have been found to associate with a suite of ericoid, endophytic and ectomycorrhizal fungi, the latter most likely providing the link between these plants and surrounding autotrophs (Robertson & Robertson, 1985; Bidartondo *et al.*, 2004; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007; Massicotte *et al.*, 2008; Vincenot *et al.*, 2008). The elucidation of the fungal associates of *P. aphylla* is of great interest for the study of myco-heterotrophy as it may provide further insight into the evolution of these intriguing plants.

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Appendix (available online)

Appendix A. Results of sequential Bonferroni-corrected Mann-Whitney U-tests for *post hoc* comparisons. MHP = myco-heterotrophic plants; Auto = autotrophic references; *P. picta* and *C. umbellata* = green pyroloids; *P. aphylla* = achlorophyllous *Pyrola*.

Comparison	^{15}N		^{13}C	
	α	*P*	α	*P*
C. umbellata / MHP	0.025	0.053	0.008	< 0.001
P. picta / MHP	0.017	0.038	0.006	< 0.001
C. umbellata / P. picta	0.05	0.933	0.017	0.085
C. umbellata / Auto	0.01	< 0.001	0.013	0.041
P. picta / Auto	0.006	< 0.001	0.05	0.873
P. aphylla / MHP	0.007	< 0.001	0.025	0.523
P. aphylla / Auto	0.008	< 0.001	0.007	< 0.001

CHAPTER 3

The nutritional mode of *Corallorhiza trifida*

The ectomycorrhizal specialist orchid *Corallorhiza trifida* is a partial myco-heterotroph

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Key words: *Corallorhiza trifida*, myco-heterotrophy, stable isotopes, ectomycorrhiza, *Tomentella*, Orchidaceae, chlorophyll content

Abstract

The leafless, circumboreal orchid *Corallorhiza trifida* is often assumed to be fully myco-heterotrophic despite contrary evidence concerning its ability to photosynthesize. Here we assess its level of myco-heterotrophy by analyzing the natural abundance of the stable isotopes ^{15}N and ^{13}C.

The mycorrhizal associates and chlorophyll contents of *C. trifida* were investigated and the C and N isotope signatures of nine *C. trifida* individuals from Central Europe were compared to those of neighboring obligate autotrophic and myco-heterotrophic reference plants.

The results show that *C. trifida* only gains 52 ± 5 % of its total nitrogen and 77 ± 10 % of the carbon derived from fungi even though it has been shown to specialize on one specific complex of ectomycorrhizal fungi similar to fully myco-heterotrophic orchids. Concurrently, compared to other *Corallorhiza* species, *C. trifida* contains a remarkable amount of chlorophyll.

Since *C. trifida* is able to supply significant proportions of its nitrogen and carbon demands through the same processes as autotrophic plants, this species should be referred to as partially myco-heterotrophic.

Introduction

Within the Orchidaceae, more than 100 species are non-photosynthetic and depend on C and N supplies from associated fungi. The majority of these 'myco-heterotrophic' orchids are entirely subterranean for most of their life cycle and their organs are well adapted to this habit in function and morphology (Leake, 1994). The orchid genus *Corallorhiza* GAGNEBIN (Epidendroideae) comprises ten leafless species of temperate-boreal terrestrial orchids. All of them are distributed in North and Central America, with exception of the circumboreal pale coral root orchid *Corallorhiza trifida* (Freudenstein, 1992). Species of the genus *Corallorhiza* are in general known to be fully myco-heterotrophic. Again, *C. trifida* poses an exception. Although the 'mycotrophic' nature of this species was already recognized in 1898 (Jennings & Hanna), many questions concerning the nutritional mode of *C. trifida* have been raised, by pigment analyses and assimilation experiments (Montfort & Küsters, 1940) and by comparative studies of the plastid DNA (Freudenstein & Doyle, 1994). However, despite these contrary evidences

C. trifida remains known as an obligate myco-heterotrophic plant (Downie *et al*., 1943**;** Zelmer & Currah, 1995; McKendrick *et al*., 2000a,b).

Natural stable isotope abundances can be used to assess the nutritional mode of orchid species (Gebauer & Meyer, 2003). This method is based on two findings that (a) fungal tissues are enriched in the heavy stable isotopes of N (Gebauer & Dietrich, 1993) and C (Gleixner *et al*., 1993) relative to accompanying autotrophic plants and (b) obligate myco-heterotrophic plants show isotope signatures similar to those of their fungal associates (Trudell *et al*., 2003). Furthermore, Gebauer & Meyer found that some green and hence putatively autotrophic orchid species are enriched in ^{15}N and ^{13}C compared to neighboring autotrophic plants but depleted in the heavy isotopes relative to obligate myco-heterotrophic plants. They concluded that these species use a mixed nutritional mode, where the acquisition of C and N through mycorrhizal fungi subsidizes the nutrient supply through autotrophic processes and referred to these plants as partially myco-heterotrophic.

Furthermore, there is evidence that the nutritional mode of orchid species is linked to association with certain functional groups of fungi. Dearnaley (2007) recently summarized the literature on orchid mycorrhiza. While roots of fully autotrophic orchids are generally associated with diverse saprotrophic, rhizoctonia-forming basidiomycete fungi (Bernard, 1909), several obligate myco-heterotrophic orchids are known to be highly specialized on a phylogenetically narrow range of fungi that simultaneously form ectomycorrhizas with roots of neighboring trees (Taylor & Bruns, 1997; Taylor *et al*., 2003; McKendrick *et al*., 2002; Selosse *et al*., 2002). In consistence with intermediate isotope signatures of partially myco-heterotrophic orchids, Bidartondo *et al*. (2004) could provide a “missing link in the evolution” and show that these putatively green orchids are also connected to ectomycorrhizal fungi of a diverse range and that some species may simultaneously associate with rhizoctonia-forming fungi. The high specialization towards certain ectomycorrhizal fungi in species belonging to the genus *Corallorhiza* (Zelmer & Currah, 1995; Taylor & Bruns, 1997; Taylor & Bruns, 1999; McKendrick *et al*., 2000b) supports the assumption that *C. trifida* is obligate myco-heterotrophic. However, if in fact, *C. trifida* is not fully myco-heterotrophic, this would be an example of a partially myco-heterotrophic species highly specialized on ectomycorrhizal fungi.

This study is the first to investigate the nutritional mode of the obviously greenish *C. trifida* by using natural stable isotope abundance analysis. The application of a linear

mixing model provides not only a qualitative conclusion but is also suited to quantitatively estimate the level of myco-heterotrophy of a plant (Gebauer & Meyer, 2003). Isotope signatures together with data of chlorophyll contents and molecular identification of fungal associates will help to answer the question of whether *C. trifida* is fully myco-heterotrophic.

Materials and Methods

Study site

Samples were collected from a forest site located in NE Bavaria, Germany (49°40' N and 11°23' E) at 522 m elevation with mean annual precipitation of 820 mm, mean annual temperature of 8 °C (German weather service, www.dwd.de). The site is a dense broadleaf forest dominated by *Fagus sylvatica* with a sparse and patchy cover of understory vegetation (Cephalanthero-Fagion). Soil is lithic leptosol originating from Jurassic dolomite with a shallow organic layer and a pH of 7.2 (0 – 5 cm) measured in H_2O.

Sampling scheme and investigated species

Sampling for isotope ratio analysis was performed in 2004 and 2005 (June). Scale-like leaves and parts of the stem including flowers or seed capsules, respectively, were collected of 17 obligate myco-heterotrophic plants (Leake, 1994; Bidartondo, 2005) (*Neottia nidus-avis*, *Monotropa hypopitys*) and of 9 individuals of *Corallorhiza trifida* (Figure 1). As reference, leaf material of three to four obligate autotrophic plant species (n = 68) was taken in close spatial proximity (within 1 m^2) to each of the *Neottia*, *Monotropa* and *Corallorhiza* individuals, following the criteria described by Gebauer & Meyer (2003). In total, 10 plant species (n = 94) were sampled, including *Neottia nidus-avis* (L.) RICH. ($n_{(2004)}$ = 4, $n_{(2005)}$ = 9), *Monotropa hypopitys* L. ($n_{(2004)}$ = 4), *Corallorhiza trifida* CHÂTEL. ($n_{(2004)}$ = 4, $n_{(2005)}$ = 5), *Fagus sylvatica* L. ($n_{(2004)}$ = 8, $n_{(2005)}$ = 10), *Convallaria majalis* L. ($n_{(2004)}$ = 8, $n_{(2005)}$ = 10), *Acer pseudoplatanus* L. ($n_{(2004)}$ = 8, $n_{(2005)}$ = 5), *Fragaria vesca* L. ($n_{(2004)}$ = 4), *Sorbus aucuparia* L. ($n_{(2005)}$ = 5), *Hieracium sylvaticum* (L.) GRUFB. ($n_{(2005)}$ = 5) and *Rubus saxatilis* L. ($n_{(2005)}$ = 5). For identification of mycorrhizal fungi, two root samples were collected from each of the four *C. trifida* individuals in 2004. Furthermore, the whole aboveground biomass of 10 *C. trifida* individuals was taken for quantitative chlorophyll analysis (May 23, 2006).

Figure 1. *Corallorhiza trifida* growing in a dense *Fagus sylvatica* forest in eastern Hesse, Germany. (a) several flowering individuals on May 5, 2005; (b) seed capsules on May 27, 2005. Courtesy of Marco Klüber.

Stable isotope abundance analysis

Leaf and stem samples were oven-dried and ground to a fine powder. Relative N and C isotope abundances were measured using a dual element analysis mode with an elemental analyzer coupled to a continuous flow isotope ratio mass spectrometer as described in Bidartondo *et al.* (2004). Measured abundances are denoted as δ values, which were calculated according to the following equation: $\delta^{15}N$ or $\delta^{13}C$ = $(R_{sample}/R_{standard}-1)$ x 1000 [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard. Standard gases were calibrated with respect to international standards by using the reference substances N1 and N2 for nitrogen isotopes and ANU sucrose and NBS 19 for carbon isotopes, provided by the International Atomic Energy Agency (Vienna, Austria).

Statistics

Variation in $\delta^{15}N$ and $\delta^{13}C$ between the two sampling years was assessed using Student's t-tests. Afterwards, pooled data were tested for differences in δ values using the Kruskal-Wallis non-parametric test and Bonferroni-corrected (Holm, 1979) Mann-Whitney U-tests for *post hoc* comparisons.

Calculation of C and N gains from fungi

As described by Gebauer & Meyer (2003), a linear two-source isotopic mixing model was used to calculate the relative contribution of nitrogen or carbon derived from fungal material to the N or C content of *C. trifida* (%x_{df} with x as nitrogen or carbon, respectively). The model is based on individual δ values of *C. trifida* (δx_{CT}), mean δ values of co-occurring autotrophic reference plants (δx_{REF}) and on the mean enrichment factor ε of sampled fully myco-heterotrophic (MH) plants ($\varepsilon_{MH\text{-}REF} = \delta x_{MH} - \delta x_{REF}$): $\%x_{df} = (\delta x_{CT} - \delta x_{REF}) / \varepsilon_{MH\text{-}REF} \times 100$. Data are given as means ± 1 SD.

Molecular identification of mycorrhizal fungi

From each of the four *C. trifida* individuals (2004), two root sections colonized by fungi were taken and placed in lysis buffer. Samples were frozen and thawed three times before grinding the softened tissue with a micropestle. Genomic DNA was extracted following methods described elsewhere (Gardes & Bruns, 1993) but using GeneClean (Q-BioGene, Carlsbad, CA, USA) for DNA binding and purification. Using PCR (polymerase chain reaction), the nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primers ITS1F and ITS4 and PCR conditions described in Gardes & Bruns (1993). Positive PCR products were purified using QIAquick 96 kits (Qiagen, Valencia, CA, USA). DNA sequencing was performed on an ABI3100 Genetic Analyzer using BigDye v.3.1 chemistry (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. Electrophoretograms were checked using Sequence Navigator v.1.0.1 (Applied Biosystems). All samples with strong PCR amplification of single templates were blasted in GenBank to ascertain taxonomic affinity. The GenBank accession number is EF471313.

Chlorophyll content

Before extraction of chlorophylls *a* and *b* (Chl *a* and *b*), stem height and mass of the ten *C. trifida* individuals was determined. N,N'-dimethylformamide was added to the fresh, ground material (whole aboveground biomass) and samples were kept in the dark at -23 °C for eight days. After centrifugation, absorbance of the supernatants was spectrophotometrically measured at 646.8, 663.8 and 750 nm. Chlorophyll concentrations (µg/ml) were calculated according to the following equations (Porra *et al.*, 1989): Chl $a = 12.00 \cdot (A_{663.8} - A_{750}) - 3.11 \cdot (A_{646.8} - A_{750})$; Chl $b = 20.78 \cdot (A_{646.8} - A_{750}) - 4.88 \cdot (A_{663.8} - A_{750})$. Chl *a*+*b* content are given in $\mu g/g_{fw}$ (fw = fresh weight).

Results and Discussion

Mycorrhizal association

Molecular identification of fungal partners revealed that all of the eight roots were exclusively colonized by fungi belonging to the obligate ectomycorrhizal basidiomycete genus *Tomentella* (Thelephoraceae). No other mycorrhizal associations were detected. These results are consistent with previous findings. Zelmer & Currah (1995) firstly isolated a clamp-bearing basidiomycete from pelotons of *C. trifida* roots and demonstrated that the same fungus is ectomycorrhizal on *Pinus contorta* seedlings. Taylor (1998) observed associations between thelephoroid fungi and *C. trifida* in North America and studies comparing DNA sequences of fungi on *C. maculata* with those of ectomycorrhizal fungi on adjacent tree roots have confirmed that these coral root orchids are indeed associated with ectomycorrhizal fungi (Taylor & Bruns, 1997). McKendrick *et al*. (2000b) found that the specificity of *C. trifida* towards fungi exclusively belonging to the *Thelephora/Tomentella* complex applies from the earliest stages of seed germination through adulthood and flowering.

C. maculata and *C. mertensiana* are known to form mycorrhizas exclusively with fungi in the Russulaceae (Taylor & Bruns, 1999; Taylor *et al*., 2004) and the desert orchid *Hexalectris spicata* is specialized on ectomycorrhizal Sebacinaceae (Taylor *et al*., 2003). The relationship of these fungi to their ectomycorrhizal partners, however, is unlikely to be as specific (Zelmer & Currah, 1995; Gardes & Bruns, 1996).

Since it has been estimated that about 15 % of net C fixation by ectomycorrhizal trees is allocated to their ectomycorrhizal fungal partners (Finlay & Söderström, 1992), McKendrick *et al*. (2000a) concluded that the switch in achlorophyllous orchids from soil-inhabiting rhizoctonia-type mycorrhizal association, to one involving ectomycorrhizal fungi might be based upon better access of the latter to C supplies.

Chlorophyll content

C. trifida individuals contained on average 26 ± 11 $\mu g/g_{fw}$ of Chl *a*+*b* (Table 1) which accounts for only 1 % of the chlorophyll amount detected in green leaves of *Cephalanthera damasonium* (Julou *et al*., 2005). However, the area-to-mass ratio of stems and leaves is highly different and relating to the same mass, stems contain considerably more non-assimilating tissues than leaves. Thus, the chlorophyll content in stems of the leafless orchid *C. trifida* must be lower than the chlorophyll content in green leaves of plants.

Table 1. Fresh weight, stem height, chlorophyll contents (Chl *a*, Chl *b*, Chl *a*+*b*) and chlorophyll ratios (Chl *a*/*b*) of the aboveground biomass of ten *Corallorhiza trifida* individuals collected from a dense *Fagus sylvatica* forest in NE Bavaria, Germany. Means ± 1 SD are shown in bold.

Fresh weight [g]	Stem height [cm]	Chl *a* [µg/g_{fw}]	Chl *b* [µg/g_{fw}]	Chl *a*+*b* [µg/g_{fw}]	Chl *a*/*b*
0.498	16.0	8.5	4.2	12.7	2.0
0.573	14.5	8.5	4.4	12.9	1.9
0.247	9.7	30.6	10.8	41.4	2.8
0.313	10.4	21.6	8.7	30.3	2.5
0.363	12.5	20.5	7.9	28.4	2.6
0.466	13.0	16.1	7.6	23.7	2.1
0.348	11.8	21.3	9.1	30.4	2.3
0.226	9.5	31.3	13.6	44.9	2.3
0.529	16.0	10.2	5.0	15.2	2.1
0.550	13.0	13.1	7.8	20.9	1.7
0.411 ± 0.128	**12.6 ± 2.4**	**18.2 ± 8.4**	**7.9 ± 2.9**	**26.1 ± 11.2**	**2.2 ± 0.3**

This also becomes apparent from the close ($P < 0.001$), negative correlations between Chl *a*+*b* content and fresh weight of each *C. trifida* individual ($r^2 = 0.896$, Figure 2), or between Chl *a*+*b* content and stem height ($r^2 = 0.893$, not shown).

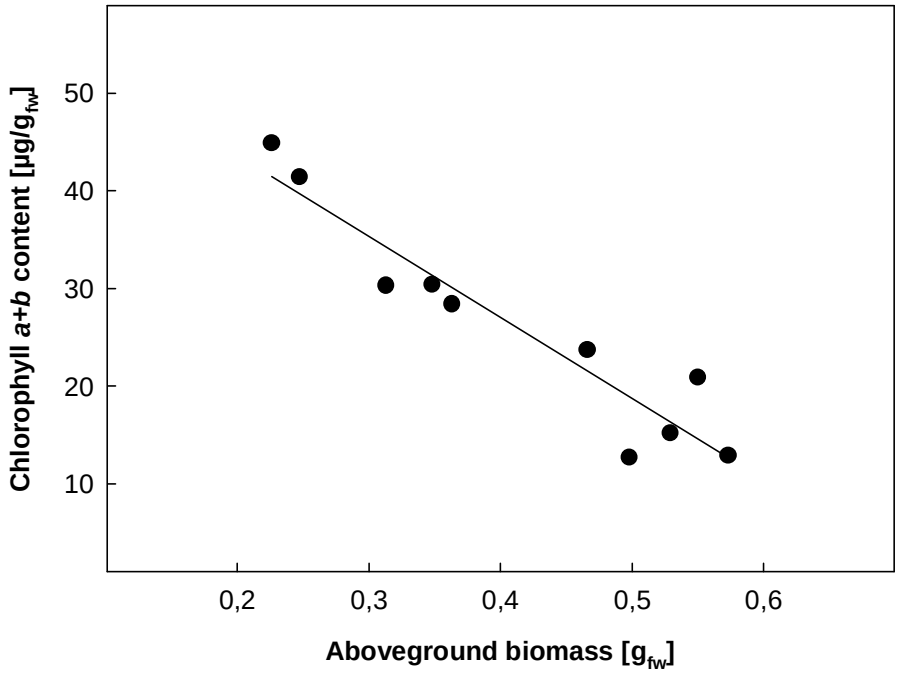

Figure 2. Correlation of chlorophyll *a*+*b* content and fresh weight of the aboveground biomass of ten *Corallorhiza trifida* individuals collected from a dense *Fagus sylvatica* forest in NE Bavaria, Germany: $y = -83.12\ x + 60.267$; $r^2 = 0.896$; $P < 0.001$.

In 1940 already, Montfort & Küsters found that inflorescences of *C. innata* R.Br. (= *C. trifida*) contained 33 % (and stems with young fruits even 60 %) of the chlorophyll content detected in inflorescences of the fully autotrophic orchid *Listera ovata*. Cummings & Welschmeyer (1998) determined the pigment composition for ten species of putatively achlorophyllous angiosperms including two orchids (*Cephalanthera austinae* and *Corallorhiza maculata*) by HPLC. They detected chlorophyll *a* in all taxa, but chlorophyll *b* was only detected in *Corallorhiza*. Compared to the total content of chlorophyll and chlorophyll-related pigments in *C. maculata* (26.75 ng/g_{fw}) (Cummings & Welschmeyer, 1998), *C. trifida* contains a three orders of magnitude higher amount of chlorophyll (Table 1). Furthermore, the mean chlorophyll *a*/*b* ratio of *C. trifida* (Table 1) is similar to that found in other C3 plants (Larcher, 2003).

If photosynthetic function of a plant is altered or absent, concomitant changes in the plastome may have occurred (Palmer *et al*., 1988). Following this assumption, Freudenstein & Doyle (1994) examined the variation in plastid DNA among species of *Corallorhiza*. They found deletions of genes relevant for photosynthesis in all investigated *Corallorhiza* species with the exception of *C. trifida* and concluded that *Corallorhiza* may be a genus "on its way" to a heterotrophic existence. However, more recent studies have shown that the deletion of photosynthesis genes in the plastid genome is not necessarily linked to the trophic strategy of the plants (Randle & Wolfe, 2005; Young & dePamphilis, 2005) and *C. trifida* frequently is still described as a non-photosynthetic plant.

Isotope signature and nutrient gain from the fungal partner

Based on Bonferroni-corrected (Holm, 1979) Mann-Whitney U-tests, highly significant differences existed between the isotope signatures of all tested groups (obligate autotrophic plants REF, *C. trifida* individuals CT, obligate myco-heterotrophic plants MH). Compared to the REF group (mean $\delta^{15}N$ = -6.1 ± 1.2 ‰, mean $\delta^{13}C$ = -32.0 ± 1.3 ‰), myco-heterotrophic species were significantly enriched by 11.2 ± 3.1 ‰ in ^{15}N and by 8.3 ± 1.0 ‰ in ^{13}C (Figure 3), which is in accordance with the enrichment factors observed in other obligate myco-heterotrophic plants relative to accompanying autotrophic plants (Zimmer *et al*., 2007). Individuals of *C. trifida* were also highly enriched in both, ^{15}N and ^{13}C, compared to the autotrophic reference plants showing the incorporation of fungal-derived organic compounds.

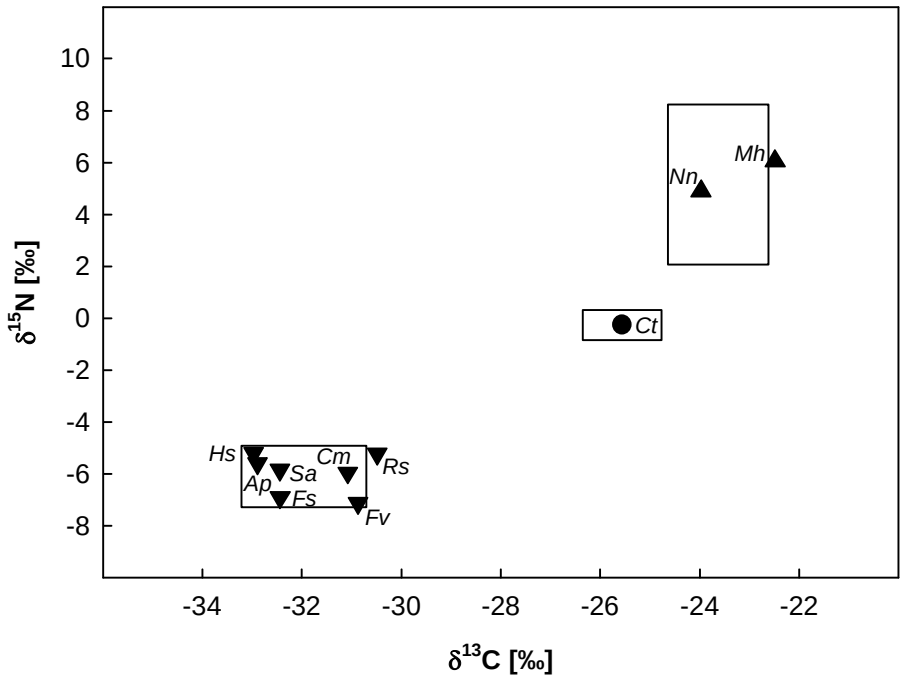

Figure 3. Mean $\delta^{15}N$ and $\delta^{13}C$ values in the aboveground biomass of *C. trifida* (CT, circle), two obligate myco-heterotrophic plant species (MH, triangle up) and in leaves of seven fully autotrophic species (REF, triangle down) collected from a dense *Fagus sylvatica* forest in NE Bavaria, Germany. Boxes are defined by standard deviations of the mean δ values from each group (REF, CT, MH). For numbers of replicates see Materials and Methods section. Plant species: *Ap, Acer pseudoplatanus*; *Cm, Convallaria majalis*; *Ct, Corallorhiza trifida*; *Fs, Fagus sylvatica*; *Fv, Fragaria vesca*; *Hs, Hieracium sylvaticum*; *Mh, Monotropa hypopitys*; *Nn, Neottia nidus-avis*; *Rs, Rubus saxatilis*; *Sa, Sorbus aucuparia*.

However, relative to obligate myco-heterotrophic plants, *C. trifida* was significantly depleted in the heavy stable isotopes of N and C (Figure 3). The latter is different to findings in *C. maculata* which has isotope signatures typical of a fully myco-heterotrophic plant (Zimmer *et al*., 2007). Hence, in contrast to other species of the genus, aboveground organs of *C. trifida* are photosynthetically active. Therefore, flowering adults of this species are not fully but only partially myco-heterotrophic. This does not exclude full myco-heterotrophy during belowground phases of this orchids' life cycle. The individuals investigated in this study also use high amounts of soil borne nitrogen. According to the linear mixing model, *C. trifida* gains 52 ± 5 % of its N from fungal association and 77 ± 10 % of its total C demand is derived from fungi. These results confirm findings by Montfort & Küsters (1940) who observed photosynthesis in *C. innata* (= *C. trifida*) by measuring the plants' CO_2 exchange. They found that inflorescences and especially stems with maturing fruits were able to compensate for respiratory losses of CO_2 through assimilation and calculated a quotient Q (Q =

assimilation / respiration) of 2.2 for the plants including young fruits, indicating a positive CO_2 balance. The detection of a higher C than N gain from fungi (Student's *t*-test, $P < 0.001$) is unusual for partially myco-heterotrophic plants (see Gebauer & Meyer, 2003; Bidartondo *et al*., 2004; Zimmer *et al*., 2007) and might be caused by a nitrogen limited fungal partner. Since several *Tomentella* species are wood inhabiting (Küffer & Senn-Irlet, 2005) and may therefore have lower N concentrations than those living on humus (Gebauer & Taylor, 1999) *C. trifida* might be forced to supply its N demand through higher uptake of soil born inorganic nitrogen.

As it has now been shown quantitatively that *C. trifida* is able to supply its nitrogen and carbon demand through autotrophic processes, this leafless orchid should no longer be referred to as fully or obligate myco-heterotrophic. A possibly similar situation has been shown for the Mediterranean orchid *Limodorum abortivum* (Girlanda *et al*., 2006). *L. abortivum* also has reduced leaves, associates predominantly with narrow clades of ectomycorrhizal fungi (*Russula* spp.) and contains photosynthetic pigments. However, its photosynthetic activity was found to be insufficient to compensate for respiration in adult plants (Girlanda *et al*., 2006).

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The chlorophyll-containing orchid *Corallorhiza trifida* derives little carbon through photosynthesis

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Abstract

While measurements of tissue stable isotope signatures and isotope mixing models have suggested that the green orchid *Corallorhiza trifida* is photosynthetically active and hence only partially myco-heterotrophic, these assumptions have not been validated by direct analysis of carbon assimilation.

The photosynthetic capabilities of three orchid species assumed on the basis of the indirect methods or chlorophyll content to have differing trophic strategies: *Neottia nidus-avis* (fully myco-heterotrophic), *Cephalanthera damasonium* (partially autotrophic), *C. trifida* (partially autotrophic), as well as saplings of an autotrophic tree, *Fagus sylvatica,* were investigated by combining the determination of chlorophyll content and fluorescence, with direct measurement of the potential for CO_2 assimilation using ^{13}C isotope tracers in the field.

Chlorophyll content and fluorescence values were indicative of ineffective photochemical processes in *Neottia* and reduced efficiency of photochemical processes in *Corallorhiza.* These differences are reflected in the mean assimilation rates of $^{13}CO_2$ of 594 ± 129, 331 ± 72, 12.4 ± 2.4 and 7.3 ± 0.9 mg g^{-1} h^{-1} for *Fagus*, *Cephalanthera*, *Corallorhiza* and *Neottia* respectively.

Our study, while confirming the fully myco-heterotrophic status of *Neottia* and the partially autotrophic condition in *Cephalanthera*, also demonstrates under field conditions that *Corallorhiza* is physiologically closer to the fully myco-heterotrophic condition than has previously been recognized.

Introduction

The overwhelming majority of plants form mutualistic symbioses with soil fungi, termed mycorrhizas, in which the plant supplies fixed carbon (C) to the fungal symbionts in return for the provision of mineral nutrients by the fungal partner (Smith & Read, 2008). The functional status of these symbioses in orchids has however, been controversial. It is accepted that all orchids begin their life cycle with a myco-heterotrophic (*sensu* Leake, 1994) growth phase in which the fungal symbionts provide C and mineral nutrients to the orchid seedling (McKendrick *et al*., 2000a; Smith, 1966) without obvious benefit to themselves. It is also thought that, with the exception of a small proportion of species (c. 1%) that retain the fully myco-heterotrophic (achlorophyllous) condition in adulthood, the green shoots of orchids emerging

aboveground have the potential for autotrophy. Cameron *et al*. (2008a & 2006) showed that the green forest orchid *Goodyera repens* was able to engage in a mutualistic symbiosis with its fungus partner, the partnership enabling the plant to repay the C invested in it during its early achlorophyllous stage. However, the generality of this observation in other green orchids remains to be elucidated. The extent to which the green shoots are actually photosynthetic is less clear in those orchid species, such as *Corallorhiza trifida* Chatel. in which leaves have been reduced to scales but green stems and capsules appear to retain some potential for autotrophic activity.

Progress towards identification of the sources of C and N acquired by orchids has been provided by measurements of the natural abundance of these elements in their tissues (Gebauer & Meyer, 2003). These reveal that fully myco-heterotrophic orchids have distinctively enriched delta ^{13}C (and $\delta^{15}N$) signatures relative to those seen in species that are autotrophic at maturity. Zimmer *et al*. (2008) showed that in *Corallorhiza* the natural abundance of tissue ^{13}C was slightly depleted ($\delta^{13}C$ = -25.6) relative to that seen in the neighboring chlorophyll-free orchid *Neottia nidus-avis* ($\delta^{13}C$ = -24) and the ericaceous herb *Monotropa hypopitys* ($\delta^{13}C$ = -22), both of which are generally accepted to be fully myco-heterotrophic. In contrast, *Corallorhiza*, *Neottia* and *Monotropa* were all shown to be significantly enriched in ^{13}C relative to co-occurring green autotrophic reference plants ($\delta^{13}C$ = -32). These findings, by themselves, are indicative of a rather low photosynthetic capability in *Corallorhiza*. However, using data obtained by the application of a two source mixing model proposed by Gebauer & Meyer (2003), Zimmer *et al*. (2008) went on to calculate that up to 23% of the carbon gained by *Corallorhiza* may be derived from photosynthetic activity. These authors recognized the need to validate such estimated values through direct measurements of photosynthetic C gain. The need for such validation has been further highlighted by Barrett & Freudenstein (2008) who confirmed the presence of the plastid-encoded RuBisCo large subunit gene, rbcL, in *Corallorhiza trifida* as well as in its closest relatives in the genus, and called for direct measurements of the potential of these plants to express photosynthetic activity.

Here, by *in situ* field measurements in the same population that was used by Zimmer *et al*. (2008), we determined the ability of *C. trifida* and of co-occurring species known to be either fully (*Neottia nidus-avis* (L.) Rich.) or partially myco-heterotrophic orchid (*Cephalanthera damasonium* (Miller) Druce) or autotrophic (small saplings of

Fagus sylvatica L.), to fix atmospheric CO_2. By coupling the measurement of chlorophyll fluorescence and content, we re-evaluate the nutritional status of this orchid.

Materials and Methods

Field site

All experiments were undertaken at a forest site located in NE Bavaria, Germany (49°40′N and 11°23′E) at 522 m elevation with mean annual precipitation of 820 mm and mean annual temperature of 8°C (German weather service, http://www.dwd.de). The site is a dense broadleaf forest dominated by *Fagus sylvatica* with a sparse and patchy cover of understory vegetation. Soil is lithic leptosol originating from Jurassic dolomite with a shallow organic layer and a pH of 7.2 (0-5 cm) measured in H_2O (Zimmer *et al.*, 2008). Total chlorophyll extractions, measurements of chlorophyll fluorescence parameters and $^{13}CO_2$ pulse chase experiments were performed in May 2008.

Chlorophyll content

The youngest fully expanded leaf was removed from four individuals of *Neottia nidus-avis* and *Cephalanthera*, four saplings of *Fagus* and the whole stem of eight individuals of *Corallorhiza*. Shoots were harvested and kept on ice in the dark until extraction of chlorophyll (within 1 hour). Dry weights were estimated using the fresh weight/dry weight ratio of additional harvested leaves/stems and the surface area estimated using the fresh weight/area ratio of the same leaves/stems (data not shown). Leaves or stems were ground in a mortar and pestle with a small amount of acid washed sand (as an abrasive) and 5 ml of 80 % ice-cold acetone. The mortar and pestle was washed out twice with a further 2 ml of acetone and transferred to a centrifuge tube. The samples were centrifuged at 8000 *g* for 5 minutes and the supernatant diluted to 10 ml total volume with 80 % ice-cold acetone. The optical density of the supernatant was measured at 645 and 663 nm using a Hitachi U-2001 spectrophotometer.

$$\text{Chlorophyll a (mg l}^{-1}) = (12.7 \times OD_{663}) - (2.69 \times OD_{645})$$

Equation 1

$$\text{Chlorophyll b (mg l}^{-1}) = (22.9 \times OD_{645}) - (4.68 \times OD_{663})$$

Equation 2

The chlorophyll concentration (mg l^{-1} of extract) was calculated according to Arnon (1949) using equations 1 and 2 above and re-expressed as mg of chlorophyll per cm^2 (surface area) to facilitate comparison of stem data collected from *Corallorhiza* with leaf data from the other species. Surface area of the *Corallorhiza* stem was calculated as a truncated cone.

Chlorophyll fluorescence

The maximum and steady state quantum yields (F_v/F_m and Φ_{PSII}, respectively) of the youngest fully expanded leaf of four individuals of *Cephalanthera* and *Fagus* or the top of the stem below the first flower of intact *Corallorhiza* and *Neottia* shoots was measured in the field using a pulse-modulated fluorimeter (FMS2, Hansatech Ltd, King's Lynn, England). F_v/F_m is defined by equation 3 and *ΦPSII* by equation 4 after Maxwell & Johnson (2000).

$$F_v/F_m = \frac{F_m - F_0}{F_m}$$

Equation 3

$$\Phi_{PSII} = \frac{F'_m - F_t}{F'_m}$$

Equation 4

Where F_0 is the minimal level of fluorescence, F_m is the maximum fluorescence (after the application of the saturating flash), F'_m is the maximum fluorescence in the light and F_t is the steady state fluorescence immediately prior to the flash.

Samples were dark-adapted for 15 minutes prior to measurements of F_v/F_m, and the intensity of the 0.7 second light pulse of 8000 μmol photons m^{-2} s^{-1}. Leaves were adapted to an actinic beam until F_0 stabilized to obtain *ΦPSII*. In the *ΦPSII* measurements, the light pulse had an intensity of 8000 μmol photons m^{-2} s^{-1} for 0.7 seconds. All fluorescence parameters were estimated as per the manufacturer's instructions.

In-situ $^{13}CO_2$ pulse labeling

Four individuals of *Neottia* and *Cephalanthera*, four saplings of *Fagus* and eight individuals of *Corallorhiza* were identified in the field and sealed into a plastic bag (that transmitted on average 95 % PAR). A PTFE vial containing 50 mg of 99 atom %

$Ca^{13}CO_3$ was attached to the inside wall of the bag (4000 cm^3) prior to labeling. A gas-tight seal was made around the stems using anhydrous lanolin. HCl (1 % v/v) was injected through the wall of the bag and into the vial to liberate $^{13}CO_2$ and the resulting hole sealed with PTFE tape (Figure 1b). Plants were maintained in the labeling bags for four hours and the photosynthetically active radiation (PAR) was recorded at canopy height every hour throughout the labeling period. Four control plants of each species studied were harvested and dried in order to establish the natural abundance ^{13}C signature of the plants.

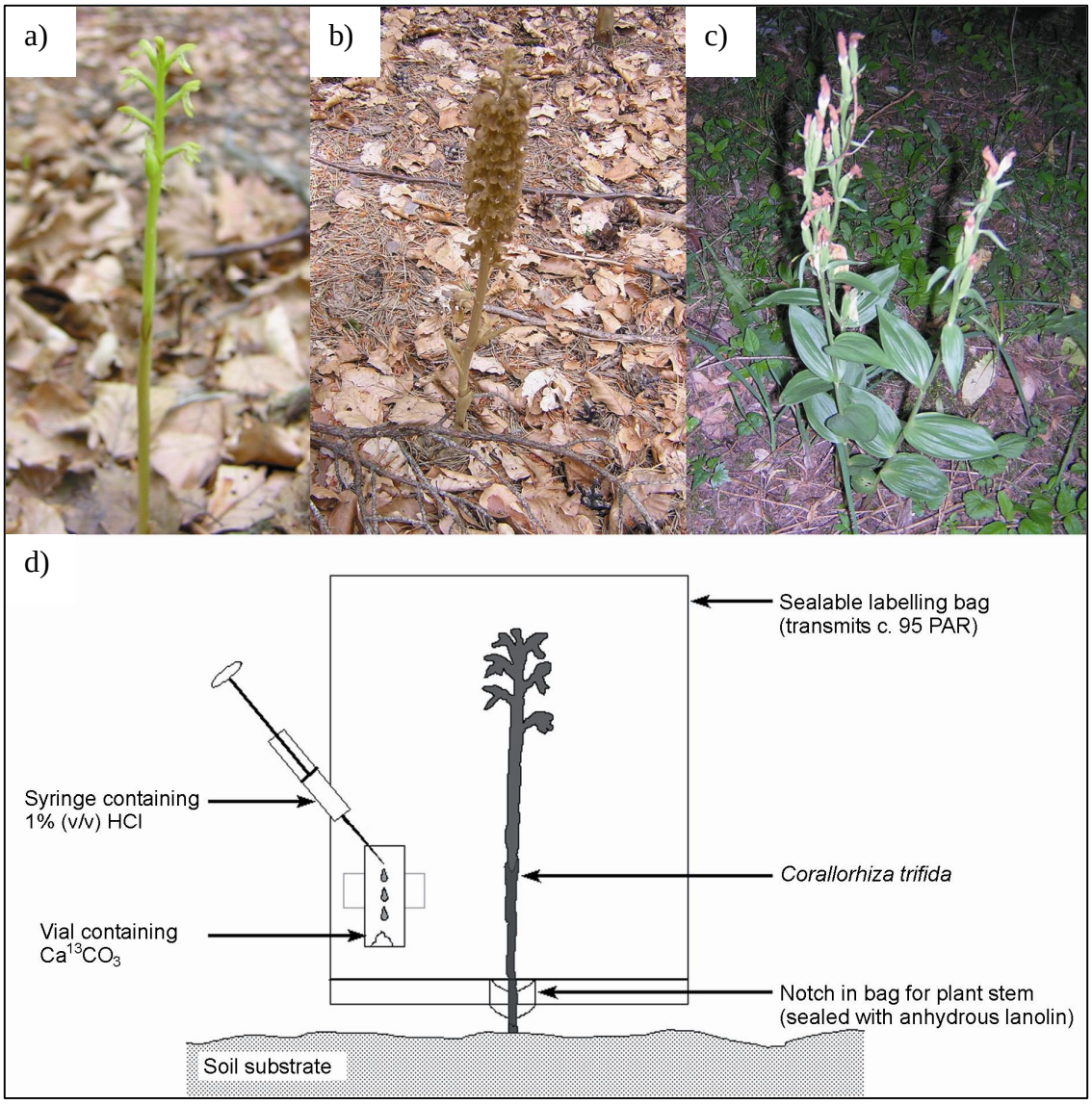

Figure 1. a) *Corallorhiza trifida*, b) *Neottia nidus-avis* and c) *Cephalanthera damasonium* growing beneath a stand of *Fagus* near Bayreuth, Bavaria, Germany and d) labeling chamber for the introduction of the $^{13}CO_2$ label.

At harvest, plants were dried at 80 ^{o}C for 48 hours and weighed (there was *c*. 1 hour between harvest and samples entering the drying oven, labeled and unlabeled samples were dried in separate ovens). The samples were homogenized separately and a 5 μg subset of each constituent part was analyzed for ^{13}C by continuous flow mass spectrometry (PDZ Europa 2020 Isotope Ratio Mass Spectrometer – IRMS coupled to a PDZ ANCA GSL preparation unit). Data were collected as δ^{13}C relative to the Pee Dee Belemnite international standard and re-expressed as atom %. The excess (above background) mass of ^{13}C was calculated using Equation 5.

$$M_{Ex} = \left(\frac{At_{lab} - At_{cont}}{100} \right) \left(M \left[\frac{\%C}{100} \right] \right)$$

Equation 5

Where M_{Ex} = Mass (excess) of the isotope in μg, At_{lab} = atom % of the isotope in labeled plant, At_{cont} = atom % of the isotope in paired control plant, M = biomass of sample (μg) and $\%C$ = percentage of carbon.

Statistical analysis

Differences between treatment means were analyzed by ANOVA followed by Fisher's multiple comparison test using Minitab 13 (Minitab Inc., PA, USA). Data were transformed either using Log_{10} or Box-Cox (Minitab 13) transformations when they failed to meet the assumptions of ANOVA. Untransformed means and associated standard errors are presented.

Results

Chlorophyll content

The total amount of chlorophyll a + b (μg cm^{-2}) was significantly different between all means (ANOVA [Log_{10}]: d.f. = 3,17; F = 368; $P < 0.001$) with *Neottia* containing the lowest amount of chlorophyll and *Cephalanthera* the highest (Figure 2a). In contrast, there was no significant difference in the chlorophyll a:b ratio of *Fagus*, *Cephalanthera* and *Corallorhiza*. The chlorophyll a:b ratio of *Neottia* was however significantly higher than that of all other species (ANOVA [Log_{10}]: d.f. = 3,17; F = 60.3; $P < 0.001$) (Figure 2b).

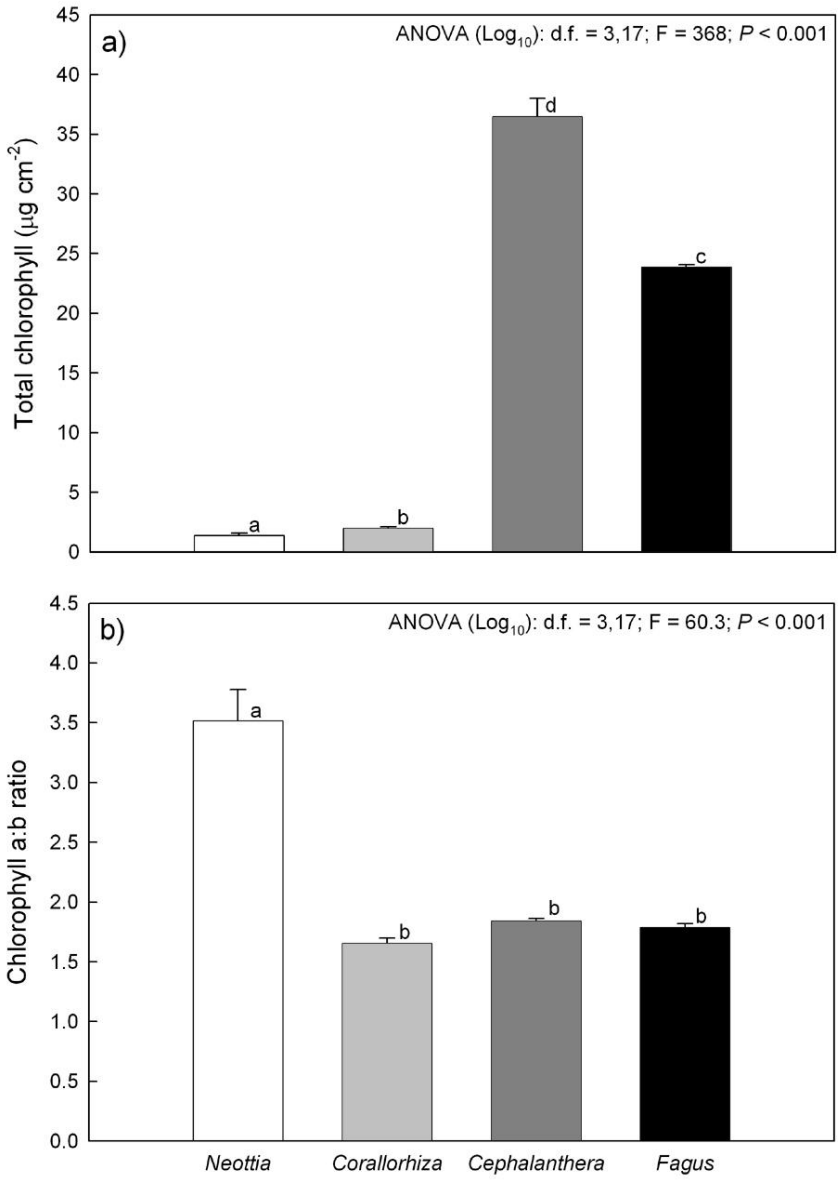

Figure 2. Total chlorophyll content (a) and a:b ratio (b) in *Neottia nidus-avis* (myco-heterotroph), *Corallorhiza trifida* (partial myco-heterotroph *sensu* Zimmer *et al*., 2008), *Cephalanthera damasonium* (partial myco-heterotroph *sensu* Gebauer & Meyer, 2003) and *Fagus sylvatica* (autotroph). Chlorophyll content is expressed as a function of surface area of the sample leaves in all cases except *Corallorhiza* which is leafless. In this latter case stems were analysed and the surface area calculated as a truncated cone. Bars with differing letters are significantly different (ANOVA: $P < 0.05$). Error bars represent + 1 SE. N = 4 - 8.

Chlorophyll fluorescence parameters (F_v/F_m and Φ_{PSII})

Maximum quantum yield (F_v/F_m) was measured for *Fagus*, *Cephalanthera* and *Corallorhiza* but could not be measured for *Neottia* as steady state F_0 was never detected (Figure 3a). F_v/F_m was highest for *Fagus* (0.85 ± 0.001) and was significantly different from *Cephalanthera* (0.81 ± 0.003) (ANOVA: d.f. = 2,12; F = 43.4; $P <$ 0.001), although both values are considered to be within the range for healthy plants

(Maxwell & Johnson 2000). F_v/F_m for *Corallorhiza* (0.71 ± 0.015) was significantly lower than that of both *Fagus* and *Cephalanthera* (ANOVA: d.f. = 2, 12; F = 43.4; P < 0.001) (Figure 3a). The steady state quantum yield of photosystem II (Φ_{PSII}) was determined for *Fagus* (0.85 ± 0.005), *Cephalanthera* (0.80 ± 0.012) and *Corallorhiza* (0.71 ± 0.020) but again could not be measured for *Neottia* (Figure 3b). Φ_{PSII} values recorded for all species were significantly different (ANOVA: d.f. = 2,12; F = 22.2; P < 0.001) (Figure 3b).

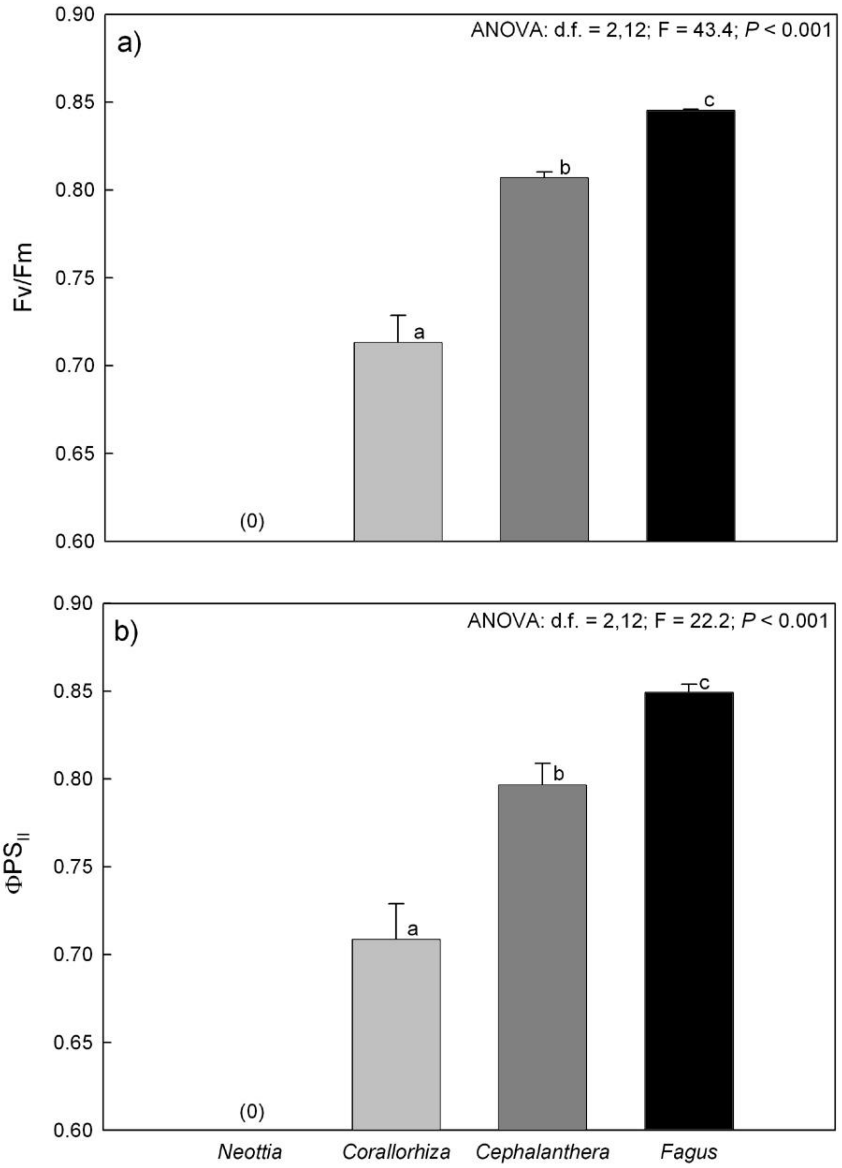

Figure 3. Maximum (F_v/F_m - a) and steady state (Φ_{PSII} - b) quantum yield of photosystem II for the stems of *Neottia nidus-avis* (myco-heterotroph) and *Corallorhiza trifida* (partial myco-heterotroph) and the leaves of *Cephalanthera damasonium* (partial myco-heterotroph) and *Fagus sylvatica* (autotroph). Bars with differing letters are significantly different (ANOVA: P < 0.05). Error bars represent + 1 SE. N = 4 - 8. NB: No values for F_v/F_m or Φ_{PSII} could be obtained for *Neottia* as steady state F_0 was not detectable following the application of the actinic beam.

In-situ $^{13}CO_2$ pulse labeling

The shoots of all target species; *Fagus*, *Cephalanthera*, *Corallorhiza* and *Neottia* contained the ^{13}C label (atom % excess and thus above background) after 4 hours of exposure (Figure 4).

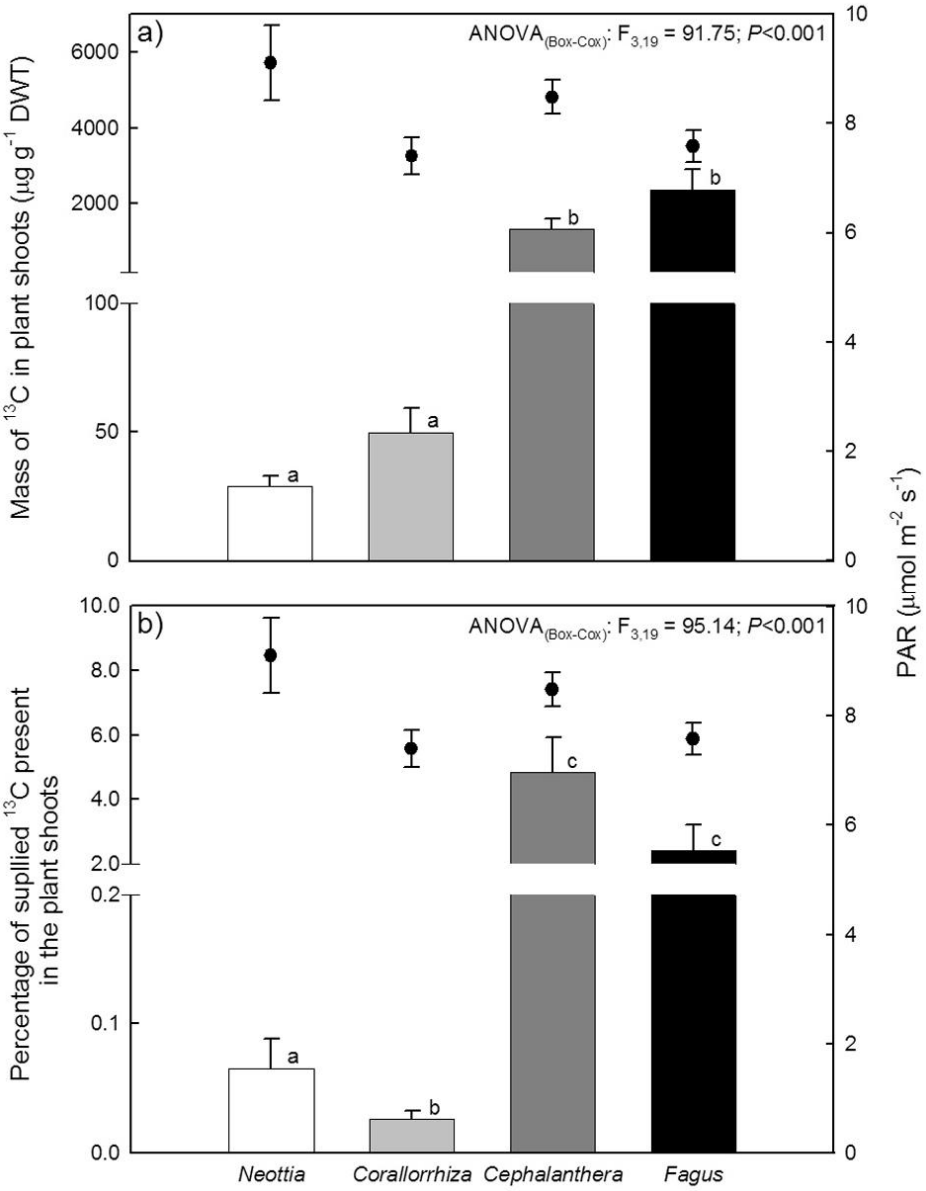

Figure 4. Total amount of ^{13}C (a) and the percentage of the supplied ^{13}C (b) present in plant shoots of *Neottia nidus-avis* (myco-heterotroph), *Corallorhiza trifida* (partial myco-heterotroph), *Cephalanthera damasonium* (partial myco-heterotroph) and *Fagus sylvatica* (autotroph) after four hours exposure to a $^{13}CO_2$ source. Mean photosynthetically active radiation (µmol photons m^{-2} s^{-1}) is given above each bar. Bars with differing letters are significantly different (ANOVA: $P < 0.05$). Error bars represent + 1 SE. N = 4 - 8.

There was significantly more of the ^{13}C label (μg g^{-1} DWT) in the shoots of *Cephalanthera* and *Fagus* than that detected in either *Corallorhiza* or *Neottia* (ANOVA [Box-Cox]: d.f. = 3,19; F = 91.8; $P < 0.001$) (Figure 4a). However, there were no significant differences in the amount of the ^{13}C label present in the tissues of *Cephalanthera* compared with *Fagus* or those of *Corallorhiza* compared with *Neottia* (ANOVA: $P > 0.05$) (Figure 4a). In terms of the proportion of the $^{13}CO_2$ label supplied that was fixed by the plant; there was no significant difference in the percentage of the label fixed by *Fagus* compared with *Cephalanthera* (ANOVA [Box-Cox]: $P > 0.05$; Figure 4b). Both *Fagus* and *Cephalanthera* fixed a significantly greater percentage of the label supplied than either *Corallorhiza* or *Neottia* whilst, somewhat surprisingly, *Neottia* contained a greater percentage of the label than *Corallorhiza* (ANOVA [Box-Cox]: d.f. = 3,19; F = 95.1; $P < 0.001$) (Figure 4b).

The amount of ^{13}C present in the plant shoots and the percentage of the label fixed are not functions of light availability as there is no relationship between photosynthetically active radiation (PAR) and ^{13}C content (Figure 4).

Discussion

A recent analysis (Zimmer *et al*., 2008) of the enrichment of the stable isotopes ^{13}C and ^{15}N in tissues of *Corallorhiza trifida* indicated a small but statistically significant depletion in the natural abundance of these two elements ($\delta^{13}C = -25.6$ and $\delta^{15}N = -0.3$) in this orchid relative to that seen in co-occurring plants of the fully myco-heterotrophic orchid *Neottia nidus-avis* ($\delta^{13}C = -24.0$; $\delta^{15}N = 4.9$). These values are similar to those recorded in the present study ($\delta^{13}C = -24.2$ and -23.15 for *Corallorhiza* and *Neottia* respectively; Appendix A). The observed difference between the two orchids was interpreted to indicate that *Corallorhiza* obtained a proportion of its carbon from photosynthesis. Indeed, Zimmer *et al*., (2008), on the basis of a stable isotope mixing model, concluded that the *Corallorhiza* plants which they analyzed had gained *c*. 23% of their carbon through autotrophic C fixation. Since the levels of C fixation by *Corallorhiza* observed in the present study were only *c*. 2 % of those seen in co-occurring *Fagus*, it would seem that its photosynthetic capacity is an order of magnitude lower than that of normal autotrophs.

Since, during their short period of development above ground, the green stems of this orchid will normally be exposed to diffuse irradiance, it is logical to expect that

some autotrophic activity could occur in their tissues. The presence of genes encoding for chlorophyll synthesis (Barrett & Freudenstein, 2008), the demonstration of the occurrence of chlorophylls a and b by Zimmer *et al*., (2008) and in the present study, as well of chlorophyll fluorescence, are also supportive of the notion that some potential for autotrophy can be expected in this orchid. However, all of these approaches to the question of the extent of photosynthetic activity in *Corallorhiza* are essentially indirect, there being only one previous report of direct analysis of its ability to assimilate C (Montfort & Küsters, 1940). This indicated that some autotrophic C fixation did occur in the orchid.

The direct measurements of $^{13}CO_2$ assimilation reported in the present study indicate that under similar conditions of irradiance, the quantitities of C fixed by photosynthesis in *Corallorhiza* are negligible relative to those seen in a co-occurring partially myco-heterotrophic (*sensu* Julou *et al*., 2005) leafy green orchid *Cephalanthera damasonium* or in saplings of *Fagus sylvatica*. Moreover, the amount of $^{13}CO_2$ assimilated by *Corallorhiza* was not significantly different from that detected in the tissues of *Neottia*. In this later case, the $^{13}CO_2$ detected must be a result of diffusion, incorporation into organic acids via the PEP carboxylase pathway or through non-photochemical processes as, by general consent, *Neottia* is unable to photosynthesize owing to a lack of critical light harvesting pigments (Menke & Schmidt, 1976; Reznik *et al*. 1969; Reznik, 1958; Montfort & Küsters, 1940; Drude, 1873).

Since our measurements were made under conditions of direct diffused solar irradiance and at the stage of maximum shoot extension in plants with fully developed green seed capsules it seems unlikely that there are other environmental or developmental conditions that would be more favorable for the expression of photosynthetic activity. Indeed, since all species examined co-occurred on identical substrates within a few meters other it is reasonable to assume that they were all experiencing the same soil conditions. Further, the phenology of this orchid is such that the opportunity for significant autotrophic accumulation of C is inevitably restricted as the flowering spikes only are exposed above ground for a very restricted period of time, normally not more than two months. Additional direct measurements of the kind described here are desirable and preferably these should be carried out over a period longer than the 4 hour duration employed in this study. Nonetheless, it is apparent from the results obtained in the parallel analyses of *Cephalanthera* that this period of exposure is sufficient to reveal C fixation when and where it is taking place. Moreover,

such exposure times are as long as or longer than those routinely employed for assessment of photosynthetic activity using infrared gas analysis techniques.

Chlorophyll fluorescence parameters for *Corallorhiza* indicate on the one hand the presence of active photosystem II reaction centers but on the other that the quantum efficiency of PS II (Φ_{PSII}) is reduced. In this orchid, the mean value of 0.7 for F_v/F_m was substantially lower than the multi species average of 0.83 recorded for healthy autotrophs by Maxwell & Johnson (2000). Such a value is potentially indicative of photo inhibition (Cameron *et al*., 2008b), though the analyses of Ritchie (2006) suggest that the values of F_v/F_m and Φ_{PSII} recorded here for *Corallorhiza* are not necessarily so depressed as to predict the absence of photosynthesis. Our failure to detect more than minimal C fixation suggests therefore that most of the excitation energy of the light harvesting complex of PSII (LHCII) is being transferred to an alternative electron acceptor, it then being dissipated as heat through non photosynthetic metabolism as described by Krause & Weis (1991). Such non photochemical quenching processes (NPQ) are known to be facilitated by xanthophylls (Johnson *et al*., 1993). Neither NPQ nor carotenoid composition of *Corallorhiza* could be measured in the present study, but in view of the observation (Bungard *et al*., 1999) that the non-photosynthetic holoparasite *Cuscuta reflexa* possesses a novel type of NPQ – related xanthophyll cycle linked with the transition from autotrophy to heterotrophy, analyses of these pathways in the orchid are called for.

Clearly, *C. trifida*, while retaining the genes encoding for chlorophyll synthesis (Barrett & Freudenstein, 2008), represents a late stage in the evolutionary development towards complete myco-heterotrophy. However, it appears from the present study that in a physiological context this orchid has moved more closely towards the fully myco-heterotrophic condition than has previously been recognized. These observations are consistent with those indicating that, in nature, *C. trifida* is routinely involved in tripartite symbiotic associations between ectomycorrhizal fungi and autotrophic overstory trees (Zelmer & Currah, 1995; McKendrick *et al*., 2000b). The mycelia of the fungal partners have been shown to provide pathways through which carbon is transferred from the trees to the large coralloid root systems which constitute the slowly developing below ground storage structures characteristic of this genus (McKendrick *et al*., 2000a). Having formed such an effective mechanism for assimilate acquisition it is perhaps not surprising that the contribution of photosynthesis to the C economy of the orchid, has, as indicated here by direct measurements, been so greatly reduced.

Acknowledgements

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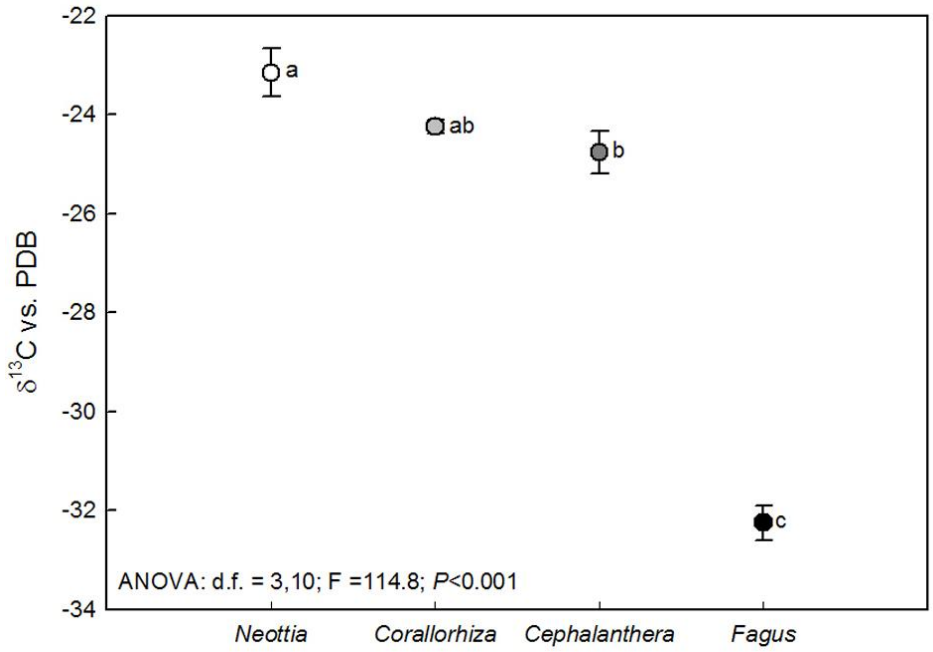
Appendix (available online)



Appendix A. Figure showing the natural abundance $\delta^{13}C$ signature (relative to the Pee Dee Belemnite international standard) of *Neottia nidus-avis*, *Corallorhiza trifida*, *Cephalanthera damasonium* and *Fagus sylvatica*. Error bars represent ± 1 SE. Points with differing letter codes are significantly different (ANOVA: d.f. = 3,10; F = 114.8; $P < 0.001$), $n = 3 - 4$.

CHAPTER 4

Confirming a new nutritinal mode in green orchids

Is it better to give than receive? A stable isotope perspective to orchid-fungal carbon transport in the green orchid species *Goodyera repens* and *G. oblongifolia*

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Key words: *Goodyera*, stable isotopes, ^{13}C, ^{15}N, mycorrhiza, orchids

Letter

In the field of orchid research species within the tribe Cranichideae have been at the center of attention due to the recent findings of Cameron *et al*. (2006, 2008) of carbon transport from adult *Goodyera repens* (L.) R. Br. orchids to their mycorrhizal fungus *Ceratobasidium cornigerum* (Bourdot) D.P. Rogers. The dependency of orchids in their early stages of development on fungi is a long recognized trait of the family (Bernard, 1909; Dearnaley, 2007). However, there has been much controversy over the potential for carbon "repayment" to the fungi once the orchid has formed leaves and is capable of assimilating its own carbohydrates through photosynthesis (Alexander & Hadley, 1985; McCormick *et al.*, 2006; Smith & Read, 2008).

Using ^{14}C labeled carbon either fed to the mycelia of the orchids' fungal symbiont or to the plant as $^{14}CO_2$ Cameron *et al.* (2008) were able to quantify the carbon transport between the orchid and fungus over an eight-day period. Their findings were that the net transfer of carbon from *G. repens* to *C. cornigerum* was over five times greater than that of carbon transported from the fungus to the plant. While this extremely well-executed study provides the "first full bidirectional C budget for any mycorrhizal association" (Cameron *et al.*, 2008), there are some limitations of their model and methods that must be taken into account. As mentioned in their recent article and the commentary by Johnson (2008), the C allocation to fungal biomass within the orchids' roots cannot be separated from that to the roots alone nor can C respiration from the plant *versus* the fungus. Furthermore, the use of radiocarbon labeling gives measurements of carbon flow within a system for only a relatively short period of time. Also, since many of these labeling experiments are carried out in the laboratory it is difficult to then relate results to any field setting. A complementary method that has been applied to examine C and N gains from fungi by partially and fully myco-heterotrophic plants associated with ectomycorrhizal (ECM) and litter or wood decaying saprotrophic (SAP) fungi is the use of naturally occurring stable isotopes of carbon and nitrogen (^{13}C:^{12}C and ^{15}N:^{14}N) (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Ogura-Tsujita *et al.*, 2008). Measured isotope abundances are denoted as δ values and are calculated according to the equation: $\delta^{15}N$ or $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard. In contrast to radiocarbon labeling, the analysis of field collected plants' bulk carbon isotope values gives an integrated view of carbon assimilation throughout the period the tissue was synthesized (Dawson *et al.*, 2002).

While there is a sub-set of orchid species that remain myco-heterotrophic for their entire life cycle and lack the ability to photosynthesize (Leake, 1994), it has been thought that green species are completely released from their dependency on heterotrophic C gain once leaves are formed (Alexander & Hadley, 1985). However, recent analysis of some green orchids' carbon and nitrogen isotope signatures has revealed that many of these putative autotrophic orchids that associate with ECM fungi actually still partially rely on these fungi to meet their carbon demands. These orchid species have been referred to as mixotrophs or partial myco-heterotrophs. Unlike obligate myco-heterotrophic orchids that have $\delta^{13}C$ signatures most similar to their fungal symbionts, mixotrophic orchids tend to have $\delta^{13}C$ signatures intermediate between those of surrounding autotrophic and myco-heterotrophic plants (Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007).

Interestingly an additional category of orchids that are depleted in ^{13}C compared to surrounding autotrophic plants is emerging from recent stable isotope analysis of species in the closely related tribes Orchideae and Cranichideae (Liebel HT *et al.*, unpublished), the latter containing the genus *Goodyera* (data herein). We collected leaf samples of *Goodyera oblongifolia* Raf. from four sites in northern California and southern Oregon, USA, and *Goodyera repens* from a single site in the Austrian Alps (Table 1).

Table 1: Sampling locations in the USA (CA, OR) and Austria (Vorarlberg) including *Goodyera* and reference species collected (*n* = number of replicates), and mean ± 1 SD $\delta^{15}N$ and $\delta^{13}C$ values [‰] in leaves of *Goodyera* and reference species.

Location	**Species (*n*)**	**$\delta^{15}N$**	**$\delta^{13}C$**
El Dorado National Forest, CA	*Goodyera oblongifolia* (4)	-2.4 ± 0.8	-32.3 ± 0.8
38˚54'01.70"N 120˚34'26.77"W	*Abies concolor* (5)	-3.4 ± 0.9	-31.0 ± 0.3
El Dorado National Forest, CA	*G. oblongifolia* (5)	-3.2 ± 0.8	-33.3 ± 1.2
38˚54'3.47"N 120˚34'28.40"W	*A. concolor* (5)	-4.0 ± 0.7	-30.6 ± 0.7
	Ribes roezlii (5)	-4.3 ± 1.2	-31.4 ± 0.6
Plumas National Forest, CA	*G. oblongifolia* (5)	-2.0 ± 1.1	-33.2 ± 0.7
40˚03'36.02"N 120˚51'32.99"W	*A. concolor* (5)	-3.8 ± 1.1	-30.4 ± 1.0
Willamette National Forest, OR	*G. oblongifolia* (1)	-1.6	-33.4
44º18'36.00"N 122º00'36.02"W	*Tsuga heterophylla* (5)	-2.2 ± 0.8	-31.9 ± 1.9
Marultal, Vorarlberg	*G. repens* (5)	-6.2 ± 1.1	-36.6 ± 1.9
47°11'44''N 9°53'57''E	*Knautia sylvatica* (5)	-8.9 ± 0.8	-32.1 ± 0.6
	Mercurialis perennis (5)	-7.7 ± 0.6	-30.3 ± 0.7
	Vaccinium vitis-idaea (5)	-9.7 ± 0.9	-30.5 ± 0.5

The site in the Alps was an open rocky outcrop habitat, while all samples collected in the USA were from the deeply shaded understories of mixed conifer forests. Altogether, leaves of 15 *G. oblongifolia* and five *G. repens* individuals were collected. In addition, at each sampling site a minimum of five autotrophic individuals from at least one species were collected for a total of 40 individuals of six species (Table 1). These collections were used as reference plants representative of the autotrophic understory.

The collected plant samples were then analyzed for carbon and nitrogen stable isotope abundances *via* elemental analyzer/continuous flow isotope ratio mass spectrometry at either the BayCEER - Laboratory of Isotope Biogeochemistry University of Bayreuth, Germany or at the Center for Stable Isotope Biogeochemistry at University of California Berkeley as described in Zimmer *et al.* (2007). Once δ values were obtained for all samples from the USA (Table 1), the $\delta^{15}N$ and $\delta^{13}C$ values of all reference plants were tested for inter-site variation with a one-way ANOVA and Tukey's HSD. Due to significant differences at an α 0.05 among $\delta^{15}N$ values of the reference plants between two sites in California ($P = 0.007$) the δ values from the USA could not be pooled to make comparisons across sites between *Goodyera* samples and their respective references. To make these comparisons δ values for both elements and all samples collected in the USA and the single Austrian site (for consistency) were converted into site-independent enrichment factors (ε) and pooled based on species identity and location (USA or Austria). The calculation of ε factors systematically eliminates the majority of the influence of spatial variation on δ values due to site-specific differences in C and N isotope abundances, thus allowing for comparisons of these values across sites (Emmett *et al.*, 1998; Gebauer & Taylor, 1999; Preiss & Gebauer, 2008). First, for each site the $\delta^{13}C$ and $\delta^{15}N$ values of reference plants were averaged. Then, on a per site basis these averages were subtracted from the *Goodyera* samples' and reference plants' $\delta^{13}C$ and $\delta^{15}N$ values to create site-independent enrichment factors ($\varepsilon = \delta x_S - \delta x_R$) for each sample where $\delta x_S = \delta^{13}C$ or $\delta^{15}N$ of individual samples per site and δx_R = mean $\delta^{13}C$ or $\delta^{15}N$ of all reference plants per site. The resulting mean of both ^{13}C and ^{15}N ε factors of the autotrophic reference plants is equal to 0 ‰. However, the enrichment factors of individual reference plants cluster around 0 ‰, reflecting the small inter- and intraspecific variations in their isotope signatures that are not significantly different between sites.

The two *Goodyera* species' ε factors separated as distinct groups for both elements based on the differences of their δ values from the mean of their respective

references (Figure 1). The variance around the mean $\delta^{13}C$ or $\delta^{15}N$ values of reference plants used to calculate $\varepsilon^{15}N$ and $\varepsilon^{13}C$ is retained by calculating ε factors for not only both *Goodyera* species, but reference plants on a site by site basis. Statistical comparisons between the individual enrichment factors of individual *Goodyera repens*, *G. oblongifolia* plants and their respective autotrophic references from either Austria or western USA were made by Mann-Whitney U tests. Both *Goodyera repens* ($P = 0.002$) and *G. oblongifolia* ($P = 0.008$) were significantly enriched in ^{15}N compared to surrounding autotrophic plants (Figure 1). In contrast, both *Goodyera* species were significantly depleted in ^{13}C in comparison to their references ($P < 0.001$; Figure 1). *Goodyera repens* plants from the open sunny habitat in the Alps were considerably more depleted in ^{13}C compared to *G. oblongifolia* from deeply shaded forests.

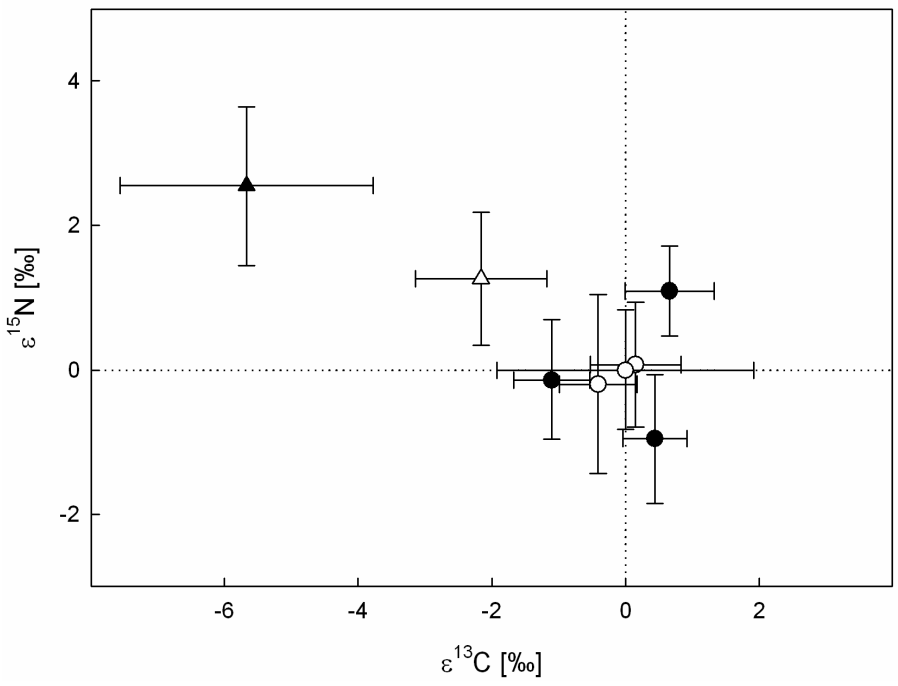

Figure 1. Mean enrichment factors (ε) of ^{13}C and ^{15}N from the leaves of *Goodyera oblongifolia* (open triangle), *Goodyera repens* (closed triangle) and each species of autotrophic reference plants collected in the USA (open circles) and in Austria (closed circles). Error bars indicate 1 SD for each *Goodyera* species and their respective reference plants.

Although the sample size of *G. oblongifolia* and *G. repens* individuals collected in this study were relatively small, the stable isotope evidence presented here shows that these orchids do not exhibit any trends toward full or partial myco-heterotrophy. In fact, these orchids' consistent depletion in ^{13}C compared to surrounding autotrophic plants reveals a distinct nutritional strategy. The physiological mechanism leading to this depletion

remains unknown, but may be related to the transfer of ^{13}C enriched C compounds from these orchids to their associated fungi (*sensu* Gleixner *et al.*, 1993). This would fit well with Cameron *et al.*'s (2006, 2008) findings of C transfer from orchid to fungus, as well as with isotope food-chain models where the source of a nutrient is left depleted in the heavy isotope compared to the sink (Fry, 2006). What is unclear is why *Goodyera* species would be significantly more depleted in ^{13}C than surrounding autotrophic mycorrhizal plants that are transferring substantial amounts of carbon to their fungal symbionts (Smith & Read, 2008).

Habitat may also play a key role in determining the ^{13}C enrichment factors of *Goodyera* species. For instance, there exists some evidence that green orchids capable of partial myco-heterotrophy increase their dependency on fungal assimilated C when in deeply shaded habitats, leading their leaf $\delta^{13}C$ values to become more enriched than those of surrounding autotrophic plants (Bidartondo *et al.*, 2004; McCormick *et al.*, 2004; Zimmer *et al.*, 2007). If *G. oblongifolia* individuals from our forested sites were at an earlier stage of seedling development more dependent on heterotrophic carbon gain than *G. repens* from open sites, then this could explain why the previous is less depleted in ^{13}C than the latter. The significant enrichment in ^{15}N (a hallmark of all myco-heterotrophic orchids studied to date) found in both *Goodyera* species supports this and Cameron *et al.*'s (2008) statement that these orchids are more parasitic upon their fungal symbionts than other mycorrhizal plants and therefore may govern the amount of nutrient exchange to the fungus. This idea of 'orchid control' over its mycorrhizal associations is further exemplified by the unique morphology of orchid mycorrhizas where fungi that are known to be saprotrophic or ectomycorrhizal when independent of orchids form intracellular coils when in association with orchids (Rasmussen, 2002).

Based on Cameron *et al.*'s (2006, 2008) work *Goodyera repens* now provides the first example of an orchid species that upon becoming photosynthetically active can transfer carbon back to its mycorrhizal fungus. Unlike other green orchids studied to date, species within the tribes Orchideae and Cranichideae including *G. repens* and *G. oblongifolia* are the first species found to be depleted in ^{13}C compared to surrounding autotrophic plants (Liebel HT *et al.*, unpublished; data herein). In summary, based on carbon stable isotope abundances and identity of their mycorrhizal associates it is now clear that terrestrial orchids can utilize at least four nutritional strategies: autotrophy, where green orchids have carbon isotope signatures indistinguishable from surrounding

autotrophs and mainly associate with *Rhizoctonia* species (a polyphyletic group of fungi); partial myco-heterotrophy, where green orchids have carbon isotope signatures intermediate between those of autotrophs and myco-heterotrophs and associate with ECM fungi; obligate myco-heterotrophy, where orchids have lost the ability to photosynthesize, are specialized on either ECM or SAP fungi, and are enriched in ^{13}C similar to their host fungi; and an additional strategy found in green orchids in the tribes Orchideae and Cranichideae, which mainly associate with ceratobasidioid and tulasnelloid fungi and are depleted in ^{13}C compared to surrounding autotrophs (Figure 1; Liebel HT *et al.*, unpublished). The variability of the ecology and physiology of orchids is not surprising for the largest plant family whose evolutionary history potentially stretches back to the late Cretaceous (Ramírez *et al.*, 2007). While there is still much to discover about the intriguing Orchidaceae, combining the use of naturally abundant isotopes and radioactive tracers along with molecular methods, especially those that allow comparisons at the genotype level (Johnson, 2008), will continue to help us understand the links between the evolutionary history of orchids, their physiology and interactions with fungi.

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CHAPTER 5

Constraints to nutritional modes in orchids

C and N stable isotope signatures reveal constraints to nutritional modes in orchids from the Mediterranean and Macaronesia

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Abstract

During early development orchids are fully dependent on mycorrhizal fungi (i.e., myco-heterotrophic). At maturity they become autotrophic, partially myco-heterotrophic or remain fully myco-heterotrophic. Using carbon (C) and nitrogen (N) stable isotope signatures and fungal DNA analyses, we compared orchids from the Mediterranean region, a hotspot of orchid diversity, and from the adjacent islands of Macaronesia in order to understand how ecological factors and habitat types determine the occurrence of different nutritional modes and impose restrictions upon orchid distribution. We hypothesized that partial and full myco-heterotrophy would be restricted to light-limited forest understories because these nutritional modes are constrained by the occurrence of suitable ectomycorrhizal fungi.

This is so far the widest assessment of orchid nutrition in natural ecosystems. Covering a range of habitats from dark forests to open sites, leaf and root samples of 35 orchid species from 14 genera were collected from 20 locations in continental Italy, Sardinia (Mediterranean) and Tenerife (Macaronesia) to test for myco-heterotrophy. Mycorrhizal fungi were identified *via* molecular analyses and stable isotope analyses were applied to test whether organic nutrients are gained from the fungal associates.

Our results show that only orchids of the tribe Neottieae growing in dark forests and associating with ectomycorrhizal fungi rely heavily or fully on myco-heterotrophy. Interestingly, orchids exhibiting this nutritional mode are missing in Macaronesia. Adult orchids of open habitats in the Mediterranean and Macaronesia show weak or no N gains from fungi and do not profit from C gain through myco-heterotrophy. Some of them may even provide C to their fungal partners.

We conclude that partial and full myco-heterotrophy in Mediterranean orchids are restricted to Neottieae of light-limited ectomycorrhizal forests, thus raising the novel hypothesis that, rather than orchid dispersal limitation, the limited diversity of host ectomycorrhizal plants and fungi is the reason for the lack of myco-heterotrophic orchids in Macaronesia.

Introduction

Early research by Bernard (1909) first described how orchids live in close mycorrhizal symbiosis with fungi. Since that time, orchid mycorrhizas have attracted much interest from plant ecologists and mycologists. Previous studies investigating orchid nutrition

have mainly focused on temperate regions with only marginal consideration of regions with Mediterranean climate (see Gebauer & Meyer, 2003; Selosse *et al.*, 2004; Girlanda *et al.*, 2006), despite the fact that the Mediterranean region shows a much higher orchid diversity (e.g., 56 species in Germany (Rothmaler, 2000) *versus* 108 orchid species in Italy alone (Ministero dell'Ambiente e della Tutela del Territorio, 2007)). In striking contrast, the adjacent climatically similar Macaronesian region is poor in orchid species (16 orchid species in Macaronesia including only eight orchid species in the Canary Islands (Eriksson *et al.*, 1979; Hohenester & Welß, 1993).

Orchids typically produce extremely light 'dust seeds' which are easily transported over large distances by the wind. Thus, orchid seed dispersal between the Mediterranean and Macaronesia is to be expected. The tiny seeds do not contain sufficient endosperm for germination and depend on nutrient supply by a fungal partner. In developing orchids (i.e., protocorms), Bernard detected easily cultivable saprotrophic or pathogenic rhizoctonia-forming fungi (belonging to the basidiomycete genera *Tulasnella*, *Thanatephorus*, *Ceratobasidium* and the biotrophic clade B of *Sebacina*; Bernard, 1909; Weiss *et al.*, 2004; Smith & Read, 2008). Perhaps with the exception of a few epiphytic tropical orchids, all orchids investigated so far remain mycorrhizal during their entire life cycle (Smith & Read, 2008).

There are some non-photosynthetic orchids that completely depend on their fungal partners throughout their life. Altogether, about 200 fully myco-heterotrophic orchids (MHO) have been described (Leake, 1994). Many MHOs depend on ectomycorrhizal (ECM) fungi that are simultaneously associated with overstorey plants (Bidartondo, 2005). Furthermore, MHOs of the tribe Neottieae often exhibit a pronounced mycorrhizal specificity towards hardly cultivable lineages of ECM fungi (Selosse *et al.*, 2002; Bidartondo *et al.*, 2004). Within other tribes of the Orchidaceae, ECM fungi have also been found to form associations with *Hexalectris* and *Corallorhiza* species (McKendrick *et al.*, 2000; Taylor *et al.*, 2003). These orchids' dependence on ECM fungi was revealed by molecular identification of fungi forming coils (i.e., pelotons) inside orchid roots (Taylor & Bruns, 1997) in combination with stable isotope natural abundance analysis (Gebauer & Meyer, 2003). The latter technique is useful to understand pathways for the acquisition of fungi-derived organic C and N based on stable isotope abundances in plant leaf tissue. The method is based on the observation that tissues from fruiting bodies of ectomycorrhizal fungi show a higher abundance of the heavy stable isotopes ^{13}C (Gleixner *et al.*, 1993; Högberg *et al.*, 1999) and ^{15}N

(Gebauer & Dietrich 1993) in comparison to neighboring autotrophic plants. MHOs relying on ECM fungi are therefore also enriched in both ^{13}C and ^{15}N similarly to ECM fungi themselves (Trudell *et al.*, 2003). Using stable isotope natural abundance analysis, some green orchids previously considered to be fully autotrophic (e.g., *Cephalanthera* and *Epipactis* spp.) were found to also have isotope signatures distinct from surrounding plants. Such orchids showing ^{13}C and ^{15}N abundances intermediate between autotrophic non-orchid neighboring plants and fully MHOs were classified as partially myco-heterotrophic (Gebauer & Meyer, 2003). This physiological phenomenon is not limited to the Orchidaceae, as a similar mechanism has recently been discovered also in pyroloids (Ericaceae) (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007; Hynson *et al.*, 2009a). Furthermore, even non-photosynthetic forms of generally green species may survive due to myco-heterotrophic nutrient supply (Julou *et al.*, 2005; Abadie *et al.*, 2006).

It has never been investigated whether the occurrence of full and partial C and/or N myco-heterotrophy (i.e., heterotrophy *sensu* Larcher, 2003 and Lüttge *et al.*, 2005) is coupled to specific types of habitats and how the diversity of ectomycorrhizal plants and fungi constrains the distribution of these nutritional modes. Here, in the widest test for myco-heterotrophy in natural ecosystems carried out to date, the nutritional mode of Mediterranean and Macaronesian orchids growing in open habitats, shrubland, forest gaps and forests was investigated in continental Italy and the islands of Sardinia (Mediterranean) and Tenerife (Macaronesia), to test whether the occurrence of full and partial myco-heterotrophy among orchids is restricted to habitats distinguished by their light regime and available fungi.

Materials and methods

Study sites

Orchids of three main regions were investigated: (1) the northern part of continental Italy (N 44.1-45.2°; E 7.1-10.1°), (2) the Mediterranean island of Sardinia (Italy, N 41.2-39.7°; E 9.4-9.8°) and (3) the Macaronesian island of Tenerife (Spain, N 28.2-28.4°; W 16.5-16.8°). The Mediterranean sites are characterized by summer droughts and a maximum of precipitation between October and May (mean annual precipitation at the sites: 800 – 1150 mm in continental Italy; 450 – 800 mm on Sardinia). The temperatures rarely reach 0 °C in winter and rise in summer to mean temperatures

around 25 °C in the months July and August. The mean annual precipitation of investigated sites on Tenerife is 400 – 700 mm (with an additional component from humidity combed out by pine trees from daily orographic fog due to the permanent stream from north easterly trade winds at a site with *Orchis canariensis*). The rainy period lasts from October to March; the mean annual temperatures vary from 10-18 °C according to altitude and exposure (Höllermann, 1982; García Canseco, 2004).

Orchids from 20 sites were investigated. Each of these sites was classified as one of the following habitat types: open habitat, shrubland, forest and forest gap. Since all orchids investigated in deciduous forests developed leaves after tree canopy development and disappeared before fall of tree leaves, a further distinction according to orchid phenology between deciduous and evergreen forests was not necessary. The different habitat types are distinguished by accompanying plant species, mycorrhizal associations and light climate. To clarify the habitat-dependent light climate, relative light availability (%) was calculated by comparing simultaneously performed PAR measurements (Quantum Sensor, Li-Cor, Lincoln, NE, USA) close to the orchid leaves and above the canopy or outside the forests, respectively. Mean relative light availability was the lowest at forest (7 ± 4 %; n = 4) and shrubland sites (7 %; n = 2) and the highest at open sites (84 ± 18 %; n = 10) whereas irradiances at forest gap sites ranged in-between (57 ± 30 %; n = 3). In continental Italy, sites had a maximum distance of 270 km and plants were collected at three open grassland sites, two deciduous broadleaf forest sites and one forest gap. Sites on Sardinia were distributed among the whole island (max. distance of 165 km) and orchids were sampled at seven open habitat sites (grassland, degraded steppe or open places in patchy macchia), two evergreen (*Quercus ilex*) forest sites and one shrubland site. Plant material on Tenerife was taken from one open grassland site, two gaps of coniferous (*Pinus canariensis*) forest and one shrubland site with a maximum distance of 40 km. Detailed site descriptions including vegetation characteristics, light availability data, geographic coordinates and details on the collected species can be found in Appendix A.

Standardized vegetation surveys per plot, recording all plant species surrounding the target orchid within $1m^2$, were set up and the mycorrhizal type of each species was investigated using the review article on the phylogenetic distribution of mycorrhizas in land plants of Wang & Qiu (2006). Plants that depend on ectomycorrhizal associations (mainly Fagaceae, Pinaceae and Cistaceae) were found in most plots irrespective of habitat type (see Table 2).

Sampling scheme and investigated species

A total of 35 orchid species were investigated (27 members of the tribe Orchideae, one of the Cranichideae and seven of the Neottieae). Five of the 35 orchid species were collected in two of the three main regions. In continental Italy, 15 orchid species of all three tribes were sampled, while the 19 orchid species collected on Sardinia belong to the tribes Orchideae and Neottieae and the six species from the Macaronesian region exclusively belong to the Orchideae. All samples were collected in April and May 2007 except for *Barlia metlesicsiana* on Tenerife (collected in 2008). Orchid species nomenclature follows Baumann *et al.* (2006) except for the island endemites of Sardinia (Delforge, 2005).

Sites having at least five individuals of an orchid species growing a minimum of two meters apart from each other (to avoid sampling orchid clones) were located. To evaluate the orchids' stable isotope signatures, each of the orchid plots (i.e., area around the orchid, max. 1 m apart) additionally had to contain three autotrophic reference plants (listed in Appendix A). For each orchid species, samples were collected from five plots yielding five replicates to allow statistical validation (except for *Cephalanthera damasonium*, $n = 2$). One to two leaves of the orchid and the reference plants were sampled. Leaf material was taken at approximately the same height as it is known that the CO_2 uptake and stomatal regulation at different heights above the surface results in different $\delta^{13}C$ values due to different CO_2 sources (soil vs. atmosphere), light climate and vapour pressure (Farquhar *et al.*, 1989; Gebauer & Schulze, 1991; Bauer *et al.*, 2000). As *Neottia nidus-avis* has only a few small bracts, a section of the above-ground inflorescence was collected instead of leaves.

Analysis of stable isotope abundance and N concentration

Leaf and stem samples were oven-dried at 105°C and ground to a fine powder. Relative C and N isotope abundances were measured using a dual element analysis mode with an elemental analyzer coupled to a continuous flow isotope ratio mass spectrometer as described in Bidartondo *et al.* (2004). Measured isotope abundances are denoted as δ values, which were calculated according to the following equation: $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard}-1) \times 1000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard. Standard gases were calibrated with respect to international standards by using the reference substances ANU sucrose and NBS 19 for carbon isotopes and N1 and N2 for nitrogen isotopes,

provided by the International Atomic Energy Agency (Vienna, Austria). Reproducibility and accuracy of the isotope abundance measurements were routinely controlled by measures of the test substance acetanilide (Gebauer & Schulze, 1991). At least six test substances with varying sample weight were routinely analyzed within each batch of 50 samples. Maximum variation of $\delta^{13}C$ and $\delta^{15}N$ within as well as between batches was always below 0.2 ‰. Nitrogen concentrations in the leaf samples were calculated from sample weights and peak areas using a daily six-point calibration curve based on the acetanilide measurements (Gebauer & Schulze, 1991). Acetanilide has a constant N concentration of 10.36 %.

Statistics

ANOVA analysis and *post hoc* comparisons based on Tukey HSD test of reference plant $\delta^{13}C$ and $\delta^{15}N$ values indicated a significant site effect for 50 % of the sites ($P < 0.001$). Thus, a normalization of δ values was necessary in order to compare data between the 20 sites. As described by Preiss & Gebauer (2008), the $\delta^{13}C$ and $\delta^{15}N$ values of the orchids and the non-orchid autotrophic reference plants were used to calculate normalized enrichment factors for each sample as $\varepsilon_S = \delta_S - \delta_{REF}$; with S as single value of a sample from an autotrophic, partially or fully myco-heterotrophic orchid and REF as mean value of all autotrophic reference plants from the respective plot. Although it has been shown that the ^{13}C and ^{15}N signature of fully autotrophic C_3 plants in temperate climates does not systematically depend on their life form or mycorrhizal status (Gebauer & Dietrich, 1993; Gebauer & Meyer, 2003; Zimmer *et al.*, 2007), we kept the spectrum of reference plants as diverse as possible (monocotyledons / dicotyledons, tree saplings / herbs, evergreen / deciduous, ectomycorrhizal / ericoid- / arbuscular- or non-mycorrhizal) to minimize errors when calculating relative enrichments of the orchids.

To test for significant differences the Kruskal-Wallis non-parametric test and Bonferroni-corrected Mann-Whitney U-tests (Holm, 1979) for *post hoc* comparisons were used. For the calculations of the enrichment factors of *Serapias cordigera* (Sardinia) only two reference species were taken into account. *Centaurium maritimum* (L.) Fritsch was excluded as a reference species as it showed surprisingly high $\delta^{13}C$ and $\delta^{15}N$ values. Some members of Gentianaceae are fully myco-heterotrophic (Imhof, 1999; Imhof & Weber, 2000); hence, a partially myco-heterotrophic nutritional mode may be expected in members of this family.

A cluster analysis (Ward's method, Euclidean distance measure) based on the relative enrichment in ^{13}C and ^{15}N of the different orchid species collected in the three sampling areas in comparison to the respective non-orchid references (ε-values) was carried out to identify groups within the dataset.

Statistical analyses were performed with SPSS v.11.5 (SPSS Inc., Chicago, IL, USA) and PC-ORD v.5.03 (MjM Software, Gleneden Beach, OR, USA). Data are given as means ± 1 SD.

Molecular identification of mycorrhizal fungi

From each of the five individuals of each orchid species two root sections colonized by fungi were sampled and placed in lysis buffer (CTAB). Roots of four orchid species from continental Italy (*Ophrys fuciflora*, *Orchis purpurea*, *Orchis laxiflora* and *Serapias vomeracea*) were analyzed at the Dipartimento di Biologia Vegetale in Torino. From these samples genomic DNA was extracted, amplified and sequenced as described in Girlanda *et al.* (2006). All other orchid root samples were analyzed at the Royal Botanic Gardens in Kew. These samples were frozen and thawed three times before grinding the softened tissue with a micropestle. Genomic DNA was extracted following methods described elsewhere (Gardes & Bruns, 1993) but using GeneClean® II Kit (Q-BioGene, Carlsbad, CA, USA) for DNA binding and purification. Using polymerase chain reaction (PCR), the nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primers ITS1F and ITS4 and PCR using conditions described in Gardes & Bruns (1993). Positive PCR products were purified using QIAquick®Multiwell PCR Purification Kit (Quiagen, Valencia, CA, USA). DNA sequencing was performed on an ABI3730 Genetic Analyzer using BigDye® v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. Electrophoretograms were checked using Sequencher v.4.5 (Gene Codes Corporation, Ann Arbor, MI, USA). All samples with strong PCR amplification of single templates were compared to GenBank using BLAST to ascertain taxonomic affinity. If impure electrophoretograms were obtained the PCR products were cloned using the TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA) and analyzed as described above. All unique DNA sequences have been submitted to GenBank (FJ688104-FJ688132 and FJ809762-FJ809770).

Results

Stable isotope abundances

The cluster analysis based on the orchids' isotope signatures revealed three categories (see Appendix B and boxes in Figure 1): (1) orchids collected in forests, (2) orchids of open habitats and forest gaps and (3) an intermediate group composed of orchids from all four habitat types (open, forest gap, shrubland and forest). While orchids from continental Italy and Sardinia cover all three clusters, the group of forest orchids is missing on Tenerife (Figure 1). Species belonging to this group of typically forest-dwelling orchids are members of the tribe Neottieae and characterized by considerable enrichments in ^{13}C and ^{15}N in comparison to non-orchids of the respective habitats (Figure 1). The highest enrichment in ^{13}C (6.4 ± 1.8 ‰) and ^{15}N (13.9 ± 1.9 ‰) was found for *Neottia nidus-avis*, the only chlorophyll-lacking orchid of this investigation, that accordingly shows enrichment factors characteristic of fully MHOs associated with ECM fungi (Preiss & Gebauer, 2008). The cluster of orchids from open sites and forest gaps is composed of species of the tribes Orchideae and Cranichideae. They are relatively enriched in ^{15}N compared to non-orchid references though their ^{15}N enrichment is considerably lower than that of orchids from forest sites. With regard to the ^{13}C signature, most of these species are statistically not distinguished from surrounding photosynthetic reference plants while some show relative ^{13}C depletion (Table 1). The intermediate orchid group comprises members of all three tribes including two neottioids in continental Italy (*Cephalanthera longifolia* and *Listera ovata*). Plants of this category do not show the typical high enrichment in ^{13}C and ^{15}N as forest orchids do but they are enriched in ^{13}C compared to non-orchids from their respective sites and to most orchids of open habitats.

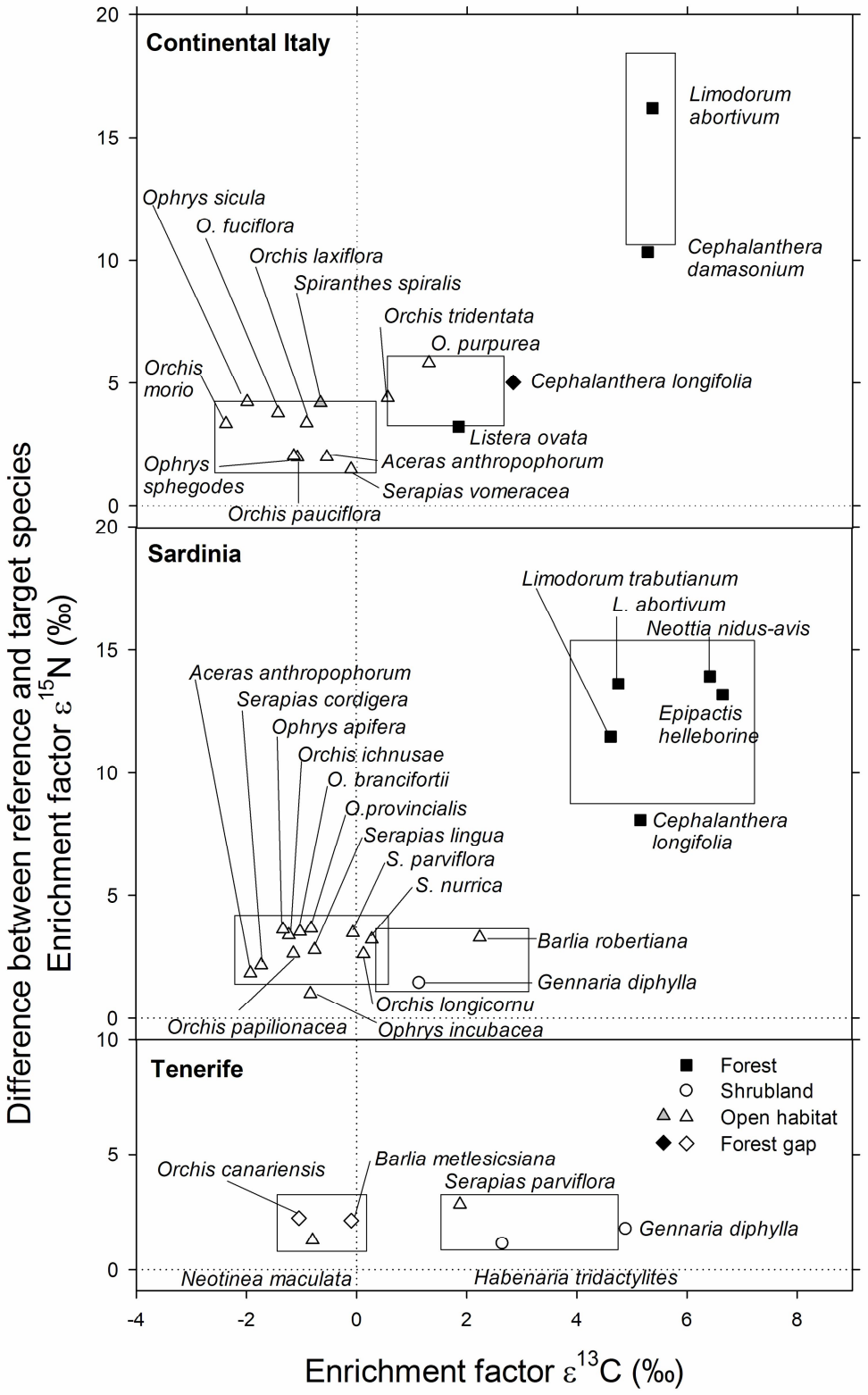

Figure 1. Enrichment factors (ε) for ^{13}C and ^{15}N of 35 orchid species collected at 20 sites in continental Italy, Sardinia and Tenerife. Orchids of the tribe Neottieae are indicated with black, Orchideae with white and Cranichideae with grey symbols. The boxes represent one SD of the mean ε values for three groups of orchids as obtained from a cluster analysis: orchids of open habitats (left boxes), typical forest orchids (right boxes, not present on Tenerife) and orchids with intermediate isotope signatures (middle boxes). After normalization, mean ε values of the autotrophic references are equal to zero. All δ values of ^{13}C and ^{15}N of orchids and reference species as well as the diagram of the cluster analysis are available in Appendix A and B.

Table 1. Nitrogen concentrations and significances for the relative enrichment or depletion in ^{13}C and ^{15}N of all investigated orchid species compared to their respective autotrophic reference plants. Several orchids show significantly higher N concentrations than the reference plants (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Species of the tribe Neottieae are given in bold. $n = 5$ for all orchid species, except for *Cephalanthera damasonium* ($n = 2$); full dataset including reference plants is available in Appendix A.

REGION / Orchid species	**Enrichment (+) or depletion (-) in ^{13}C**	**Enrichment (+) or depletion (-) in ^{15}N**	**N conc. ± 1 SD [mmol/g_{DM}]**
CONTINENTAL ITALY			
Cephalanthera damasonium	+	+	2.92
Cephalanthera longifolia	+ **	+ ***	3.10 ± 0.41 ***
Limodorum abortivum	+ ***	+ ***	2.40 ± 0.40 *
Listera ovata	+ ***	+ ***	3.13 ± 0.37 ***
Aceras anthropophorum	-	+ *	1.59 ± 0.17
Ophrys fuciflora	-	+ ***	2.52 ± 0.26 ***
Ophrys sicula	- *	+ ***	1.03 ± 0.17
Ophrys sphegodes	- *	+ *	1.86 ± 0.33 *
Orchis laxiflora	- *	+ **	1.84 ± 0.13
Orchis morio	-	+ ***	1.53 ± 0.26
Orchis pauciflora	- *	+ *	1.11 ± 0.19
Orchis purpurea	+ **	+ ***	1.66 ± 0.13
Orchis tridentata	+	+ ***	1.70 ± 0.25
Serapias vomeracea	-	+ ***	1.24 ± 0.20
Spiranthes spiralis	-	+ ***	2.43 ± 0.29 ***
SARDINIA			
Cephalanthera longifolia	+ ***	+ ***	3.37 ± 0.22 ***
Epipactis helleborine	+ ***	+ ***	3.68 ± 0.44 ***
Limodorum abortivum	+ ***	+ ***	2.16 ± 0.27 ***
Limodorum trabutianum	+ ***	+ ***	2.35 ± 0.20 ***
Neottia nidus-avis	+ ***	+ ***	2.59 ± 0.12 ***
Aceras anthropophorum	- **	+ **	1.48 ± 0.24
Barlia robertiana	+ ***	+ ***	0.94 ± 0.21
Gennaria diphylla	+	+ *	1.61 ± 0.43 ***
Ophrys apifera	- *	+ **	1.68 ± 0.06 ***
Ophrys incubacea	-	+ *	1.50 ± 0.46
Orchis brancifortii	-	+ ***	1.39 ± 0.11
Orchis ichnusae	- **	+ ***	2.01 ± 0.05
Orchis longicornu	+	+ **	1.73 ± 0.27 *
Orchis papilionacea	-	+ ***	1.73 ± 0.31 **
Orchis provincialis	- *	+ ***	1.89 ± 0.20 ***
Serapias cordigera	-	+	1.12 ± 0.13
Serapias lingua	-	+ ***	1.44 ± 0.20 *
Serapias nurrica	+	+ *	1.93 ± 0.38 ***
Serapias parviflora	-	+ ***	1.40 ± 0.33

(Continued)

Table 1. Continued

REGION / Orchid species	Enrichment (+) or depletion (-) in ^{13}C	Enrichment (+) or depletion (-) in ^{15}N	N conc. ± 1 SD [mmol/g_{DM}]
TENERIFE			
Barlia metlesicsiana	-	+ **	2.03 ± 0.47 *
Gennaria diphylla	+ ***	+ *	2.09 ± 0.16
Habenaria tridactylites	+ **	+	1.90 ± 0.30
Neotinea maculata	-	+	2.27 ± 0.27
Orchis canariensis	- *	+ **	1.59 ± 0.13 ***
Serapias parviflora	+	+ ***	1.60 ± 0.19 ***

Cephalanthera longifolia – one of the five species that were sampled at two different sites - falls into the group of forest orchids (Sardinia) or the intermediate group (continental Italy), depending on the respective habitat type. In continental Italy *Cephalanthera longifolia* was collected at a forest gap with relative light availability of 23 % and on Sardinia in a densely shaded forest with only 2 % of irradiance reaching the understorey plants. *Serapias parviflora* was also collected on two different sites with varying relative light availability of 62 % and 90 % and belongs to the group of orchids of open habitats on Sardinia while individuals collected at the more exposed grassland terraces on Tenerife are slightly enriched in ^{13}C (not significantly, Table 1) and therefore fall into the intermediate group (Figure 1).

Regarding the orchids' taxonomy, it becomes apparent that all neottioids are significantly enriched in ^{13}C and ^{15}N compared to autotrophic reference plants (Figure 1, Table 1) - some of them (e.g., *Epipactis helleborine* on Sardinia) even as strong as obligate myco-heterotrophs. Most representatives of the Orchideae and Cranichideae show significant relative enrichments in ^{15}N as well, but only a few members of the Orchideae (i.e., *Gennaria diphylla* from Tenerife, *Barlia robertiana*, *Orchis purpurea*, and *Habenaria tridactylites*) are additionally enriched in ^{13}C. For some species of the genera *Ophrys*, *Orchis* and *Aceras* (all Orchideae), a significant depletion in ^{13}C in relation to their autotrophic reference plants was found (Table 1).

Nitrogen concentrations

The total N concentrations in leaf material of the neottioids (2.85 ± 0.58 mmol/g_{DM}, DM: dry mass, n = 42) are significantly (P < 0.001) higher than in leaves of non-neottioid orchids (1.67 ± 0.44 mmol/g_{DM}, n = 155). However, the group of non-neottioid orchids still has significantly (P < 0.001) higher leaf total N concentrations than the

group of autotrophic reference species (1.40 ± 0.53 mmol/g_{DM}, n = 513) though this latter effect was not always significant on a species level based on plot comparisons (Table 1).

Molecular identification of mycorrhizal fungi

All investigated orchid roots contained fungal pelotons inside their root cortex cells and mycorrhizal fungi could be identified from 50 % of these roots. All neottioids from which fungal DNA could be extracted and analyzed are associated with ECM fungi (Table 2). The highest specificity to ECM partners is found in *Neottia nidus-avis* and *Limodorum* species. In only few species of forest gaps and shrublands obligate ECM fungi could be found (*Orchis canariensis* and *Gennaria diphylla*) and the majority of orchids of open habitats are associated with rhizoctonia-forming basidiomycetes (e.g., *Ceratobasidium* and *Tulasnella*) and ascomycetes (e.g., *Leptodontidium*).

Table 2. Mycorrhizal fungi of orchids from continental Italy, Sardinia and Tenerife and presence (+) / absence (-) of ectomycorrhizal (ECM) plants at the respective sites. All roots were collected in 5 replicates. Obligate ectomycorrhizal fungi are indicated in bold

Tribe	REGION/ Orchid species	Mycorrhizal fungi	ECM plants
	CONTINENTAL ITALY		
Orchideae	*Ophrys fuciflora*	*Ceratobasidium*† (2), *Tulasnella**† (5)	+
	Ophrys sphegodes	*Tulasnella**† (2)	-
	Orchis laxiflora	*Ceratobasidium*† (2), *Tulasnella**† (5)	-
	Orchis purpurea	*Ceratobasidium*† (2), *Tulasnella**† (4)	-
	Serapias vomeracea	*Ceratobasidium*† (1), *Sebacina**† (1), *Tulasnella**† (5)	+
	SARDINIA		
Neottieae	*Cephalanthera longifolia*	***Hebeloma*** (1), ***Russula*** (2), ***Tomentella*** (1)	+
	Epipactis helleborine	*Leptodontidium* (1), *Pyronemataceae** (3), ***Tuber*** (2)	+
	Limodorum abortivum	***Russula*** (5)	+
	Limodorum trabutianum	***Russula*** (4), *Sebacina** (1)	+
	Neottia nidus-avis	*Sebacina** (5)	+
Orchideae	*Barlia robertiana*	*Thanatephorus* (3)	-
	Gennaria diphylla	***Cenococcum*** (1), ***Lactarius*** (3), ***Russula*** (1)	+
	Ophrys apifera	*Tulasnella** (4)	+
	Ophrys incubacea	*Thanatephorus* (1), *Tulasnella** (4)	+
	Orchis ichnusae	*Tulasnella** (1)	-
	Orchis longicornu	*Ceratobasidium* (2), *Leptodontidium* (1)	+
	Orchis papilionacea	*Ceratobasidiaceae* (1), *Tulasnella**† (1)	+
	Orchis provincialis	*Tulasnella** (3)	+
	Serapias lingua	*Ceratobasidium*† (1), *Thanatephorus*† (1)	-
	Serapias parviflora	*Leptodontidium* (1)	+

(Continued)

Table 2. Continued

Tribe	REGION/ Orchid species	Mycorrhizal fungi	ECM plants
	TENERIFE		
Orchideae	*Gennaria diphylla*	*Leptodontidium*† (1), *Pezizaceae**† (3)	?
	Habenaria tridactylites	*Ceratobasidium*† (1), *Leptodontidium*† (2)	?
	Neotinea maculata	*Leptodontidium*† (3), *Ceratobasidiaceae*† (3), *Ceratobasidium*† (1), *Tulasnella**† (1)	+
	Orchis canariensis	***Russula***† (1), *Tulasnella** (4)	+
	Serapias parviflora	*Leptodontidium*† (2)	+

Notes: *, taxa that contain some ECM lineages; †, taxa detected by cloning PCR products; ( ), number of orchid individuals in which a fungus was detected; ?, presence of species that are not classified for their mycorrhizal condition but are phylogenetically closely related to ectomycorrhizal plants

Discussion

Nutritional modes in orchids from the Mediterranean and Macaronesia

All fungal partners successfully identified in neottioids, solely forest orchids, turned out to be ectomycorrhizal fungi, either exclusively (e.g., *Limodorum abortivum*) or together with root endophytic saprotrophs (e.g., *Epipactis helleborine*) (Table 2). *Neottia nidus-avis*, *Limodorum abortivum* and *L. trabutianum* show high mycorrhizal specificity towards only one or two fungal genera. The only fully MHO of this investigation, *Neottia nidus-avis,* is restricted to the genus *Sebacina* in the area that we have examined. This is consistent with investigations on this orchid in other parts of Europe showing that *N. nidus-avis* is associated with fungi belonging to the ECM clade of *Sebacina* (McKendrick *et al.*, 2002; Selosse *et al.*, 2002). Both *Neottia nidus-avis* and *Limodorum* species were regarded as fully myco-heterotrophic orchids at a site in France (Gebauer & Meyer, 2003). The study of Girlanda *et al.* (2006), however, suggested partial myco-heterotrophy in *L. abortivum* as chlorophyll is formed in the stem and the small leaves of this orchid and photosynthesis was detected. Isotope data in our present work confirm the latter finding. Investigated individuals of *Limodorum abortivum* and *L. trabutianum* of this study are less enriched in ^{13}C than obligate myco-heterotrophic plants (Figure 1). It thus can be concluded that these *Limodorum* plants are not solely using the organic fungal source but additionally assimilate C through photosynthesis, as it was recently described for the leafless *Corallorhiza trifida* (Zimmer *et al.*, 2008; but see also Cameron *et al.* 2009). *Cephalanthera longifolia* and *Epipactis helleborine* collected at a forest site on Sardinia are characterized by strong

enrichments in ^{13}C and ^{15}N showing that they are mainly nourished *via* myco-heterotrophic means. Since enrichment factors of *Epipactis helleborine* are even within the range of fully myco-heterotrophic plants, we suggest that this orchid almost completely relies on fungal nutrient supply under extreme dark conditions. In summary, all investigated neottioids of the Mediterranean region turned out to be strongly (or even fully) myco-heterotrophic. There were only four species besides the tribe Neottieae (*Orchis purpurea* in continental Italy, *Barlia robertiana* on Sardinia, *Habenaria tridactylites* and *Gennaria diphylla* on Tenerife) with apparent organic C and N gain from their fungal partners. Only the latter was associated with ECM fungi but we know from investigations on some fully myco-heterotrophic orchids that saprotrophic fungi can also be an effective nutrient source, at least in warm and humid climates (Yamato *et al.*, 2005; Yagame *et al.*, 2007; Ogura-Tsujita *et al.*, 2009).

In orchids of open habitats, root endophytes are abundant and diverse. Most of the mycorrhizal associates are part of the saprotrophic rhizoctonia-forming clades. Some of them (e.g., *Ceratobasidium* spp. and *Thanatephorus* spp.) occur in the roots of several orchid species and have the potential to link different orchid species through their hyphal network. Orchids from exposed sites were frequently associated with members of the cosmopolitan family Tulasnellaceae (Roberts, 1999) which is in accordance with global investigations of orchid mycorrhizas (Dearnaley, 2007). It has to be mentioned that the ecology of supposedly saprotrophic fungi could be more complex than generally thought. For instance, a few *Tulasnella*, commonly regarded as exclusively saprotrophic lineage, have been shown to form ectomycorrhizas with non-orchid plants (e.g., Bidartondo *et al.*, 2003) and very exceptionally, *Ceratobasidium* may also be ectomycorrhizal (Yagame *et al.*, 2008). Isotope data of some *Aceras*, *Orchis* and *Ophrys* species (tribe Orchideae) show significant depletion in ^{13}C relative to their autotrophic references (Table 1). This phenomenon occurs in both the Mediterranean and Macaronesian region and has already been found for two *Goodyera* species (Hynson *et al.*, 2009b) and (though statistically not significant) for some other *Orchis* species (Gebauer & Meyer, 2003). Depletion in ^{13}C might be a consequence of a specific flux of organic C compounds from the orchid to the fungus as it has been shown experimentally for the green orchid *Goodyera repens* (Cranichideae) by Cameron *et al.* (2006, 2008). They demonstrated that *in vitro* the C flux from *Goodyera repens* to its non-ectomycorrhizal fungus (*Ceratobasidium cornigerum*) is over five times higher than the fungus-to-plant C transfer. Depletion in ^{13}C together with enrichment in ^{15}N (as found

for some Orchideae of open habitats in this study) could result from two simultaneous processes: (1) organic nutrient gain from fungi leading to enrichment in both ^{13}C and ^{15}N and (2) the plant-to-fungus flux of sugars assimilated through photosynthesis and thus enriched in ^{13}C compared to leaf bulk C (Gleixner *et al.*, 1993). Thus, while the ^{15}N signal from heterotrophic nutrient gain remains within the plant, the ^{13}C enrichment can dissolve - and if more C flows from the plant to the fungus (supposedly under high light availability) it can even turn into a relative ^{13}C depletion.

Nitrogen concentrations

The strikingly high N concentrations of neottioids may be caused by nutrient gain from obligate ECM fungi. Such high N concentrations are in the range usually found for legumes associated with N_2-fixing bacteria (Gebauer *et al.*, 1988). Fungi have similar C concentrations, but considerably higher N concentrations than plants (see e.g., Gebauer & Dietrich, 1993; Gebauer & Taylor, 1999). Thus, the incorporation of fungal metabolites after lysis of the pelotons inside the root cells of myco-heterotrophic orchids could produce an N surplus. Orchid species of open habitats display lower N concentrations though they are in many cases still significantly increased compared to autotrophic reference plants (Table 1). Previous studies on orchids from Central Europe and Estonia reported similar ranges of leaf N concentrations (Gebauer & Meyer, 2003; Abadie *et al.*, 2006). Because orchids, as well as the majority of reference plants, presumably receive their N through association with mycorrhizal fungi, there must be physiological differences in how this occurs. For instance, the orchids could gain organic N compounds (e.g., amino acids) from their fungi while other plants under temperate climate conditions may be supplied preferentially with mineral N compounds (Gebauer & Dietrich, 1993; Schulze *et al.*, 1994).

Constraints on orchid nutrition and distribution

Gebauer (2005) suggested that light availability can determine the degree of myco-heterotrophy since the contribution from photosynthesis should be reduced at very dark sites. At a dense *Quercus ilex* forest on Sardinia *Cephalanthera longifolia* is mainly nourished *via* myco-heterotrophic means. When growing at more exposed forest gaps, *Cephalanthera longifolia* is less enriched in the heavy stable isotopes of C and N (Figure 1) and thus less dependent upon organic nutrient supply from mycorrhizal fungi, fitting Gebauer's hypothesis. At open sites where orchids were rarely associated with

(potential) ECM fungi, we found depletion in ^{13}C for some members of the Orchideae. Our findings indicate that a net plant-to-fungus C flux may occur in these species and that this phenomenon might be coupled to open light-saturated habitats just as like strong partial and full myco-heterotrophic nutrition is coupled to light-limited forest understories.

There are no reports for any occurrence of neottioid orchids in the Macaronesian region (Eriksson *et al.*, 1979; Hohenester & Welß, 1993). The limited number of ectomycorrhizal plants in the Macaronesian region might be a reason for this observation. A maximum of 20 ECM plant species are reported to occur on Tenerife, mostly belonging to the family Cistaceae (Hohenester & Welß, 1993). Despite the large number of ECM fungi that are linked to *Cistus* spp. (Comandini *et al.*, 2006), it is questionable whether these shrubs are able to act as efficient host plants in tripartite symbioses between ECM plants, ECM fungi and orchids in Macaronesia. *Orchis canariensis* was the only orchid on Tenerife that was found to associate with obligate ECM fungi (Table 2). Nonetheless, a C gain from partial myco-heterotrophy is likely for the Macaronesian species *Habenaria tridactylites* and *Gennaria diphylla*, the latter associating with Pezizaceae that contain some ECM lineages.

Conclusions

Based on the wide spectrum of species and habitats investigated, we conclude that high dependence on myco-heterotrophy in orchids is related to only some taxonomic groups (i.e., Neottieae) and to the light-limited understorey of forest sites. Even though forests are present on the Macaronesian islands, fully myco-heterotrophic orchids are lacking and the occurrence of partial myco-heterotrophy is rare. Our results raise the hypothesis that this pattern is caused by the low diversity of ectomycorrhizal plants and/or suitable ectomycorrhizal fungi. In order to test this hypothesis, we need to investigate whether fully and partially myco-heterotrophic neottioids are able to germinate in the Macaronesian region.

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Appendix

Appendix A. Site characteristics and $\delta^{15}N$, $\delta^{13}C$ and nitrogen concentrations ± 1 SD in the leaves of orchid species and their autotrophic reference species (n = 5, except for *Cephalanthera damasonium* where n = 2) collected on sites of the following habitat types: forest (F), forest gap (FG), shrubland (SL) or open habitat (O) in continental Italy, Sardinia (Mediterranean region) and Tenerife (Macaronesian region). Asterisked orchid species belong to the tribe Neottieae.

CONTINENTAL ITALY **Site description /** **Orchids and autotrophic references**	**$\delta^{15}N$** **[‰]**	**$\delta^{13}C$** **[‰]**	**N conc.** **[mmol/g_{DM}]**
Site 1 (O): N 44.3825°; E 8.25623° Grassland dominated by *Brachypodium pinnatum*, *Bromus erectus* and *Carex flacca* **relative light availability: 96 %**			
Aceras anthropophorum (L.) R.Br.	-2.85 ± 0.14	-30.07 ± 0.90	1.59 ± 0.17
Pimpinella saxifraga L.	-3.65 ± 2.90	-29.15 ± 0.53	1.56 ± 0.15
Salvia pratensis L.	-4.74 ± 0.97	-29.08 ± 0.60	1.87 ± 0.18
Helianthemum apenninum (L.) Miller	-6.71 ± 0.72	-30.56 ± 0.29	1.66 ± 0.16
Ophrys fuciflora (Crantz) Moench	-2.36 ± 0.92	-30.49 ± 0.81	2.52 ± 0.26
Orchis morio L.	-2.73 ± 0.75	-31.36 ± 1.69	1.53 ± 0.26
Plantago lanceolata L.	-6.38 ± 0.42	-30.11 ± 0.50	1.40 ± 0.16
Helianthemum nummularium (L.) Miller	-5.63 ± 0.72	-30.23 ± 0.60	1.54 ± 0.21
Bromus erectus Hudson	-6.56 ± 0.81	-27.08 ± 5.21	1.89 ± 0.15
Ophrys sicula Tineo	-2.69 ± 1.44	-31.43 ± 1.11	1.03 ± 0.17
Thymus vulgaris L.	-7.99 ± 1.36	-30.54 ± 0.88	1.04 ± 0.23
Teucrium polium L.	-8.36 ± 1.63	-28.91 ± 1.41	1.33 ± 0.36
Carex hallerana Asso	-4.44 ± 0.78	-28.86 ± 0.81	1.10 ± 0.12
Ophrys sphegodes (Miller)	-2.82 ± 0.63	-29.68 ± 1.13	1.86 ± 0.33
Sanguisorba minor Scop.	-5.64 ± 0.57	-28.96 ± 0.36	1.30 ± 0.12
Prunus spinosa L.	-3.39 ± 0.48	-28.38 ± 0.60	1.77 ± 0.10
Bromus erectus Hudson	-5.56 ± 0.87	-28.27 ± 0.61	1.28 ± 0.09
Orchis laxiflora Lam.	2.80 ± 2.01	-30.02 ± 0.58	1.84 ± 0.13
Ranunculus bulbosus L.	-1.56 ± 2.56	-29.19 ± 0.56	1.68 ± 0.22
Filipendula vulgaris Moench	-1.23 ± 2.18	-29.22 ± 0.68	1.66 ± 0.27
Carex hirta L.	1.07 ± 0.64	-28.92 ± 0.56	1.65 ± 0.34
Orchis purpurea Hudson	1.64 ± 0.81	-28.25 ± 0.70	1.66 ± 0.13
Pimpinella saxifraga L.	-3.33 ± 1.00	-29.24 ± 0.60	1.67 ± 0.18
Plantago lanceolata L.	-5.23 ± 0.47	-29.73 ± 0.42	1.58 ± 0.24
Linum strictum L.	-3.98 ± 0.53	-29.71 ± 0.50	1.53 ± 0.21
Serapias vomeracea (Burm.) Briq.	-3.38 ± 0.37	-29.46 ± 0.37	1.24 ± 0.20
Bromus erectus Hudson	-4.16 ± 0.25	-28.89 ± 1.07	1.53 ± 0.30
Sherardia arvensis L.	-5.04 ± 0.39	-29.57 ± 0.20	1.38 ± 0.12
Crepis vesicaria L.	-5.42 ± 0.59	-29.62 ± 0.43	1.17 ± 0.23

(Continued)

Appendix A. Continued

CONTINENTAL ITALY Site description / Orchids and autotrophic references	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	N conc. [mmol/g_{DM}]
Site 1 (O): N 44.3825°; E 8.25623° Grassland dominated by *Brachypodium pinnatum*, *Bromus erectus* and *Carex flacca* **relative light availability: 96 %**			
Spiranthes spiralis (L.) Chevallier	-2.07 ± 0.84	-29.48 ± 0.82	2.43 ± 0.29
Teucrium polium L.	-7.30 ± 0.50	-30.40 ± 0.39	1.08 ± 0.07
Potentilla neumanniana Rchb.	-5.96± 0.78	-28.39 ± 0.50	1.32 ± 0.07
Bromus erectus Hudson	-5.53 ± 0.24	-27.65 ± 0.25	1.12 ± 0.12
Site 2 (O): N 44.0655°; E 10.1410° Grassland dominated by *Brachypodium pinnatum* and *Teucrium botrys* **relative light availability: not measured**			
Orchis pauciflora Ten.	-0.33 ± 1.42	-29.89 ± 0.76	1.11 ± 0.19
Brachypodium pinnatum (L.) Beauv.	-3.52 ± 1.11	-28.44 ± 0.79	1.72 ± 0.06
Teucrium botrys L.	-2.23 ± 0.42	-28.26 ± 0.82	1.92 ± 0.37
Artemisia spp.	-1.22 ± 0.43	-29.73 ± 0.43	2.41 ± 0.44
Site 3 (FG): N 45.1457°; E 7.1247° Forest gap in a deciduous forest dominated by *Quercus pubescens* **relative light availability: 23 %**			
Cephalanthera longifolia (Hudson) Fritsch*	0.02 ± 1.48	-26.87 ± 0.96	3.10 ± 0.41
Quercus pubescens Willd.	-3.37 ± 0.78	-27.65 ± 1.55	1.56 ± 0.22
Teucrium botrys L.	-5.59 ± 0.43	-30.67 ± 0.24	1.82 ± 0.18
Cichorium intybus L.	-6.05 ± 0.74	-30.80 ± 0.95	2.15 ± 0.50
Site 4 (F): N 45.1457°; E 7.1247° Shaded areas of a deciduous forest dominated by *Quercus pubescens* **relative light availability: 11 %**			
Limodorum abortivum (L.) Swartz.*	11.74 ± 2.93	-23.52 ± 0.30	2.40 ± 0.40
Cichorium intybus L.	-4.88 ± 0.26	-30.17 ± 1.58	2.14 ± 0.26
Quercus pubescens Willd.	-4.16 ± 0.85	-27.32 ± 0.37	1.78 ± 0.24
Hedera helix L.	-4.35 ± 0.95	-29.19 ± 0.52	1.25 ± 0.68
Site 5 (O): N 45.1457°; E 7.1247° Grassland dominated by *Festuca* spp. and *Koeleria* spp. **relative light availability: 89 %**			
Orchis tridentata Scop.	-1.33 ± 0.94	-30.03 ± 0.72	1.70 ± 0.25
Teucrium polium L.	-5.76 ± 0.62	-31.18 ± 0.38	1.13 ± 0.19
Leontodon spp.	-6.20 ± 0.89	-30.24 ± 0.36	1.78 ± 0.14
Helianthemum apenninum (L.) Miller	-5.27 ± 0.58	-30.35 ± 1.19	1.47 ± 0.17

(Continued)

Appendix A. Continued

CONTINENTAL ITALY **Site description /** **Orchids and autotrophic references**	**$\delta^{15}N$** **[‰]**	**$\delta^{13}C$** **[‰]**	**N conc.** **[mmol/g_{DM}]**
Site 6 (F): N 45.151°; E 7.1744° Mixed deciduous forest dominated by *Acer campestre, Tilia platyphyllos* and *Fraxinus excelsior* **relative light availability: 6 %**			
Cephalanthera damasonium (Miller) Druce*	6.11	-25.39	2.92
Acer campestre L.	-3.49	-29.52	1.42
Hedera helix L.	-3.70	-29.01	1.35
Prunus spinosa L.	-1.62	-28.40	1.52
Listera ovata (L.) R.Br.*	-2.30 ± 0.74	-30.05 ± 0.59	3.13 ± 0.37
Ligustrum vulgare L.	-5.65 ± 0.95	-32.47 ± 0.70	1.47 ± 0.21
Hedera helix L.	-6.44 ± 0.50	-31.93 ± 0.22	0.97 ± 0.19
Tamus communis L.	-4.49 ± 0.64	-31.28 ± 0.91	2.53 ± 0.37
SARDINIA **Site description /** **Orchids and autotrophic references**	**$\delta^{15}N$** **[‰]**	**$\delta^{13}C$** **[‰]**	**N conc.** **[mmol/g_{DM}]**
Site 7 (O): N 40.0054°; E 9.6667° Degraded steppe dominated by *Asphodelus aestivus* **relative light availability: 97 %**			
Barlia robertiana (Loisel.) Greuter	1.30 ± 0.43	-28.01 ± 0.40	0.94 ± 0.21
Anagallis foemina Miller	-1.46 ± 0.36	-30.60 ± 0.29	0.66 ± 0.11
Scandix pecten-veneris L.	-2.94 ± 0.47	-29.61 ± 0.91	0.79 ± 0.17
Asphodelus aestivus Auct. non Brot.	-1.56 ± 0.48	-30.54 ± 0.23	1.17 ± 0.18
Serapias lingua L.	-0.03 ± 1.08	-31.50 ± 0.77	1.44 ± 0.20
Asphodelus aestivus Auct. non Brot.	-1.70 ± 0.83	-29.95 ± 0.52	1.37 ± 0.15
Scandix pecten-veneris L.	-4.16 ± 0.77	-30.59 ± 0.26	0.89 ± 0.19
Anagallis foemina Miller	-2.59 ± 0.39	-31.71 ± 0.58	0.83 ± 0.11
Site 8 (O): N 40.1595°; E 9.5072° Grassland intensively grazed by cattle **relative light availability: 100 %**			
Orchis ichnusae (Corrias) J. Devillers-Terschuren & P. Devillers	2.38 ± 0.50	-30.51 ± 0.81	2.01 ± 0.05
Santolina chamaecyparissus Auct. non L.	-1.11 ± 0.58	-28.66 ± 0.70	2.64 ± 0.40
Teucrium marum L.	-0.31 ± 0.67	-29.25 ± 0.52	1.53 ± 0.08
Anemone hortensis L.	-1.61 ± 0.45	-29.95 ± 0.45	2.48 ± 0.37

(Continued)

Appendix A. Continued

SARDINIA Site description / Orchids and autotrophic references	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	N conc. [mmol/g_{DM}]
Site 9 (O): N 39.8786°; E 9.5092° Grassland dominated by *Linum bienne* and *Lavandula stoechas* **relative light availability: 99 %**			
Orchis provincialis Balb.	0.59 ± 1.15	-29.90 ± 0.48	1.89 ± 0.20
Orchis papilionacea L.	-0.43 ± 0.39	-30.23 ± 1.32	1.73 ± 0.31
Anthoxanthum odoratum L.	-4.22 ± 0.74	-29.63 ± 0.45	1.10 ± 0.08
Asterolinum linum-stellatum (L.) Duby	-2.88 ± 1.06	-28.43 ± 1.01	0.99 ± 0.26
Cistus crispus L.	-2.11 ± 0.63	-29.20 ± 0.43	1.37 ± 0.13
Orchis longicornu Poiret	-1.05 ± 1.10	-29.57 ± 0.44	1.73 ± 0.27
Cistus crispus L.	-2.62 ± 1.22	-29.58 ± 0.70	1.37 ± 0.08
Sherardia arvensis L.	-3.08 ± 0.85	-29.78 ± 0.74	1.45 ± 0.12
Anthoxanthum odoratum L.	-5.33 ± 2.74	-29.74 ± 0.47	1.14 ± 0.13
Site 10 (F): N 39.8869°; E 9.5113° Evergreen forest dominated by *Quercus ilex* **relative light availability: 2 %**			
Epipactis helleborine (L.) Crantz*	11.65 ± 3.27	-27.17 ± 1.23	3.68 ± 0.44
Cephalanthera longifolia (Hudson) Fritsch*	6.54 ± 0.67	-28.67 ± 1.81	3.37 ± 0.22
Geranium robertianum L.	-3.69 ± 2.28	-35.34 ± 0.62	2.60 ± 0.50
Quercus ilex L.	0.08 ± 0.36	-31.89 ± 1.25	0.97 ± 0.05
Hedera helix L.	-0.97 ± 1.75	-34.26 ± 0.44	1.45 ± 0.35
Neottia nidus-avis (L.) L.C.Rich.*	11.61 ± 1.67	-27.29 ± 1.65	2.59 ± 0.12
Cyclamen repandum S. et S.	-4.41 ± 2.36	-34.32 ± 0.82	2.40 ± 0.13
Mycelis muralis (L.) Dumort.	-1.81 ± 0.98	-34.73 ± 0.25	2.17 ± 0.16
Quercus ilex L.	-0.73 ± 0.60	-32.08 ± 0.34	0.87 ± 0.08
Site 11 (O): N 39.7014°; E 9.4699° Degraded steppe dominated by *Asphodelus aestivus* **relative light availability: 48 %**			
Aceras anthropophorum (L.) R.Br.	-0.40 ± 1.00	-30.36 ± 0.45	1.48 ± 0.24
Valantia hispida L.	-2.81 ± 0.83	-28.94 ± 0.46	0.93 ± 0.09
Asphodelus aestivus Auct. non Brot.	-2.46 ± 0.69	-28.04 ± 0.54	1.73 ± 0.19
Teucrium marum L.	-1.45 ± 0.60	-28.34 ± 1.05	1.59 ± 0.23
Orchis brancifortii Bivona-Bernardi	1.83 ± 1.59	-30.11 ± 1.18	1.39 ± 0.11
Asphodelus aestivus Auct. non Brot.	-1.66 ± 0.65	-28.84 ± 0.70	1.47 ± 0.11
Cistus monspeliensis L.	-1.29 ± 0.82	-28.99 ± 0.93	1.14 ± 0.10
Leontodon tuberosus L.	-2.11 ± 0.82	-29.45 ± 0.52	1.63 ± 0.13

(Continued)

Appendix A. Continued

SARDINIA Site description / Orchids and autotrophic references	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	N conc. [mmol/g_{DM}]
Site 12 (O) N 40.4840°; E 9.7773° Open places in a patchy shrubland dominated by *Erica arborea* **relative light availability: 91 %**			
Serapias cordigera L.	-1.28 ± 0.44	-31.77 ± 0.97	1.12 ± 0.13
Centaurium maritimum (L.) Fritsch	-0.91 ± 0.60	-30.30 ± 0.27	0.97 ± 0.08
Cistus monspeliensis L.	-4.31 ± 0.63	-31.31 ± 1.02	1.09 ± 0.20
Erica arborea L.	-2.58 ± 0.70	-28.77 ± 1.22	0.76 ± 0.19
Site 13 (O): N 39.7405°; E 9.5729° Grassland dominated by *Aira caryophyllea*, *Lophochloa cristata* and *Vulpia bromoides* **relative light availability: 62 %**			
Ophrys apifera Hudson	2.24 ± 0.78	-33.37 ± 0.85	1.68 ± 0.06
Plantago lanceolata L.	-1.10 ± 0.75	-31.57 ± 0.50	1.04 ± 0.15
Leontodon spp.	1.17 ± 5.40	-31.95 ± 0.78	1.25 ± 0.15
Cistus crispus L.	-1.96 ± 2.23	-32.61 ± 1.24	0.92 ± 0.12
Ophrys incubacea Bianca	1.01 ± 0.83	-31.49 ± 3.57	1.50 ± 0.46
Asphodelus aestivus Auct. non Brot.	0.54 ± 0.95	-29.56 ± 0.87	1.42 ± 0.15
Anagallis foemina Miller	-0.12 ± 0.68	-31.19 ± 1.59	0.87 ± 0.15
Sherardia arvensis L.	-0.42 ± 0.52	-31.22 ± 1.52	0.94 ± 0.16
Serapias parviflora Parl.	2.93 ± 1.30	-30.83 ± 0.68	1.40 ± 0.33
Parentucellia viscosa (L.) Caruel	-0.66 ± 0.19	-30.73 ± 0.22	2.64 ± 0.48
Lophochloa cristata (L.) Hyl.	-0.26 ± 0.62	-30.96 ± 0.22	0.68 ± 0.06
Plantago lanceolata L.	-0.71 ± 0.78	-30.63 ± 0.62	1.48 ± 0.42
Erica arborea L.	-2.57 ± 0.82	-27.99 ± 0.89	1.05 ± 0.05
Site 14 (F): N 39.7405°; E 9.5729° Evergreen forest dominated by *Quercus ilex* **relative light availability: 8 %**			
Limodorum abortivum (L.) Swartz.*	12.81 ± 2.24	-27.89 ± 0.39	2.16 ± 0.27
Carex distachya Desf.	0.28 ± 3.25	-34.03 ± 0.53	1.05 ± 0.15
Quercus ilex L.	-0.46 ± 1.03	-31.87 ± 0.90	1.05 ± 0.12
Viburnum tinus L.	-2.22 ± 0.85	-32.05 ± 0.86	0.82 ± 0.21
Limodorum trabutianum Batt.*	10.42 ± 3.13	-27.02 ± 0.60	2.35 ± 0.20
Arum pictum L. fil.	-1.82 ± 1.55	-32.55 ± 0.72	1.92 ± 0.26
Quercus ilex L.	-1.00 ± 1.18	-31.09 ± 0.97	1.07 ± 0.11
Smilax aspera L.	-0.35 ± 1.14	-31.28 ± 0.48	1.01 ± 0.28

(Continued)

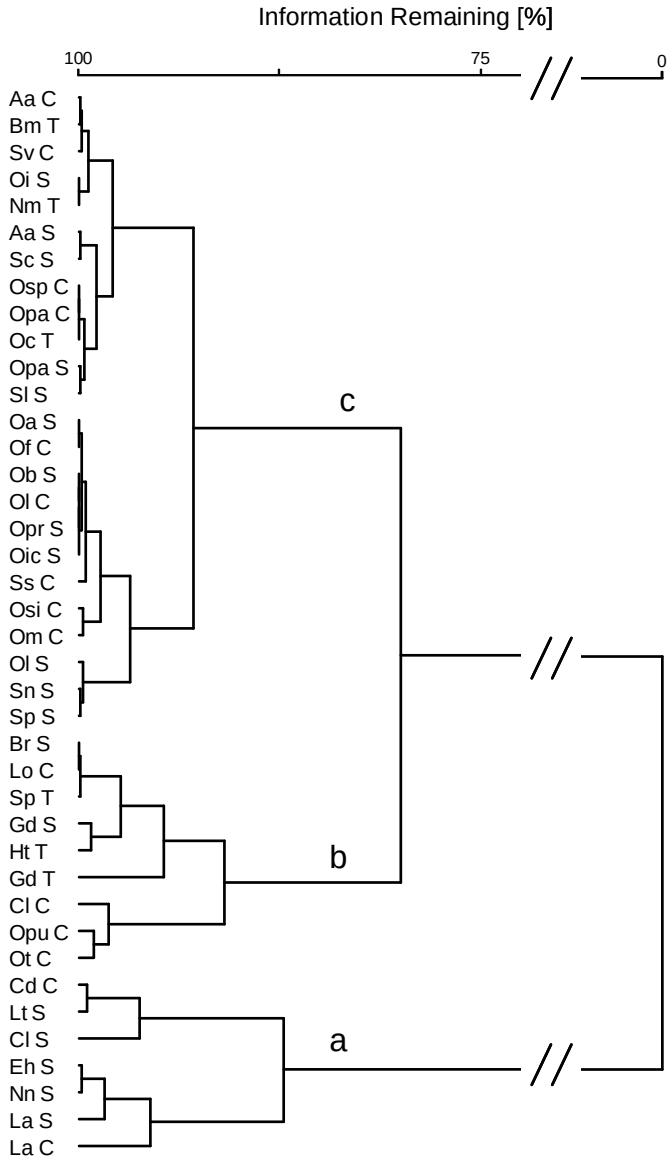
Appendix A. Continued

SARDINIA **Site description /** **Orchids and autotrophic references**	**$\delta^{15}N$** **[‰]**	**$\delta^{13}C$** **[‰]**	**N conc.** **[mmol/g_{DM}]**
Site 15 (SL): N 41.1755°; E 9.3873° Under shrubs in a patchy macchia dominated by *Cistus monspeliensis*, *Erica arborea* and *Arbutus unedo* **relative light availability: 2 %**			
Gennaria diphylla (Link) Parl.	-2.16 ± 1.05	-29.91 ± 1.00	1.61 ± 0.43
Erica arborea L.	-3.96 ± 0.89	-31.88 ± 1.47	0.85 ± 0.25
Quercus ilex L.	-3.49 ± 1.36	-31.09 ± 0.68	0.82 ± 0.12
Arbutus unedo L.	-3.38 ± 1.44	-30.19 ± 2.37	0.84 ± 0.15
Site 16 (O): N 41.1755°; E 9.3873° Open places in a patchy macchia dominated by *Cistus monspeliensis*, *Erica arborea* and *Arbutus unedo* **relative light availability: 67 %**			
Serapias nurrica Corrias	-0.15 ± 1.61	-29.45 ± 0.57	1.93 ± 0.38
Cistus monspeliensis L.	-5.07 ± 1.26	-31.21 ± 1.19	1.13 ± 0.12
Lavandula stoechas L.	-2.71 ± 2.06	-29.52 ± 1.24	0.91 ± 0.16
Erica arborea L.	-2.57 ± 0.82	-27.99 ± 0.89	1.05 ± 0.05
TENERIFE **Site description /** **Orchids and autotrophic references**	**$\delta^{15}N$** **[‰]**	**$\delta^{13}C$** **[‰]**	**N conc.** **[mmol/g_{DM}]**
Site 17 (O): N 28.3284°; W 16.7845° Grazed grassland **relative light availability: 90 %**			
Neotinea maculata (Desf.) Stearn.	1.17 ± 0.81	-31.43 ± 0.80	2.27 ± 0.27
Erica arborea L.	-1.43 ± 1.01	-29.39 ± 0.85	0.95 ± 0.23
Allium roseum L.	0.07 ± 0.89	-32.24 ± 1.12	2.16 ± 0.29
Andryala pinnatifida Ait.	1.04 ± 1.08	-30.25 ± 0.92	2.33 ± 0.17
Serapias parviflora Parl.	1.91 ± 0.86	-28.03 ± 0.88	1.60 ± 0.19
Erica arborea L.	-1.10 ± 0.94	-27.62 ± 1.35	1.02 ± 0.09
Plantago lagopus L.	-0.63 ± 1.21	-31.15 ± 0.74	1.04 ± 0.20
Avena barbata Pot. ex Link	-1.02 ± 0.59	-30.93 ± 0.51	1.02 ± 0.09
Site 18 (FG): N 28.3560°; W 16.4976° Rocky outcrop in a *Pinus canariensis* forest with *Erica arborea*, *Cistus monspeliensis* and *Cistus symphytifolius* **relative light availability: 71 %**			
Orchis canariensis Lindl.	-1.98 ± 1.25	-29.86 ± 1.05	1.59 ± 0.13
Erica arborea L.	-3.63 ± 1.22	-28.87 ± 0.95	0.92 ± 0.30
Cistus monspeliensis L.	-4.63 ± 0.46	-28.75 ± 0.43	0.92 ± 0.06
Cistus symphytifolius Lam.	-4.38 ± 0.77	-28.81 ± 0.46	0.98 ± 0.10

(Continued)

Appendix A. Continued

TENERIFE **Site description /** **Orchids and autotrophic references**	**$\delta^{15}N$** **[‰]**	**$\delta^{13}C$** **[‰]**	**N conc.** **[mmol/g_{DM}]**
Site 19 (SL): N 28.4429°; W 16.4547° *Erica arborea* dominated shrubland **relative light availability: 12 %**			
Gennaria diphylla (Link) Parl.	-0.01 ± 1.23	-28.12 ± 0.80	2.09 ± 0.16
Habenaria tridactylites Lindl.	-0.66 ± 0.56	-30.36 ± 0.85	1.90 ± 0.30
Erica arborea L.	-2.55 ± 0.95	-31.04 ± 0.62	0.84 ± 0.16
Hypericum canariense L.	-1.24 ± 1.60	-33.46 ± 0.33	2.13 ± 0.22
Micromeria varia Benth.	-1.58 ± 1.10	-34.51 ± 0.38	1.57 ± 0.49
Site 20 (FG): N 28.2323°; W 16.7608° Open *Pinus canariensis* forest with wide gaps **relative light availability: 77 %**			
Barlia metlesicsiana Teschner	1.58 ± 0.31	-27.55 ± 0.78	2.03 ± 0.47
Pinus canariensis Sweet ex. Spreng.	-0.63 ± 0.74	-24.49 ± 0.55	0.84 ± 0.13
Asphodelus aestivus Brot.	-1.76 ± 0.52	-28.30 ± 0.67	1.25 ± 0.12
Rumex lunaria L.	0.74 ± 0.65	-29.57 ± 0.44	1.13 ± 0.09



Appendix B. Cluster analysis based on the enrichment factors for ^{13}C and ^{15}N of the different orchid species collected in continental Italy (C), on Sardinia (S) and Tenerife (T); three important groups are labelled: a, forest orchids; b, orchids with intermediate isotope signatures; c, orchids of open habitats and forest gaps. Habitat types in brackets: (**F**) forest, (**FG**) forest gap, (**O**) open habitat, (**SL**) shrubland.

Orchideae: Aa, *Aceras anthropophorum* ((**O**) in C and on S); Bm, *Barlia metlesicsiana* (**FG**); Br, *B. robertiana* (**O**); Gd, *Gennaria diphylla* ((**SL**) on S and T); Ht, *Habenaria tridactylites* (**SL**); Nm, *Neotinea maculata* (**O**); Oa, *Ophrys apifera* (**O**); Of, *O. fuciflora* (**O**); Oi, *O. incubacea* (**O**); Osi, *O. sicula* (**O**); Osp, *O. sphegodes* (**O**); Ob, *Orchis brancifortii* (**O**); Oc, *O. canariensis* (**FG**); Oic, *O. ichnusae* (**O**); Ol C, *O. laxiflora* (**O**); Ol S, *O. longicornu* (**O**); Om, *O. morio* (**O**); Opa S, *O. papilionacea* (**O**); Opa C, *O. pauciflora* (**O**); Opr, *O. provincialis* (**O**), Opu, *O. purpurea* (**O**); Ot, *O. tridentata* (**O**); Sc, *Serapias cordigera* (**O**); Sl, *S. lingua* (**O**); Sn, *S. nurrica* (**O**); Sp, *S. parviflora* ((**O**) on S and T); Sv, *S. vomeracea* (**O**). **Neottieae:** Cd, *Cephalanthera damasonium* (**F**); Cl, *Cephalanthera longifolia* ((**FG**) in C and (**F**) on S); Eh, *Epipactis helleborine* (**F**); La, *Limodorum abortivum* ((**F**) in **C** and on **S**); Lt, *L. trabutianum* (**F**); Lo, *Listera ovata* (**F**); Nn, *Neottia nidus-avis* (**F**). **Cranichideae:** Ss, *Spiranthes spiralis* **(O)**.

CHAPTER 6

The major determinant for the degree of myco-heterotrophy

Irradiance governs exploitation of fungi: Fine-tuning of carbon gain by partially myco-heterotrophic orchids

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Key words: partial myco-heterotrophy, Orchidaceae, stable isotopes, carbon gain, irradiance, mycorrhiza

Abstract

While all members of the Orchidaceae are fully dependent on mycorrhizal fungi during their achlorophyllous juvenile stages, mature plants may remain fully myco-heterotrophic, become fully autotrophic or develop a nutritional mode where the carbon gain through photosynthesis is complemented by organic carbon from fungal partners. The latter, so-called partial myco-heterotrophy, is an intriguingly complex form of mixotrophy. Current knowledge indicates a large range in the proportion of fungi-derived carbon between and within partially myco-heterotrophic plant species. However, the driving factors for this variation are hitherto mostly unknown. Here we show for two green species of the orchid genus *Cephalanthera* that light availability is the major determinant for the degree of myco-heterotrophy. Using leaf stable isotope natural abundance analysis together with time-integrated micro-scale light climate monitoring we could show that there is a sensitive reaction to varying light availability within forests. Low light levels result in strong myco-heterotrophy while higher irradiances successively drive the orchids towards full autotrophy. Our results demonstrate that partial myco-heterotrophy is not a static nutritional mode but a surprisingly flexible mechanism driven by light availability which allows a well balanced utilization of carbon resources available in nature.

Introduction

Since the recent discovery of a novel nutritional mode in the world's largest plant family, the Orchidaceae, a dogma in plant sciences meaning that green plants are autotrophic is no longer valid (G. Gebauer in Whitfield, 2007). Although green plants are able to photosynthesize, some specialized terrestrial orchids have recently been shown to additionally use an underground carbon source - their mycorrhizal fungi (Gebauer & Meyer, 2003). A switch of their mycorrhizal associates from typical *Rhizoctonia* species (a polyphyletic group of fungi) to ectomycorrhizal partners that are simultaneously associated with trees enables the looting of organic nutrients (Bidartondo *et al.*, 2004). Analogue mechanisms have in the meantime also been found in some green pyroloids (Ericaceae) (Zimmer *et al.*, 2007; Tedersoo *et al.*, 2007; Hynson *et al.*, 2009) and ongoing investigations continually reveal further species that exhibit this exciting and complex form of mixotrophy (Bidartondo *et al.* 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Zimmer *et al.*, 2008) which is more precisely referred to

as partial myco-heterotrophy (Gebauer & Meyer, 2003). Although it can be hypothesized that many more green plants from diverse taxa may up to now unnoticeably gain organic compounds through myco-heterotrophic means, we know very little on the mechanisms behind this ecologically relevant phenomenon.

Natural stable isotope abundances in organism tissues are a convenient tool to study the utilization of isotopically distinguished nutrient sources. The incorporation of fungi-derived carbon, e.g., is reflected by the green plants' leaf isotope signature since fungal tissues are enriched in the heavy carbon stable isotope ^{13}C relative to accompanying fully autotrophic plants (Högberg *et al.*, 1999). Previous studies indicate a large range in the proportion of fungi-derived carbon between and within partially myco-heterotrophic species (Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007, 2008; Hynson *et al.*, 2009; Cameron *et al.*, 2009) but the driving factors for this variation remain mostly unknown.

A comparison of three independent investigations on the trophic status of green orchids at different forest types raised the hypothesis that the exploitation of mycorrhizal fungi might be affected by the prevalent light climate (Gebauer, 2005). To test this hypothesis experimentally, we combined leaf stable isotope natural abundance analysis with time-integrated micro-scale light climate monitoring and investigated two partially myco-heterotrophic orchid species (*Cephalanthera damasonium* and *C. rubra*) together with 12 fully autotrophic and one fully myco-heterotrophic reference species.

Materials and Methods

Study sites and investigated species

Plant samples were collected in 2007 and 2008 from three forest sites in NE Bavaria: an open *Pinus sylvestris* stand, a forest dominated by *Fagus sylvatica* and a mixed stand composed of several conifer (e.g., *Pinus sylvestris*, *Picea abies*) and broadleaf species (e.g., *Fagus sylvatica*, *Acer campestre*). All sites are located at 480 - 520 m a.s.l. and characterised by mean annual precipitation of 700 - 1000 mm and mean annual temperatures of 6 - 9 °C. In total, 224 understory plant samples were collected from a fully myco-heterotrophic (*Neottia nidus-avis*, n = 11), a fully autotrophic (*Cypripedium calceolus*, n = 9) and two partially myco-heterotrophic orchid species (*Cephalanthera damasonium*, n = 18; *C. rubra*, n = 18); and from 11 diverse (monocotyledons /

dicotyledons, tree saplings / herbs, evergreen / deciduous, ectomycorrhizal / arbuscular- or nonmycorrhizal) autotrophic non-orchid species (*Acer campestre*, *n* = 9; *A. pseudoplatanus*, *n* = 3; *Anthericum ramosum*, *n* = 12; *Carex flacca*, *n* = 20; *Convallaria majalis*, *n* = 11; *Euphorbia cyparissias*, *n* = 20; *Fagus sylvatica*, *n* = 48; *Fragaria vesca*, *n* = 3; *Galium odoratum*, *n* = 10; *G. verum*, *n* = 10; *Polygala chamaebuxus*, *n* = 22).

Micro-scale light climate monitoring

For each of the 56 orchid individuals, a 1-m^2 plot including two to four autotrophic non-orchids was selected. As soon as the young orchid shoots could be identified, a calibrated light sensor (silicon photodiode BPW 21, Infineon, Germany) connected to a mini data logger (HOBO H8, ONSET, USA) was installed right next to each shoot at about 15 cm height. Irradiance was logged every 15 min from the day of sensor installation until the development of seed capsules (2007: May 9 - June 20, 2008: Mai 18 - July 6). Measured values were converted into photosynthetically active radiation (µmol photons m^{-2} s^{-1}) and averaged as daily means (from sunrise to sunset). Due to the equal global solar radiation from May to July in the two sampling years (2007: 488 kWh m^{-2}, 2008: 494 kWh m^{-2}; weather station of the Ecological-Botanical Garden Bayreuth), the measured relative light availability of understorey plants had not to be adjusted.

Carbon stable isotope abundance analysis

Leaf samples (and stem samples of the leafless *N. nidus-avis*) were taken following the criteria described by Gebauer & Meyer (2003). The plant material was oven-dried at 105 °C and ground to a fine powder. Relative C isotope abundances were measured with an elemental analyzer coupled to a continuous flow isotope ratio mass spectrometer as described in Bidartondo *et al.*, (2004). Measured abundances are denoted as δ values, which were calculated according to the following equation: $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard. Standard gases were calibrated with respect to international standards by using the reference substances ANU sucrose and NBS 19, provided by the International Atomic Energy Agency (Vienna, Austria).

Data preparation and statistics

To facilitate precise data comparisons between sites and plots, δ values were normalised according to Preiss & Gebauer (2008): $\delta^{13}C$ values of the orchids and the non-orchid autotrophic reference plants were used to calculate ^{13}C enrichment factors (ε) of every plant against the mean of the autotrophic plants for each plot: $\varepsilon_S = \delta_S - \delta_{REF}$; with S as single value of a sample from an autotrophic, partially or fully myco-heterotrophic orchid and REF as mean value of all autotrophic reference plants from the respective plot.

To test for significant ($\alpha = 0.05$) correlations between measured light availability and $\delta^{13}C$ values or enrichment factors (ε), respectively, regression analyses were performed using SigmaPlot v. 11.0 (Systat Software, Inc., USA). Means are given ± 1 SD.

Results and Discussion

Responses of $\delta^{13}C$ on varying irradiance

$\delta^{13}C$ values in leaves of autotrophic non-orchids ranged from -34.2 to -26.3 ‰ (Figure 1) and showed a significant, positive correlation with light availability ($F_{1,166} = 70.2$, $R^2_{adj.} = 0.293$, $P < 0.001$). These $\delta^{13}C$ values and their dependence on light climate are based on the carbon isotope discrimination during C_3 photosynthesis (fractionation during carboxylation by Rubisco) and on stomatal regulation which affects the intercellular partial pressure of CO_2 (Farquhar *et al.*, 1989). Leaf isotope signatures of the fully autotrophic orchid *Cypripedium calceolus* responded in the same way as autotrophic non-orchids ($F_{1,7} = 7.5$, $R^2_{adj.} = 0.518$, $P = 0.029$), demonstrating that members of the Orchidaceae *per se* do not show any peculiarity in carbon nutrition. This is consistent with findings by Zimmerman & Ehleringer (1990) who analysed the carbon isotope composition of a Panamanian epiphytic C_3 orchid (*Catasetum viridiflavum*) and found higher $\delta^{13}C$ values with increasing irradiance due to increasing stomatal limitation to photosynthesis.

The achlorophyllous orchid *Neottia nidus-avis* showed the highest $\delta^{13}C$ values of all investigated species (-23.1 ± 1.07 ‰ on average). Such a relative ^{13}C enrichment is characteristic of all fully myco-heterotrophic plants that associate with ectomycorrhizal fungi (Preiss & Gebauer, 2008) and fits the food-chain model (Trudell *et al.*, 2003).

Since these plants' carbon demand is exclusively covered through organic compounds supplied by fungi, $\delta^{13}C$ values of *N. nidus-avis* are not correlated with the micro-scale light climate ($F_{1,9} = 0.7$, $R^2_{adj.} < 0.001$, $P = 0.411$; Figure 1).

A quite interesting pattern was found for the two *Cephalanthera* species. Although these green orchids are able to photosynthesize, their carbon isotope signatures do not respond on varying light availability ($F_{1,34} = 0.2$, $R^2_{adj.} < 0.001$, $P = 0.637$; Figure 1). Their mean $\delta^{13}C$ values (*C. damasonium*: -28.1 ± 1.4, *C. rubra*: -28.6 ± 1.6) range between those of fully autotrophic and fully myco-heterotrophic plants as typical for partial myco-heterotrophs. At higher irradiances (above 250 µmol m^{-2} s^{-1}) 95 % confidence intervals of *Cephalanthera* individuals and fully autotrophic plants overlap (Figure 1) indicating a complete autotrophic nutrition at sufficiently light-exposed sites. However, since it has been shown that irradiance-dependent physiological effects can strongly influence leaf $\delta^{13}C$ values, isotope data have to be related to a fine spatial scale before assessing the question whether partial myco-heterotrophy is a flexible or a static nutritional mode.

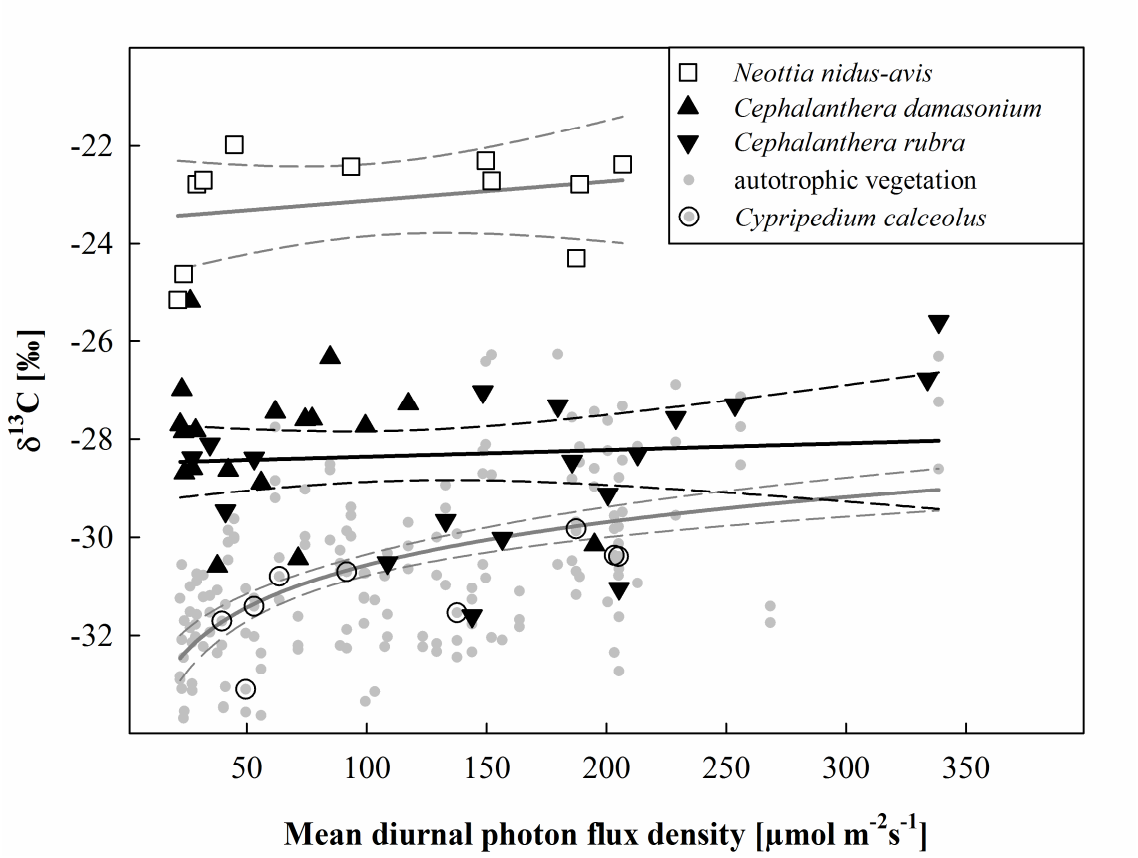

Figure 1. $\delta^{13}C$ values of 12 autotrophic plant species ($n = 177$) including the orchid *C. calceolus*, two partially myco-heterotrophic *Cephalanthera* spp. ($n = 36$) and the fully myco-heterotrophic orchid *N. nidus-avis* ($n = 11$), plotted against relative light availability. Regression curves (solid lines) are given with 95 % confidence intervals (dashed lines).

Effects of irradiance on partial myco-heterotrophy

Regarding the normalized ^{13}C enrichment of the *Cephalanthera* individuals after relating the isotope data to references of the respective plot, the existence of a true relation between enrichment factor ε and micro-scale light availability becomes obvious ($F_{1,34} = 56.6$, $R^2_{adj.} = 0.614$, $P < 0.001$; Figure 2). Thus, the darker a habitat is, the more fungi-derived carbon is incorporated (reflected by the proportional enrichment in ^{13}C). One could have the impression, that there also is a light-dependent reaction in the fully myco-heterotrophic species ($F_{1,9} = 18.9$, $R^2_{adj.} = 0.641$, $P = 0.002$), but this is the effect resulting from referencing against autotrophic plants whose $\delta^{13}C$ values increase with increasing irradiance while $\delta^{13}C$ values of *N. nidus-avis* remain constant (cp. Figure 1). Under low light conditions, *Cephalanthera* individuals receive about half as much of fungi-derived carbon as achlorophyllous plants while the proportion of heterotrophic nutrition decreases with increasing irradiance (pointed out by arrows in Figure 2). At sufficiently high irradiances, adult *Cephalanthera* plants cover their carbon demands completely through assimilation of atmospheric CO_2 as like as fully autotrophic non-orchids and orchids (e.g., *C. calceolus*).

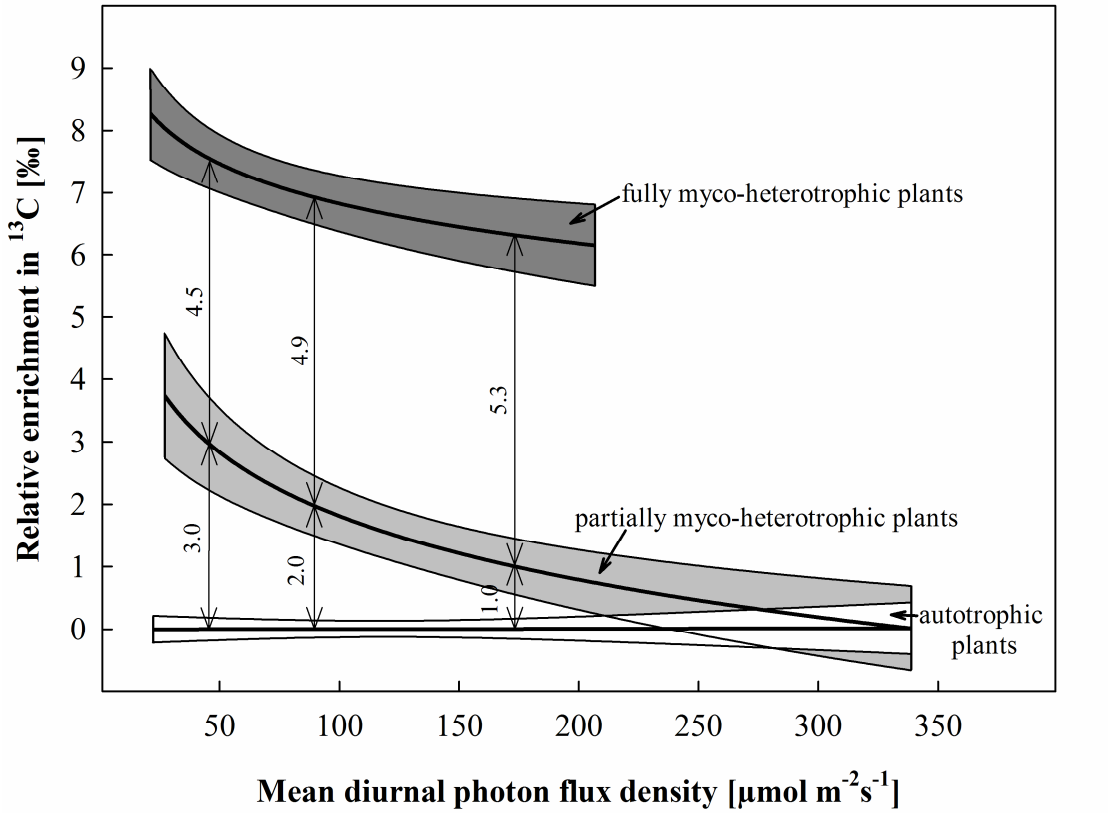

Figure 2. Correlation between relative enrichments in ^{13}C (ε) calculated per plot (Preiss & Gebauer, 2008) and relative light availability based on the data shown in Figure 1. Regression lines (± 95 % confidence intervals) represent the range of isotope signatures of autotrophic, partially myco-heterotrophic and fully myco-heterotrophic plants.

Conclusions

Here we show that partial myco-heterotrophic *Cephalanthera* species strongly supplement their carbon gain through photosynthesis by organic carbon from fungal partners under low light conditions but nourish completely autotrophic when they are exposed to sufficiently high irradiances. This demonstrates that partial myco-heterotrophy is not a static nutritional mode but a surprisingly flexible mechanism allowing a well balanced utilization of carbon resources available in nature. The fact that the degree of myco-heterotrophy may successively change - driven by the prevalent micro-scale light climate - could explain several discrepancies between previous studies that investigated the trophic status of numerous green Orchidaceae and Ericaceae.

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LIST OF FURTHER PUBLICATIONS

ON THIS TOPIC

Zimmer (Preiss) K, Hynson NA, Gebauer G, Allen EB, Allen MF, Read DJ. 2007. Wide geographical and ecological distribution of nitrogen and carbon gains from fungi in pyroloids and monotropoids (Ericaceae) and in orchids. *New Phytologist* 175: 166-175.

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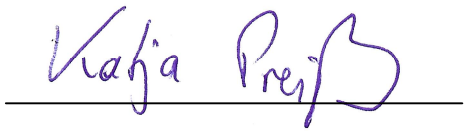
ON ANOTHER TOPIC

Fischer K, Zimmer (Preiss) K, Wedell N. 2009. Correlated responses to selection on female egg size in male reproductive traits in a butterfly. *Evolutionary Ecology* 23: 389-402.

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst verfasst und keine anderen als die von mir angegebenen Hilfsmittel und Quellen verwendet habe. Ferner erkläre ich, dass ich diese Arbeit an keiner anderen Universität zur Erlangung des Doktorgrades vorgelegt habe. Ich habe noch kein Promotionsverfahren endgültig nicht bestanden.



Katja Preiß