



# Recruitment ecology and fungal interactions in mycoheterotrophic Ericaceae

Veronika A. Johansson

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Cover: Dust seeds of *Pyrola chlorantha* (Ericaceae) in relation to a  
Swedish krona. Photo by: Veronika Johansson

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If the wind will not serve, take to the oars.



Doctoral dissertation  
Veronika A. Johansson  
Department of Ecology, Environment and Plant Sciences  
Stockholm University  
SE-106 91 Stockholm

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### Abstract

There are generally two contrasting alternatives to what limits recruitment in plants, namely the availability of seeds (seed limitation) or the quality or quantity of suitable sites (microsite limitation). Dust seeds, the smallest existing seeds, lack or have minimal nutrient reserves. During germination and initial development they consequently parasitize on mycorrhizal fungi. This is called mycoheterotrophy, and can vary in degree of fungal dependency in adult plants from full, partial or initial mycoheterotrophy.

The aim of this thesis was to investigate the recruitment ecology of mycoheterotrophic Ericaceae (tribe Pyroleae) species with dust seeds, and to determine what limits their recruitment. The investigated species were: *Chimaphila umbellata*, *Moneses uniflora*, *Orthilia secunda*, *Pyrola chlorantha*, *P. minor* and *P. rotundifolia*. This aim was achieved by combining field experiments (seed sowing) with isotope analysis and fungal host pyrosequencing.

Results provide evidence that the species in Pyroleae are heterogeneous, not only with regard to their degree of mycoheterotrophy, but also concerning germination and early seedling development. A combination of microsite and seed limitation is thus likely to be of importance for all studied species, but the relative importance of these limitations varies among species. Despite having adaptations for wind dispersal the majority of the seeds were deposited in close vicinity of the seed source. But with high seed production at least some seeds should be able to disperse long-distance. Seedlings of all studied species were found to associate with a wide range of ectomycorrhizal fungi, at least during their initial developmental stages. There seems to be a tendency for host narrowing in some Pyroleae species, but not as strict as the host specialization seen in fully mycoheterotrophic *Monotropa hypopitys*, supporting the hypothesis of geographical and developmental host shifts.

**Keywords:** 454 Pyrosequencing, Dispersal limitation, Dust seeds, Ectomycorrhiza, Ericaceae, Microsite limitation, *Monotropa hypopitys*, Mycoheterotroph, Pyroleae, Seed limitation, Stable isotopes, Subterranean seedling, Symbiotic germination, *Tricholoma*



# List of Papers

The Thesis is comprised of a summary and four papers, which are referred to by their Roman numerals:

- I.** Johansson VA, Eriksson O (2013) Recruitment limitation, germination of dust seeds, and early development of underground seedlings in six Pyroleae species. *Botany* 91: 17-24.
- II.** Johansson VA, Müller G, Eriksson O (2014) Dust seed production and dispersal in Swedish Pyroleae species. *Nordic Journal of Botany* 32: 209-214.
- III.** Johansson VA, Mikusinska A, Ekblad A, Eriksson O (2015) Partial mycoheterotrophy in Pyroleae - nitrogen and carbon stable isotope signatures during development from seedling to adult. *Oecologia, In press*. Available online. doi: 10.1007/s00442-014-3137-x
- IV.** Johansson VA, Bahram M, Tedersoo L, Kõljalg U, Eriksson O. Diversity of ectomycorrhizal fungi associated with Pyroleae and *Monotropa hypopitys* during germination and seedling development. *Manuscript*

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# Abbreviations and Glossary

- Achlorophyllous** – Without chlorophyll and hence the capacity to photosynthesize.
- Arbuscular mycorrhiza (AM)** – A type of mycorrhiza in which the fungus penetrates the cells of the roots of a vascular plant. Arbuscular mycorrhizas are characterized by the arbuscules and vesicles by fungi of the phylum Glomeromycota.
- Autotroph** – An organism that can synthesize the organic material it requires from inorganic sources, e.g. using light as a source of energy.
- Dust seed** – The smallest type of seeds in angiosperms, often with immature embryos and no significant energy reserves.
- Ectomycorrhiza (EcM)** – A type of mycorrhiza in which the fungus does not penetrate their host's cell walls. The ectomycorrhizal fungi tend to be predominantly from the phyla Basidiomycota and Ascomycota, although a few are represented in the phylum Zygomycota.
- Fully mycoheterotrophic plant** – A plant solely dependent on carbon compounds from fungi during its entire life cycle.
- Initially mycoheterotrophic plant** – A plant that is fully dependant on associated fungi for its carbon supply during the early stages of development.
- Isotope** – Any of two or more forms of an element where the atoms have the same number of protons, but a different number of neutrons.
- MHP** – Mycoheterotrophic plant
- Microsite** – A small area with distinctive ecological characteristics
- Mixotrophy** – A term previously used for partial mycoheterotrophy (see below). Mycoheterotrophy is rather a kind of mixotrophy, implying several nutritional sources (not necessary a fungal source).
- Mycoheterotrophy** – The ability of a plant to obtain carbon from fungi.
- Mycorrhiza** – A form of symbiotic relationship that occurs between a fungal symbiont and the roots of various plant species.
- Parasitism** – Interaction between two organisms, in which one organism (the parasite) benefits and the other (the host) is usually harmed (but sometimes also unaffected).
- Partially mycoheterotrophic plant** – The ability of a plant to obtain carbon simultaneously through autotrophy and mycoheterotrophy during at least one stage of its life cycle.
- Rhizome** – A modified underground stem of some plants that often sends out roots and shoots from its nodes.
- Seed sowing** – In this thesis seed sowing refers to seed bag sowing.
- Seedling** – In this thesis this term refers to a subterranean root-like structure similar to orchid protocorm.
- Symbiosis** – An association between two or more organisms of different species that benefits both members.

# Introduction

## Mycoheterotrophy

Plants interact with fungi in multiple ways, forming a range of interactions from mutually beneficial (mutualistic) to those that are detrimental for one part (parasitic; Johnson et al. 1997). One of the most widespread and important symbiotic relationship is the formation of mycorrhiza. Approximately 80% of all terrestrial plants are associated with mycorrhizal fungi (Smith & Read 2008). This symbiosis is based on an exchange of resources where the fungi supply plants with mineral nutrition and increased area for water uptake, and in return the fungi receive plant assimilates. A single plant can associate simultaneously with multiple fungi and a single fungus can associate simultaneously with multiple plants, resulting in complex networks.

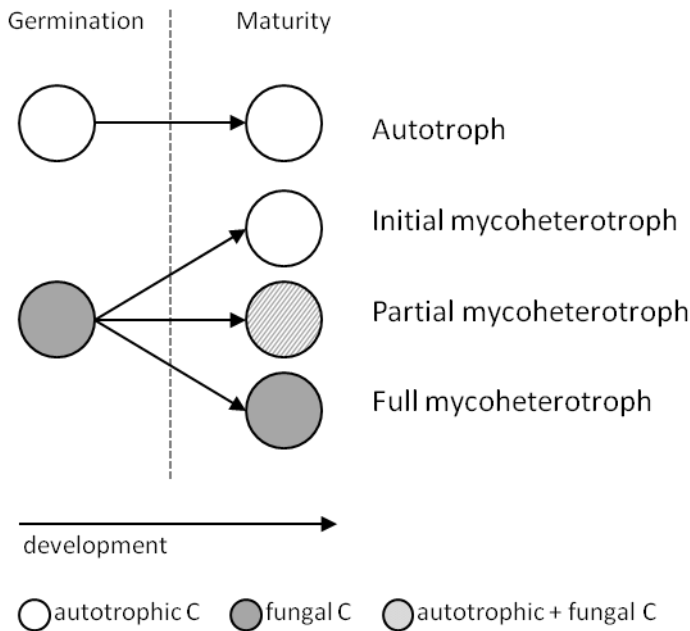
From a theoretical viewpoint, mutualisms are expected to run the risk of becoming invaded by “cheaters”, i.e. exploiters that obtain benefits offered to mutualists without reciprocating (Bronstein 2001; Yu 2001). One example is mycoheterotrophic plants (MHP) (Leake 1994). These plants use fungi as their source of organic carbon (C), and are considered cheaters in mycorrhizal symbioses (Björkman 1960; Cullings et al. 1996; Taylor & Bruns 1997; Bidartondo et al. 2002; Bidartondo 2005; Smith & Read 2008). Some MHP are known to associate with saprotrophic fungi, which obtain carbon from dead or decaying organic matter, but the large majority of MHP associate with either arbuscular (AM) or ectomycorrhizal (ECM) fungi (Smith & Read 2008; Merckx 2013).

Mycoheterotrophy is divided into full, partial or initial mycoheterotrophy, depending on the life cycle stage during which the plant is mycoheterotrophic (Fig. 1; Merckx 2013). Fully mycoheterotrophic plants are characterized by some convergent traits like the reduction of leaves to scales, few or no stomata, a loss of chlorophyll, small dust-like seeds, and modified root systems or rhizomes that are heavily colonized with mycorrhizal fungi (Leake 1994). Fully mycoheterotrophic plants are dependent on fungal associations throughout their entire life cycle due to the lack of chlorophyll. More than 400 fully MHP species have so far been described (Leake 2004).

Partial mycoheterotrophy is when adult plants partially use fungi as carbon source (Selosse & Roy 2009), occurring for example in many orchids

and some Ericaceae. Similar characters as for fully mycoheterotrophic plants can also be found in related partially mycoheterotrophic species but usually not as progressed. They develop green leaves (although sometimes reduced) and are thus capable of photosynthesis.

All MHP have exceptionally small dust seeds which after germination develop subterranean “seedlings” obtaining all their carbon from fungi (Eriksson & Kainulainen 2011). Thus, mycoheterotrophic plants, irrespective of whether they as adults are full or partial mycoheterotrophs are initial mycoheterotrophs as seedlings (Smith & Read 2008; Merckx 2013).



**Figure 1.** Schematic representation of the different types of mycoheterotrophic plants known, based on life stage and their primary source of carbon (C) (adapted from Merckx 2013).

### Fungal specificity

Mycorrhizal interactions are usually not very specific; plants and fungi often associate with many different species (Smith & Read 2008; Rasmussen & Rasmussen 2009). However, although there are exceptions (e.g. Hynson & Bruns 2009; Roy et al. 2009; Selosse et al. 2010; Merckx et al. 2012), most full mycoheterotrophs have been found to be highly specific in their use of fungal hosts, for example in the Ericaceae (e.g. Cullings et al. 1996; Kretzer et al. 2000; Bidartondo & Bruns 2001; Leake et al. 2004; Bidartondo &

Bruns 2005), in orchids (e.g. Taylor & Bruns 1997; 1999; Kennedy et al. 2011), and in the Gentianaceae (Bidartondo et al. 2002). The fungal hosts of mycoheterotrophs with high host specificity are restricted to certain fungal species or genera, and sometimes to certain fungal families (e.g. Bidartondo et al. 2002; Smith & Read 2008; Hynson & Bruns 2010), in contrast to generalists which associate with a phylogenetically wide range of fungi. Among partial mycoheterotrophs most species have been found non-specific in their host use (Tedersoo et al. 2007; Zimmer et al. 2007; Selosse & Roy 2009; Jacquemyn et al. 2010), but there are exceptions also to this pattern. For example, some orchid genera that are partially mycoheterotrophic include both host specific and generalist species (e.g. Shefferson et al. 2010).

Most of what we know of the associations between MHP and fungi come from studies of root samples from adult plants. The associations between seedlings and fungi are generally less well known. It has been found that the range of host fungi may change during development from germinated seed, through seedlings in different stages, to adults. Some studies have documented that the range of fungi decreases from germination to later stages of juvenile ontogeny, e.g. in the Monotropoideae (Bidartondo & Bruns 2005) and in orchids (Bidartondo & Read 2008), suggesting a “narrowing” in associations between seedling and fungi. Other studies have, however, found indications of the reverse, i.e. seedlings are more host specific than adults, as was found for *Pyrola asarifolia* (Hashimoto et al. 2012). Another pattern was reported by Hynson et al. (2013b) who found a similar overall diversity of fungi associated with seedlings and adults in two other Pyroleae species, *Pyrola chlorantha* and *Orthilia secunda*, but also that the composition of fungal associates differed between these life cycle stages. Regardless of whether fungal host specificity appears in early germination or later during seedling development, it could result in possible bottle-necks during seedling ontogeny, leading to recruitment limitations.

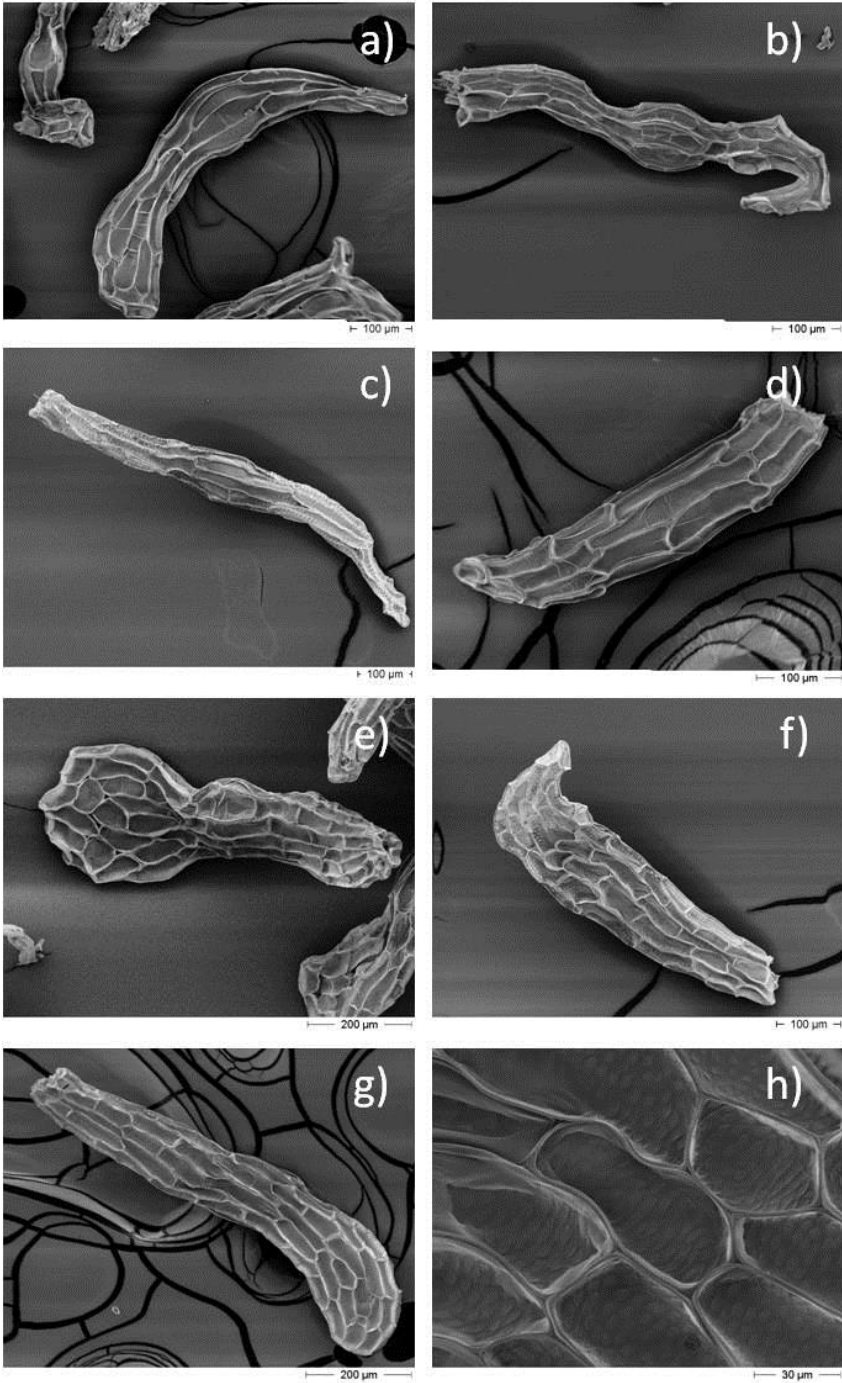
## Dust seeds

One characteristic trait of all mycoheterotrophic plants (but also some parasitic plants) is the possession of dust seeds. These are the smallest existing seeds in angiosperms (Leake 2004), usually below 1 mm in size (Eriksson & Kainulainen 2011). Although most commonly found in orchids (Arditti & Ghani 2000), dust seeds occur in at least 12 different families, most likely representing no less than 15 independent evolutionary origins (Eriksson & Kainulainen 2011). Apart from Orchidaceae, a circumglobal plant family, dust seeds are most commonly found in tropical plant families e.g. Triuridaceae and Burmanniaceae but also in Gentianaceae, Orobanchaceae and Ericaceae (Eriksson & Kainulainen 2011). In the Ericaceae, dust seeds are found in the subfamily Monotropoideae, including

the tribe Pyroleae (*Pyrola*, *Moneses*, *Orthilia*, and *Chimaphila*) which contain full, partial and initial mycoheterotrophs, and *Monotropa* (Syn. *Hypopitys*), which is fully mycoheterotrophic.

Dust seeds have convergent features such as an elongated shape and large internal air spaces, possibly beneficial to enhance buoyancy in air and/or water (Fig. 2), and some even have wing-like structures (e.g. Takahashi 1993; Arditti & Ghani 2000; Fig. 2e). Due to the size and characteristics of dust seeds, they may also be transported in and on animals (e.g. in fur, mud on feet, and possibly also following ingestion; Arditti & Ghani 2000; pers. obs.). In order to establish, the seeds of MHP must be infected by a mycorrhizal fungus. Developing seedlings then remain subterranean, probably for several years, during which they act as parasites on mycorrhizal networks (McKendrick et al. 2000; McKendrick et al. 2002; Eriksson & Kainulainen 2011).

Most dust seeds either lack or have very small endosperms and undifferentiated embryos, in some species only consisting of two cells (Pyykkö 1968). Eriksson & Kainulainen (2011) suggested that the probable driver of dust seed evolution is the selection for maximal fecundity at the expense of reducing maternal resources per seed. An important finding supporting this is that growth programs determining seed size, for example embryo growth, are controlled by endosperm-derived signals (Luo et al. 2005). Reductions of endosperm would thus have led to the evolution of undifferentiated embryos due to endosperm mediated control of embryo development. This would eventually have resulted in seed size reductions passing a threshold where external hosts became necessary for resource acquisition (Eriksson & Kainulainen 2011).



**Figure 2.** SEM photos of dust seeds of a) *Chimaphila umbellata*, b) *Monotropa hypopitys*, c) *Moneses uniflora*, d) *Orthilia secunda*, e) *Pyrola chlorantha*, f) *Pyrola minor*, g) *Pyrola rotundifolia*, and h) cell surface of *P. rotundifolia* (paper II).

## Recruitment limitation

An interest in recruitment limitations in plant populations developed during the early 1990s as a result of a growing awareness that the recruitment phase in plant populations could hold the key to understand plant species distribution and abundance (e.g. Eriksson & Ehrlén 1992). This insight was in conflict with the previous understanding that plants were not limited in their recruitment but inhabited practically all available suitable habitats.

Now it is known from a number of studies that plants often are recruitment limited, meaning that processes from seed production, through dispersal, to germination and establishment of juveniles commonly have strong impacts on population growth rate (e.g. Ehrlén et al. 2006; Clark et al. 2007; Myers & Harms 2009). Generally, studies on recruitment limitations have been performed using a “black box” thinking, i.e. experiments are designed to establish *if* recruitment is limited by the availability of seeds. However, few studies have examined the actual *mechanisms* behind recruitment limitation (which in principle may consider “source related” mechanisms, i.e. seed production, dispersal processes, or processes acting during germination and establishment).

There are generally two contrasting alternatives to what limits recruitment of plants, namely seed limitation or microsite limitation (e.g. Eriksson & Ehrlén 1992; Turnbull et al. 2000a; Münzbergová & Herben 2005; Clark et al. 2007). Microsite limitation (or establishment limitation) occurs when populations are limited in their recruitment by the quantity and quality of available sites suitable for recruitment while seed limitation occurs when seeds fail to disperse to suitable sites (dispersal limitation) or when too few seeds are produced to occupy potential recruitment sites, even though they are able to disperse there (source limitation) (Clark et al. 1998; Nathan & Muller-Landau 2000).

As MHP with dust seeds are parasites on fungi, in at least early life cycle stages, dust seed recruitment is closely associated with finding suitable microsites where associating fungal hosts are present. Apart from factors that concern the interactions between germinating seeds and fungi, two other factors contribute to patterns of recruitment limitation of MHP: seed production and seed dispersal ability. Seed production is an important part of the actual seed dispersal potential, since increased numbers of seeds from a seed source in itself improves dispersal (Eriksson & Jakobsson 1999). Few studies have estimated the dispersal range of plants with dust seeds, but results of these studies suggest that the majority of seeds are deposited very close to the mother plant (Machon et al. 2003; Diez 2007; Jacquemyn et al. 2007; Jersáková & Malinová 2007; paper II). It is however reasonable that a small fraction of dust seeds travel long distances due to the often immense number of seeds produced by single plants (Arditti & Ghani 2000). This

could result in at least some long-dispersed offspring, provided that seeds reach suitable microsites with appropriate fungal hosts present.

Few studies have addressed the question of seed versus microsite limitation in species with dust seeds. Ackerman et al. (1996) concluded that seed (source) limitation was the main factor determining recruitment in an epiphytic orchid. In a study of *Pyrola asarifolia*, Hashimoto et al. (2012) found that germination after seed sowing was high at sites without adults, indicating dispersal limitation in this species. Jacquemyn et al. (2007) concluded that seed dispersal was very limited for the orchid, *Orchis purpurea*, and that suitability for germination varied among sites. Studies investigating the role of mycorrhizal specificity for successful recruitment suggest that MHP may be limited by microsite availability where the presence of suitable fungi determines the germination rate (e.g., Swarts et al. 2010; but see Phillips et al. 2011). Germination of MHP have in some cases also been shown to be more successful in the presence of conspecific adults (e.g., Batty et al. 2001; McKendrick et al. 2002; Diez 2007; Jacquemyn et al. 2007). Apart from the assumption that germinating seeds associate with the same fungal hosts as adult plants, this could also suggest a possible microsite limitation involving soil properties like pH, nutrient content, or moisture, which changes into becoming more unfavourable at some distance from the main population, as shown in a study by Diez (2007). Other studies have found no relationship between germination and adult presence (McKendrick et al. 2000; Hashimoto et al. 2012; Těšitelová et al. 2012), indicating a possible seed limitation.

Field studies of orchids, monotropes and Pyroleae species have revealed relatively high rates of germination and initial development (e.g. Rasmussen & Whigham 1998a; Bidartondo & Bruns 2005; paper **I**; **IV**) but very low numbers of seedlings have been shown to reach larger developmental stages (e.g. McKendrick et al. 2000; Leake et al. 2004; paper **I**, **IV**). These studies clearly illustrate that the chance of successful recruitment is exceptionally small, even for dust seeds that successfully reach suitable sites for germination.

# Aim of the thesis

The overall aim of this thesis was to investigate the recruitment of plants with dust seeds and to determine the relative importance of microsite limitation and/or seed availability as well as possible underlying mechanisms. This was achieved by combining field experiments (seed sowing) with isotope analysis and fungal pyrosequencing of species within the sub-family Monotropoideae (Ericaceae). The specific aims of this thesis were:

- 1) To describe the recruitment process and examine mechanisms determining recruitment in the target species (paper **I** and **IV**) and investigate whether recruitment is limited by microsite or seed availability/dispersal (paper **I**).
- 2) To examine the seed production and dispersal abilities of the target species (paper **II**).
- 3) To investigate the degree of mycoheterotrophy throughout the target species lifecycle: from germination and seedling ontogeny to adult plants (paper **III**).
- 4) To identify the fungi associating with the target plant species and investigate the degree of specialization/generalization of respective species (paper **IV**).
- 5) To investigate whether recruitment limitation and the degree of heterotrophy as well as fungal associates can be connected to the rarity of the target species and how this implements the conservation of these species (paper **I**, **II**, **III** and **IV**).

# Methods

## Study species

The choice of study organisms was motivated by the very poor knowledge we have on recruitment among the plants which produce the smallest existing seeds in nature. In total, six Swedish species of the tribe Pyroleae (Ericaceae) were included in the study: *Chimaphila umbellata* (L.) W.P.C Barton, *Moneses uniflora* (L.) A. Gray, *Orthilia secunda* (L.) House, *Pyrola chlorantha* Sw., *Pyrola minor* L., *Pyrola rotundifolia* L., as well as the closely related full mycoheterotroph *Monotropa hypopitys* L. (synonymous to *Hypopitys monotropa* Crantz) of the tribe Monotropeae (Ericaceae) (Fig. 3). Different sets of species have been included in the four studies presented in this thesis.

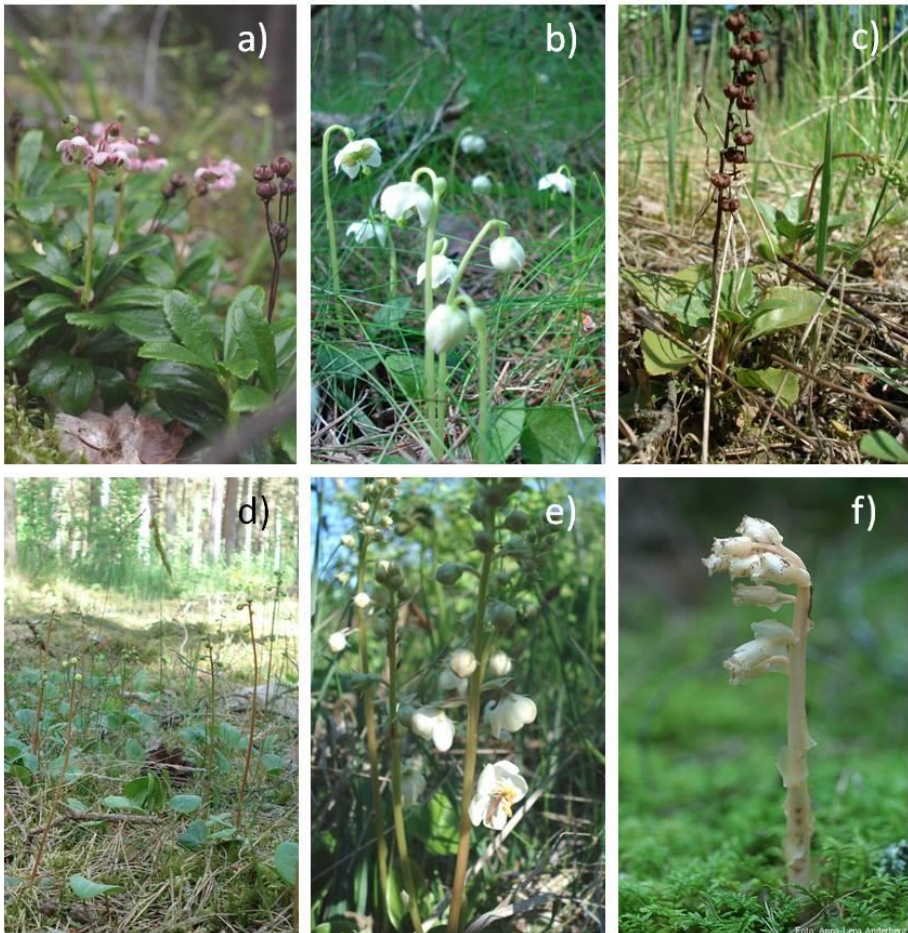
The investigated Pyroleae species are perennial, wintergreen herbs or small shrubs (*C. umbellata*), usually with rosette leaves, that are distributed in a range of habitats in the Northern hemisphere from boreal coniferous forests to deciduous forests (Kron et al. 2002). They produce a large number of minute dust seeds (paper II) and are mycoheterotrophic during their germination and initial development and therefore completely dependent on fungal interactions for nutrient acquisition (Eriksson & Kainulainen 2011). *Monotropa hypopitys* has a similar distribution but unlike the other studied species it is achlorophyllous and hence fully dependent on fungal nutrition throughout its entire life cycle. All study species are capable of reproducing both by seeds and clonally by rhizomes. A population could thus consist of a single or several genetic individuals (genets) that are producing vegetative units (ramets).

The study species are mainly pollinated by bumblebees (so called buzz pollination), where pollen is the sole pollinator reward, but are also to a large extent self pollinated (Knudsen & Olesen 1993). Of the species included, only *C. umbellata* and *O. secunda* produce nectar. Similar to orchid pollinia, Pyroleae species pollen grains are packed into units of monads in *O. secunda*, tetrads in *M. uniflora* and *Pyrola* spp., or polyads in *C. umbellata* (Knudsen & Olesen 1993).

Species of Pyroleae have previously been found to be general in their fungal associations (Tedersoo et al. 2007; Zimmer et al. 2007; Vincenot et al. 2008; Toftegaard et al. 2010; Matsuda et al. 2012; Hynson et al. 2013b), whereas *H. monotropa* has been found to possess specialized interactions

with fungi, mainly associating with fungi of the genus *Tricholoma* (e.g. Leake et al. 2004; Bidartondo 2005; Bidartondo & Bruns 2002, 2005; paper IV).

Three of the investigated species are currently declining in Sweden, *C. umbellata*, *M. uniflora* and *P. chlorantha* (Rydberg & Wanntorp 2001; Maad et al. 2009; Jonsell et al. 2010; Lundell et al. 2015) and *C. umbellata* is red-listed as endangered. *Monotropa hypopitys* is in addition inherently rare due to its cryptic population dynamics with sometimes large annual differences in the production of inflorescences.



**Figure 3.** Photos of study species, a) *Chimaphila umbellata* (Photo: Anna Lundell), b) *Moneses uniflora*, c) *Orthilia secunda* with year-old inflorescence, d) *Pyrola chlorantha*, e) *Pyrola rotundifolia*, and f) *Monotropa hypopitys* (Photo: Anna-Lena Anderberg).

## Study areas

The study took part mainly on the island Öland (56° 41'N, 16° 36'E) situated in the southern parts of the Baltic Sea, Sweden (paper **I**, **II**, **III**, **IV**). In paper **I** seed sowing experiments were conducted in three different regions of Sweden. The majority of the sites were situated on Öland, but also in the provinces of Uppland (60° 16'N, 18° 5'E) and Södermanland (58° 53'N, 17° 1'E). Sampling of plant material for paper **III** was performed exclusively around Lake Hornsjön (57°11'N, 16°57'E), Öland.

The sites where *C. umbellata*, *H. monotropa* and *P. chlorantha* were present were usually drier open old growth boreal/temperate coniferous forests, dominated by *Pinus sylvestris* L. with sparse ground vegetation mainly consisting of *Vaccinium myrtillus* L., *Vaccinium vitis-idaea* L., *Calluna vulgaris* (L.) Hull. Sites where *M. uniflora*, *P. minor* and *P. rotundifolia* were present were usually dry to mesic mixed coniferous/deciduous forests (including *P. sylvestris*, *Picea abies* (L.) H. Harst, *Betula pendula* L., *Salix* spp., *Quercus robur* L., *Populus tremula* L. and *Corylus avellana* L.) with more species-rich herbal ground vegetation. *Orthilia secunda* could be found in both types of forests described above.

## Seed sowing

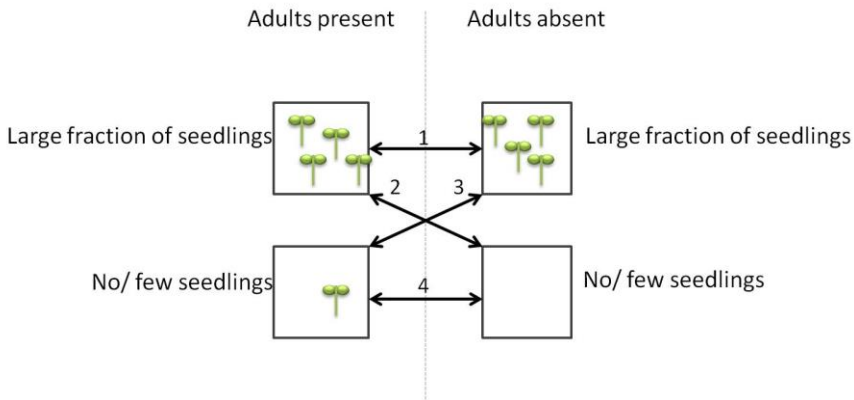
The most common way to study recruitment limitation is by seed sowing or seed augmentation experiments. Seeds are simply added to specified plots and the numbers of emerging seedlings are then counted and compared to plots where no seeds have been added (e.g. Eriksson & Ehrlén 1992; Turnbull et al. 2000b). If there is no increase in seedling density in plots where seeds have been added one can conclude that recruitment is not seed limited but instead limited by the quantity or quality of the microsites. In contrast, if there is an increase in recruitment after seed addition one can conclude that recruitment is at least to some extent seed limited.

Dust seeds cannot, however, be treated in this manner in recruitment studies, since they are so small and have subterranean seedlings which are more or less impossible to locate. Thus, we used a method first implemented by Rasmussen & Whigham (1993), where seed bags were constructed of 50µm mesh-cloth in which seeds were placed and sealed in (paper **I**, **III** & **IV**). This method prevents loss of seeds and seedlings and at the same time allows fungal hyphae, necessary for germination, to penetrate the seed bags. The seed bags were “sown” at occupied plots where adult plants were present and at unoccupied plots where adults were absent, and retrieved in sequence in order to follow the recruitment process (paper **I** & **IV**). Collected seeds were stored dark and dry and the time between seed collection and seed burial was kept to a minimum.

## Interpreting seed sowing results

There are several scenarios to which the results of germination frequency in relation to adult presence could be applied. Seeds can germinate solely, more, equally or less successful at sites where adults are present compared to where adults are absent (Fig. 4). If seeds germinate exclusively at sites where adults are present it would suggest a scenario where microsite is the sole limiting factor (Fig. 4 scenario 2), which is the only unambiguous outcome. In contrast, equal or varying germination frequency between plots where adults are present or absent can be interpreted as a gradient of the impact of seed limitation. An increased effect of seed limitation is then expected, the higher germination is at sites where adults are absent (Fig. 4 scenario 1, 3, and 4). In these scenarios microsite limitation cannot be disregarded, and is probably of varying importance for recruitment. An extreme scenario would be if no seeds germinated at plots where adults are present but instead germinated at plots where adults are absent (Fig. 4 scenario 3). This might occur for example if there are negative density dependence or allelopathy effects. Such effects may also lie behind scenarios where generally larger fractions of seeds germinate in plots where adults are absent compared to where adults are present. It is, however, also possible that this pattern is due to temporal changes in habitat quality, where for example successional changes lead to decreased microsite suitability for recruitment. Adult presence would hence in this case indicate microsites that were once suitable for recruitment but with time has changed into becoming unsuitable.

When comparing the results with other studies on recruitment limitation, one should notice that the Pyroleae are unusual in the sense that these species are dependent on host fungi for germination, in contrast to most other species where microsite suitability reflects abiotic conditions.



**Figure 4.** Possible outcomes of germination of seed sowing experiments in plots where adults are present and absent.

## Stages of germination

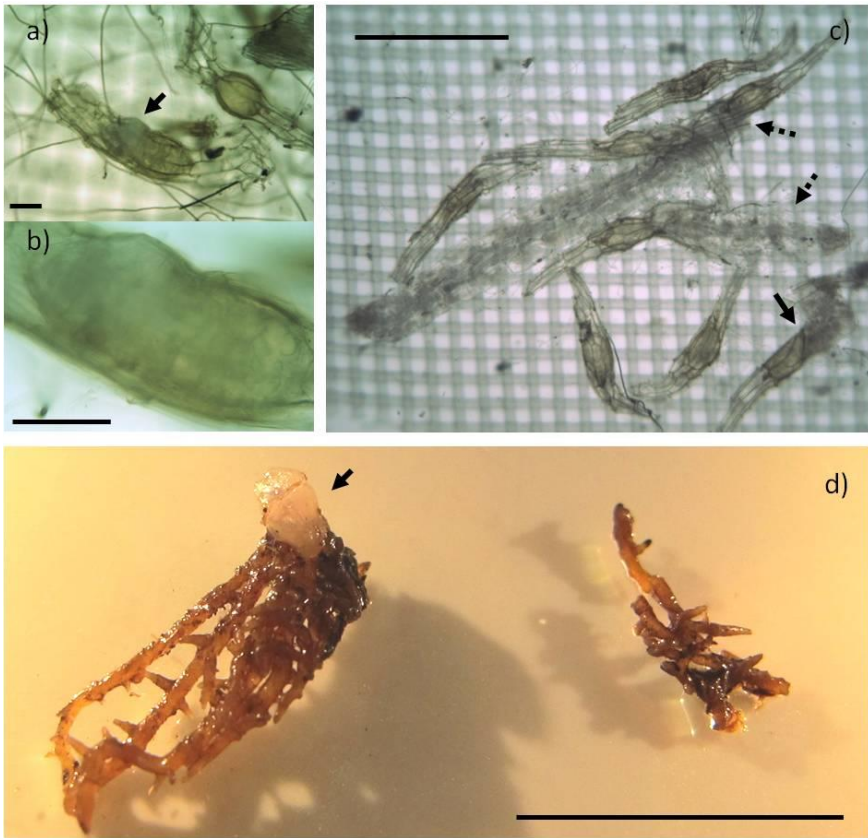
The visual evidence that germination had been initiated took the form of an increase in translucence of the embryo, from which a root-like structure emerged, usually from one side of the embryo (Fig. 5). In paper **IV** we used a modification of a stage classification used by Hashimoto et al. (2012) to distinguish between stages of germination: stage 0, ungerminated seeds; stage 1, growth cracking the testa (< 2 mm); stage 2, 2–10-mm seedling without branching; stage 3, elongated 10–20-mm seedling without branching; stage 4, branched seedling.

Intracellular microscopic investigations of whether seeds were infected by fungi were not performed due to the large number of seeds used in the seed sowing experiments (paper **I**, **III** & **IV**). However, it is likely that proximity of appropriate fungi is a prerequisite for germination, and fungal hyphae were always present within the seed bags, often surrounding or growing on the seeds and seedlings. Aborted seeds were not included in the analyses (paper **I** & **IV**); neither were decomposing seeds/seedlings (paper **IV**). Germination status (paper **I** & **IV**) and seedling size (paper **I**) was assessed using an eye piece microscope camera (Eurecam 3.0 and 10.0, BEL Engineering, Milano, Italy) connected to a computer.

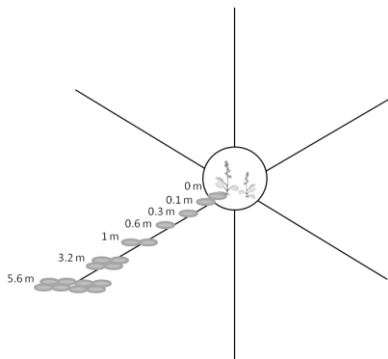
We used dissecting microscopes when sampling seedlings used in the molecular identification of fungal hosts of the target species (paper **IV**) and divided seedlings in three developmental stages: germinated (stage 1 and 2 in the description above), elongated (stage 3) or branched (stage 4; Fig. 5 d).

## Seed baiting

Seed baiting traps were used to examine the dispersal ability of dust seeds produced in Ericaceae (paper **II**). We used *P. chlorantha* as a model species, partly due to practicalities concerning timing of seed set, but also because this species is usually growing in open forests with sparse ground vegetation that could disrupt the possible dispersal ability. The traps were constructed by wrapping sticky tape around Petri dishes. The numbers of seed traps were increased with increasing distance to the seed source to keep sampling effort proportional (Fig.6). This method was based on Jersáková & Malinová (2007), Machon et al. (2003) and Jacquemyn et al. (2007), to allow comparisons between studies. In these studies maximum distance of dispersal was found to be between 0.2 m and 1.6 m, which is why the maximal distance from the seed source where seed traps were placed was c. 6 m. The traps were placed along six transects in different directions from the seed source. The long-distance 'tail' was not measured for practical reasons although species with dust seeds are likely to have some long-distance dispersal.



**Figure 5.** Stages of germination. a) Germinated seed with emerging seedling (arrow) and ungerminated seed (above) of *Chimaphila umbellata*. Note the fungal hyphae surrounding the seeds. b) Close-up of emerging seedling to the left of the embryo from seed of *C. umbellata* (scale bars = 0.05 mm). c) Various stages of germination for *Moneses uniflora*. Germinated seeds (arrows) elongated seedlings (dotted arrows) (scale bars = 0.5 mm). d) Branched seedlings of *Monotropa hypopitys* with floral bud formation (arrow) (scale bar = 1.25 cm).



**Figure 6.** Seed baiting experimental set up. Lines indicate transects where seed traps were placed.

## Isotope analysis

To investigate the degree of mycoheterotrophy from germination and seedling ontogeny to adult plants we analyzed carbon (C) and nitrogen (N) isotopic content of leaf and seedling material (paper **III**). The majority of biologically important elements occur as two or more stable isotopes, with one being more abundant than the other(s). Fractionation of the isotopes by different physiological and biological processes leads to a small but measurable concentration difference in biological materials and substances which can provide insights about natural fluxes among organisms and between organisms and their abiotic environment (e.g. Dawson et al. 2002; Fry 2006). Together, C and N isotopes can be utilized to study the degree of mycoheterotrophy since organisms usually have similar isotopic signatures (especially  $^{13}\text{C}$ ) to their food source (“you are what you eat”).

Studies of C and N enrichment in partial MHP have so far depended on the use of full MHP as reference material, which is by necessity another species. In paper **III** we use the subterranean seedlings of the target species as reference material for full mycoheterotrophy, a method first implemented by Stöckel et al. (2014). This might be associated with some advantage by reducing any unknown source of variation resulting from comparing the physiology of different species.

The autotrophic reference plants were chosen in accordance with Gebauer & Meyer (2003), based on the following criteria: 1) that they grew within the plot (to account for differences in the microhabitat possibly affecting isotopic signatures), 2) that they have a plot-abundance, enabling us to sample five replicates, and 3) that they were part of the understory vegetation (i.e. no larger trees were used).

To compare the isotope abundances of the study species and seedlings collected from different plots, the data was normalized according to standard, i.e. enrichment factors ( $\epsilon$ ) were calculated per plot (Preiss & Gebauer 2008; Hynson et al. 2013a). This is done to easier be able to compare results of different isotope studies since  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values usually vary with site.

The ambition was also to include measurements of the light environment at each plot since the grade of partial mycoheterotrophy has been shown to increase in more shaded habitats (Zimmer et al. 2007; Preiss et al. 2010; Hynson et al. 2012). However, since only one site was used (with several plots) in a relatively homogenous forest, measurements of canopy cover produced no relevant information and was thus not used.

## Pyrosequencing

To estimate the diversity and determine the fungal associates of our study species during seedling development we used a molecular sequencing method called pyrosequencing (paper **IV**). Pyrosequencing (Ronaghi 2001; Margulies et al. 2005) is one of the leading sequencing technologies replacing Sanger sequencing for comparative genomics and metagenomics. The method allows sequencing of genetic material from environmental samples where e.g. all species of fungi in a community can be simultaneously sequenced.

Pyrosequencing is based on the detection of released pyrophosphate during DNA synthesis. This ‘sequencing by synthesis’ is performed by synthesizing the complementary strand along a single strand of DNA, one base pair at a time, and detecting which base was actually added at each step. In a cascade of enzymatic reactions, visible light is generated that is proportional to the number of incorporated nucleotides (e.g. Ronaghi 2001).

There is currently a limitation of the method where the lengths of individual reads of DNA sequences are quite short (between 300-500 nucleotides). Another important caveat with this method is that sequencing errors could artificially increase the estimated diversity (e.g. Huse et al. 2007; Dickie 2010; Kunin et al. 2010). This is, in contrast to genome projects, especially problematic for studies of microbial diversity since they cannot take advantage of consensus assemblies to detect and minimize incorrect base calls (e.g. Huse et al. 2007). This problem highlights the importance of bioinformatics for ‘cleaning’ among resulting sequences as well as appropriate data bases for identification of the sequence diversity (see Tedersoo et al. 2014; paper **IV**, for details on bioinformatics). Another important object, implemented in paper **IV**, is to include positive as well as negative controls throughout the molecular analysis. Positive controls are not always included, but can be very useful in e.g. chimera checks. Two well-known previously sequenced tropical fungi were used as positive controls in paper **IV**.

We used NP-MANOVA analysis (often referred to Adonis analysis from the function used in R) to compare EcM fungal genera composition in seedlings at different developmental stages and between species. This was also illustrated by nonmetric multidimensional scaling (NMDS) plots. Analysis were performed on EcM lineages only (Tedersoo et al. 2013; although including Sebaciniales clade B) since they are essential for mycoheterotrophic plants and hence of focus.

# Results and Discussion

## Microsite vs. seed limitation

In general, the results of this study suggest that a combination of microsite and seed limitation is of importance for all studied species, but the extent of these limitations varies among species (paper I).

*Orthilia secunda* was the only species where recruitment proved to be almost exclusively limited by microsites, a conclusion also corroborated by increased seedling development at sites with conspecific adult presence. Since associated fungi were not determined for this species (paper IV) we can only speculate on why microsite was the main limiting factor. *Orthilia secunda* is the most common species used in this thesis and further has the widest distribution in the study area (Sweden). The clear pattern of increased germination and development of *O. secunda* in proximity to conspecific adults could suggest that similar hosts are involved in seedling germination and development as in adult roots. Whether this involves several fungal hosts or narrower host range remains untold, either way it is likely commonly distributed fungi that is involved considering the distribution of *O. secunda*. Similar results of germination success in relation to adult presence were found for *M. hypopitys* (paper IV); a species highly specialized on a single, widely distributed, genus of fungi (*Tricholoma*). It is however also possible that adult plants themselves create or indicate facilitative effects other than just the presence of suitable fungi. One major limiting factor for germination and seedling development is likely soil moisture, where summer droughts can cause bottle-necks in seedling survival (e.g. Leake 2004).

The remaining species can be placed in an interval where the extent of microsite and seed limitation varies in importance. Microsite availability was found to be the main limiting factor for *C. umbellata* and *P. chlorantha*, where adult presence influenced germination rates positively while increased soil nutrients had a negative effect on seedling development (paper I). This is corroborated by results from paper IV where microsite parameters such as EcM diversity and adult presence had a positive effect on germination. *Chimaphila umbellata*, *P. chlorantha* as well as *M. hypopitys* usually inhabit drier pine forest where soil moisture may be of large importance. Adult presence as well as higher EcM diversity could then perhaps be indicative for suitable microclimates. Higher EcM diversity may however be a more

direct indication of suitable microsite quality than merely adult presence, as adult presence might indicate microsites previously suitable for germination, but where suitability has presently declined. One mechanism may relate to soil nutrient availability since seedling development of *C. umbellata* and *P. chlorantha* was found to be negatively affected by increased soil nutrients (paper I). EcM fungi have further been shown to decrease with higher levels of soil nitrogen to be replaced by AM fungi (Kranabetter & MacKenzie 2010).

Seed availability was found to be the main limiting factor for *P. minor* since no significant differences between plots could be found, however, microsite quality/availability is likely of some importance. The results for *M. uniflora* and *P. rotundifolia* gave no clear indication of whether microsite or seed limitation dominated, suggesting that microsite and seed limitation may be of equal importance. The latter three species are more commonly found in mesic habitats where droughts are less frequent and hence may not be of larger importance for microsite suitability.

## Dispersal and seed production

The average number of seed per capsule ranged from 1,024 (*O. secunda*) to 7,882 (*C. umbellata*). The actual seed production potential of the Pyroleae species is however higher for those species that produce several capsules per shoot (all species except *M. uniflora*). The average seed production per shoot ranged from 7,324 (*M. uniflora*) to 60,487 (*P. minor*). This is a rather low seed production compared to e.g. most orchids (e.g. Eriksson & Kainulainen 2011). Aborted seeds (not included in the analysis) ranged between 16% in *C. umbellata* to 30% in *P. chlorantha*.

The results concerning dispersal ability of Pyroleae dust seeds followed an inverse power law function ( $f(x) = 3.18x^{-0.21}$ ) where the vast majority of seeds (82.5%) were deposited close to the seed source, i.e. with a dispersal distance less than 1 m, and 95.7% were deposited within ca 5 m from the seed source (paper II). There are always some seeds that escape the immediate vicinity of the mother plant, however, and the high seed production ensures that the number of potentially long-dispersing seeds may nevertheless be relatively high. With germination and developmental rates (paper I & IV) a calculation example of germination success considering possible long distance dispersal can be produced (Box 1).

**Box 1.** Calculation example of germination success in relation to dispersal ability:  
Model species: *Chimaphila umbellata*

- *C. umbellata* produce c. 27,000 seeds per inflorescence (paper **II**)
- If 1% disperse long distance it equals 270 seeds
- 13.35% of seeds germinates (mean germination in occupied and unoccupied plots; paper **I**) = 36 seedlings
- 0.0035% develops into branched seedlings (paper **IV**) = 0.01 seedling(s)

Each adult flowering individual of *C. umbellata* could thus in theory produce 0.01 seedlings, each time flowering, which reaches the largest developmental stage (branched) at a site far from the mother plant. This calculation example does not however consider possible stochastic or environmental events that the seedling may be subjected to that could stunt further development or survival. As previously mentioned droughts may for example act as bottle-necks on seedling survival.

One of the investigated species, *P. minor*, deviates from the other species in having a very high seed production (paper **II**), possibly reflecting both its presumable self-pollination, and its capacity to produce many capsules per shoot. This is perhaps a bit surprising since this species found to mainly exhibit seed limited recruitment (paper **I**). However, this may reflect that *P. minor* has a comparatively wide range of potential recruitment sites (e.g. due to a comparatively broad range of host fungi or being generalistic regarding soil conditions), so that even such high number of seeds is not sufficient to saturate the recruitment microsites in the vicinity of natural populations.

## Nutritional status and degree of mycoheterotrophy

Our findings suggest that there is a variation among the studied Pyroleae species concerning partial mycoheterotrophy in adults (paper **III**; Fig. 7). Adult autotrophy (i.e. initial mycoheterotrophy) may be most common in Pyroleae species, and these species may not be as dependent on fungi-derived nutrients as for example some partially mycoheterotrophic orchids (e.g. Bidartondo et al. 2004; Julou et al. 2005; Cameron et al. 2008; but see Gonneau et al. 2014).

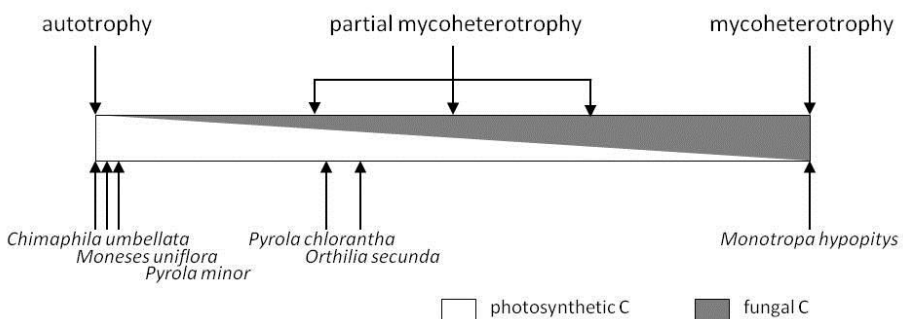
All investigated Pyroleae species were enriched in  $^{15}\text{N}$ , with  $\epsilon^{15}\text{N}$  values ranging between  $-2.9\text{‰}$  and  $7.7\text{‰}$ , which is between those of autotrophic and fully mycoheterotrophic reference plants. This does not necessarily indicate fungal N dependency, since plants in general receive some N from associating fungi, but rather that they either are processing N differently than

other mycorrhizal species and/or that the fungus is transferring a different form of N to these plants.

Only two species, *P. chlorantha* and *O. secunda*, were found to be partially mycoheterotrophic as adults. These two species had significantly higher  $\epsilon^{13}\text{C}$  values than the cluster of autotrophic reference plants and significant fungi-derived C. The remaining studied species, *C. umbellata*, *M. uniflora* and *P. minor* showed no enrichment in  $^{13}\text{C}$ , suggesting adult autotrophy. These results are congruent with previous studies (except for *M. uniflora* for which we present the first results of trophic status; paper III) made of Pyroleae (Tedersoo et al. 2007; Zimmer et al. 2007; Hynson et al. 2009b; Hynson et al. 2012)

The estimates of C and N gains in the Pyroleae showed similar patterns when using mycoheterotrophic seedlings and other mycoheterotrophic species as references, but with more pronounced differences. Despite this it may still be valuable to use seedlings as references since it would eliminate effects resulting from comparisons based on another species with perhaps different physiology. We further find that a previously unknown interspecific variation in isotope signature occurs during early ontogeny, from seed production to developing seedlings. The reasons for the differences between species C and N isotope content in different plant organs are further discussed in paper III.

Even though the majority of the studied Pyroleae plants are shown to be autotrophic as adults it is possible that the degree of fungal C dependency may be masked by accumulated temporal changes in C gain and/or variations in light or soil conditions. This could be particularly true for wintergreen forest plants such as the Pyroleae.



**Figure 7.** Schematic representation of the trophic strategies autotrophy, mycoheterotrophy, and partial mycoheterotrophy based on the carbon (C) source a plant uses, and the positioning of the studied species relative degree of mycoheterotrophy as adults (adapted and modified from Merckx 2013).

## Diversity of fungal associates

Similar to previous studies on adult plants, seedlings of all studied Pyroleae species and *M. hypopitys* were found to associate with a wide range of EcM fungi, at least in their initial developmental stages (table 1; paper IV). These mainly include EcM species in the genera *Cortinarius*, *Inocybe*, *Tomentella*, *Piloderma*, *Thelephora*, *Russula*, *Amphinema* and, in addition, Sebaciniales, all of which have been found in adult Pyroleae species (Tedersoo et al. 2007; Zimmer et al. 2007; Vincenot et al. 2008; Toftegaard et al. 2010; Hashimoto et al. 2012; Matsuda et al. 2012) and to some extent in seedlings (Hashimoto et al. 2012; Hynson et al. 2013).

Hynson et al. (2013b) found seedlings of *O. secunda* and *P. chlorantha* to be associating with a wide range of fungal hosts but that the dominating fungus, and also the only fungus shared by the two Pyroleae species, was of unknown trophic status in the order Sebaciniales. The majority of Sebaciniales taxa found in paper IV belongs to Sebaciniales clade A and was present in seedlings of all studied species. Taxa belonging to Sebaciniales clade B were far from dominating but were found in all studied species except *M. uniflora*. Among the studied species, Sebaciniales (clade A and B) were most frequent in *P. chlorantha* and *P. rotundifolia*. It is however notable that taxa belonging to Sebaciniales clade B were almost exclusively found in seedlings collected at occupied plots where adults were present (with the exception of *P. chlorantha*) which could suggest that taxa from Sebaciniales clade B are more important for adult plants or more frequently associated with adult plants.

Furthermore, ordination plots and adonis-analysis revealed that *C. umbellata*, *M. hypopitys* and *M. uniflora* changed composition of fungal associates during seedling ontogeny. While still generalized, in the sense that a wide array of fungi associated with the seedlings of *C. umbellata* and *M. uniflora*, the actual composition narrowed from germination and onwards. A similar pattern was found for *M. hypopitys* during seedling development from initial germination to elongated seedling but the narrowing of fungal associates was even more pronounced in branched seedlings. Congruent with previous studies on fungal associates of *M. hypopitys* (e.g. Bidartondo & Bruns 2001; 2002), a specialization on the genus *Tricholoma* (Tricholomataceae) was found. However, unlike Bidartondo & Bruns (2002), who found *T. columbetta*, *T. saponaceum* and *T. portentosum* to be dominating in their Swedish populations of *H. monotropa*, we found *T. equestre* to be the main taxa present. Our results thus support the hypothesis that there may be rapid shifts in fungal host species geographically (Bidartondo & Bruns 2002; 2005).

**Table 1.** The most common fungal genera associated with the studied species at different stages of development (paper IV) and with adult plants (from literature).

Study species	Dominant* fungal associates of seedlings (%) (paper IV)	Fungal associates found in adult plant roots (from literature)	Reference
<i>Chimaphila umbellata</i> <sup>a</sup>	<b>Cortinarius</b> (100%), <b>Piloderma</b> (100%), <b>Amphinema</b> (86%), <b>Inocybe</b> (86%), <b>Tylospora</b> (86%), <b>Tomentella</b> (71%), <b>Tylopilus</b> <sup>c</sup> (29%), <b>Tricholoma</b> (14%)	<i>Tricholoma</i> , <i>Amphinema</i> , <i>Laccaria</i> , <i>Tomentella</i> , <i>Inocybe</i>	Tedersoo et al 2007; Zimmer et al. 2007
<i>Moneses uniflora</i> <sup>a</sup>	<b>Atheliaceae</b> (35%) <b>Meliniomyces</b> (30%), <b>Russula</b> (30%), <b>Tylospora</b> (30%), <b>Piloderma</b> (25%)	Na	
<i>Monotropa hypopitys</i> <sup>b</sup>	<b>Tricholoma</b> (100%), <b>Hydnellum</b> (56%)	<i>Tricholoma</i>	e.g. Bidartondo & Bruns 2001; 2002
<i>Orthilia secunda</i>	Na (but see Hynson et al. 2013b)	<i>Phialocephala</i> , <i>Heliotales</i> , <i>Wilcoxina</i> , <i>Inocybe</i> , <i>Tomentella</i> , <i>Tricholoma</i> , <i>Piloderma</i> , <i>Sebacina</i>	Tedersoo et al 2007; Zimmer et al. 2007
<i>Pyrola chlorantha</i>	<b>Inocybe</b> (47%), <b>Piloderma</b> (39%), <b>Cortinarius</b> (35%), <b>Tomentella</b> (35%), <b>Sebacinales</b> (31%), <b>Russula</b> (27%)	<i>Tricholoma</i> , <i>Suillus</i> , <i>Sebacinales</i> , <i>Heliotales</i> , <i>Tomentella</i> , <i>Russula</i>	Tedersoo et al 2007; Zimmer et al. 2007
<i>Pyrola minor</i>	Na	<i>Laccaria</i>	Zimmer et al. 2007
<i>Pyrola rotundifolia</i>	<b>Cortinarius</b> (42%), <b>Sebacinales</b> (36%), <b>Tomentella</b> (36%), <b>Tricholoma</b> (24%)	<i>Tomentella</i> , <i>Cortinarius</i> , <i>Russula</i> , <i>Tricholoma</i> , <i>Sebacina</i>	Vincenot et al. 2008

\* Dominant refers to the fungal genera/family/order most frequently present in seedlings of respective species  
<sup>a</sup> Fungal associates of elongated seedlings are presented  
<sup>b</sup> Fungal associates of branched seedlings are presented  
<sup>c</sup> Unique fungal genera in larger developmental stages

## Conservation biology of Pyroleae

Irrespective of the underlying mechanisms behind fungal host range, the range of fungal hosts will have consequences for the distribution of mycoheterotrophic plants. The chances of successful recruitment (both short and long-distance) will likely increase with fungal host generalization, specialization on commonly distributed fungal host, and partial mycoheterotrophy. Species with narrower host range that are initial mycoheterotrophs will thus likely be less successful in their recruitment due to decreased quantities of suitable recruitment sites.

A likely process behind the decreased quality and quantity of suitable microsites is the loss of disturbance regimes in forests. Decreased fire frequency and forest grazing along with a modernized forest industry may have increased the competition from other plants, but also made it less likely

for these species to disperse to suitable recruitment sites and get in contact with appropriate fungi. Grazing animals (and fire) would e.g. remove competing plants, help with seed dispersal and by trampling disturb the soil. It is also possible that increased inputs of nutrients, both from agricultural and silvicultural sources as well as atmospheric downfall, may deprive these species of their advantage of being adapted to low nutrient levels. Densely planted monoculture forests further involves decreases in fungal diversity as well as more closed canopy covers, likely disfavoring initially mycoheterotrophic species. Habitat fragmentation and loss leads to fragmented populations and has been shown to entail resource allocations in plants from sexual reproduction to vegetative reproduction (e.g. Beatty & Provan 2011a). Increased shading has furthermore been found to negatively affect the flowering frequency in *C. umbellata* (Lundell et al. 2015), and thus also the possible seed production, i.e. genetic propagation. Since increased seed production from a seed source in itself improves dispersal it is likely to have major effects on recruitment.

Both *C. umbellata* and *P. chlorantha* were found to mainly be microsite limited (paper I & IV). These species are adapted to dry habitats like pine forests, are dependent on adult presence for successful recruitment, and are also negatively affected in their seedling development by higher levels of soil nutrient content (paper I). This could imply that current land use and forest management are causing decreased suitability of microsites for recruitment. In addition, climatic changes like warmer summers could involve severe droughts which could decrease the chances of seedling survival, especially for species already growing in dry habitats. The very dry summers (on Öland) of 2012 -2014 may be the reason for the lack of larger seedlings of *P. chlorantha*. The low number of more developed seedlings found could also be a result of long-lived seed/seedling bank, where seedlings await more suitable conditions for further development (Hynson et al. 2013b).

One should note that the three species exhibiting host narrowing, *C. umbellata*, *M. hypopitys* and *M. uniflora* are also decreasing in Sweden. It would be interesting to further study mechanisms connected to recruitment limitations in relation to plant demography. The diversity and especially the distribution of associating fungi would be of interest for such studies since it is likely to have large effects on plant demography. The two largest populations of *C. umbellata* used in this thesis (Rälla and Skedås), likely to be the most stable populations and suitable for recruitment, were also the populations with highest germination rates and EcM diversity.

To more specifically determine which life stages that are most sensitive of these plants is crucial for strategic long-term conservation. Management actions such as to increase the amount of light, decrease competition from other species and reduce the nutrient load are of great importance for both short- and long-term preservation.

## Concluding remarks

This thesis has examined the recruitment ecology and possible mechanisms behind recruitment limitations of mycoheterotrophic Ericaceae. The majority of studies included in this thesis focus on mechanisms controlling and affecting microsite quality, such as soil chemistry (paper **I**), the presence of conspecific adult MHP (paper **I** & **IV**) and the dependency on and diversity of fungal hosts present (paper **III** & **IV**). Seed limitations such as the availability and production of seeds, and dispersal capacity were also investigated (paper **II**).

The results provide evidence that the species in Pyroleae are heterogeneous, not only with regard to their degree of mycoheterotrophy (paper **III**), but also concerning the germination and early seedling development (paper **I** & **IV**). Despite the high production of seeds (not comparing with orchids) the majority of seeds are dispersed in close proximity to the seed source (paper **II**). This may be suitable for MHP that share fungal hosts with their conspecific adults or in other ways are benefitted by facilitative effects of adult plants. Nevertheless, some seeds will likely disperse long-distance and could, with the relatively high germination frequency found for some of the studied plants (paper **I** & **IV**), lead to new establishments. The chances of successful long-distance recruitment will however likely increase with fungal host generalization, partial mycoheterotrophy or specialization on commonly distributed fungal host.

Apart from a conspicuous variation in germination rate and seedling development, we find evidence that there is a tendency for host narrowing in some of the Pyroleae species, even if this is not as strict as the specialization seen in the full mycoheterotroph *H. monotropa* (paper **IV**). For the latter species, we provide further supporting evidence of host specialization on *Tricholoma*, and, by finding dominance of *T. equestre* as a host, also further evidence for geographical and developmental host shifts. Much remains, however, before we fully understand the intricate relationships between recruitment ecology and the evolution of fungal interactions in mycoheterotrophic plants.

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# Errata

**Paper I:** In Table 1 seed size should be given in mm not cm.  
The scale bar in Figure 2c corresponds to 0.5 mm not 0.05 mm.

**Paper II:** The scale bars in Figure 1 corresponds to 100 $\mu$ m.

# Svensk sammanfattning

Växter interagerar med svampar på flera olika sätt, från symbiotiska (mutualistiska) till parasitiska förhållanden. Ett av de mest utbredda och allra viktigaste symbiotiska förhållandet är bildandet av mykorrhiza. Ungefär 80% av alla landväxter lever i symbios med mykorrhizasvampar. Denna symbios bygger på ett utbyte av resurser där svampen förser växten med mineraler och ökat område för vattenupptag och i gengäld får svampen kol som assimilerats genom växtens fotosyntes. Växt-svamp interaktioner kan leda till komplexa nätverk eftersom en enda växt parallellt kan associera med flera svampar och en enda svamp kan samtidigt associera med flera växter. I den här avhandlingen studeras växter som "fuskar" till sig näring genom att parasitera på mykorrhizasvampar. Dessa växter kallas för mykoheterotrofer. Majoriteten av mykoheterotrofa växter associerar antingen med arbuskulär (AM) eller ektomykorrhiza (EcM), men det finns även de som associerar med saprotrofiska (nedbrytande) svampar. Graden av mykoheterotrofi kan variera beroende på under vilken del i livscykel som växten är mykoheterotrof. Växter som är fullt mykoheterotrofa, under hela livscykeln, saknar klorofyll och därmed förmågan att fotosyntetisera. Andra, så kallade partiellt mykoheterotrofa växter utvecklar gröna blad som vuxna plantor och kan därför delvis utnyttja de associerade svamparna samtidigt som de fotosyntetiserar. Alla mykoheterotrofa växter har exceptionellt små frön, så kallade dammfrön. Dammfrön är den minsta typ av frön man kan hitta bland angiospermer. Dessa frön saknar eller har minimala näringsreserver och är därför helt beroende av en extern näringskälla under groningen och tidig utveckling då den underjordiska groddplantan mest ser ut som en liten rot. Det betyder att oavsett om mykoheterotrofa växter är fullt eller partiellt mykoheterotrofa så är alla initialt mykoheterotrofa. Orkidéer är den grupp växter som kanske är mest känd för att ha dammfrön men sådana frön är också vanligt förekommande i Gentianaceae, Orobanchaceae och Ericaceae (ljungväxter) samt flera tropiska växtfamiljer.

Interaktioner som involverar mykorrhiza är i allmänhet inte specialiserade. Både växter och svampar kan oftast associera med ett flertal olika arter även om de såklart finns undantag. Tidigare studier har visat att fullt mykoheterotrofa växter oftast är väldigt specialiserade på specifika släkten av svampar medan partiellt mykoheterotrofa växter oftast är generalister. Tallört (*Monotropa hypopitys*), som är fullt mykoheterotrof, har till exempel visat sig vara strikt specialiserad på svampsläktet *Tricholoma*

(musseroner). Många orkidéer och arter inom gruppen *Pyrola*-växter har däremot visat sig associera med ett större antal olika svampgrupper. Dessa studier är dock huvudsakligen utförda på vuxna plantor och kunskapen om vilka svampar som är involverade under groningen och tidig utveckling hos dessa växter är ytterst begränsad. Det är möjligt att en del arter är specialiserade under tidiga livscykelstadier för att senare bli alltmer generaliserande, eller tvärt om.

Syftet med avhandlingen var att undersöka rekryteringsekologin hos mykoheterotrofa växter med dammfrön; dvs. studera vad som begränsar rekryteringen och vilka mekanismer som kan ligga bakom eventuella begränsande faktorer. Det finns i allmänhet två kontrasterande alternativ till vad som begränsar rekryteringen hos växter, nämligen tillgången på frön (fröbegränsning) eller mängden och/eller kvaliteten på lämpliga etableringsplatser ("microsite"-begränsning). Eftersom mykoheterotrofa växter med dammfrön parasiterar på svampar, åtminstone i de tidiga livscykelstadierna, så är deras rekrytering nära förknippat med att hitta lämpliga platser där svamparna de associerar med också är närvarande. Utöver de faktorer som påverkar interaktionerna mellan groende frön och svampar så finns det två andra faktorer som också påverkar rekryteringen av mykoheterotrofa växter, nämligen fröproduktionen och fröspridningsförmågan. Fröproduktionen är en viktig del av den faktiska fröspridningspotentialen eftersom en ökad fröproduktion i sig förbättrar den möjliga spridningen.

I avhandlingen studerades växter inom underfamiljen *Monotropoideae* (*Ericaceae*). Främst användes arter av *Pyrola*-växter som är partiellt eller initialt mykoheterotrofa: *Chimaphila umbellata* (ryl), *Moneses uniflora* (ögonpyrola), *Orthilia secunda* (björkpyrola), *Pyrola chlorantha* (grönpyrola), *P. minor* (klotpyrola) och *P. rotundifolia* (vitpyrola). Men också *Monotropa hypopitys* (tallört) som är släkt med *Pyrola*-växterna och är fullt mykoheterotrof studerades.

Metoderna som användes i de olika delstudierna var huvudsakligen fältexperiment i form av frösåningsförsök kombinerat med kol- och kväveisotopanalys för att undersöka graden av mykoheterotrofi samt pyrosekvensering av de mykorrhizasvampar som är involverade i studiearternas groningen och tidiga utveckling. Dessutom uppskattades fröproduktion, och även fröspridningsförmåga med hjälp av fröfällor.

Resultaten visar att *Pyrola*-växterna är heterogena, inte bara när det gäller graden av mykoheterotrofi, utan också i gröningsfrekvens och tidig utveckling. Det är troligtvis en kombination av mängden och/eller kvaliteten av lämpliga etableringsplatser och fröbegränsning som begränsar utbredningen och rekryteringen av samtliga studerade arter. Omfattningen av dessa begränsningar varierar dock mellan arterna. Vissa är mer beroende av kvaliteten på etableringsplatserna medan andra är mer beroende av fröproduktionen/fröspridningsförmåga. Trots att dammfrön är anpassade för

vindspridning så hamnar den största delen av de frön som produceras i närheten av frökällan. Den höga produktionen av frön gör det dock troligt att åtminstone ett fåtal sprider sig en längre distans från modersplantan.

Studier av stabila kol- och kväveisotoper visade att endast grönpyrola och björkpyrola var partiellt mykoheterotrofa som vuxna plantor medan ryl, ögonpyrola och klotpyrola var autotrofa (dvs. initialt mykoheterotrofa) som vuxna plantor.

Groddplantor från samtliga undersökta arter associerade med ett fylogenetiskt brett utbud av ektomykorrhizasvampar, åtminstone i de inledande utvecklingsstadierna. Några svampsläkten som var vanligt förekommande var exempelvis: *Amphinema* (skinn), *Cortinarius* (spindelskivlingar), *Inocybe* (trådskevlingar), *Piloderma* (skinn), *Russula* (kremlor), *Tomentella*, *Tricholoma* (musseroner) samt svampar inom ordningen Sebaciales. För några arter (ryl och ögonpyrola) verkar det finnas en tendens till en minskning i antalet svampsläkten de associerar med från groning till mer utvecklade groddar, även om de fortfarande är generalister i förhållande till tallört. Precis som i tidigare studier var tallört starkt förknippad med svampsläktet *Tricholoma*. Skillnaden från tidigare studier var dock att *T. equestre* (riddarmusseron) var den dominerande arten. *Tricholoma columbetta* (silkesmusseron), *T. saponaceum* (såpmusseron) och *T. portentosum* (streckmusseron) har annars tidigare huvudsakligen funnits associera med tallört i Sverige. Våra resultat stödjer därför hypotesen om att det kan ske snabba skiften i vilka svampar mykoheterotrofa arter associerar med, såväl geografiskt som under de tidiga livscykelstadierna.

Tre av de studerade arterna (ryl, grönpyrola och ögonpyrola) minskar för närvarande i Sverige och ryl är rödlistad som starkt hotad. Det ligger troligtvis flera faktorer bakom dessa arters minskning men en viktig faktor kan vara bristen på störning i skogar. Minskad störning medför en ökad konkurrens från andra arter samt sämre ljusinsläpp vilket speciellt missgynnar de arter som är autotrofa som vuxna (t.ex. ryl). Fragmenterade habitat i kombination med den minskade fröproduktionen som också är associerad med sämre ljusinsläpp minskar dessutom chansen att frön sprids till lämpliga habitat. Det är också troligt att ökat näringsinnehåll i marken påverkar artsammansättningen av svampar samt även minskar studiearternas fördel av att vara anpassade till habitat med lägre näringsinnehåll. Skötselåtgärder som ökar ljusinsläppet samt minskar utsläpp av näringsämnen är därför av stor vikt för långsiktigt bevarande. En högre brandfrekvens i skogar samt mer skogsbete skulle gynna dessa arter. Fortsatta studier med fokus på mykoheterotrofa växters demografi i kombination med begränsande mekanismer är av intresse eftersom det är viktigt att fastställa vilka livscykelstadier som är känsligast.

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