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The interplay between *Pinus sylvestris*, its root hemiparasite, *Melampyrum pratense*, and ectomycorrhizal fungi: Influences on plant growth and reproduction

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Abstract: Despite the extensive literature on mutual interactions between plants and mycorrhizal fungi, and host plants and parasitic plants, little is known about the outcomes of interactions when the three organisms exist in concert with one another. We investigated, in a microcosm experiment, the interactions between *Pinus sylvestris* L. and the root hemiparasitic *Melampyrum pratense* L. in the presence/absence of ectomycorrhizal (EM) fungi. Mycorrhizal infection significantly increased the biomass of *P. sylvestris*, whereas the parasitic infection decreased it. Concentration of host phosphorus was greatly increased by the presence of EM fungi. *M. pratense* plants attached to mycorrhizal pines had higher biomass and produced more flowers than those growing with non-mycorrhizal pines. We attribute the stimulation of parasite performance in the presence of EM fungi to enhanced nutrient availability to the host individuals, and to increased photosynthetic capacity of hosts as a result of increased above-ground biomass.
Keywords: ectomycorrhiza, host-hemiparasite-EM fungi interaction, parasitic angiosperms, *Pinus sylvestris*, *Melampyrum pratense*.

Résumé : En dépit d'une documentation considérable à propos des interactions mutuelles des plantes hôtes, soit avec les champignons mycorhiziens ou soit avec les plantes parasites, il existe peu d'informations concernant les interactions des trois organismes lorsqu'ils coexistent l'un avec l'autre. Nous avons étudié dans des microcosmes les interactions entre *Pinus sylvestris* L. et l'hémiparasite *Melampyrum pratense* L. en présence ou pas des champignons ectomycorhiziens (EM). La colonisation mycorhizienne a augmenté d'une façon significative la biomasse de *P. sylvestris* par rapport à l'infection parasitaire qui l'a diminuée. La concentration en phosphore dans l'hôte est grandement accrue en présence de champignons ectomycorhiziens. Les plantes parasitaires de *M. pratense* qui se sont fixées aux pins mycorhizés ont développé une biomasse plus élevée et ont produit plus de fleurs que celles qui ont poussé en présence des pins non mycorhizés. Nous attribuons ce meilleur rendement des plantes parasitaires en compagnie des champignons ectomycorhiziens à une augmentation de la disponibilité des éléments minéraux envers les plantes hôtes, de même qu'à la hausse de leur capacité photosynthétique due à l'accroissement de la partie aérienne.
Mots-clés : ectomycorhize, hôte-hémiparasite, interaction champignon-ectomycorhize, angiospermes parasitaires, *Pinus sylvestris*, *Melampyrum pratense*.

Introduction

Ecological literature has increasingly emphasized the importance of mycorrhizal fungi in mediating interactions between their plant associates and other organisms, such as other plants (Perry *et al.,* 1989; Hartnett *et al.,* 1993; Moora & Zobel, 1996; West, 1996), herbivores (Gange & West, 1994; Gehring & Whitham, 1994; Larsen & Jakobsen, 1996; Borowicz, 1997) and soil pathogens (Newsham, Fitter & Watkinson, 1995). The three-level interaction among parasitic plants, mycorrhizal fungi, and their common host plants has also received growing attention (Weber, 1987; Gehring & Whitham, 1992; Sanders, Koide & Shumway, 1993; Davies & Graves, 1998), but so far only associations with arbuscular mycorrhizal (AM) fungi have been studied. However, some of the generalist root hemiparasites frequently parasitize hosts living symbiotically with ectomycorrhizal (EM) fungi, which are known to differ from AM in several of their functional characteristics (Connell & Lowman, 1989).

Ectomycorrhizal association with fungi can be vitally important for the supply of phosphorus and nitrogen for woody plants growing in the often infertile soils of northern coniferous forests (Harley & Smith, 1983; Allen, 1991; Finlay, Frostegård & Sonnerfeld, 1992; Marscher & Dell, 1994). In this mutualistic association, the fungus obtains carbohydrates from the plant (Allen, 1991). The amount of hyphae of EM fungi in coniferous forest soils is extensive, enabling a rapid establishment of mycorrhiza between a plant seedling and the fungus.

Among parasitic plants, a distinction is commonly made between holoparasites lacking chlorophyll and photosynthesis, and hemiparasites which have green, photosynthetic leaves (Musselman & Press, 1995). The hemiparasites are capable of extracting nutrients, water, and organic substances from the root xylem of their hosts by means of haustoria (Govier & Harper, 1965; Press, Graves & Stewart, 1990; Tennakoon & Pate, 1996), and when so doing, can markedly reduce photosynthesis, growth, and reproduction of their hosts (Gibson & Watkinson, 1991; Gurney, Press & Ransom,

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1995; Matthies, 1995a, b; 1996; Marvier, 1996; Puustinen & Salonen, 1999). From the host's point of view the hemiparasite clearly differs from the fungal associates: the former acts only as a resource sink, whereas the mycorrhizal fungi also benefit the plant. However, the fungi also require energy from the host plant. Thus in the tripartite association, the host plant may become exposed to two simultaneous external sinks, both reducing the ability of the plant to satisfy the demands of its own resource sinks. Moreover, a hemiparasitic infection may cause a decline in mycorrhizal colonization of host roots by fungi (Gehring & Whitham, 1992; Davies & Graves, 1998). Hence, these three organisms are likely to interact with one another.

In this study, we used laboratory microcosms to examine the effects of simultaneous colonization by several species of EM fungi and a root hemiparasite, *Melampyrum pratense*, on the performance of the host, *Pinus sylvestris*. We were especially interested in the effects of the host's mycorrhizal status on the performance of the hemiparasite. We presumed that mycorrhizal association will be advantageous for the pine seedlings, and that this advantage will be perceptible as enhanced growth. Consequently, we predicted that growth and flower production of the *M. pratense* plants should be stimulated more following attachment to mycorrhizal hosts than it would following attachment to non-mycorrhizal hosts.

Material and methods

The parasitic plant *Melampyrum pratense* is an annual herb, common in various types of boreal forests. It is a generalist root hemiparasite attacking the roots of a large variety of both herbaceous and woody plants. In nutrient-poor pine forests, where potential herbaceous or graminoid hosts are often totally lacking, *M. pratense* parasitizes the roots of woody plants (V. Salonen, pers. obs.). Both coniferous and deciduous trees, as well as ericaceous shrubs have been reported as suitable hosts for *M. pratense* (see references in Gauslaa, 1990). Seeds of *M. pratense* usually germinate soon after being dropped on a moist soil surface in late summer, and overwinter in the cotyledon stage in moss carpets. The plants start to grow in late April/early May, and flowering begins at the end of May.

Seeds of Scots pine (*Pinus sylvestris*) were collected from cones produced by trees growing in a pine plantation in Nurmijärvi, southern Finland. Prior to sowing, the seeds were surface-sterilized to eliminate mycorrhizal hyphae and spores by keeping them in 30% hydrogen peroxide for 20 minutes, after which they were carefully washed with sterile water. The surface-sterilized seeds were allowed to germinate, and the emerged seedlings were grown in aseptic conditions in vermiculite. The vermiculite was soaked in nutrient solution (modified Melin-Norkran's) enriched with glucose (1.25 g/L) and malt extract (5.0 g/L) to provide energy for the six species of EM fungi (*Cenococcum geophilum, Laccaria bicolor, Hebeloma sp., Piloderma croceum, Suillus bovinus and S. variegatus*) growing in the vermiculite. The pine seedlings were allowed to become infected by the EM fungi for ten weeks at room temperature before being transplanted to the microcosms. Seedlings to be transplanted into microcosms without mycorrhiza were treated similarly to the method described above, except that the vermiculite in the glass jars did not contain mycorrhizal fungi.

The experiment was conducted in transparent plastic bottles (microcosms; volume 1.5 L, height 28 cm, diameter 8.5 cm). The cap of the bottle was replaced by a cotton plug to provide ventilation in the system. The soil used was a mixture of sieved (4-mm sieve) raw humus collected from a pine forest in Jyväskylä and sieved (1-mm sieve) sand (ratio 1:1 by volume). On average, the content of soluble phosphorus and ammonium nitrogen in the soil was 3.5 mg/kg dry mass and 0.76 mg/kg dry mass, respectively. Prior to adding 300 g (fresh mass) of soil into the microcosms it was sterilized using an autoclave. A cut was made on the wall of the bottle in order to facilitate introduction of the soil and seedlings into the microcosms. The *P. sylvestris* seedlings were individually transplanted into these microcosms at the age of ten weeks (counted from the day the seeds were sown).

Two days after planting the *P. sylvestris* seedlings, an inoculum of soil microbes with 10 species of bacteria and 10 species of saprophytic fungi was added into each microcosm. The fungi and bacteria were cultivated on agar plates, and the biomass growing on the surface of agar was scraped with a sterilized knife and transferred into jars containing sterilized water. Both the bacteria and fungi were added into the microcosms along with 2 mL of sterilized water. Three weeks later, an inoculum of bacterial-feeding nematodes (*Mesodiplogaster sp.*) was added into the microcosms to improve nutrient mineralization. On average, 170 nematodes per microcosm were added along with 2 mL of sterilized water. The aim of the addition of soil microbes and their predators was to establish a simple decomposer food web to promote nutrient mobilization in the microcosms.

The microcosms were incubated in a climate chamber for 13 weeks. A daily cycle of 18 hours of daylight (*ca* 300 $\mu E/m^2/s$) at 20°C and 6 hours of darkness at 12°C was maintained in the climate chamber. Artificial winter (4 weeks) with constant darkness at + 1°C was created by gradually lowering the temperature and illumination over a period of 4 weeks. After the artificial winter, the temperature and illumination were gradually increased to mimic spring (4 weeks), whereafter conditions (similar to those during the first summer) for the second summer were created.

In the two treatments with parasitic plants, two *M. pratense* seedlings (*ca* 4 cm in length) were transplanted in the microcosm at the beginning of the second summer. After two weeks, the number of parasite seedlings was thinned to one per microcosm. The *M. pratense* transplants were over-wintered seedlings collected from a nutrient-poor pine forest in Säynätsalo, central Finland. Since the seedlings were potentially contaminated with EM fungi, they were treated with 0.1% carbendazim fungicide for 20 minutes to eliminate the fungi, and then carefully washed with sterile water. In this experiment, all plant transplantations were conducted aseptically using a laminar-flow chamber.

The experiment had a 2×2 factorial design with mycorrhizal infection of the host plant (non-mycorrhizal/mycorrhizal) combined with hemiparasitic infection of the host (non-parasitized/parasitized). The four treatment combina-

tions, *i.e.,* (*i*) a non-mycorrhizal pine seedling growing alone ($M^- P^-$), (*ii*) a mycorrhizal pine seedling growing alone ($M^+ P^-$), (*iii*) a non-mycorrhizal pine seedling growing with a *M. pratense* seedling ($M^- P^+$), and (*iv*) a mycorrhizal pine seedling growing with a *M. pratense* seedling ($M^+ P^+$), were replicated ten times. An additional test was conducted to see if the parasite seedlings were capable of growing and flowering without connection to the host. Twelve *M. pratense* seedlings were individually transplanted in similar microcosms as described above, but without the fungicide treatment.

After running the experiment for 14 weeks following transplantation of the *M. pratense* seedlings, roots of each plant were carefully washed free of debris, and the hosts and the hemiparasites were separated from each other. To ascertain that the non-mycorrhizal pines had remained uninfected, and that the mycorrhizal pines had become infected, roots of each seedling were carefully checked for the presence of EM using a microscope. Furthermore, the presence of haustorial connections between host roots and parasite roots was checked. Dry biomass (dried at 80°C for 48 hours) of both roots and above-ground parts was determined for each plant, and the number of flowers produced by the *M. pratense* plants was counted.

Concentrations of phosphorus, nitrogen, and potassium in the host above-ground tissue were analyzed from five replicates in each treatment. After oven-dried needle and stem tissue was cut into small pieces, the Kjeldahl technique was applied for the analysis of nitrogen concentration. Potassium concentration was determined using an atom absorption spectrophotometer, and the concentration of phosphorus was analyzed spectrophotometrically.

Statistical analyses were performed using SPSS for Windows (Norusis/SPSS Inc. 1992). Data sets for the host responses were analyzed using two-way ANOVA, and data for the parasite responses by *t*-test for two independent samples. Host plants in replicates from which the parasitic plant had died during the experiment were excluded from the statistical analyses.

Results

Host responses

Total biomass of the host plant was significantly increased by mycorrhiza (Table I, Figure 1). The mycorrhizal pines produced 12% (unparasitized) and 10% (parasitized) more biomass than the non-mycorrhizal pines. In contrast, parasitic plant infection resulted in a significant suppression of host growth (Table I, Figure 1). The total biomass of unparasitized *P. sylvestris* plants was 19% (mycorrhizal) and 17% (non-mycorrhizal) higher than that in treatments where the parasitic plant was present. A significant reduction of host above-ground biomass due to parasitic infection exclusively accounted for the reduction of host total biomass, since parasitism had no effect on host root biomass (Table I). No significant interactions between mycorrhizal and parasitic infections in host root, shoot, or total biomass were found.

Concentrations of N, P, and K in host above-ground tissue at the end of the experiment are shown in Table II. As revealed by two-way ANOVAs, the concentrations of host nitrogen and potassium were unaffected by mycorrhiza ($F_{1,17} = 0.859$, $P = 0.370$ for N; $F_{1,19} = 0.795$, $P = 0.386$ for K), but they were significantly decreased by the presence of the parasitic plant ($F_{1,17} = 6.008$, $P = 0.028$ for N; $F_{1,19} = 13.209$, $P = 0.002$ for K). Mycorrhizal infection of the host had a highly significant effect ($F_{1,19} = 22.485$, $P < 0.001$) on the phosphorus content, whereas the parasitic infection had a marginally significant negative effect ($F_{1,19} = 3.735$, $P = 0.071$). No significant mycorrhiza × parasite interactions on concentrations of N, P, or K were found.

Only non-quantitative observations of the performance of the EM fungi were carried out. The microscopic examination revealed that a conspicious mantle had developed on the roots of pines, indicating that the mycorrhizal fungi had become well established. The fungal sheath covering the roots of the parasitized hosts was as dense as that found on roots of the unparasitized hosts, indicating that the parasitic plants did not prevent the establishment of mycorrhiza. Likewise, the percentage of root tips infected with EM did not differ between parasitized and non-parasitized pines; over 90% of the short roots in both treatments were mycorrhizal.

Parasite responses

Mycorrhizal status of the pine hosts had a significant effect on both the biomass ($t = 4.215$, df = 11, $P = 0.001$), and number of flowers ($t = 4.215$, df = 11, $P = 0.001$) produced by the hemiparasitic *M. pratense* plants. The biomass of *M. pratense* plants growing with the mycorrhizal hosts was on average 81% higher than that of the plants attached to the non-mycorrhizal plants (Figure 2a). The *M. pratense* plants hosted by the mycorrhizal pines produced three times more flowers than the parasitic plants growing with the non-mycorrhizal pines (Figure 2b).

In each treatment with the hemiparasitic plants, some parasites died soon after they had emerged. Mycorrhizal status of the host plants did not affect the survival of the parasites, since the number of dead *M. pratense* in the treatment with mycorrhizal and non-mycorrhizal hosts was three and four, respectively. The death of the parasitic plants occurred during the first half of the experiment, indicating that these plants probably failed to establish a haustorial connection to the host. Microscopic examination at the end of the experiment revealed that each of the living parasitic plants had formed haustorial connections to their hosts.

A total of eight out of the twelve parasite seedlings transplanted in the microcosms without hosts emerged. Each of these seedlings died well before the end of the experiment, suggesting that the haustorial connections between parasites and hosts in the actual experiment were functional.

Discussion

Davies & Graves (1998) showed that mycorrhizal colonization of host (*Lolium perenne*) roots by AM fungi stimulated the growth and reproduction of the attached root hemiparasite (*Rhinanthus minor*). In that study, an increase in both the biomass and the number of flowers of the hemiparasite was found, even though the mycorrhizal infection of host roots affected neither the growth nor the nutritional status (K, P, and N content of the plants) of the hosts. Results of the present study are consistent with that of

TABLE I. ANOVA results showing the effects of infection by the root hemiparasitic *Melampyrum pratense* (parasitized/unparasitized) and the host's (*Pinus sylvestris*) mycorrhizal status (mycorrhizal/non-mycorrhizal) on root, shoot and total biomass of the host

Source	Root biomass				Shoot biomass				Total biomass			
	df	MS	*F*	*P*	df	MS	*F*	*P*	df	MS	*F*	*P*
Parasite	1	0.003	0.767	0.388	1	0.142	30.185	< 0.001	1	0.170	14.319	0.001
Mycorrhiza	1	0.011	2.861	0.101	1	0.014	3.077	0.090	1	0.061	5.160	0.031
Parasite × mycorrhiza	1	0.000	0.020	0.888	1	0.005	0.42	0.524	1	0.002	0.146	0.705
Residual	29	0.004			29	0.005			29	0.012		

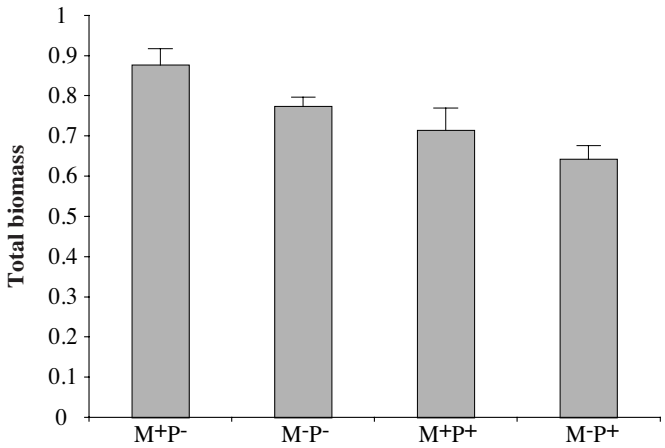

FIGURE 1. Mean (with SD) biomass (g dry mass) of *Pinus sylvestris* seedlings infected (P^+) or non-infected (P^-) with a root hemiparasitic *Melampyrum pratense* plant, and infected (M^+) or non-infected (M^-) with EM fungi.

TABLE II. Mean concentrations (mg/kg dry mass) of nitrogen (N), phosphorus (P), and potassium (K) in host (*Pinus sylvestris*) above-ground tissue at the end of the experiment. Host plants were either mycorrhizal (M^+) or non-mycorrhizal (M^-) and parasitized (P^+) or unparasitized (P^-) with one hemiparasitic *Melampyrum pratense* plant. The concentration values were analyzed from five plants in each treatment

	$M^- P^-$		$M^- P^+$		$M^+ P^-$		$M^+ P^+$	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
N	8.47	1.58	6.67	1.03	10.18	0.71	6.88	0.73
P	1.05	0.11	0.75	0.06	1.46	0.07	1.35	0.16
K	6.40	0.58	5.48	0.41	7.84	0.51	4.97	0.57

Davies & Graves (1998) in that the performance of a parasitic plant was greater when associated with a mycorrhizal host; this time with an ectomycorrhizal host plant. Despite the similar responses of the parasitic plants in these two studies, the actual mechanism behind them seems to differ.

Under nutrient-poor conditions in the microcosms, the association with EM fungi proved to benefit the pine hosts by enhancing nutrient availability, particularly phosphorus. In contrast to our results, Davies & Graves (1998) found mycorrhizal colonization by AM fungi to have no significant effect on either P or N content of the graminoid hosts, and no stimulation of growth of the hosts was found. Whereas the graminoid host (*Lolium perenne*) used in their study is known to be poorly responsive to mycorrhizal infection (Koide, 1991), the short roots of Scots pines are nearly always colonized by EM fungi in nature (Wallander, 1992), and especially when growing in nutrient-poor soil conditions pine is responsive to this infection (Setälä,

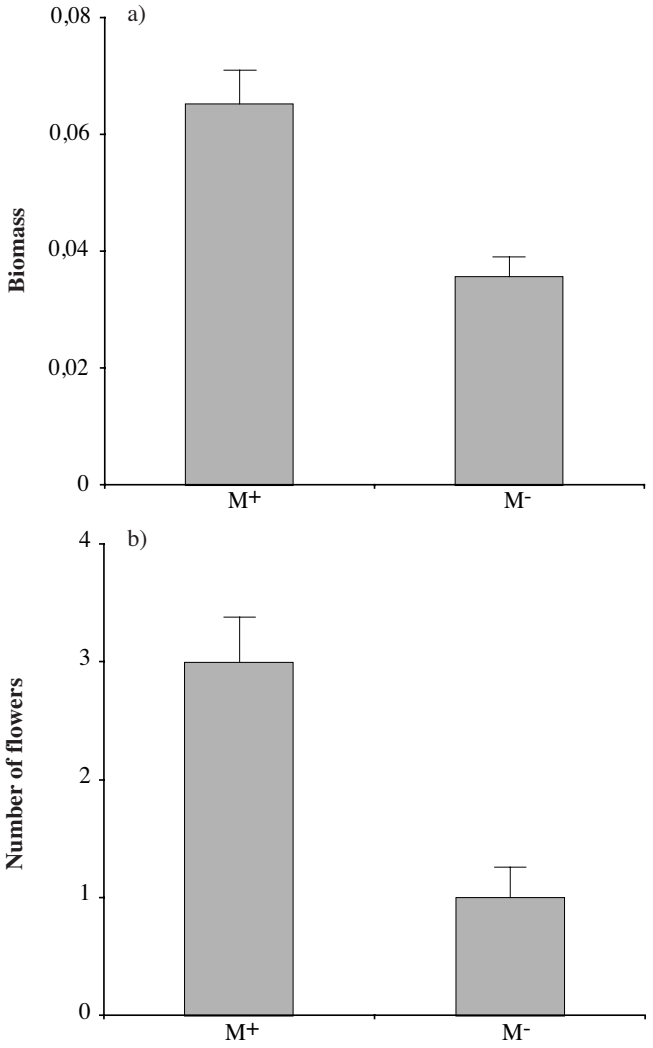

FIGURE 2. Mean (with SD) biomass (g dry mass) (a), and number of flowers (b) produced by *Melampyrum pratense* plants grown for three months in microcosms with Scots pine (*Pinus sylvestris*) hosts. The pine seedlings were either mycorrhizal (M^+) or non-mycorrhizal (M^-).

Rissanen & Markkola, 1997). We therefore believe that the better performance of the parasitic plants in the presence of EM fungi in the present study can be attributed to the nutritional benefit obtained by the host plant from its association with the fungi.

The enhanced growth and flower production of *M. pratense* in the presence of EM fungi could also be related to other benefits obtained by the host plant from its association with the fungi. In addition to water and nutrients, hemiparasitic plants are known to receive a proportion of their carbon from their hosts (Press *et al.,* 1990; Tennakoon

& Pate, 1996). The increased above-ground biomass of both hosts and hemiparasites in the presence of EM fungi indicates a higher photosynthetic capacity by these plants. It is therefore likely that the total availability of host C for the uptake by *M. pratense* was higher in mycorrhizal than in non-mycorrhizal pines. A chance for a larger uptake of carbohydrates by the hemiparasites connected to mycorrhizal pines may account for the increased growth and flower production of these plants. Overall, when compared to the non-mycorrhizal hosts, the mycorrhizal hosts appeared to have a larger pool of resources (P and C) to be exploited by the hemiparasite.

Without a functional haustorial connection to the host, hemiparasitic plants usually grow poorly and are unable to produce flowers (Cantlon, Curtis & Malcolm, 1963; Seel, Cooper & Press, 1993). However, production of flowers by plants grown without a host in experimental conditions with a sufficiently fertile soil and in absence of competitors have been reported (Yeo, 1964; Matthies, 1998). In this study, the growth medium was so nutrient poor that without access to resources from hosts, the hemiparasites were able to survive for no longer than a few weeks (as revealed by the additional treatment in which we allowed *M. pratense* plants to grow in conditions similar to those for the experimental plants, but without a host). Being strongly dependent on their host, the *M. pratense* parasites could have been expected to put more stress on the non-mycorrhizal hosts than the mycorrhizal hosts. However, the biomass of the parasitized mycorrhizal hosts did not differ from that of the parasitized non-mycorrhizal hosts. It is obvious that this similarity in pine biomass resulted from the fact that the parasitic plants grew larger when associated with mycorrhizal pines, and the intake of host resources by these plants was accordingly increased.

Although no accurate quantification of the growth of the EM fungi was made, in microscopic examination it was easy to observe that a fungal mantle had been established on the roots of mycorrhizal pines. This mantle was equally well-developed on the roots of pines infected with and without a hemiparasite, proving that the parasitic plants did not prevent the establishment of mycorrhiza. This is in contrast to the results by Gehring & Whitham (1992) and Davies & Graves (1998) who found a decline in host mycorrhizal colonization by fungi as a result of host parasitism. In these two studies, AM fungi were involved. In the present study, however, the relationship between the hemiparasite and the EM fungi appeared commensalistic. Since the biomass production and fecundity of the hemiparasite increased with enhanced growth of its host, and because pine growth was enhanced by the presence of EM fungi, it is reasonable to deduce that the hemiparasite benefits, although indirectly, from the presence of EM fungi. Based on the observation of equal development of EM fungi on parasitized and unparasitized pine roots, the relationship between *M. pratense* and EM fungi appears to be neutral.

In this study, as in others having various species compositions (Gibson & Watkinson, 1991; Matthies, 1995a,b; Marvier, 1996), infection by the hemiparasite retarded the growth of the host. Futhermore, in the present study, the host plants were found to suffer only in terms of above-ground biomass from parasitism, whereas their root biomass was unaffected. This response is not a general rule, although some other studies (Graves *et al.,* 1990; Matthies, 1997) have revealed a similar increase in relative allocation of resources to host roots due to a hemiparasitic infection. In some other hemiparasite-host associations, the pattern of biomass allocation of the host has remained unaffected by parasitism (Matthies, 1997; Puustinen & Salonen, 1999), and studies with leguminous hosts have shown that the impact of a hemiparasitic infection can be even more negative on the host root biomass than on shoot biomass (Matthies 1995a, b). Thus the effect of root hemiparasitism on host biomass allocation appears to depend on the specific host-parasite association.

In conclusion, the three-level association amongst the host plant, EM fungi, and hemiparasitic plant can be seen to represent a set of biological relationships of three different kinds. First, under nutrient-deficient conditions in the microcosms, the host plant-fungi association was clearly beneficial for the pine, indicating a mutualistic relationship between the pine and the EM fungi. Second, as the hemiparasite reduced the growth of its host, a true parasitic interaction took place between *M. pratense* and *P. sylvestris*. Finally, although we were unable to measure the actual performance of the EM fungi, the association between the hemiparasitic *M. pratense* and the EM fungi seem to fullfil the criteria set for commensalism. Our study nevertheless corraborates recent findings that the association between EM fungi and the plant can affect other taxa external to the symbiosis. Such taxa can be soil heterotrophic microbes (Ingham & Molina, 1991; Grayston, Vaughan & Jones, 1997), parasitic nematodes (Little & Maun 1996) and herbivorous insects (Gange, Brown & Sinclair, 1994), each of which have been shown to react differently to mycorrhizal and non-mycorrhizal plants. The observed increase in growth and reproduction of the hemiparasites attached to mycorrhizal hosts implies that, in the field, growth of the parasite populations would be faster at sites where the proportion of suitable hosts with well-functioning mycorrhiza is high. With such an increase in parasite density, the changes that parasitic plants can have on habitat productivity and species composition (Davies *et al.,* 1997) would be accelerated.

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