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The influence of ammonium nitrate on the root growth and ericoid mycorrhizal colonization of *Calluna vulgaris* (L.) Hull from a Danish heathland

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Abstract Conversion of European heathlands to grassland has been reported as a response to increased nutrient availability, especially of nitrogen; a direct effect upon mycorrhizal colonization has been proposed as an likely explanation.This hypothesis was tested in a random block experiment with four blocks and four replicates on a Danish inland heath, Hjelm Hede. Ammonium nitrate was applied (0, 35, 50 and 70 kg N ha^{-1} $year^{-1}$) to a stand of *Calluna vulgaris* (L.) Hull four times annually for 2 years. *Calluna* roots were sampled on four occasions in the 2nd year of the nitrogen treatment. The extent of ericoid mycorrhizal colonization was determined by direct observation of the roots using a line-intersection method. The nitrogen content of the current-year shoots of *Calluna* increased when they were treated with nitrogen. Nitrogen fertilization had no significant effects on ericoid mycorrhizal colonization of *Calluna* nor on root biomass. The seasonal variation in mycorrhizal colonization of the *Calluna* roots was highly significant. The spatial variability of mycorrhizal colonization, both in replicated plots and in the two contrasted soil horizons – the mor layer and the bleached sand – within the plots, were considerable. I conclude that heather decline under enhanced nitrogen input is unlikely to be caused by a direct impact on the ericoid mycorrhizae of *Calluna*.

Key words Ericoid mycorrhiza · Ammonium nitrate · *Calluna vulgaris* · Heathland · Roots

Introduction

Calluna vulgaris (L.) Hull is the most important component of the northwest European heathland vegetation (Gimingham 1972). Ericoid mycorrhizal colonization of *Calluna* roots undoubtly contributes to this success, as the activities of these symbiotic fungi are central in the ecology and physiology of *Calluna* (Read et al. 1989). The main fractions of nitrogen in the sandy and nutrient poor heathland soils are organic and hydrolysable (Abuarghub and Read 1988). The ericoid mycorrhizal fungi can utilize these nitrogen compounds for growth by means of extracellular proteolytic enzymes (Bajwa and Read 1986). Ericoid mycorrhizal plants thus have access to a considerable nitrogen resource which is inaccessible to non-mycorrhizal ericaceous roots and roots of other competing plants (Michelsen et al. 1996).

During the past decades there has been a widespread conversion of the northwest European heathlands into grasslands (Bobbink et al. 1990). Destabilisation of these nutrient-poor ecosystems has occurred as a result of changed land use and increased atmospheric nitrogen deposition (Aerts and Heil 1993). Increasing nitrogen availability may alter the competitive relationship between dominant plant species favouring the growth of more nitrogen-demanding species like *Deschampsia flexuosa* (L.) Trin. and *Molinia caerulea* (L.) Moench at the expense of the ericaceous dwarf shrubs (Berendse and Aerts 1984; Heil and Diemont 1983). The decline of *Calluna* may be accelerated by outbreaks of heather beetles responding to changes in the host plants induced by the increased nitrogen supply (Berdowski and Zeilinga 1987; Riis-Nielsen 1997) or by increased frost sensitivity (Lee et al. 1992). In addition, herbaceous species like *Arnica montana* L. are becoming increasingly rare in the heathland vegetation. Their decline, as well as reduced numbers of ectomycorrhizal sporocarps, are associated with increasing levels of nitrogenous air pollutants such as ammonia (Arnolds 1988; van Dam et al. 1986).

Nutrient conditions influence the development of ericoid mycorrhizae; high levels of nitrogen in particular resulted in a decline of the ericoid mycorrhizal colonization in *Rhododendron* spp. (Moore-Parkhurst and Englander 1982). Stribley and Read (1976) observed that the ericoid mycorrhizal colonization of *Vaccinium macrocarpon* Ait. was inhibited with increasing concentrations of ammoni-

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um. Heathland vegetation dynamic changes could be mediated through nitrogen effects on the ericoid mycorrhizal symbionts, as inhibition of ericoid mycorrhizal colonization may reduce the competitive ability of *Calluna*. Only a few studies of the effects of nitrogen on the symbiosis in the field have been reported. Caporn et al. (1995) and Lee et al. (1992) studied an upland British heath and failed to any find negative effects of 0–120 kg N ha^{-1} $year^{-1}$ on the amount of colonization of *Calluna*, using ergosterol and chitin analysis, respectively. However, these chemical methods do not reliably distinguish between mycorrhizal and saprotrophic fungi in the roots (e.g. Johansson 1994). In contrast, based on direct observations of the roots, Johansson (1992) found that 35 kg N ha^{-1} stimulated colonization of *Calluna* on a Danish heathland. This study was not carried out in replicated plots and was only based on one harvest after 6 months of nitrogen treatment. The result is therefor regarded as preliminary. Thus, at the moment it is not possible to draw conclusions about ericoid mycorrhizal responses to increased nitrogen avaliability across the heathlands of Europe.

In this paper I present results from a field experiment that evaluated the influence of increased nitrogen availability on the root growth and colonization of ericoid mycorrhizal fungi of *Calluna vulgaris* (L.) Hull growing on a typical Danish inland heath, Hjelm Hede. Seasonal and spatial variability were studied in replicated plots in two soil horizons within the plots by microscopic analysis of the *Calluna* roots.

Materials and methods

The experiment was carried out on Hjelm Hede (UTM 32V MH 938 604). History and soil descriptions are given by Nørnberg et al. (1994). A random block experiment with four blocks (I, I, III and IV) and four replicates, each plot 5×5 m, was established in an even-aged stand of 10-year-old *C. vulgaris*. Nitrogen was supplied four times annually as NH_4NO_3 dissolved in deionized water. The blocks received 0, 35, 50 and 70 kg N ha^{-1} $year^{-1}$, respectively.

In the 2nd year of nitrogen treatment five soil cores (diameter 5 cm) were sampled at random from the top 20 cm of the soil close to the centre of individual *Calluna* plants growing within an area of 4×4 m of each plot. Soil cores were sampled in November 1992 and in February, May and August 1993. The soil cores were kept at 4°C until handling within a week.

The surface litter was removed and the soil cores divided in two parts: The mor layer and the bleached sand. The water content was determined in subsamples after oven drying at 70°C for 48 h. In order to separate the roots from the adhering soil, each sample was preliminarily separated in water in a Sorwal Omnimixer for 20 s at low speed (step 3) to avoid damage to the roots. The samples were subsequently soaked in water for 12–20 h at 4°C until thoroughly dispersed.

The mor layer samples were washed over sieves with mesh sizes 2.0 and 0.5 mm. The coarse roots were retained in the 2.0-mm sieve. Organic material, dead roots and sandy sediment were discarded. The subsample in the 0.5-mm sieve was resuspended in 2 l water for further cleaning and left for 30 s. The suspended fine roots were collected in the 0.5-mm sieve. The precipitate was then resuspended in 2 l water and the procedure repeated twice. All subsamples of fine roots and the coarse roots were mixed. Roots from grasses and other plants were removed. *Calluna* roots were easily recognizable by the absence of root hairs and a characteristic white or brown colour depending on age.

Samples from the bleached sand were washed in the 0.5-mm sieve only, as the roots could easily be separated from the sandy sediment and recollected in the 0.5-mm sieve. Roots from the grasses were removed as well.

The cleaned root samples were cut into 0.5 cm segments and mixed. Random subsamples used to estimate ericoid mycorrhizal colonization were stained in 0.2% cotton blue dissolved in lactoglycerol followed by a short period of heating in a microwave oven. Destaining followed in lactoglycerol. The root biomass was determined after oven drying the rest of the root sample (70°C, 48 h).

The extent of ericoid mycorrhizal colonization was determined by a line-intersection method (Newman 1966). The percentage of colonization (scored as cortex cells with hyphal coils) was calculated from 200 intersections of the roots and vertical lines at 100× magnification. Each sample was counted twice. Only the fine roots (diameter less than 150 µm) were counted. Dead and damaged roots were not counted.

The nitrogen content in the roots and the soil sampled in November 1992 and in random samples of the current-year shoots of *Calluna* plants (October 1992) were determined. The dried plant material was ground, the dried soil passed through a 2.0-mm sieve and the total nitrogen content determined using an automatic analyser (LECO FP-428) after combustion. Nitrogen content in the soil, root and shoot tissue is given as percentage of the dry weight.

Data were statistically analysed using SAS System for Windows (SAS Institute 1994) for ANOVA based on a mixed model type II to examine the nitrogen, seasonal and block effects. If appropriate the data were transformed prior to the analysis. The data were tested for normality. The homogeneity of variance was tested by Bartletts test. Correlation coefficients (r) between the data are based on the Spearman rank correlation test. Descriptive statistics used SigmaPlot and SigmaStat, network version 1994, Jandel Corporation.

Results

The soil was strongly podsolized; pH of the mor layer was 3.9 and of the bleached sand 4.4. The mor layer depth, calculated as an average of all samples taken during the field experiment, was 3.5 cm. The nitrogen contents of the mor layer and the bleached sand were 1.0% and 0.1%, respectively. The nitrogen concentrations of the mor layer soil and the bleached sand were not significantly influenced by the nitrogen treatments. The nitrogen content of the soil had no significant influence on root colonization (Table 1).

The biomass of *Calluna* roots (g dry weight m^{-2}) in the upper horizons, the mor layer and 10 cm of the bleached sand, exhibits a highly significant seasonal pattern (P=0.001). In the untreated control plots the root biomass decreased from 1263 g dry weight m^{-2} in November to 856 g dry weight m^{-2} in February. During the growing season the root biomass increased to 1126 g dry weight m^{-2} in May and 1445 g dry weight m^{-2} in August. The increase in total root biomass was largely due to the doubling of the root biomass in the bleached sand from May 1993 to August 1993 (Fig. 1). The root biomass in the mor layer and in the bleached sand, respectively, were not influenced by the root colonization level (Table 1). Roots of other plants, especially grasses, contributed no significant root biomass. The root density (g dry weight cm^{-3}) in the mor layer was 5 times the density in the bleached sand in November. In February

Table 1 Correlation coefficients (r) between the data based on the Spearman rank correlation test

Data	Data		Correlation coefficient (r)	Probability (P)
Colonization (%) of roots in the mor layer	Colonization (%) of roots in the bleached sand	Nov	0.3452	0.0018
		Feb	0.2577	0.0219
		May	0.2425	0.0302
		Aug	0.5745	0.0001
Root tissue nitrogen (%) November	Colonization (%) of roots	Mor	–0.2401	0.0331
		Bleached sand	–0.1392	0.2338
Soil nitrogen (%), November	Colonization (%) of roots	Mor	–0.0165	0.8361
		Bleached sand	0.0257	0.8219
Water content	Colonization (%) of roots	Mor	–0.2386	0.0001
		Bleached sand	–0.3108	0.0001
Colonization (%) of roots	Root biomass (g dry weight m^{-2})	Mor	–0.1465	0.0087
		Bleached sand	0.1122	0.0462
Colonization (%) of roots	Root density (g dry weight m^{-2})	Mor	–0.1785	0.0013
		Bleached sand	0.1087	0.0535

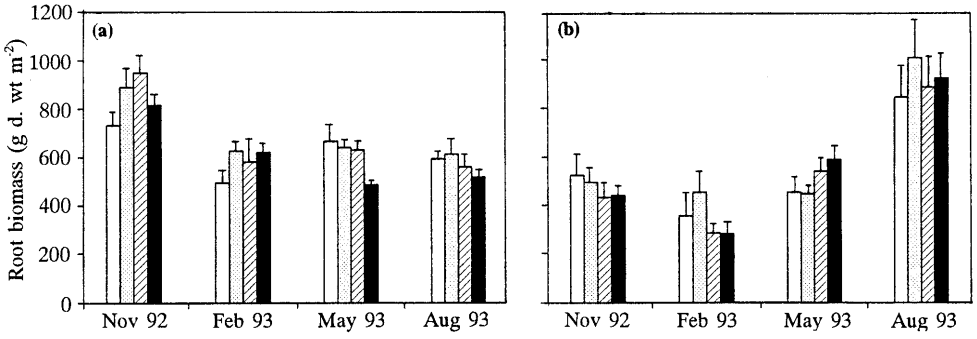

Fig. 1 Root biomass (g dry weight m^{-2}) of *Calluna* in **a** the mor layer and **b** the bleached sand to a depth of 10 cm treated with ammonium nitrate (□ control, ▒ 35 kg N ha^{-1} $year^{-1}$, ▨ 50 kg N ha^{-1} $year^{-1}$ and ■ 70 kg N ha^{-1} $year^{-1}$). Each *column* represents the average dry weight of roots in 20 soil samples from each of four replicate blocks at four sampling occasions, Hjelm Hede. *Vertical bars* represent 1 SEM

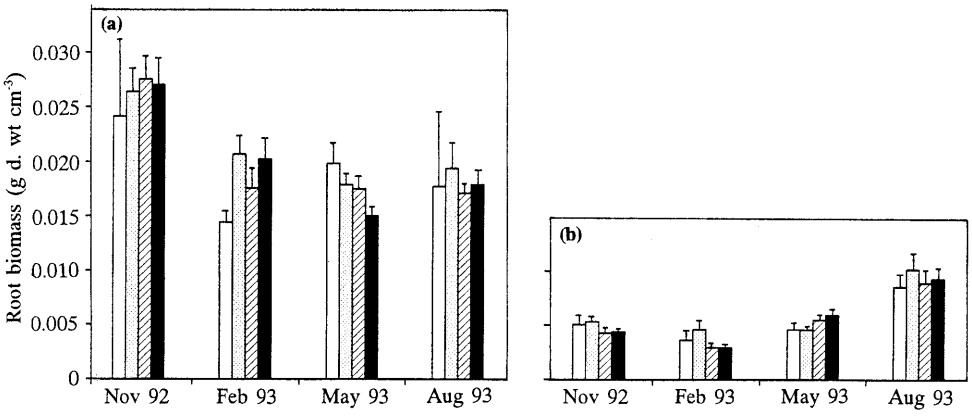

Fig. 2 Density (g dry weight cm^{-3} soil) of *Calluna* roots in **a** the mor layer and **b** the bleached sand treated with ammonium nitrate (□ control, ▒ 35 kg N ha^{-1} $year^{-1}$, ▨ 50 kg N ha^{-1} $year^{-1}$ and ■ 70 kg N ha^{-1} $year^{-1}$). Each *column* represents the mean of 20 samples from each of four replicate blocks at four sampling occasions, Hjelm Hede. *Vertical bars* represent 1 SEM

and May fourfold and in August twofold (Fig. 2). The root density is not correlated with the root colonization (Table 1). The root biomass and density were not significantly affected by the nitrogen treatments (Figs. 1, 2).

The nitrogen content of the roots from the mor layer was 1.35% and from the bleached sand 0.82%. The content in the roots from the mor layer was approximately 50% higher than in the roots from the bleached sand (Fig. 3). Nitrogen amendments did not significantly influence the root tissue nitrogen content of *Calluna* roots in either horizon as measured 1 year after initiation of the application. Root colonization in the mor layer was weakly but significantly negatively correlated with the nitrogen content in the root tissue (Table 1). In the bleached sand no significant correlation was found (Table 1).

The content of nitrogen in the current-years shoots of the *Calluna* plants on the untreated control plots was

1.34%. Application of 35 kg N ha^{-1} over 2 years increased the nitrogen content significantly to 1.49%, which is an increment of 11.7% compared to the control. The nitrogen content in shoots from the plants treated with 50 kg N ha^{-1} and 70 kg N ha^{-1} increased to 1.56% and 1.61%, increments of 17.2% and 20.9%, respectively.

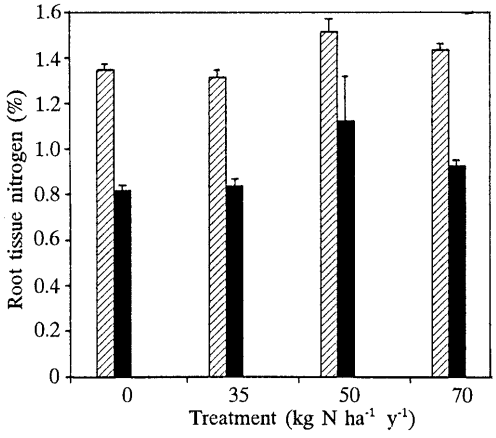

Fig. 3 Relationship between the average *Calluna* root nitrogen content (percentage of dry weight) in the mor layer (▨) and in the bleached sand (■) and applied nitrogen. Each *column* represents 20 samples. *Vertical bars* represent 1 SEM

Significant seasonal variability was seen in the colonization of *Calluna* roots with ericoid mycorrhiza in both the mor layer (P=0.001) and the bleached sand (P=0.0012) (Fig. 4). Root colonization in the mor layer and the bleached sand, respectively, was 28% on average in November. During the growth season the mean values increased to around 40%. Within the experimental period a minimum of 10% was measured in the mor layer in November, and a maximum of 64% measured in the bleached sand in May.

Average root colonization values in the mor layer and the bleached sand given as average values were not significantly different (Fig. 4) because there is considerable spatial variability in *Calluna* root colonization. Colonization differed in the roots from the mor layer and the bleached sand within the same soil core. The correlation between colonization in the mor layer and the bleached sand was weak despite a significant positive correlation in August (Table 1).

The nitrogen amendments at all application levels did not significantly influence the ericoid mycorrhizal colonization, either in the roots of the mor layer or in the bleached sand (Fig. 4). This pattern was obtained at all harvests. Significant interaction effects between harvest, block and treatment (P=0.0001) indicate that the root colonization is affected by unidentified local differences in different ways in the four blocks as demonstrated by the results from November (Fig. 5).

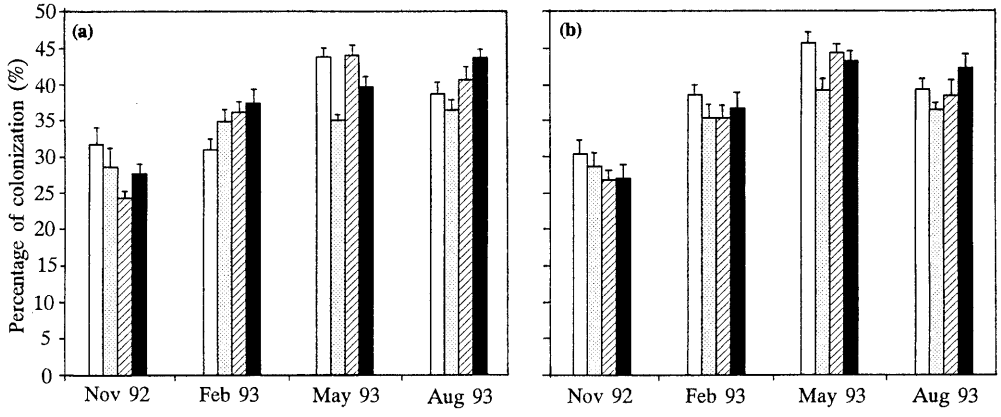

Fig. 4 Ericoid mycorrhizal colonization (measured as percentage root length colonized) of *Calluna* roots at four sampling seasons from **a** the mor layer and **b** the bleached sand treated with ammonium nitrate (□ control, ▩ 35 kg N ha^{-1} $year^{-1}$, ▨ 50 kg N ha^{-1} $year^{-1}$ and ■ 70 kg N ha^{-1} $year^{-1}$). Each *column* represents the mean of 20 samples from each of four replicate blocks. *Vertical bars* represent 1 SEM

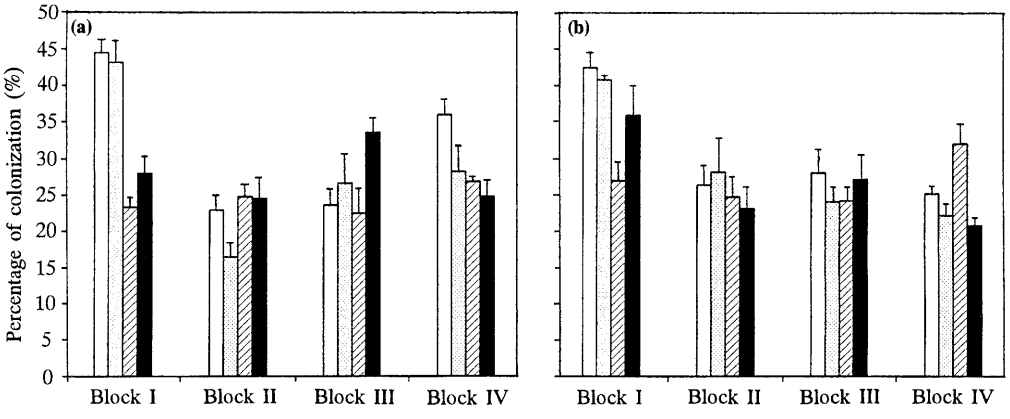

Fig. 5 Ericoid mycorrhizal colonization (measured as percentage root length colonized) of *Calluna* roots from four blocks from **a** the mor layer and **b** the bleached sand treated with ammonium nitrate (□ control, ▩ 35 kg N ha^{-1} $year^{-1}$, ▨ 50 kg N ha^{-1} $year^{-1}$ and ■ 70 kg N ha^{-1} $year^{-1}$). Each *column* represents the mean of 5 samples collected in November, Hjelm Hede. *Vertical bars* represent 1 SEM

The roots with low mycorrhizal colonization were confined to soil cores with high water content; although the correlations are weak they are highly significant (Table 1).

Discussion

The influence of nutrient conditions on the development and activity of ericoid mycorrhizae has so far largely been investigated under laboratory conditions. In the present experiment, conducted under field conditions, the ericoid mycorrhizal colonization of *Calluna* had not changed after 2 years of fertilization with ammonium nitrate; this result is contrary to expectations based on findings in the laboratory (Moore-Parkhurst and Englander 1982; Stribley and Read 1976). In the field different regions of heathlands may be affected by past and present land use, management practices and patterns of atmospheric nitrogen deposition. Based on the experiment on Hjelm Hede, I conclude that the effect of nitrogen on the mycorrhizal colonization of *Calluna* is unlikely to provide an explanation of heather decline under enhanced nitrogen input. These findings are supported by a field experiment where Lee et al. (1992) also reported a lack of response in ericoid mycorrhizal colonization, measured by a chitin assay, of *Calluna* fertilized with 40, 80, 120 and 200 kg N ha^{-1} $year^{-1}$. The chitin assay unfortunately does not distinguish between mycorrhizal and saprotrophic fungi in the roots (Johansson 1994), which makes a direct comparison difficult. Recent field experiments indicate that phosphorus limits plant growth on Hjelm Hede (Riis-Nielsen 1997). Thus, if important nutrient limitations beside nitrogen prevail, the amount of ericoid mycorrhizal colonization of *Calluna* is likely to be a poor indicator of excess nitrogen in these heathlands.

Separated peaks of colonization by several ericoid mycorrhizal fungi (Johansson 1995) could mask the uniform levels of colonization reported from this experiment. Furthermore, the estimates of percentage of colonization do not reflect the mycorrhizal fungal biomass over a range of colonization densities. Caporn et al. (1995) estimated the ericoid mycorrhizal biomass in *Calluna* roots by the concentration of ergosterol. They could not, apart from a single measurement, demonstrate significant changes in the mycorrhizal biomass due to repeated nitrogen fertilization (0, 40, 80 and 120 kg N ha^{-1} $year^{-1}$ over 3 years). Furthermore various responses to increases in nutrient availability could be expected due to different sensitivities between host plant and fungus combinations. Haselwandter (1987) reported variations in colonization intensity within and between ericaceous plant species in climatically and nutritionally stressed alpine plant communities. Heijne et al. (1992, 1994) reported no decrease in the arbuscular mycorrhizal colonization of several herbaceous heathland species from nutrient-poor soils under natural conditions, in contrast to increased colonization in *Antennaria dioca* (L.) Gaertner with increasing nitrogen availability.

The spatial variation in ericoid mycorrhizal colonization in my experiment was related more to seasonality and the heterogeneity of the plots than to the nitrogen treatments. Ericoid mycorrhizal colonization may be influenced by unidentified local differences in soil or microclimate as indicated by the significant interaction effects between blocks, treatments and harvests. Belowground patchiness, as shown for root biomass of *Calluna*, can be considerable even when the aboveground vegetation appears homogeneous (Tinhout and Werger 1988). Teuben and de Jong (1982) measured colonization of *Calluna* fine roots and found on average 25% (SD=7) of the root length colonized in January, with minimum values of 9% and maximum values of 51%. Colonization of *Calluna* roots by mycorrhizal fungi with different sensitivities to nitrogen could contribute to the observed pattern, as seen for ectomycorrhizal fungi (Arnebrandt 1994). Haselwandter (1979) suggested regulatory mechanisms to explain mycorrhizal colonization intensity in ericaceous plants growing in the Austrian Alps. Ericoid dwarf shrubs of heathlands under nutrient-poor conditions are heavily dependent on ericoid mycorrhizal colonization for absorption of nutrients from the soil (Read and Bajwa 1985; Read et al. 1989). If the resource costs to the host plants of carrying the mycorrhizal symbionts are small compared to the cost of regulatory mechanisms to exclude the fungi under less beneficial conditions then no response in colonization level would be measured.

Expected vertical differences in the mycorrhizal colonization (Caporn et al. 1995; Johansson 1992, 1994) were not seen in this experiment. Instead, roots within the same soil core differed in colonization, irrespective of depth. Comparative studies of ericoid mycorrhizal fungi from localities differing in soil pH and prevailing nutrient compounds show that variability in performance may be related to the differences in the natural environment and there may even be ecologically distinct fungi (Leake and Read 1990; Mitchell and Read 1985). On the other hand, differences in colonization would imply selective regulation in different parts of the root system within individual host plants. Such mechanisms have not yet been confirmed for ericoid mycorrhizae.

In agreement with Reed (1989), who found that the mycorrhizal colonization of an Australian ericoid host plant was not correlated with the nitrogen status of the plants, the results from the present experiment did not confirm any significant relations between ericoid mycorrhizal colonization and root tissue nitrogen alone; a result seen in roots of both the mor layer and the bleached sand irrespective of differences in root nutrient status. The response of ericoid mycorrhizal colonization to different nutrient regimes may instead be influenced by the ratio of nutrients in the host plant tissue, as suggested for arbuscular mycorrhizae (Hepper 1983). The levels of nutrients in the host tissue thus have an important influence on the level of mycorrhizal colonization rather than a direct effect on the soil phase of the fungi (Ratnayake et al. 1978). Phosphorus measurements of *Calluna* tissue

should be included in order to confirm these types of mechanisms for ericoid mycorrhiza under field conditions. Thus, the ratio of nutrients could play a crucial role in the observed spatial variability where the differences in ericoid root colonization between the sets of plots were considerable.

The negative correlation between soil water content and mycorrhizal colonization seen in my experiment is very important as this could be a major determinant of the block, profile and seasonal effects on root activity and mycorrhizal colonization.

The seasonal pattern in colonization is important in assessing the dynamics of nutrient cycling in the heathland system. Studies of root growth dynamics and environmental conditions are needed to understand the seasonal dynamics of root colonization. The decrease in root biomass coincides with low winter temperatures. The increase in colonization during the winter months can be explained by the decrease in root growth. The colonization increase during the growing season, despite root biomass increase, suggests a fungal response to environmental conditions. Water stress and temperatures have been reported to influence the levels of mycorrhizal colonization (Haselwandter 1987; Read et al. 1976). Reed (1989), in a study of the seasonal mycorrhizal colonization of *Leucopogon juniperus*, found a low proportion of uninfected root tips in the cold months. It is not possible from this experiment to say whether the observed seasonal differences are consistent from year to year.

Additional nitrogen could change the rate of root growth, thereby influencing the proportion of the roots colonized. The root biomass in this experiment was not affected by the nitrogen amendments, which, together with the lack of response to nitrogen on colonization, indicates that application of nitrogen did not have a direct effect on the spread of the ericoid mycorrhizal fungi in the root system.

The main pathway for applied nitrogen, as indicated by the increased tissue content, could be by the direct uptake by the *Calluna* shoots bypassing the soil-root pathway. Bobbink et al. (1990) reported that a significant proportion of deposited ammonia and ammonium was assimilated by canopy exchange processes in *Calluna* vegetation; the increases in tissue nitrogen concentrations may have profound effects on herbivorous insects which probably play an important role in changing the heathland vegetation dynamic towards gramineous dominance (Aerts and Heil 1993). The consequences of increasing nitrogen status will apparently be widespread as alterations of vegetation can change soil development and also the soil microflora in a sandy sediment within decades (Nielsen et al. 1987a, 1987b; Nørnberg et al. 1993). The data presented suggest that nitrogen input from e.g. atmospheric deposition may influence dwarf shrub communities even if there are no instant detrimental effects on the mycorrhizal symbionts. The microflora present in the soil beside the mycorrhizal fungi could also benefit from uptake of additional nitrogen (Michelsen et al. 1996). The detection of these changes in the heathland vegetation dynamic will require sustained experimentation over many growing seasons.

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