Temperature responses of the arbuscular mycorrhizal symbiosis

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Abstract

The AM fungal communities of the Faroes were characterised. Open Top Chambers were used to examine the impact of passive warming, and from the same site samples were taken for a controlled warming experiment, looking at differences in root colonisation and fungal communities at a temperature close to the mean summer temperature in the Faroes, and another temperature within the upper limit of what is measured in the Faroes.

The main finding from this project is that arbuscular mycorrhizal (AM) fungi from the Faroe Islands respond to temperature, and do so in the same way as other field studies have shown: less root colonisation at elevated temperatures. When field samples were exposed to controlled temperatures in a laboratory, the results were not so conclusive, as there were considerable differences in colonisation responses from the different sites. However, pre-warming followed by low temperatures reduced colonisation, indicating that, after 2-week exposure to high temperatures, the fungi were not able to respond once low temperatures were restored.

There was evidence for a temperature-related change in the composition of the fungal assemblage in roots. Some of the fungal types were only found at low altitude. Moreover, species richness of the fungi was a function of the temperature range.

The impact of temperature on symbiotic function was examined in controlled conditions with a cultured fungal isolate. The main effect was that temperature increased fungal phosphate concentration, while arbuscule frequency was dependent on both substrate phosphate and temperature. Further there was a relationship between fungal sugars and arbuscular colonisation which implies that with more arbuscules the plant should receive more phosphate, and the fungus more sugars.

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Dedication

This work is dedicated to the memory of Sólja Ingunnardóttir Mortensen (28/5-1990 – 1/1-2005). Her name Sólja means in Faroese either marsh marygold (*Caltha palustris*) or a buttercup (*Ranunculus* sp.). One of the plants used in this study was buttercup, is highly mycorrhizal, and needs special attention to survive without a mycorrhizal network. The meaning of being mycorrhizal has got a new meaning for me after Sólja left us. People are not physically connected to each other, but we do indeed have tight relationships. It hurts if they are severed. People might not be obligate symbionts, but our relationships might resemble what we call faculative symbionts.

Acknowledgments

This project was supervised by Prof. Alastair Fitter and funded by the Faroese Research Council. The Faroese Museum of Natural History has provided office, laboratory equipment, and other necessary facilities while I have been in the Faroes during field sampling and writing the thesis.

A special thank to Alastair Fitter for his incredible patience, guidance and support. Always able to guide me into the right direction after I had been far out chasing red herrings. You never showed any sign of annoyance when I made the same mistakes over and over again.

I also acknowledge the help, support and friendship that I have experienced in the York laboratories. A special thank to Karyn Ridgway who guided me through the mysteries of PCR, gels and T-RFLP.

Anna Maria Fosaa at the Faroese Museum of Natural History, a thank to you for encouraging me to take this project on. Also I thank Michael Reveal for the proof-reading. And finally I wish to thank Angela Hodge and Robin Sen for a critical review of my thesis, with valuable comments and input.

Author's declaration

The research presented in this thesis is entirely my own work and currently unpublished.

Although the thesis has been written as a whole, the chapters are designed to be understood individually, and therefore some repetition occurs between the various introductory, methods and discussion sections.

Chapter 1

General introduction

Arbuscular mycorrhiza

It is the rule rather than the exception for a plant to be mycorrhizal. Some of the different types of mycorrhiza have evolved in specialised groups of plants. Thus heathland biomes are characterised by plants such as *Calluna vulgaris, Erica* spp. and *Vaccinium* spp. with hair roots colonised by ericoid mycorrhizal fungi, while boreal and cool-temperate forests are dominated by trees colonised by ectomycorrhizal fungi (Smith and Read, 1997, pp. 413 and 422). The most common form, however, is arbuscular mycorrhiza. Arbuscular mycorrhizal (AM) fungi (Phylum Glomeromycota) are ubiquitous symbionts associating with around two-thirds of plant species (Fitter and Moyersoen, 1996) and in many cases are critical to a plant's survival (Smith and Read, 1997, p. 440).

It is known that the symbiosis can provide a range of benefits to plants, including protection from pathogens, uptake of micronutrients, and possibly improved drought resistance (Newsham *et al.*, 1995b). Also, a relationship between plant diversity and fungal diversity has been suggested. For example, Helgason *et al.* (1998) found a much higher AM fungal diversity in samples from woodland compared with samples from arable sites. In the same vein, van der Heijden *et al.* (1998) found higher plant diversity when a higher number of AM fungal species was present.

Traditionally, the fungi are identified by morphology, especially of the spores, though structures found in the internal phase of the colonisation are sometimes also used as part of the identification. Spore identification typically involves culturing from single spores. This requirement means that spores from the field that fail to germinate or colonise a bait plant cannot be properly identified. Thus, morphology-based identification might be of limited use in ecological settings, because spore production is highly dependent on physiological parameters (Redecker *et al.,* 2000). For example, Merryweather and Fitter (1998b) did not find a significant relationship between AM fungal populations in roots and populations of dominant glomalean spores recovered from associated soil and their conclusion was that spore assemblage does not reflect the extent of colonisation. Those fungi that have been cultured show very limited specificity, at least in culture, and many fungal taxa will colonise a wide range of host plants indiscriminately (Smith and Read, 1997, p. 31), as might be expected from the ratio of plant species (~250,000) to fungal species involved in the symbiosis.

One striking feature of the association is the low diversity of the fungal partner. Approximately 150 species of AM fungi have been described (Walker and Trappe, 1993), although this is certainly an underestimate of the true

diversity of the group as revealed by both traditional methods (Bever *et al.*, 2001) and molecular techniques (e.g. Helgason *et al.,* 1999; Husband *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002, 2003; Öpik *et al.*, 2003). Paradoxically, evidence from spore collections, trap culturing, and the use of molecular techniques to identify fungi in roots suggests that the local diversity is high relative to the described low global diversity. Natural communities typically support 10-20 AM fungal taxa, and sometimes more, suggesting that these co-existing taxa may display niche differentiation, either in relation to the hosts they colonise (implying specificity) or in relation to environmental factors.

Historic overview

Symbiosis was defined by De Bary in 1879 (Meyer, 1966) as "phenomena of a living together of dissimilar organisms". This definition could be considered overly wide, allowing species living "side-by-side" to be deemed symbiotic. Therefore, De Bary also distinguished various forms of living together – from mutualism to parasitism – making it clear that these concepts are linked together by all types of transitions (Meyer, 1966). Knowledge of mycorrhiza dates from the year 1885, when Frank first applied the term to roots of trees showing a regular and characteristic infection by fungal mycelium (Rayner, 1926a). It was also Frank that in 1887 differentiated two categories of mycorrhiza, ecto- and endotrophic (Meyer, 1966). Originally, Frank used the term "endotrophic mycorrhiza" to describe the two types of mycorrhiza characteristic of Orchidaceae and Ericaceae (Rayner, 1926b). In 1889, Schlicht, working in Frank's laboratory, published an account of his research on endotrophic mycorrhiza in herbaceous plants. He described in detail that found in *Paris quadrifolia, Ranunculus acris, Holcus lanatus* and *Leontodon autumnali*s, noting the endotrophic mycorrhiza's restriction to the actively absorbing roots and the position of the infected tissue between the absorbing and conducting regions (Rayner, 1926b).

Schlicht's type of mycorrhiza was that now known as arbuscular mycorrhiza. The early mycorrhizal researchers were in the main looking for plant pathogens, and their methods where those plant pathologists were using. However, they found out that most herbaceous species had mycorrhiza, but that the root-fungal association was absent from certain plant groups. Some of these very early mycorrhizal workers favoured the view of a mutualistic symbiosis. In 1900, Stahl published his theory of nutrition, suggesting that the incidence of fungus infection was directly related to the difficulty of procuring mineral salts, and hence, to the

efficiency of the mechanism for their absorption (Rayner, 1926c).

The early mycorrhizal researchers also dealt with the formation of mycorrhiza by arctic and alpine plants, and they found that a large number of species from high latitudes possess well-developed mycorrhiza (Rayner, 1926c).

This very brief overview of the early mycorrhizal research has mostly concentrated on the account by M.C. Rayner of the progress from 1885-1900. It is striking how similar the problems they dealt with over a century ago are to present days questions. In a note to a reviewer of her book, *Mycorrhiza,* M.C. Rayner clarified of what she meant by mycorrhiza. It is a very concise description of ecological issues that still require attention. One of these is temperature:

> "In general, I regard mycorrhiza as an ecological phenomenon resulting directly from the inevitable competition in soil between roots of vascular plants and the mycelium of numerous soil fungi. […] Regarding the incidence of fungus attack simply as one of a number of biologic soil factors, it is clear that, like others, it is subject to great variation in relation to differences in external conditions, viz. temperature, moisture, and the soil content of humus and nutritive salts, all these operating directly and also perhaps indirectly by affecting the capacity for attack on the one hand and resistance on the other."
>
> M.C. Rayner, 1928

During the following years the main focus was more on how to identify these fungi, and decide whether they were beneficial and if so, how. Only recently, since the 1960s, has ecological research on mycorrhizas been undertaken, at approximately the same time as the relevance of symbioses for agriculture was accepted (Koide and Mosse, 2004). The acknowledged benefits for the plants have been the main focus, and therefore mycorrhizal research to a great extent has measured the increase in plant biomass, plant phosphate concentration, etc. compared with non-mycorrhizal controls, and used these parameters as estimates for the mycorrhizal symbiosis. Methods for AM fungal identification and quantification have been limited, as they are obligate symbionts and thus cannot be separated from the plant. Therefore, the fungi usually are quantified by measuring the length of the plant root colonised or by measuring the external mycelium, and until recently the identification has relied on spore identification.

Arbuscular mycorrhiza and temperature

Temperature is a key environmental variable both because it exhibits wide variation in the natural environment and because of its central role in climate change. Soils show particular well-marked differentiation in temperature conditions: surface soils experience wide fluctuations whereas deeper soil layers remain relatively constant, providing a strong spatial heterogeneity. Temporal variation is represented most importantly by the seasonal pattern. There is therefore a clear environmental pattern to which AM fungi might show niche differentiation.

Over the years a range of studies has dealt with arbuscular mycorrhiza and temperature (Table 1.1). Most of them found an increase in root length colonisation with increased temperature, but in some the colonisation decreased with increased temperature. This applies to experiments done in the field (Rabatin, 1979; Black and Tinker, 1979), as well as to Monz *et al.* (1994) who brought large intact soil cores into a growth-chamber, where the temperature followed the growing season temperature. The named AM fungal types, apart from *Glomus tenue* (the name given to 'fine endophyte' in roots), were all found to have a positive response to increased temperature. The fact that they are named indicates that it is possible to culture them under laboratory conditions. Thus, the studies presented in Table 1.1 do not conclusively suggest any direction of fungal response to temperature, but imply that there might be a distinction between the response of culturable fungi and other native fungi.

Most mycorrhizal work has been done with temperate zone plant and fungal species, for example all the studies shown in Table 1.1., though a few studies have dealt with mycorrhiza in the Arctic, and from these studies some information is available on mycorrhizas in cold environments.

In arctic environments the soil temperature during growth season can be so low that it prevents or seriously limits physiological activities of fungal hyphae (Kytöviita, 2005). In a study from Svalbard, Väre *et al.* (1992) found that AM fungal colonisation was restricted compared with ecto- and ericoid mycorrhizal fungi. They found no AM root colonisation, only one glomalean spore, but both ecto- and ericoid mycorrhizal plant roots. AM fungal performance may be more constrained by low growing season temperatures than ectomycorrhizal; while the ectomycorrhizal fungal range may be limited by host constraints, the AM host range may be limited due to inability of the AM fungi to grow in cold arctic soils (Kytöviita, 2005).

Another indication that AM fungi might be more restricted by temperature than ectomycorrhizal comes from a study that examined the soil fungal

Author	Year	Plant species	Fungal species	Temperature measured	Fungal responses to increased temperature	Mycorrhiza and temperature	Experiment type
Hayman	1974	*Allium cepa* L. var. James Keeping	Non-specified inoculum	14°C and 23°C	Increase	Lower arbuscular colonisation at low temperature.	Growth chamber
Rabatin	1979	Native grasses	*Glomus tenue*	Only seasonal variation	Decrease	Highest colonisation in spring, lower colonisation in summer	Field
Black and Tinker	1979	*Hordeum distichon* L.	Native	No measurements presented; however, a span of 7.7-18.4 is considered	Decrease		Field
Schenck and Smith	1982	*Glycine max (L.) Merr.*	*Gigaspora gregaria, Gigaspora pellucida, Glomus mosseae, Acaulospora laevis, Glomus clarum, Glomus claroideum*	18°C, 24°C, 30°C, 36°C	Increase	Highest colonisation at 30°C, then decreasing colonisation and number of spores.	Glasshouse
Borges and Caney	1989	*Fraxinus pennsylvanica* Marsh.	*Glomus macrocarpum, Glomus fasciculatum*	15°C, 25°C, 35°C	Increase	Highest %RLC at 25°C, followed by 15°C.	Glasshouse
Monz *et al.*	1994	*Pascopyrum smithii*	Native	Field average (13-31°C), 4°C elevated	Decrease	Long-term experiment using field material	Growth chamber
Rillig *et al.*	2002b	Annual non-native grasses	Native	Canopy warming = +1.5-2°C, soil surface +1°C	Increase	The results indicate that ecosystem warming may have stimulated carbon allocation to AMF	Warming field site
Gavito *et al.*	2003	*Pisum sativum*	*Glomus caledonium* and inoculum from field soil	10°C or 15°C	Increase	Extraradical mycelium (even northern isolates) is more sensitive to low temperatures than the intraradical mycelium.	Growth chamber

Table 1.1. A selection of studies examining the AM response to temperature.

community assembly in a newly deglaciated area. A small dormant spore bank with several other types of biotrophic fungal species was found, of which some were ectomycorrhizal, but no AM fungi were found (Jumpponen, 2003). At the same site, but further away from the retreating glacier, AM colonisation was found in an area that was exposed 35 years ago; the data indicated that the AM spores seemed to arrive in stochastic events (Cázares *et al.,* 2005).

Soil microbial biomass consists of bacteria, saprotrophic fungi and mycorrhizal fungi, but the ratio between these microorganisms is not well studied. In boreal forests, ectomycorrhizal fungi have been shown to constitute one-third of the microbial biomass in soil (Högberg and Högberg, 2002). The AM fungal contribution to the microbial biomass has been estimated to be between 5 and 50% of the total soil microbial biomass (Olsson *et al.*, 1999).

The specific respiration rate of mycorrhizal hyphae is greater than that of roots, but the non-mycorrhizal fraction of the microbial biomass has an even higher respiration rate than the mycorrhizal (Langley *et al.*, 2005). Kirschbaum (1995) found the Q10 of microbial respiration to range from 1 to 12.9, with highest values at low temperature. If the mycorrhizal fraction can be as high as 50% of the total microbial biomass, and the temperature response of this fraction is similar to the microbial response as a whole, then profound impact on the soil microbial environment might be expected especially in cold regions. Kirschbaum (1995) also found that a 1°C increase in temperature could lead to a loss of three times more soil organic carbon in cold regions compared with warm regions. Temperature is, however, not the only climatic factor predicted to change; precipitation is also predicted, in general, to increase due to increased evaporation from the oceans (Houghton, 2004, p. 125), thus increasing soil moisture. Both temperature and moisture affect soil respiration; increased temperature would likely increase soil respiration, whereas drought would decrease it (Kirschbaum, 1995), and increased soil moisture would likely increase it (Davidson *et al.*, 1998). The impact of soil moisture on AM fungi was assessed in a field-study by Staddon *et al.* (2003b) who found a decline in %RLC with water addition, but did not account for the change in plant composition due to the treatments.

Elevated soil temperatures facilitate the uptake of nutrients by the plant in several ways: by increasing the length of new roots, by physiologically improving nutrient uptake by the roots and by accelerating nutrient mineralisation in the soil (Domisch *et al.*, 2002). Hence indirect temperature responses may be mediated via changes in plant photosynthesis rate, plant and soil nutrient concentrations, and soil moisture, in particular because of increased carbon allocation to the roots

when available nutrients are few or soil moisture content is low (Pendall *et al.*, 2004).

In a study testing whether plants growing in the Arctic compete with soil microorganisms for nutrients, Jonasson *et al.* (1996) found that short-term availability of phosphorus to the plants depends more on changes in population sizes of soil microorganisms than on changes of mineralisation rates, with a flush of phosphorus to the inorganic pool when the microbial population declined, suggesting that the microbial organisms are a regulating factor for the nutrient supply for the plants in the Arctic.

The study by Väre *et al.* (1992) suggested that ectomycorrhizal and ericoid fungal species were less sensitive to low temperatures than AM fungi. This could be explained by the findings of Domisch *et al.* (2002) which suggested that the soil temperature had affected the formation of mycorrhizas relatively more than their function. The relative ectomycorrhizal fungal diversity did not respond to a 4°C increase in temperature when the entire morphotype community was considered, although some morphotypes were affected seasonally by temperature (Rygiewicz *et al.*, 2000).

Although ericoid mycorrhizal fungi might be less sensitive to temperature, a study from a subarctic region (Abisco, Sweden) found that the ericoid mycorrhizal colonization responded to a 5°C above ambient treatment with a seasonally greater colonisation, but this was interpreted as a treatment effect on ecosystem photosynthesis and carbon allocation (Olsrud *et al.*, 2004).

Climate change

The global temperature has, over the 20th century, increased by about 0.6°C and this temperature increase is expected to continue due to anthropogenic climate change, mostly caused by increased carbon dioxide in the atmosphere from burning of oil, coal, etc. Some have predicted an increase from 1.4°C to 5.8°C over the period 1990 to 2100 (Houghton *et al.,* 2001). Most focus has been on climate warming, which is what happens on a global scale. However, studies of ocean currents indicate that local cooling might occur, and that parts of northern Europe might be colder (Hansen *et al.*, 2004). The temperatures in parts of the northern oceans are warmer than average for the same latitude, especially in Iceland, Norway and Scotland (Fig. 1.1). This increase in temperature is due to the so-called Gulf Stream or more specifically the North Atlantic Current, which is a driving mechanism for ocean currents.

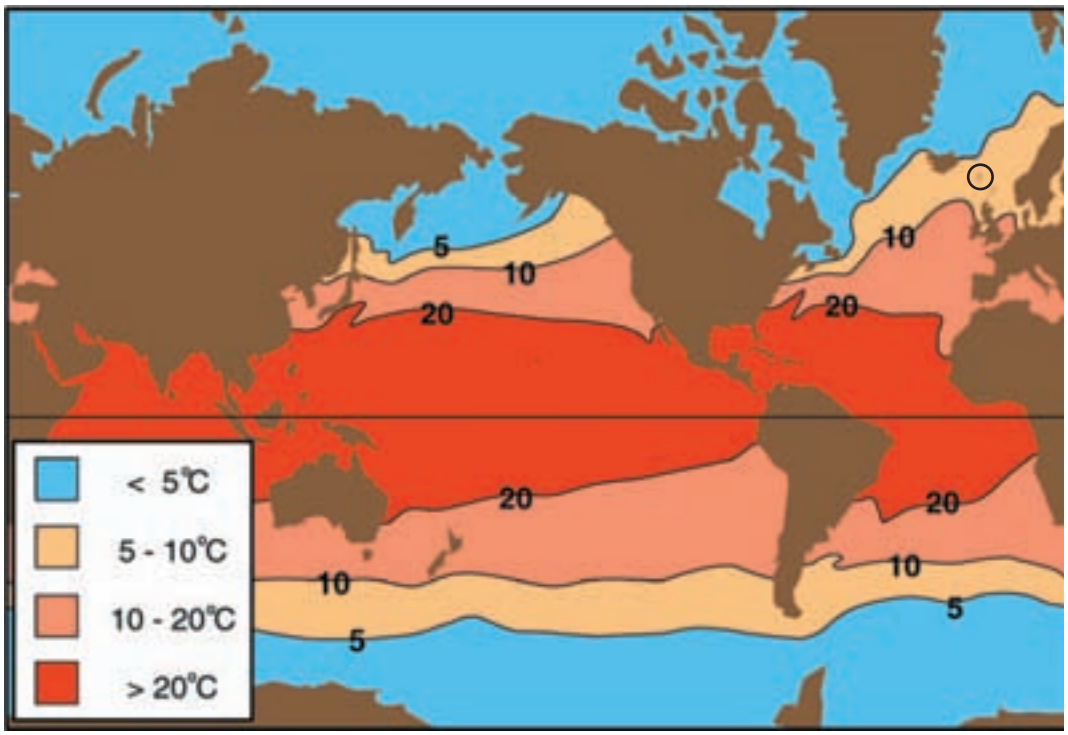


Fig. 1.1. Mean temperature on the ocean surface. Circle shows the Faroe Islands. Courtesy of Bogi Hansen, Faroese Fisheries Laboratory.

The Faroe Islands have a unique position in the midst of the North Atlantic Current. The islands are located on the Greenland-Scotland ridge that separates the Nordic Seas from the Atlantic Ocean (Fig. 1.2). Just south of the islands is one of the main overflows of the North Atlantic Deep Water. The driving force of the thermohaline circulation is evaporation that increases the density of surface waters sufficiently to cause them to sink. This sinking occurs at the poles. Because water with different densities do not easily mix, as cold polar water sinks, the cold dense water escapes along the bottom through the deepest points in the Greenland-Scotland ridge, of which one is the Faroe Bank Channel located just south of the Faroe Islands. The surface water that sank has to be replaced, and therefore warm Atlantic water is drawn north to the Nordic Seas, warming the entire area (Fig. 1.1).

If the amount of overflow water declined, that would mean a local cooling. In fact there are measurements that show that the overflow through the Faroe Bank Channel has decreased by 20% during the period 1950-2000 (Fig. 1.2; Hansen *et al.*, 2001). This decrease might be due to natural fluctuations, but it might also be an indication that the overflow has started to diminish. The Faroe Islands lie in relative warm waters close to considerably colder waters (Fig. 1.3).

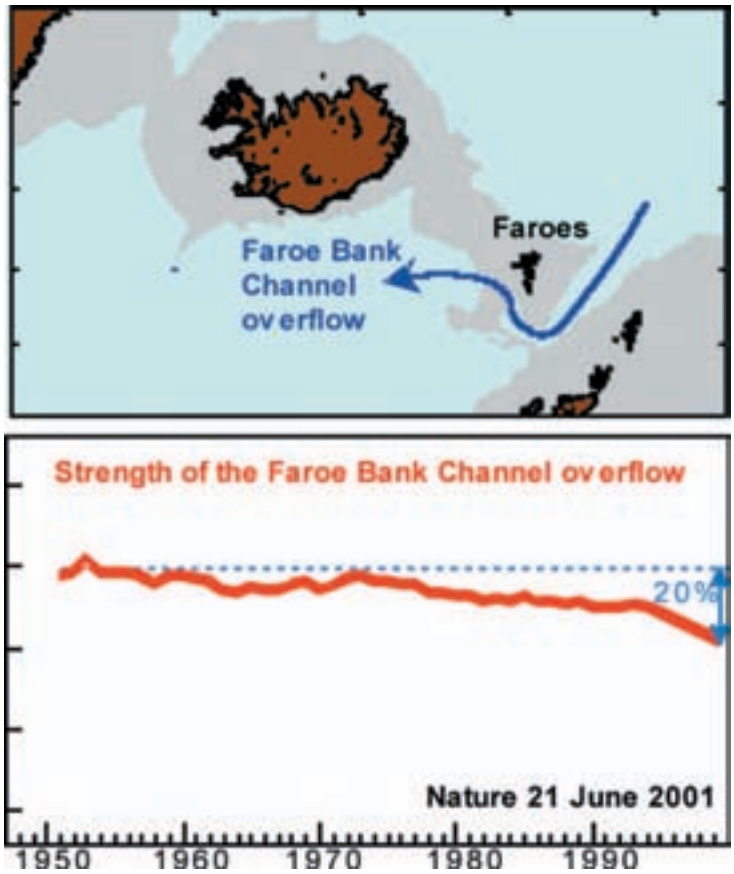


Fig. 1.2. The location of the Faroe Islands on the Greenland-Scotland ridge with the Faroe Bank Channel just south of the islands. The overflow has decreased 20% over the last 50 years. The units of the overflow (bottom picture) are in million cubic metres per second. Courtesy of Bogi Hansen, Faroese Fisheries Laboratory.

If the overflow diminishes it is likely that the cold waters shown in blue in Fig. 1.3 will move further south, and on reaching the Faroes, a temperature decrease of 5°C might be a possibility (Hansen, 2003). The Faroe Islands thus lie in a perfect position to undertake a study of potential effects of climate change, because any change in circulation and temperatures of North Atlantic surface water or in generation of deep water is apt to have significant and immediate impact on the local Faroese climate. However, two scenarios have to be considered, namely a warming scenario, where the ocean currents will not be affected, and a cooling scenario, where the ocean currents are affected. There might be a third scenario, which might have more complex biological consequences: A delayed cooling scenario, where the region first experiences warming and after that a cooling.

Previous vegetational research in the Faroes related to climate

There was a minor increase in the global mean temperature in the 1930-1940s. At the same time, the increase in mean temperature in the Faroes was considerably higher than the global mean increase (Fig. 1.4). During the period 1960-1980,

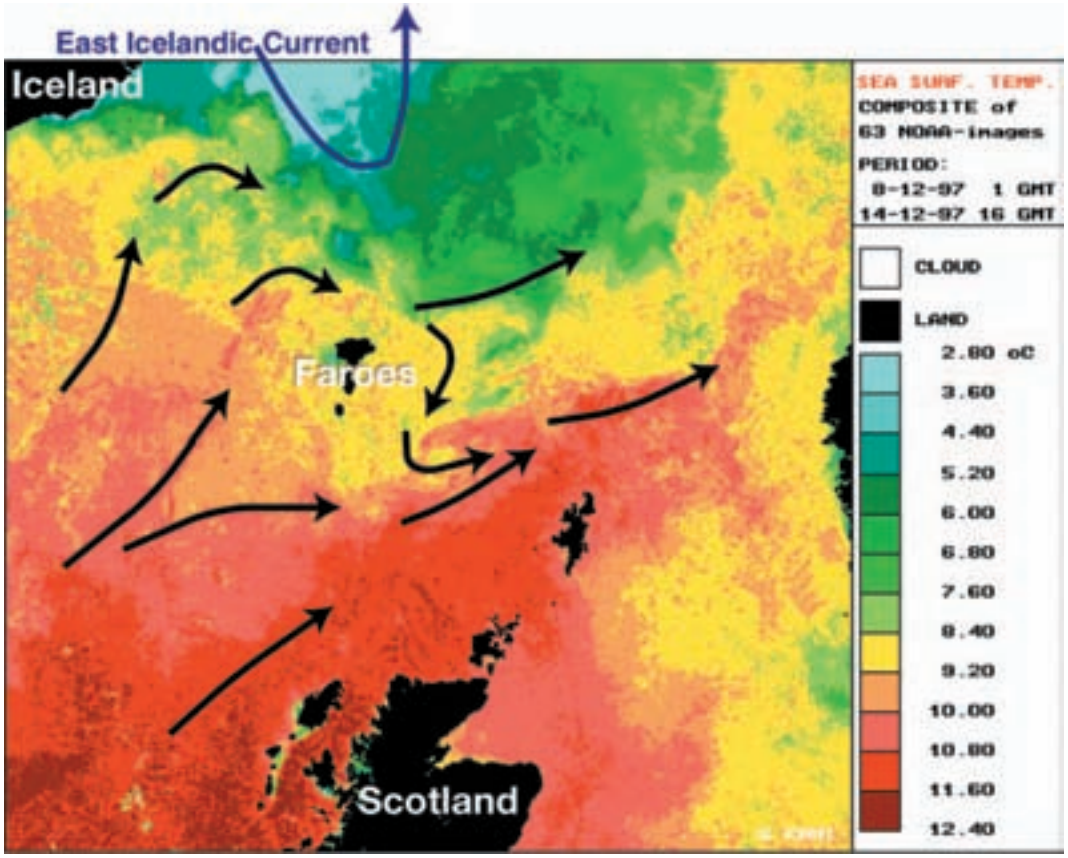


Fig. 1.3. The surface temperature around the Faroes measured from 8 Dec. to 14 Dec. 1997. Satellite picture from the National Oceanic and Atmospheric Administration (NOAA). Courtesy of Bogi Hansen, Faroese Fisheries Laboratory.

there was actually a cooling in the Faroes, so the air temperature now is below the global mean air temperature. During the last decade there has been a slight increase in mean air temperature, but not as profound as the change in the global mean air temperature.

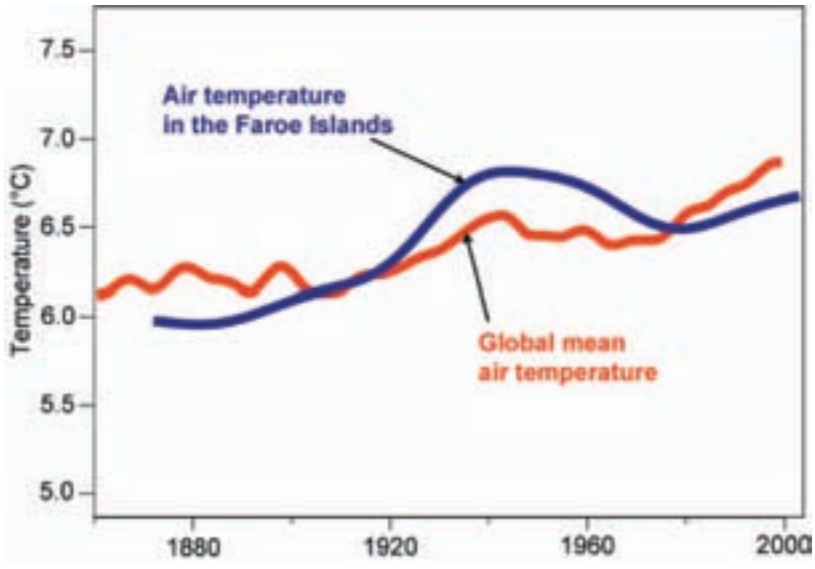


Fig. 1.4. Air temperature, globally, and on the Faroe Islands. Courtesy of Bogi Hansen, Faroese Fisheries Laboratory.

Original in colour

A vegetational study in 1937 defined vegetational zones in the Faroes (Böcher, 1937). In 1999-2000, a similar study was carried out (Fosaa, 2003). The 60 years between the two studies should be sufficient time to allow the plants to acclimate to new conditions and to migrate to new zones. According to the study from 1999-2000 plant zones have changed; Fosaa (2003) concluded that, after accounting for differences in methods and locations the boundaries of the vegetational zones seems to be at a lower altitude now than in the study from 1937 (Fig. 1.5).

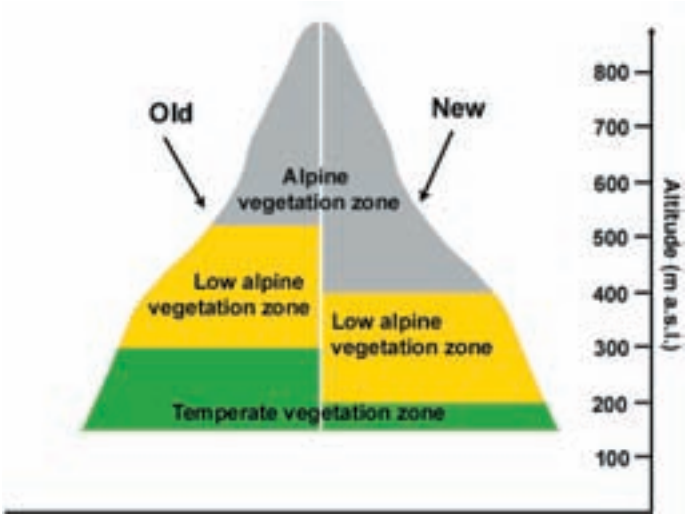


Fig. 1.5. Altitudinal differences in vegetation zones from a study from 1937 (Old) and a study from 1999-2000 (New). Courtesy of Anna Maria Fosaa, Faroese Museum of Natural History.

The plant species richness increased downslope (Fig. 1.6; Fosaa, 2004b), but nutrient availability, humus content, pH etc. also changed with altitude (Fig. 1.7) – all factors that might affect the plant community and diversity. If the species

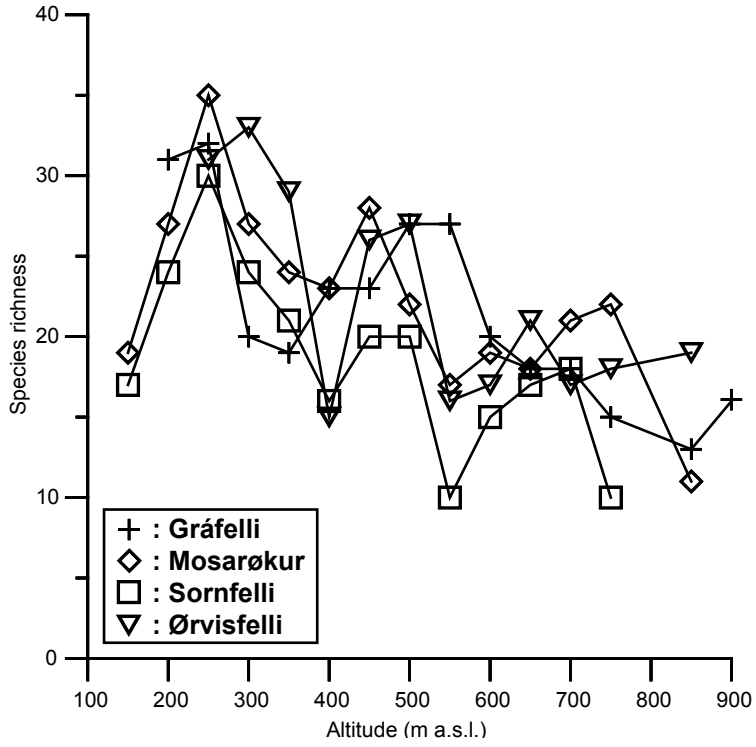


Fig. 1.6. The variation in plant species richness in altitudinal intervals from the study by Fosaa (2004b). The markers indicate five different mountains. Courtesy of Anna Maria Fosaa, Faroese Museum of Natural History.

Original in colour

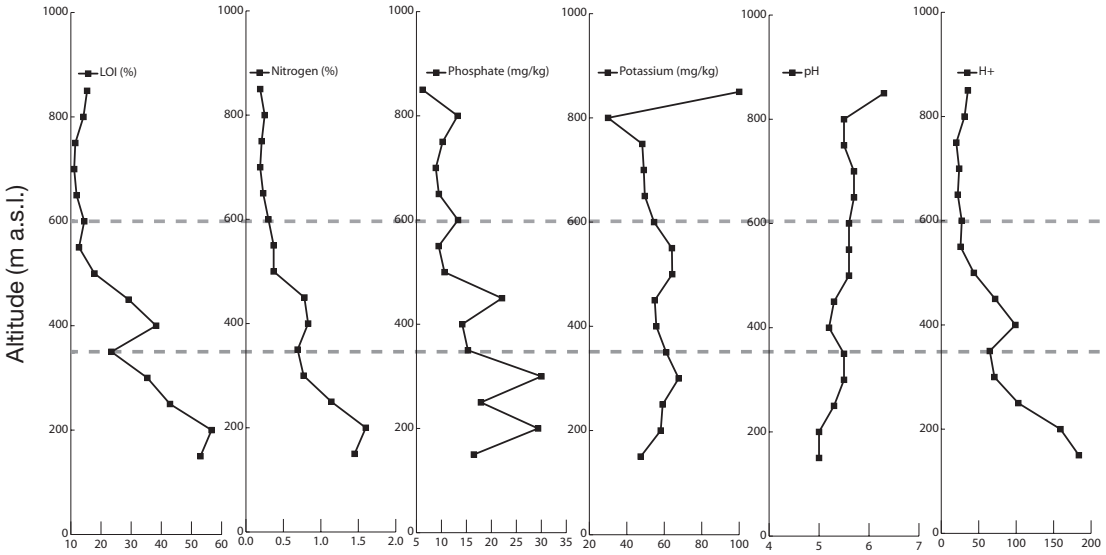


Fig. 1.7. Soil nutrients, humus and pH differ along an altitudinal gradient. Values are mean values of the same four slopes that were used in the fieldwork. The dashed horizontal lines at 600 m and 350 m indicate the altitudes used in the fieldwork (Chapter 2).

richness of plant and AM fungal communities is correlated as suggested by van der Heijden *et al.* (1998) then fewer fungal types would be expected at high altitude.

A mycorrhizal study used the checklist from Harley and Harley (1987; 1990) and the Ecoflora database from the University of York (Fitter and Peat, 1994) as the main source to determine whether the plants from the 1999-2000 study were mycorrhizal or not, and if mycorrhizal, what type of mycorrhiza the plant species was likely to associate with. The findings were, as expected, that arbuscular mycorrhiza is the most common type of mycorrhiza in the Faroe Islands, with around 70% of the plant species arbuscular mycorrhizal, regardless of altitude (Olsen and Fosaa, 2002).

Another study related to one of the slopes in the vegetational study from 1999-2000 analysed the amount of chitin in dried soil samples taken at 50 m altitudinal intervals (E. Olsen, unpublished data). This study found that the amount of chitin was positively correlated to the loss of ignition, with highest values at low altitude. Though several studies have used chitin determinations to estimate hyphal biomass in soil (e.g. Pacovsky and Bethlenfalvay, 1982; Ahmadsad, 1984; Bethlenfalvay and Ames, 1987), and other have used chitin for estimating the intensity of AM fungal colonisation in roots (Hepper, 1977), this approach suffers from the disadvantage that the exoskeleton of insects also contains chitin (Sylvia, 1992) and that the chitin-content represents both alive and dead material (Nylund and Wallander, 1992). Especially when using field

samples background interference can affect the correlation between chitin content and hyphal length (Frey *et al.*, 1994). Therefore the amount of AM fungi can not be estimated by the correlation between soil chitin and soil carbon content and soil carbon without further examinations. In an attempt to only measure live fungal biomass newer studies have measured ergosterol (e.g. Grant and West, 1986; Frostegård and Bååth, 1996; Hart and Reader, 2002). Ergosterol is an important component of the plasma membranes of most fungi, but the amount of ergosterol in soils does not reflect AM fungal biomass, but is rather a reflection of saprotrophic or ectomycorrhizal fungal biomass (Olsson *et al.* 1996; 1998). As ergosterol is not a specific AM metabolite (Frey *et al.*, 1994), other approaches have been used to estimate AM fungal biomass. For example the fatty acid 16:1ω5 has been used as a biomarker for AM fungi (Olsson *et al.*, 1995; Olsson, 1999; Balser *et al.*, 2005), but with this method the estimation of biomass can be affected by differences in AM fungal species composition (Olsson *et al.*, 2003). These chemical attemps to estimate AM fungal biomass indicate that especially when dealing with field material containing unknown AM fungal species as well as non-AM fungi, the traditional examination by microscopy probably is more consistent than chemical methods for quantification.

To conclude, previous studies have shown contradictory responses of AM fungi to temperature, but acknowledge the importance of the symbiosis for the plants in terms of nutrients and protection from pathogens. Also, it has been suggested that AM fungi are key determinants of the diversity and functioning of plant communities. In a global climate change context, the climate of the Faroe Islands is expected to show an immediate response to changes in the Gulf Stream, with consequences for vegetation zones, and both plant and AM fungal diversity.

The research project and the hypotheses

The goal of the project was to determine whether the communities of AM fungi found in thermally distinct environments in the Faroe Islands differ, and whether those differences can be explained by the response of the fungi themselves to temperature.

The following hypotheses were tested in this project:

- The diversity of the AM fungal community will be greatest where the thermal heterogeneity of the habitat is greatest, since this will permit the co-existence of species with distinct thermal tolerance. ***Chapter 2***
- Plants from a single habitat that differ in mycorrhizal dependency will be colonised by distinct fungal communities because the benefits they acquire from colonisation will also differ. ***Chapter 2***
- Open Top Chambers can be used to measure the responses of AM fungi to climate warming. ***Chapter 3***
- Fungi from colder sites have a greater ability to maintain growth at low soil temperature. ***Chapter 4***
- The growth of fungi from cold sites is affected by prolonged periods of elevated temperature. ***Chapter 4***
- The benefits for the two symbionts in the AM symbiosis are dependent on both soil temperature and soil phosphate. ***Chapter 5***

Chapter 2

The responses of root colonisation and arbuscular mycorrhizal fungal communities to changes in altitude and plant host

Abstract

- Field samples from two plant species from thermally distinct environments were examined to see whether they were colonised by distinct fungal communities.
- Two plant species, *Agrostis capillaris* and *Ranunculus acris* from four mountain slopes in the Faroe Islands were examined for percentage root length colonisation (%RLC) and the arbuscular mycorrhizal (AM) fungal types in the roots identified by cloning and sequencing.
- Greater %RLC but fewer AM fungal types were found at high altitude in *Agrostis capillaris*. The %RLC in *Ranunculus acris* did not respond to altitude, but instead to aspect, which *Agrostis capillaris* did not have a significant response to. The AM fungal diversity in *Ranunculus acris* was lower than in *Agrostis capillaris*.
- In *Agrostis capillaris* temperature explained less than 10% of the variation in %RLC. However, when also nutrients and root morphology were taken into account, more than 20% of the variation were explained. In *Ranunculus acris* nutrients and root morphology explained more than 30% of the variation, but the %RLC showed no response to temperature.
- Species richness increased with increased temperature range, suggesting a possible niche differentiation of co-existing fungal types in response to temperature.
- Statistical analysis suggested that the AM fungal communities colonising the two plant species were distinct.

Introduction

Mycorrhizas are beneficial symbioses between fungi and plants. Several kinds of fungi form different types of mycorrhiza. The most abundant of these mycorrhizas is the arbuscular mycorrhiza (AM), as about two-thirds of the mycorrhizal plants form AM (Fitter *et al.*, 2000). These estimates are biased towards temperate zone plant species, as there is limited data from arctic and sub-arctic ecosystems.

A literature survey suggested that in the Faroe Islands, where the present study was conducted, the AM mycorrhizal symbiosis is the most frequent, as 70% of the native plant species are AM mycorrhizal, while other forms of mycorrhiza account for 15%. In relation to altitude, the types of mycorrhizas are relativly stable, apart from the summit zone where non-mycorrhizal and ectomycorrhizal plants increase at the expense of AM and ericoid mycorrhizal plants (Olsen and Fosaa, 2002).

The AM fungal symbiosis is thought to be non-specific, though some plants are known to be non-mycorrhizal. Smith and Read (1997, p. 26-32) state that an AM fungus isolated from one species of host plant will colonise any other species that has been shown to be capable of forming arbuscular mycorrhiza, and this lack of specificity has important consequences for ecological interactions in plant communities. Though only 150 or so AM fungal species have been described, it is increasingly apparent that there are very many more species of fungi in natural ecosystems, and that many of these are highly selective as to the host they will colonise (Fitter *et al.*, 2004).

Even though Smith and Read (1997) consider the symbiosis to be non-specific they acknowledge the fact that there are differences in the extent to which species of plants become colonised by mycorrhizal fungi. Thus the term selectivity rather than specificity for the plant-fungal interaction as suggested by Helgason *et al.* (2002) might be more appropriate. According to Smith and Read (1997, p. 28) one of the more significant traits of AM fungi is their occurence in plant communities with high species diversity, allowing individual fungi to colonise a broad range of host plants.

In general the vegetation changes with both latitude and altitude. Thus, a lowland Faroese site would correspond better to a high-altitude, rather than to a lowland British site, due to its more northern location. These latitudinal and altitudinal changes are due to several factors, such as light intensity and day-length, but one of the main reasons is temperature. Temperature is one of the most powerful factors affecting living organisms, for example by changing the kinetics of enzymes (Elliott and Elliott, 1997, p. 7), and can affect both growth

and development of a plant (Fitter and Hay, 2002, p. 197). However, the impact of the oceanic climate in the Faroes must also be taken into consideration, which, according to Crawford (2000), is characterised by late springs, cool summers and relatively warm, wet winters with strong winds that can impoverish natural habitats in terms of species abundance.

Although declining temperature is the most obvious climatic correlate of altitude, temperature does not always correlate with root growth, and photosynthetically active radiation (PAR) and nutrients may be more important factors than temperature in determining root production and turnover (Fitter *et al.*, 1999). Increased root growth means that more carbon is allocated to the root system, and a higher mycorrhizal colonisation might be expected, because carbon allocation to the root is the only carbon source for the AM fungi.

Using altitudinal gradients as a surrogate for a change in mean temperature is a method that has been used in several field studies, e.g. Ineson *et al.* (1998) followed nitrogen dynamics in a climate change context, Fitter *et al.* (1998) estimated the impact of temperature on root turnover, and Ruotsalainen *et al.* (2004) investigated colonisation of roots by both AM fungi and other fungal endophytes. Although altitudinal gradient studies have not shown a direct correlation between temperature and mycorrhizal colonisation, other studies have indicated that fungi might be adapted to distinct edaphic niches. For example, Merryweather and Fitter (1998b) found a distinct seasonality in the types of AM fungi colonising the winter active geophyte *Hyacinthoides non-scripta*.

Several studies have shown that an AM fungal community can consist of a considerable number of fungal types. The traditional method of identifying these fungi is by identifying spores found in the soil around plant roots. For example, Merryweather and Fitter (1998a) found eight glomalean spore taxa from soils around one plant species (*Hyacinthoides non-scripta*); Bever *et al.* (2001) examined a one-hectare field and identified 37 different species of AM fungi. The site Bever *et al.* (2001) used had a high plant diversity with approximately 50 plant species, and the high number of fungal species together with the high plant diversity that Bever *et al.* (2001) found could be another indicator of that there is a relationship between fungal diversity and plant diversity as suggested by van der Heijden *et al.* (1998).

Recently molecular methods have given a fuller picture of AM fungal diversity in natural ecosystems. For example, the fungi colonising *Hyacinthoides non-scripta* have been examined by cloning and sequencing: the number of fungal types found was similar to that found with spore identification, namely

8-10 types (Helgason *et al.*, 1999). The sampling intensity in the latter's study was not high – 35 roots, of which 33 amplified, and 141 clones were examined. Thus, from 141 clones they identified 8-10 types. Other studies have used a higher number of clones, e.g. Vandenkoornhuyse *et al.* (2002) analysed 2001 cloned fragments from 47 root samples, and found 24 AM fungal types, while Husband *et al.* (2002) analysed over 1300 cloned fragments from 48 plants and found 30 AM fungal types. In contrast to these studies that used a high clone number, Öpik *et al.* (2003) examined 58 plant roots and found 19 sequence groups from only 128 cloned fragments by using denaturating gradient gel electrophoresis (DGGE) before the cloning step, eliminating the need for a high number of clones.

AM fungi enhance plant acquisition of poorly mobile nutrients in soil by enabling the uptake of ions beyond the depletion zones that surround plant roots. They can do this cost-efficiently because fungal hyphae are much finer than roots (Smith and Read, 1997, p. 131). Other mechanisms have been proposed, including the ability to detect and respond to nutrient patches that the plant roots cannot detect (Hodge, 2004). In the case of arctic soils, the nutrients are often present in complex organic form, possibly increasing the dependence of plants on the symbiosis. Increased phosphate uptake is the best documented benefit that has been demonstrated for plants in association with arbuscular mycorrhizal fungi (Smith and Read, 1997, p. 126-147). The uptake of other nutrients has also been demonstrated. For example, Hodge *et al.* (2001) has shown that the symbiosis facilitated the uptake nitrogen by the plant from organic sources. However, these nutritional benefits to plants have been demonstrated principally in controlled experiments. In a field experiment, Fitter (1986) concluded that the symbiosis is only beneficial under certain circumstances.

Plants differ in their mycorrhiza-dependency (Fitter and Peat, 1994). One "rule of thumb" is that plants with coarse roots, such as *Hyacinthoides non-scripta*, are dependent on mycorrhiza for nutrient uptake (Merryweather and Fitter, 1996), while plants with fine roots, for example the grass *Vulpia ciliata* ssp. *ambigua*, are less dependent on mycorrhiza for nutrient uptake, but might benefit for other reasons, such as pathogen defence (Newsham *et al.*, 1995a). Those findings led to the hypothesis of multi-functionality and biodiversity in AM (Newsham *et al.*, 1995b). The hypothesis states that different AM fungi have different functions: some might be good for nutrient uptake, others for protection against pathogens, and still others for drought, just to mention a few examples. All these functions might be sensitive to temperature, so that, for example, a particular fungal type might be most efficient in obtaining phosphate at a certain

temperature. Support for the multifunctionality and biodiversity hypothesis comes from the work of van der Heijden *et al.* (1998), who found that with more AM fungal taxa the plant biodiversity, plant biomass and plant phosphate content all increased.

We can therefore expect that most plant communities support numerous species of AM fungi, which may be functionally distinct. But can we further expect these fungi to be specialised to edaphic niches? For example, do cold sites have specialised mycorrhizal fungi adapted for maintaining growth, with the ability to colonise and to provide nutrients at low temperatures? Moreover, can we expect these fungi also to show plant host selectivity?

The goal of the study was to determine whether the communities of AM fungi found in thermally distinct environments in the Faroe Islands are different, and whether those differences can be explained by the response of the fungi themselves to temperature. Achieving these objectives allows the testing of the following hypotheses:

- The diversity of the AM fungal community will be greatest where the thermal heterogeneity of the habitat is greatest, since this will permit the co-existence of species with distinct thermal tolerance.
- Plants from a single habitat that differ in mycorrhizal dependency will be colonised by distinct fungal communities because the benefits they acquire from colonisation will also differ.

Material

The data presented here consist of climatic data, mycorrhizal colonisation data obtained by measuring the percent root length colonised (%RLC), and AM fungal diversity obtained by molecular methods. The samples are from *Agrostis capillaris* and *Ranunculus acris* and taken from four mountain slopes in the Faroes. Two of the slopes face south-west, Gráfelli and Mosarøkur, and two face north, Sornfelli and Ørvisfelli (Fig. 2.1 and Table 2.1). The sites are all open grassland (Plate 1) and range from temperate zone (south-facing low altitude) to alpine zone (all high altitude sites) (Fosaa, 2004a).

The Faroes and the vegetation in general

The Faroese vegetation is mostly perennial and is influenced by several factors. The two most obvious factors are the oceanic climate and the ubiquitous sheep.

The landscape is dominated mostly by steep mountains.

The temperature is relatively constant in both winter and summer, with an average of 4°C in the coldest month (January) and 11°C in the warmest month (July). The climate is oceanic with much precipitation, clouds and wind. The annual mean precipitation is 1,500 mm at 50 m a.s.l.

Sheep are the most important herbivore, with an average number of around 44 ewes/km^2 (Thorsteinsson, 2001). The impact of geese and hares, however, cannot be ignored (Fosaa, 2004a). In general the area is eroded, and according to farmers the situation has become worse during the last decades. Probably both weather conditions and overgrazing are to blame for the erosion. The vegetation is largely grassland, although some dwarf shrubs occur. Trees are only present as single trees planted in gardens or in plantations at some very sheltered locations.

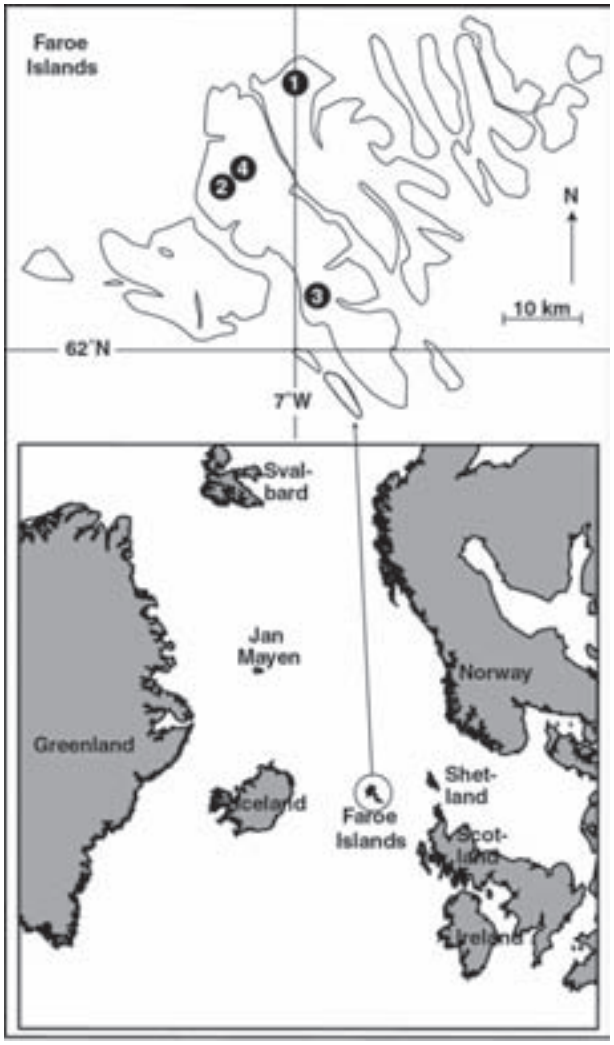


Fig. 2.1. The location of the Faroe Islands (62°N, 7°W) and location of the four studied mountains in the Faroe Islands.
Name, location (latitude, longitude) aspect and altitude for each mountain are as follows:

❶ Gráfelli (62°18'N, 6°59'W)
South-west • 856 m a.s.l.

❷ Mosarøkur (62°11'N, 7°10'W)
South-west • 756 m a.s.l.

❸ Sornfelli (62°04'N, 6°57'W)
North • 749 m a.s.l.

❹ Ørvisfelli (62°12'N, 7°09'W)
North • 783 m a.s.l.

Plate 1. Faroese vegetation. The study sites at 350 m a.s.l. are in the uppermost part of the low-alpine zone. Therefore it contained species from the low-alpine- and the alpine vegetation zone described as moist grassland vegetation and open grassland vegetation. The study sites at 600 m a.s.l. are located within the upper alpine vegetation zone and contained species from the main vegetation type described as open grassland vegetation as well as *Racomitrium* vegetation.
A: Pictures from Gráfelli, 600 m
B: Gráfelli, 350 m
C: Mosarøkur, 120 m

Original in colour

Plant material

The two plant species selected for investigation were *Agrostis capillaris* L. and *Ranunculus acris* L. The grass *Agrostis capillaris* is one of the most common plant species in the Faroes, found both at high and low altitude. It has a relatively fine root system and flowers in June-July (Jóhansen, 2000). The dicot *Ranunculus acris* is also found at both low and high altitudes, although at high altitudes it is mainly *Ranunculus acris* var. *pumilus*. The root system is relatively coarse and flowering time is May-July (Rasmussen, 1952).

Methods

Soil parameters

Soils were sampled in 2000 on each of the selected mountains at 50 m altitudinal intervals from the top down to where the uncultivated land meets the cultivated land. Eight cores of soil (5 cm diameter, 10 cm deep) from each sample site were mixed, dried at 70°C for 5 days, then sieved through a 2 mm sieve and analysed at the University of the Faroe Islands, Tórshavn according to the Danish Plant Directorate's manual (Sørensen and Bülow-Olsen, 1994).

Loss of ignition (LOI) was determined by ashing a soil sample at 550°C in a muffle furnace for three hours.

The pH in a water solution (pH) was determined by mixing one part dried soil with 2.5 parts distilled water, left overnight before measurement with a Radiometer PHM 240 (Radiometer Analytical, Villeurbanne, France).

Exchangeable cations (Ca, Mg, K) were determined by adding 50 cm^3 1 M ammonium acetate (CH_3COONH_4) solution to 10 g dried soil. The solution was left overnight, filtered and measured by flame spectrometry (Milton Roy Spectronic 1200, Ivyland, Pennsylvania, US).

Total Kjeldahl-N was determined by digestion of 10 g dried soil sample with sulphuric acid (H_2SO_4) and a Cu catalyst. The ammonium concentration in the distillate (ammonium-nitrogen) was estimated by steam titration with HCl.

Phosphorus was determined following Olsen *et al*. (1954) but with poly-acrylamide used to replace carbon (Banderis *et al.,* 1976). 5 g of soil were diluted in a 100 ml solution of 0.5 M sodium-bicarbonate ($NaHCO_3$) with polyacrylamide added (0.25 g/l). After extraction for 30 min. the phosphorus was determined by a phosphate reagent with ammonium-molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$) and potassium antimonyl tartate ($C_4H_4O_7KSb$) and ascorbic acid. The phosphate in the extract was determined by spectrometry at 890 nm wavelength.

Exchangeable H (H_3O^+) was determined by dissolving two 5 g soil samples in 100 ml m-nitrophenol ($C_6H_5O_3N$) at pH 8.33 and 8.16, respectively and titrated with HCl to pH 4.0. The two volumes of HCl were used to calculate the amount of H+ released by the soil solution.

Temperature data

There was ongoing temperature-logging on the sites from 1999-2002, using Tiny-Tags *Plus* dataloggers (Gemini Data Loggers, Chichester, UK) recording the soil temperature at 1 cm below the surface at hourly intervals. However, there were substantial problems with missing data from the on-site logging, especially because the inserted probes corroded and thus started to leak. This problem became worse each year, as the probes were used again and became increasingly corroded. For example, it was only possible to get data from two of the slopes in 2002. The only continuous data are the climatic data from the Danish Meteorological Institute (DMI). Therefore, the temperature in Tórshavn was used as the standard. Thus, when dealing with "missing data" from sites where previously temperature measurements had been obtained, the temperature on the site was extrapolated from the relationship between the data from Tórshavn and the previous data measured on the site by a regression equation. When no data were available from the site, the estimated lapse rate from the specific mountain was combined with a regression from a site on the same slope as nearby as possible (Table 2.1; Appendix 1). The lapse rate for each slope was calculated from available data measured along the whole slope (data not shown), and then the lapse rate was used to estimate the temperature on a site with missing data based on data measured on a nearby site (Appendix 1).

The temperature data are thus based on three different datasets:

1. Temperature measurements from two of the slopes, at 150, 350 and 600 m altitude, measured in 2002 (Gráfelli and Mosarøkur 2002 in Appendix 1).
2. Mean monthly temperature measured in Tórshavn (the Faroese capital) at approximately 50 m altitude by DMI (Cappelen and Jørgensen, 2002, 2003).
3. Mean monthly temperature measurements from the slopes used in this study, measured from 1999-2001 at 50 m altitudinal intervals.

Site and calculated lapse rate	Altitude m a.s.l.	Soil temperature		Air temperature	Relationship between soil and air temperature (regression)		
		months measured on site	months estimated from another site (site in brackets)	months estimated from Tórshavn	Tórshavn and measured soil temperature	R^2	month/year used in the regression
Gráfelli Lapse rate = 0.4°C/100 m	60	0	30 (G350)	4	$F_{1,34} = 126$	0.78	7/99- 7/02 (8/01 missing)
	350	30	0	4			
	600	11	0	23	$F_{1,9} = 229$	0.96	9/01- 7/02
Mosarøkur Lapse rate = 0.6°C/100 m	120	31	0	3	$F_{1,36} = 437$	0.92	7/99- 8/02
	350	31	0	3	$F_{1,36} = 206$	0.85	7/99- 8/02
	600	24	0	10	$F_{1,23} = 167$	0.87	8/00- 8/02
Sornfelli Lapse rate = 0.4°C/100 m	70	0	6 (S150)	28	$F_{1,9} = 51$	0.83	8/99- 6/00
	350	22	0	12	$F_{1,26} = 432$	0.94	7/99-11/01 (10/00 missing)
	600	0	23 (S500)	11	$F_{1,23} = 348$	0.93	7/99-11/01
Ørvisfelli Lapse rate = 0.8°C/100 m	50	0	16 (Ø250)	18	$F_{1,20} = 245$	0.92	7/99- 4/01
	350	0	16 (Ø250)	18			
	600	0	16 (Ø550)	18	$F_{1,20} = 259$	0.93	7/99- 4/01

Table 2.1. The temperature measurements and the statistics behind the estimated values used in this study. All p-values are below 0.001 (not shown).

Sufficient data were obtained from both 1 cm and 5 cm depth at two sites only, both from the same slope on Mosarøkur. The site at 350 m is the most exposed site used in this study, with very sparse vegetation. There was a very good correlation between temperatures measured at the two depths at 600 m altitude, but the correlation was not as good at the more exposed site at 350 m altitude (Table 2.2). The maximum soil temperature at 5 cm depth at 600 m was higher than at 1 cm (Table 2.2), which was unexpected as most of the shorter time series indicated a higher temperature at 1 cm than at 5 cm depth; there might be several explanations for this difference, including soil structure, moisture, vegetation, how the probe was placed etc., but as there were no replicates it is not possible to isolate any factor.

Altitude m a.s.l.	Soil temperature 1 cm below surface (°C)			Soil temperature 5 cm below surface (°C)			Regression between 1 cm and 5 cm	
	Max	Min	Range	Max	Min	Range		R^2
350	27.2	-6.8	34.0	24.2	-2.0	26.1	$F_{1,9062} = 56999$	0.86
600	26.4	-2.2	28.6	28.7	-1.4	30.2	$F_{1,9060} = 242177$	0.96

Table 2.2. The relationship between soil measurements at 1 cm soil depth and at 5 cm soil depth. All p-values are below 0.001 (not shown). Both sites are on Mosarøkur. The measurements shown are based on hourly measurements from 2nd Aug. 2001 to 14th Aug. 2002.

Root samples

During the summer period (June-August) 2001 and 2002, three conical cores of soil, approximately 5-6 cm wide and 8 cm deep, were taken from each site on each sampling day; the sampling days are listed in Table 2.3. Three altitudinal sites were used, one site at low altitude (50-120 m a.s.l.; Table 2.1), one at 350 m a.s.l. and one at 600 m a.s.l. Samples were taken from each mountain once a month during the summer, weather conditions permitting. The core was selected by the combination of vegetation: *Ranunculus acris* and *Agrostis capillaris* in the same core was preferred. *Agrostis capillaris* is ubiquitous, while *Ranunculus acris* is patchily distributed. Since the two plants came from the same core, the same fungi should have had the opportunity to colonise both plant species.

	June		July		August	
	2001	**2002**	**2001**	**2002**	**2001**	**2002**
Gráfelli		30.	7.	21.[1)]	2. 31.	9.[2)]
Mosarøkur		7.	19.	2. 26.[1)*)]	1. 3. Sep.	9.[2)]
Sornfelli	1.	5.	8.	1. 22.[1)]	1. Sep.	11.[2)]
Ørvisfelli	18.	29.		26.[1)*)]	9.	3.[2)]

Table 2.3. Sampling dates in 2001 and 2002 with respect to location, month and year.
1) First sampling for molecular analysis. 2) Second sampling for molecular analysis *) Only low altitude sampled.

Field work was carried out from May to September 2001 and from April to August in 2002. However, sampling was only possible on north-facing slopes in May 2001 and only on south-facing slopes in May 2002, due to weather conditions – especially snow at high altitude. Little growth was observed in either species before the end of May. Therefore, only data from June to August were used in the analysis presented here.

The roots from 2001 were washed in tap water and put into a 10% KOH solution. The roots from 2002 were divided into two: The roots for %RLC were washed and then transferred to KOH directly, while the roots for the molecular work were washed, dried at 60°C for a week and stored at room temperature until extraction.

Percent root length colonisation

The root clearing was a modified procedure after Grace and Stribley (1991), Koske and Gemma (1989) and Walker and Vestberg (1994). The staining procedure followed Vierheilig *et al.* (1998). After washing, the roots were put directly into tubes with 10% KOH (w/v) and left at room temperature for 5-7 days, roots from each plant in a separate tube. Thereafter, the samples were rinsed in tap water, acidified with 4-5% vinegar for 24 hrs, then stained in a lactic acid (90%)/glycerol (85%) 60/40 (v/v) solution with 1% blue ink (Parker, Quink) for 24 hrs, rinsed in tap water and then left for 3 days in a destaining solution (lactic acid (90%)/glycerol (85%) 60/40 (v/v)).

After this, the samples were mounted on microscope slides in destaining solution and examined with 200 x magnification; colonisation was measured following McGonigle *et al.* (1990). One hundred intersections were examined and mycorrhizal structures recorded for each intersection. The mycorrhizal structures were external AM hyphae, entry point, hyphae connected to arbuscules, hyphae connected to vesicles, hyphae with pegs that are likely to be AM, arbuscules, arbuscular coils, fine endophytes, vesicles. If one, two or more mycorrhizal structures were found in one intersection, they accounted for 1% RLC. In addition to mycorrhizal structures, an estimate of whether the plant root at the intersection point was coarse or fine was recorded (percent fine roots, %FR). A typical fine root was between 0.1 and 0.15 mm diameter, but a diameter up to 0.20 mm could be considered as a fine root if the stele was poorly developed.

Molecular analyses

The roots from two sampling days in 2002 (Table 2.3) and from low and high altitude were examined for which types colonise the roots, as well as %RLC.

DNA extraction: Roots were ground in liquid nitrogen, and then the DNA was extracted by the CTAB method (Gardes and Bruns, 1993). After washing with ethanol the extract went through an extra purification with StrataPrep PCR Purification Kit from Stratagene, Cedar Creek, Texas, US. These purifications are necessary to get rid of inhibitors.

PCR: TAQ polymerase (Invitrogen, Carlsbad, California, US) was used together with the primers NS31 (Simon *et al.*, 1992), AM1 (Helgason *et al.,* 1998) and AM2 (Karyn Ridgway, unpubl.; see Appendix 2). NS31 is a general primer, valid for all eucaryotes, while AM1 is a specific primer, amplifying especially glomalean fungal types, but not Paraglomalean and *Archaeospora* types. AM2 amplifies a shorter segment and a wider range of glomalean fungi, including

Paraglomalean and *Archaeospora* types. The drawback in using AM2 is that more ascomycetes are amplified together with the glomalean fungi.

The primers used were always NS31, and either AM1 (Sornfelli) or AM2 (all other sites). The reactions were performed using 0.2 mM dNTPs, 10 pmol of each primer and the supplied reaction buffer to a final volume of 50μL. The PCR-amplification was done on a PTC-200 (the DNA engine cycler developed by MJ Research, now Bio-Rad Laboratories, Hercules, California, US), 94°C for 3 min, then 30 cycles for 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. The final step was 10 min at 72°C.

Cloning: The PCR product was ligated into a pGEM-T vector (Promega, Madison, Wisconsin, US), and then the recombinant DNA molecule was transferred into competent *E. coli* cells (DH5α from Gibco BRL Life Technologies, now Invitrogen Corporation, Carlsbad, California, US). All steps followed strictly the manufacturers' manuals. The competent cells were grown on solid medium, forming colonies of identical clones. At least 16 clones, but typically 24, were used for each sample, giving a total of 376 clones. Of these, 137 were glomalean types; the rest either had no insert, were ascomycetes or gave unclear results.

RFLP (Restriction Fragment Length Polymorphism): The clones were grouped by using the enzymes *Hin*f1 and *Hsp*92II (Promega, Madison, Wisconsin, US) mainly to distinguish between the glomalean types, while *Stu*I and *Hpa*II were used to detect amplified ascomycetes. *Hpa*II also distinguished between some *Acaulospora* types.

Sequencing: Only RFLP types that occurred more than twice were sequenced. Of these, one clone of each RFLP from each site and plant species was sequenced on a Beckman Coulter CEQ8000 (Fullerton, California, US) analyser at the Technology Facility, University of York, UK. Only one strand was sequenced. In total, 28 cloned fragments were sequenced, some of them were ascomycetes, and hence omitted.

Statistics

ClustalX (Thomson *et al.,* 1997) was used for multiple alignment and neighbour-joining phylogenetic analysis (Saitou and Nei, 1987), using *Corallochytrium limacisporum*, a putative choanozoan (Cavalier-Smith and Allsopp, 1996) as the outgroup. The phylogenetic tree was drawn in TreeView (Page, 1996).

All other statistical analyses were carried out using SPSS version 11.03 for Mac OS X. The samples were tested for normal distribution using the One-Sample Kolmogorov-Smirnov test. Parameters that failed the normal distribution test were re-tested in an attempt to fit them into a normal distribution by using the

square root of the sample; this approach worked for the %RLC. All correlations are Pearson's if not otherwise stated. The entry/removal criteria for stepwise-regression was the probability of F (enter 0.05, removal 0.1). The sampling period in the statistical analysis was divided into weeks (1-52 per year). For post-hoc tests the Bonferroni test was used.

Log-linear modelling is an analogue to multiple regression, examines all possible interactions, and is a type of multi-way frequency analysis. A saturated model is generated with all the relevant factors included; such a saturated model by definition has the probability of 1. Thereafter, by using backward elimination, models with fewer factors are generated and the 0-hypothesis is that the new model does not differ significantly from the saturated model. A p-value below 0.05 indicates that a factor that changed the saturated model has been removed.

Diversity tests used were Shannon-Weiner and species richness. Shannon-Weiner was calculated by the formula: $H_0 = -\Sigma p_i \ln p_i$
where p_i in this case was used as the frequency of the different types of clones.

Species richness was calculated as number of "number of types"/"number of clones".

Similarity tests were calculated by using the formula: $S = 2N_{ab}/(N_a + N_b)$
This is a pairwise test known as the Sørensen similarity coefficient, estimating the similarity between two samples based on presence/absence values. N_{ab} is the number of clone types that are shared between the two samples, while N_a and N_b is the total number of clone types in each sample. This is a simple test that can be done in a spreadsheet, but as the number of calculations quickly adds up with increasing numbers of samples, the software EstimateS (Colwell, 2005) was used.

Results

Edaphic and climatic data

The summer of 2002 was on average 1.5°C warmer than 2001 (Table 2.4), while the altitudinal difference in temperature was more than 3°C from low altitude to high altitude. Both these effects were significant. Though the mean values for southfacing slopes were slightly higher than northfacing, the difference was not significant. This might be due to differences in wind direction, as the main wind direction June-August 2001 was south-east to south, while in 2002 it was south-west (wind data provided by "Landsverk" – Faroe Islands Office of Public Works). The reason for choosing north- and southfacing slopes was to get an extra indicator of temperature, as it was expected that it would be warmer on southfacing slopes.

Altitude	Aspect		Year	
	North	South	2001	2002
Low	11.4 ±0.49	11.9 ±0.44	10.9 ±0.46	12.4 ±0.38
350 m	9.9 ±0.39	10.1 ±0.59	9.4 ±0.40	10.6 ±0.53
600 m	7.6 ±0.41	8.4 ±0.40	7.5 ±0.47	8.6 ±0.28

Table 2.4. Mean temperature values for June-August 2001 and 2002 per altitude in relation to aspect and year. These are the factors used in ANOVA analysis as substitutes for temperature.

The extractable phosphate levels are low compared with the values the Danish Plant Directorate has suggested as typical of Danish soils (Table 2.5). Soil nitrogen was on average 0.5% and is a measure of both ammonium and organic nitrogen. The humus content was relatively high. The loss on ignition was on average 19%. Further to this the correlation between soil nitrogen and loss on ignition was high (Spearman's rho = 0.976, $p < 0.001$, $N = 8$), suggesting that the bulk of nitrogen in Faroese soils is organically bound and thus not available for the plants.

Site	Alti-tude	Slope	P	N	pH	LOI	K	Mg	Ca	H+
	m a.s.l.		ppm	% d.w.		% d.w.	ppm	ppm	ppm	*)
Typical DK range			0-200	0.1-0.2	4-7.8	2-4	80	20-150	500-5000	0-50
Gráfelli	350 m	40°	19.9	0.72	5.3	24.15	60	189	991	95.9
	600 m	13°	22.4	0.55	5.4	23.04	69	176	739	22.2
Mosarøkur	350 m	13°	13.6	0.50	5.8	19.80	69	229	777	38.3
	600 m	20°	12.7	0.15	6.0	10.91	62	105	641	21.1
Sornfelli	350 m	5°	12.8	0.92	5.3	26.50	29	142	212	94.0
	600 m	10°	9.7	0.21	5.6	12.80	65	341	1635	43.1
Ørvisfelli	350 m	35°	15.0	0.60	5.6	23.40	87	440	1730	31.1
	600 m	25°	8.6	0.29	5.2	11.05	22	175	194	23.0

Table 2.5. Site description and soil nutrient status. All measurements are exchangeable ions apart from N, where the total nitrogen was measured. The row named "Typical DK range" contains the values that, according to the Danish Plant Directorate, are typical for Danish agricultural soils, and may differ significantly from what might be expected in Faroese outfield soils.
*) exchangeable acidity expressed in meq/100 g soil dw.

Percent root length colonisation

The colonisation pattern in the two plants differed considerably. While the colonisation increased with altitude in *Agrostis capillaris*, it slightly decreased in *Ranunculus acris* (Fig. 2.2). There was a significant difference in the extent of colonisation between the two plant species, with *Ranunculus acris* averaging 31% and *Agrostis capillaris* 23% ($F_{1,405} = 7.830$; $p = 0.005$).

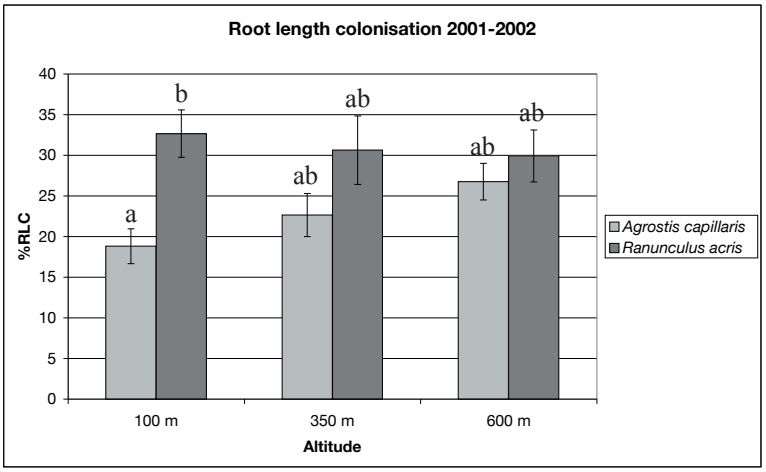


Fig. 2.2. The %RLC in *Agrostis capillaris* and *Ranunculus acris* at the three altitudes investigated in this study. Values are means (n = 407) ±SE. Different letters indicate significantly different means.

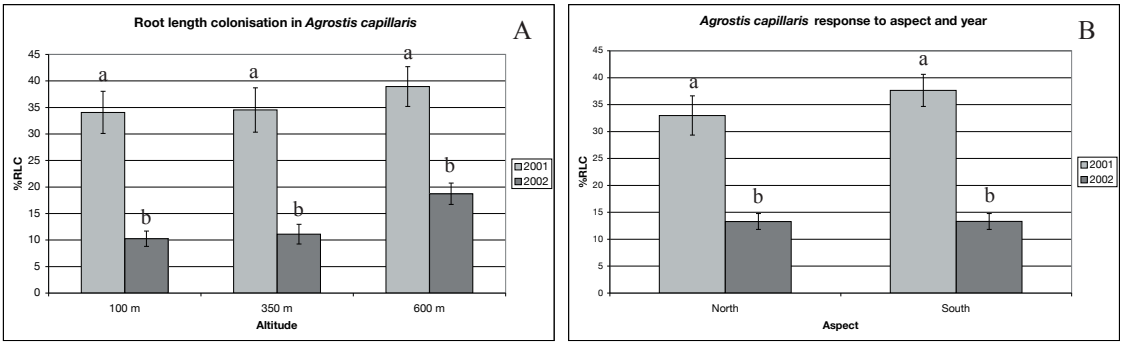


Fig. 2.3. The %RLC in *Agrostis capillaris* at the three altitudes (A) investigated in this study and (B) the differences between years and aspect shown. Values are means (n = 212) ±SE. Different letters indicate significantly different means.

There was a large difference in colonisation in *Agrostis capillaris* between the two sampling years with %RLC higher in 2001, which was colder than 2002 (Fig. 2.3A). In both years, the colonisation was greater at high altitude, again where it is colder (Fig. 2.3A). In 2001 the mean colonisation was slightly, but insignificantly, higher on southfacing slopes than on northfacing slopes, while there was no difference in the response to aspect in 2002 (Fig. 2.3B).

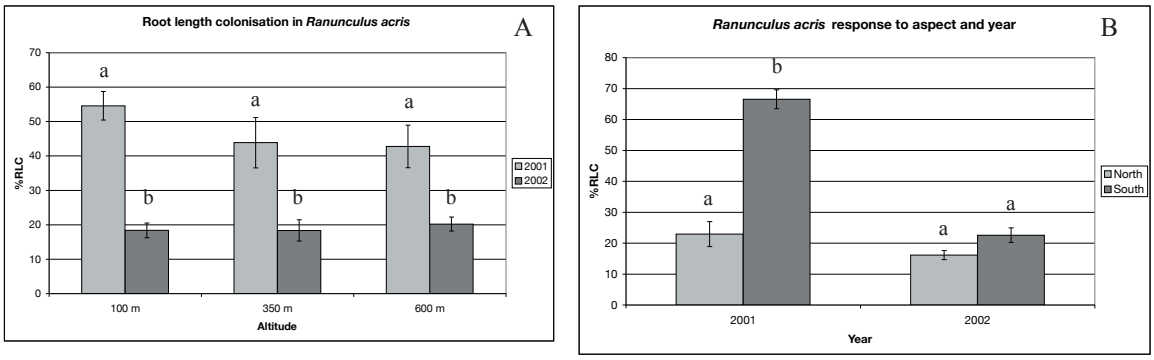


Fig. 2.4. The %RLC in *Ranunculus acris* at the three altitudes (A) investigated in this study and (B) the difference between years and aspect shown. Values are means (n = 195) ±SE. Different letters indicate significantly different means.

An identical response between years was seen in *Ranunculus acris* (Fig. 2.4A); in addition colonisation was consistently higher on the southfacing slopes (Fig. 2.4B). The differences between years and aspects were significant (Table 2.6).

%RLC	***Agrostis capillaris***	***Ranunculus acris***
Factor:		
Altitude	$F_{2,200}$ = 4.960; p = 0.008	n.s.
Year	$F_{1,200}$ = 71.782; p < 0.001	$F_{1,183}$ = 43.593; p < 0.001
Aspect	n.s.	$F_{1,183}$ = 95.026; p < 0.001
Altitude * year	n.s.	$F_{2,183}$ = 5.848; p = 0.003
Altitude * aspect	$F_{2,200}$ = 7.110; p = 0.001	$F_{2,183}$ = 19.838; p < 0.001
Year * aspect	n.s.	$F_{1,183}$ = 33.331; p < 0.001
Altitude * year * aspect	n.s.	n.s.

Table 2.6. Univariate Analysis of Variances of the %RLC. Data were square root transformed.

Table 2.6 shows an analysis of variance of %RLC in *Agrostis capillaris* and in *Ranunculus acris*. The colonisation in *Agrostis capillaris* responded significantly to altitude and year, but not to aspect, while the colonisation in *Ranunculus acris* responded significantly to year and aspect. For *Agrostis capillaris* the yearly variation was the most significant, followed by the altitudinal variation; for *Ranunculus acris* the aspect was the most important single factor, followed by the yearly variation. The altitudinal variation was only significant in *Agrostis capillaris*. The interaction between altitude and aspect in *Agrostis capillaris* arose because at low altitude there was a higher colonisation on northfacing slopes, while at the two higher altitudes the %RLC was higher at southfacing slopes (not shown). In *Ranunculus acris* all two-way interactions

were significant: %RLC was greater at low altitude in 2001 but not in 2002 (Fig. 2.4A), and on southfacing slopes in 2001 only (Fig. 2.4B), while the interaction between aspect and altitude is because the %RLC at 350 m altitude was considerably greater on southfacing and considerably lower on northfacing slopes than at the two other altitudes (not shown).

There was a negative relationship between %RLC and temperature in *Agrostis capillaris* (Fig. 2.5), meaning higher colonisation at low temperature, though less than 10% of the variation was explained by the regression. *Ranunculus acris* did not show any significant relationship.

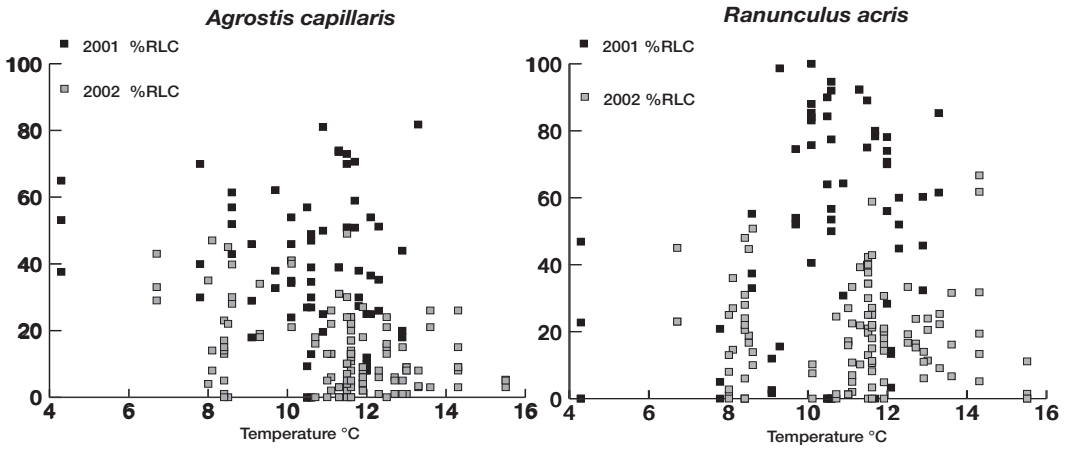


Fig. 2.5. %RLC for the two plant species explained by temperature. 2001 labelled by black squares, 2002 by grey squares. The regression for *Agrostis capillaris*: $F_{(1,210)} = 20.557$, $p < 0.001$, $R^2 = 0.085$. The relationship between temperature and %RLC in *Ranunculus acris* was not significant.

Factors that might account for the unexplained variation include seasonal variation and plant characteristics, which here are represented by the percentage fraction of fine roots (%FR). The seasonal variations of %RLC differ between the two plant species. *Agrostis capillaris*, flowering in June-July, has its lowest colonisation in July, while *Ranunculus acris*, flowering in May-June, has its lowest colonisation in June (Fig. 2.6). The percentage fraction of fine roots accounted for close to 10% of the variation in the %RLC in *Ranunculus acris,* but less than 3% in *Agrostis capillaris* (Table 2.7).

	Dependent	Independent	Statistics	R^2
Ranunculus acris	%RLC	%FR	$F_{1,184} = 20.702$; $p < 0.001$	0.096
Agrostis capillaris	%RLC	%FR	$F_{1,189} = 6.154$; $p = 0.014$	0.026

Table 2.7. The relationship between root colonisation and fraction of fine roots explained by linear regression.

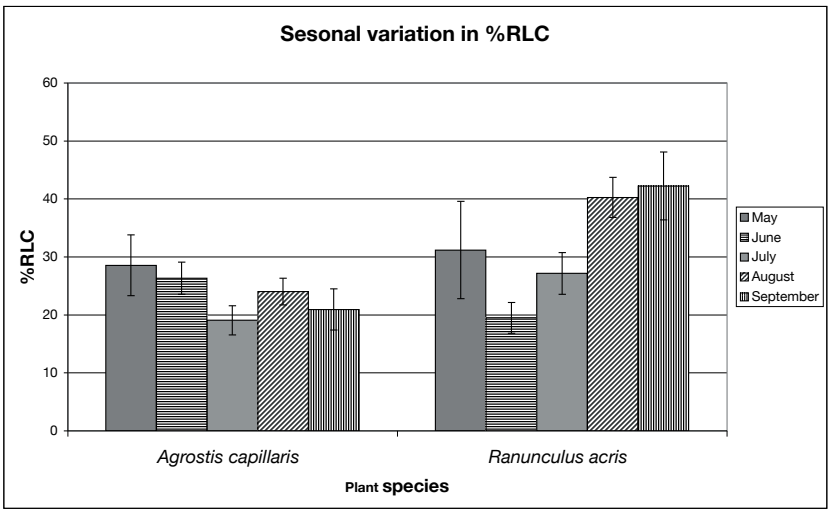


Fig. 2.6. The seasonal variation for %RLC for the two plant species. Values are means for the summer period 2001 and 2002 (n = 433) ±SE.

To find a statistically reliable suggestion for which parameters explain most of the %RLC variation, a stepwise regression was carried out (Table 2.8). In *Ranunculus acris*, temperature did not enter the regression model, but %FR and nutrients were the most important, explaining up to 34.5% of the variation. In *Agrostis capillaris,* temperature entered the regression model, but only after soil nitrogen and LOI. This might indicate that decomposition of organic nitrogen is of importance for the colonisation in particular in *Agrostis capillaris.* Soil nitrogen and LOI are closely correlated and might therefore both enter the model because of autocorrelation. However, if LOI is removed, only nitrogen enters the model for *Agrostis capillaris*.

Dependent	Plant species	Sample size	Stepwise Enter:	Statistics	R^2
%RLC (Square root transformed)	*Ranunculus acris*	N = 133	1: %FR	$F_{1,132} = 13.196$; $p < 0.001$	0.084
			2: %FR, P	$F_{2,131} = 11.751$; $p < 0.001$	0.139
			3: %FR, P, N	$F_{3,130} = 15.971$; $p < 0.001$	0.252
			4: %FR, P, N, H	$F_{4,129} = 16.550$; $p < 0.001$	0.319
			5: %FR, P, N, H, Ca	$F_{5,128} = 15.006$; $p < 0.001$	0.345
	Agrostis capillaris	N = 155	1: N	$F_{1,154} = 21.418$; $p < 0.001$	0.116
			2: N, LOI	$F_{2,153} = 14.669$; $p < 0.001$	0.150
			3: N, LOI, Temp	$F_{3,152} = 13.105$; $p < 0.001$	0.190
			4: N, LOI, Temp, H	$F_{4,151} = 12.547$; $p < 0.001$	0.230

Table 2.8. Stepwise regression with the %RLC (square root) as the dependent, and soil nutrients, soil acidity, temperature and %FR as variables.

Abbreviations: %FR = percentage intersections with fine roots, P = soil phosphate, N = soil nitrogen, H = exchangeable acidity, LOI = loss on ignition (carbon), Temp = mean temperature during the sampling month.

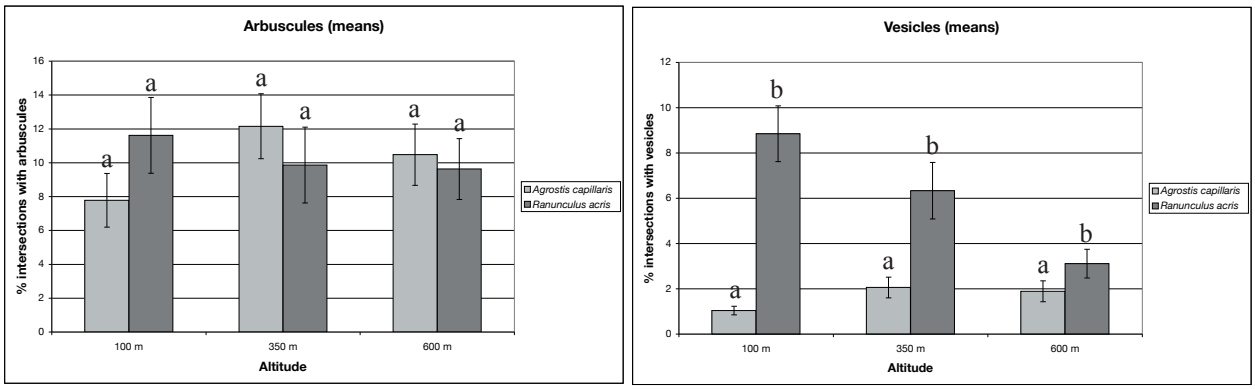


Fig. 2.7. The percentage intersections with either arbuscules or vesicles. Values are means (n = 407) ±SE.

Mycorrhizal structures

The mycorrhizal structures (arbuscules and vesicles) displayed different patterns in relation to plant species and their responses to the temperature related factors such as altitude (Fig. 2.7). Arbuscules showed little variation among species or altitudes, but vesicles were more abundant in roots of *Ranunculus acris* and in particular in *Ranunculus acris* from low altitude. When analysing both plant species together, there was a consistent negative correlation between arbuscules and temperature, while vesicles had a positive correlation with the soil phosphate concentration (Table 2.9). Taking into account the plant species, in *Ranunculus acris* there is a strong correlation between vesicles and soil phosphate, while in *Agrostis capillaris* there is a strong correlation between arbuscules and temperature. Soil phosphate increases at low altitude, where it is also warmer, and this might be responsible for the relatively weak correlation found between vesicles and temperature in *Ranunculus acris*.

	Both plant species		*Ranunculus acris*		*Agrostis capillaris*	
	Temp. N = 395	Phosphate N = 290	Temp. N = 195	Phosphate N = 140	Temp. N = 200	Phosphate N = 150
Arbuscules	r = -0.111 p = 0:027	n.s.	n.s.	n.s.	r = -0.222 p = 0.002	r = -0.175 p = 0.032
Vesicles	n.s.	r = 0.135 p = 0.022	r = 0.141 p = 0.050	r = 0.265 p = 0.002	r = -0.160; p = 0.023	r = -0.176 p = 0.031

Table 2.9. Correlations between the arbuscules and vesicles and mean monthly temperature (temp) and soil phosphate.

Molecular data

Only roots sampled on two sampling days in 2002 were used for molecular analysis (Table 2.3), and only for low (100 m a.s.l.) and high altitude (600 m a.s.l.). Samples were taken from all 4 locations used in this study. The samples were pooled, resulting in 14 samples for molecular analyses for each plant species, in total 28 samples. The amplification success-rate was low: only 15 samples amplified; of these, 10 were from *Agrostis capillaris* and only 5 from *Ranunculus acris*.

Only RFLP types with several occurrences were sequenced, using one clone from each type, site and date where possible. The number of clones used in this analysis was 137, 101 from *Agrostis capillaris* and 36 from *Ranunculus acris* (Table 2.10). There were insufficient data to allow analysis by sampling date.

The clones obtained from these samples represented 6 main types, from which sequences were obtained; a further 11 RFLP patterns were only found once; none of these were sequenced and they might therefore be undetected ascomycetes, since the AM2 primer that was used amplifies some ascomycetes. One RFLP type had 7 occurrences, of which 2 clones were sequenced, and they turned out to be different types, one that clusters distantly to the glomalean types (Type E2-RC, Fig. 2.9), and one that clusters outside all the glomalean types (E1-RC, Fig. 2.9). As the identity of these types is uncertain, they are not included in the diversity analysis performed on the data, but are included in Fig. 2.8 and Table 2.10 as 'Other' types.

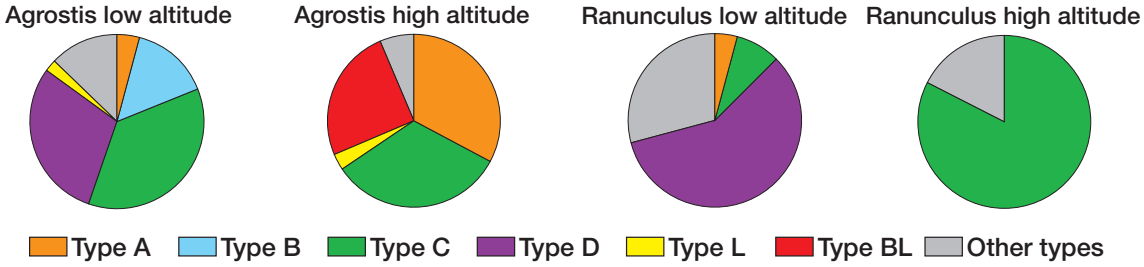


Fig. 2.8. The sequenced fungal types and their distribution according to plant species and altitude. The data are the number of clones shown in Table 2.10.

Number samples	Plant species		Type								Number clones
		Altitude	A	B	C	D	L	BL	Other	Shannon	
6	*Agrostis capillaris*	Low	2	7	17	14	1	0	6	1.27	47
4	*Agrostis capillaris*	High	21	0	21	0	2	16	4	1.20	64
2	*Ranunculus acris*	Low	1	0	2	14	0	0	7	0.58	24
3	*Ranunculus acris*	High	0	0	19	0	0	0	4	0.00	23

Table 2.10. The distribution of the main AM fungal types at low and high altitude. For the Shannon-Weiner calculations only the main types are used; 'Other' types were omitted.

The most frequent type found in this study is Type C, found in both plant species and at both altitudes. Type C accounted for 37% of the clones in Table 2.10. Type D was the second most frequent type, accounting for 18% of the total number of clones; this type is only found at low altitude where it accounted for 39% of the total clones. Type A was found at both low and high altitude and in both plant species, though most at high altitude and most in *Agrostis capillaris*. Type B was only found at low altitude, and Type BL only at high altitudes. Type B, BL and L were only found in *Agrostis capillaris*. The group "Other" includes 12 types of which 11 had only 1 occurrence each, distributed quite evenly between the samples; 1 type was found in 7 cloned fragments.

Of the clones, 70% were obtained from *Agrostis capillaris*, which also had the highest diversity (Table 2.10).

The different clone-types found in this study are presented in a phylogenetic tree (Fig. 2.9) together with named sequences obtained from identified spores submitted to Genebank by Schwarzott *et al.* (2001) and sequences submitted by members of the J.P.W. Young Laboratory at the University of York. According to the phylogenetic tree the most common type found in this study, Type C, is identical to Glo3, a *Glomus* type that has been found in almost every study, and has a worldwide distribution.

Type D also clusters within the genus *Glomus*, but forms a distinct group, together with one form of Type L. The other form of Type L is an *Acaulospora* type close to *Acaulospora laevis*. To be able to distinguish between the two types of L it would be necessary to use an additional restriction enzyme.

Type B clusters together with Glo9, which is another common type found to be most closely related to *Glomus hoi* though they are not identical (Helgason *et al*., 2002).

Two Acaulospora types are found, Type L and Type BL, while Type A clusters towards Archaeosporaceae.

Type E (E1-RC and E2-RC in Fig. 2.9) was not used in the analysis, because they are not the same fungal type, and E1-RL is unlikely to be a glomalean fungus. The number of cloned fragments from each site differs both because of the low amplification success rate, and due to the many clones that had not taken up a glomalean fragment. This caused an uneven number of cloned fragments per site, making it difficult to compare samples quantitatively. Therefore it was not possible to analyse for fungal types per sampling day. Instead, for each altitude and site the number of clones and the number of glomalean types for the two sampling days were pooled.

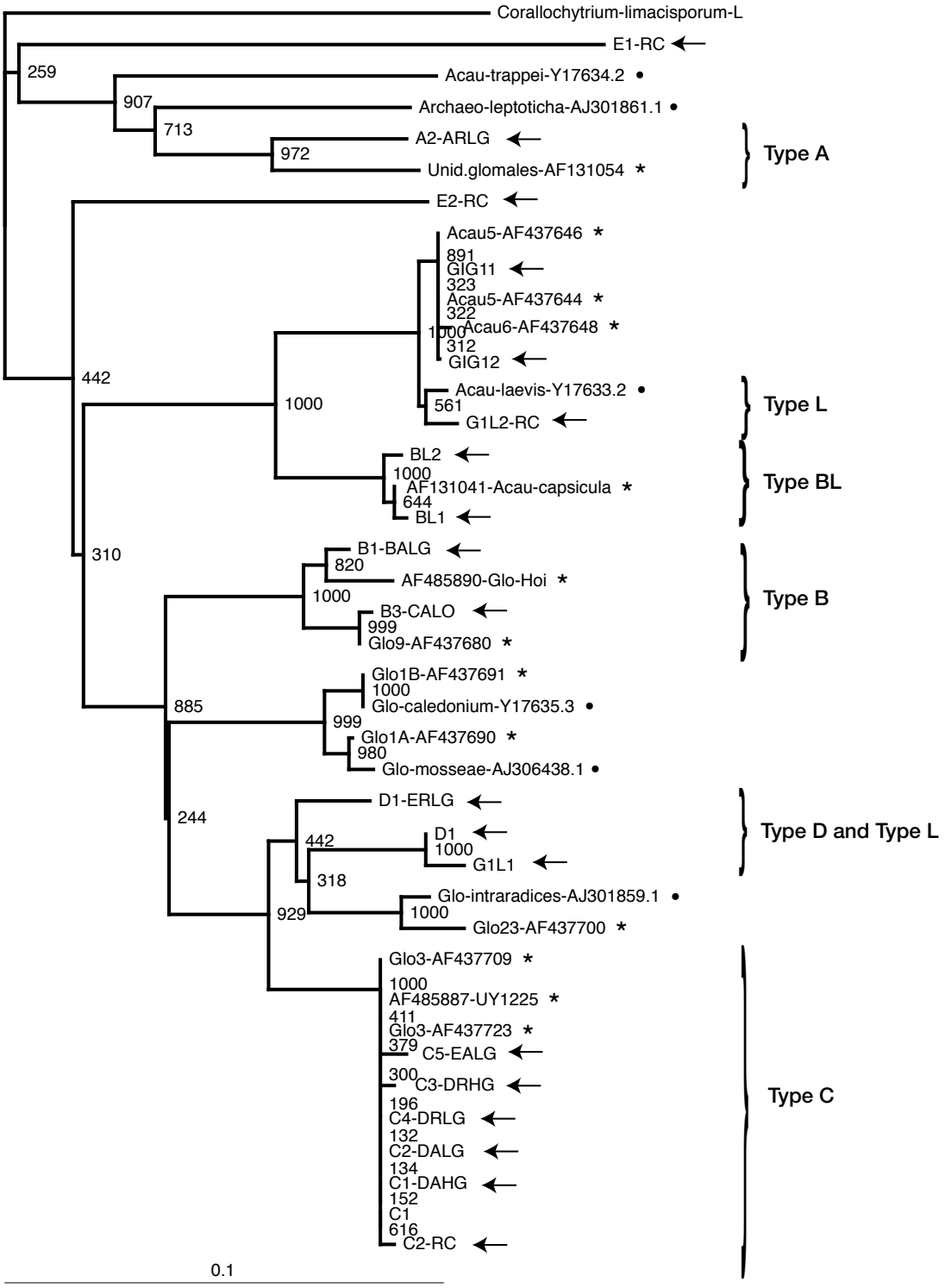


Fig. 2.9. Phylogenetic tree. The sequences found in this study combined with named sequenced submitted to Genebank (Schwarzott *et al.*, 2001) and sequences submitted by members of the J.P.W. Young lab at the University of York. Symbols: Arrows: Sequences from this study. • Named sequences obtained from identified spores from Schwarzott *et al.* (2001). * Sequences submitted by the mycorrhiza team in J.P.W.Young's lab. The Type signature indicates RFLP-types. The numbers at the nodes are bootstrapped values (1000 replicates).

To analyse the response of fungal diversity to temperature, the number of fungal types per number of cloned fragments was used as the diversity parameter. A stepwise regression suggested that the best temperature fit was the temperature range found on each site (based on mean monthly values) calculated as the difference between the mean monthly maximum and mean monthly minimum temperature for the period Jan 2001 to Sep 2002. This temperature measurement expresses the temperature variation that the sites experienced through the whole sampling period (Table 2.11). The relationship between these two factors explains 84% of the variation (Fig. 2.10).

Some of the temperature values used in Table 2.11 were obtained from extrapolated values, based on temperature loggings from another site from the same slope (Table 2.1). Two of the sites were based on a site from a considerably higher altitude, namely Gráfelli low altitude (300 m above the sampling site) and Ørvisfelli low altitude (200 m above the sampling site). These two sites were also those that had the worst fit in Fig. 2.10. By omitting these two sites, the fit was considerably improved ($R^2 = 0.989$).

A similar relationship would have been obtained by using Shannon-Weiner diversity indices, but as the Shannon-Weiner calculations give zero in samples with only one fungal type, only 31% of the fungal diversity would have been explained by the temperature range.

Site	Altitude	Temperature range (°C)	Number fungal types	Number clones	Number types / number clones
Gráfelli	Low	14.5	4 (7)	11 (20)	0.364
Gráfelli	High	11.3	1 (4)	10 (10)	0.100
Mosarøkur	Low	12.0	2 (3)	15 (16)	0.133
Mosarøkur	High	11.1	1 (3)	11 (13)	0.091
Sornfelli	Low	12.7	3 (3)	20 (20)	0.150
Sornfelli	High	10.8	3 (7)	35 (41)	0.081
Ørvisfelli	Low	12.3	3 (5)	12 (14)	0.250
Ørvisfelli	High	10.0	1 (1)	21 (21)	0.048

Table 2.11. Diversity index. Only the six main types are included, and the fungal types from both plant species are included. The low altitude sites have the highest temperature range, and in general also most fungal types. The numbers in brackets under "Number fungal types" and "Number clones" indicate the total number of types or clones, as well as those where no sequence is obtained, and which only occurred once or twice; these numbers were not used when calculating diversity.

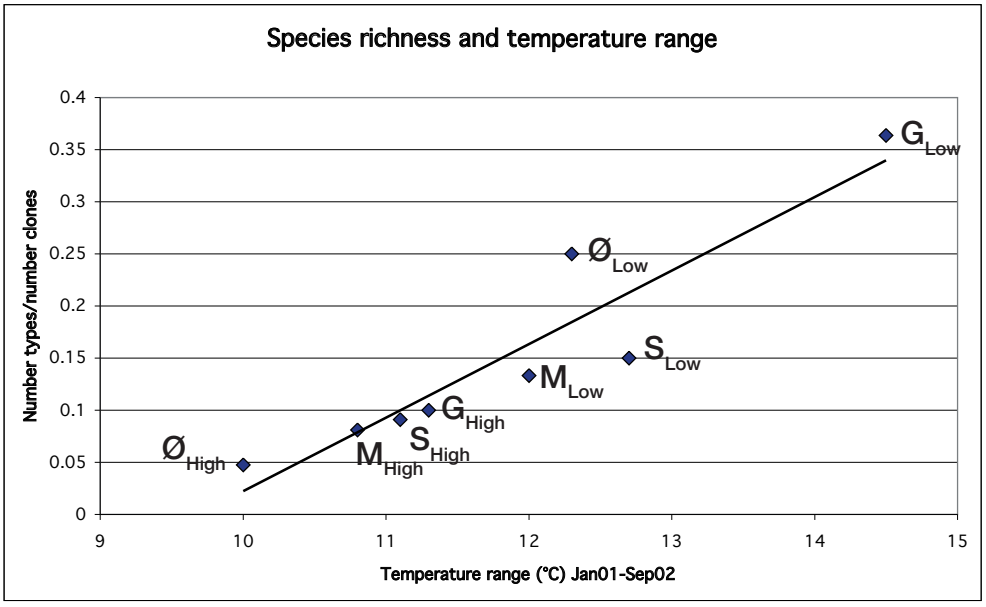


Fig. 2.10. The species richness was positively and significantly correlated to the range of temperature (°C) measured on site from January 2001 to September 2002. The values used in this graph are from Table 2.11. "Number types/number clones" is the number of AM fungal types divided by the number of clones. Statistics: Regression $F_{1,6} = 37.56$; $p < 0.001$; $R^2 = 0.839$.

Sørensen's similarity indices were used to estimate the similarity between fungal communities in roots of *Agrostis capillaris* or *Ranunculus acris*. The values found were rather low, approximately 0.4 (Table 2.12), indicating large variation within host plants. The lowest similarity was found in *Agrostis capillaris* from high altitude, and the highest in *Ranunculus acris* at high altitude.

	Alti-tude	*Agrostis capillaris*		*Ranunculus acris*	
		Low altitude	High altitude	Low altitude	High altitude
Agrostis capillaris	Low	0.42 ±0.085	0.30 ±0.069	0.43 ±0.082	0.44 ±0.066
	High		0.19 ±0.125		0.42 ±0.131
Ranunculus acris	Low			0.50 ±*)	0.25 ±0.112
	High				1.00 ±0.000

Table 2.12. Sørensen's similarity values showing the similarity between samples origin from *Agrostis capillaris* **Agrostis capillaris*, *Ranunculus acris* * *Ranunculus acris* and *Agrostis capillaris* **Ranunculus acris* in combination with low and high altitude. Only the main RFLP types are used in the calculations. *) Only two samples from *Ranunculus acris* at low altitude amplified. Values are means (n = 105) ±SE.

The Sørensen similarity indices indicated that the fungal types colonising the two plant species are similar; however, as Sørensen's similarity is based upon presence/absence values the extent of colonisation of each type is not taken into account.

As the frequency of the fungal types found in this study varied with plant species, altitude, and aspect, an hierarchical loglinear analysis was carried out. Log-linear modelling has previously been used with similar data (e.g. Helgason *et al.*, 1999). The models suggested by the hierarchical loglinear analysis shown in Table 2.13 have a very high probability (> 0.9) for a good fit. For the individual combinations, the partial chi-square values shown in Table 2.14 might be more informative. None of the factors used as single factors give a good fit, nor do any of the two-way interactions. However, two of the three-way interactions had a very good fit. In both cases, it was the interaction Plant*Type in combination with either Aspect or Altitude. Type*Altitude*Aspect was not significant, indicating that the Plant factor is crucial for the model. Thus, loglinear analysis suggests that the pattern of fungal types in the two plant species differ, but that it is in interaction with the habitat.

Backwards eliminations	Factors included	df	Likelihood ratio chi-square	Probability
Step 1	Plant*Altitude*Type Plant*Altitude*Aspect Plant*Type*Aspect Altitude*Type*Aspect	4	0.00007	1.000
Step 2	Plant*Altitude*Aspect Plant*Type*Aspect Altitude*Type*Aspect	8	0.00757	1.000
Step 3	Plant*Altitude*Aspect Altitude*Type*Aspect Plant*Type	12	5.93957	0.919

Table 2.13. Hierarchical loglinear analysis showing models of the interactions between the factors 'plant species', 'altitude', 'fungal type' and 'aspect'. Saturated model: Plant * Altitude * Type * Aspect. Type stands for Fungal types and is the factor in question, therefore combinations without the Type factor are not relevant for this study. The best fit of factors interacting for each step is not used in the following step. The data used in this loglinear analysis were those presented in Table 2.10 with aspect as an additional factor. Type L had too few occurrences, and did not enter the analysis. 'Other' types were not included.

Factor	df	Partial Chi-sq.	Probability
Plant*Type*Altitude	4	0.008	1.0000
Plant*Type*Aspec	4	0.486	0.9748
Plant*Altitude*Aspect	1	2.577	0.1084
Altitude	1	2.996	0.0835
Altitude*Aspect	1	3.674	0.0553
Aspect*Type	4	11.135	0.0251
Altitude*Type*Aspect	4	13.997	0.0073
Plant*Altitude	1	8.608	0.0033
Aspect	1	12.124	0.0005
Plant*Type	4	23.228	0.0001
Plant*Aspect	1	25.346	< 0.0001
Altitude*Type	4	94.376	< 0.0001
Plant	1	29.809	< 0.0001
Type	4	54.973	< 0.0001

Table 2.14. Partial chi-square values from the loglinear analysis in Table 2.13.

Discussion

The expectations were that the diversity of the AM fungal community would be greatest where the temperature range of the habitat is greatest. The highest temperature range was found at low altitude, as was the highest fungal diversity. Low altitude seems to have more nSSU rDNA sequence types than high altitudes. Further, it is very likely that the number of AM fungal types increases with increased temperature range, according to Fig. 2.10. This could be explained by niche differentiation of co-existing fungal types in response to temperature. Different fungal types might have different temperature optima and therefore a site with a wider temperature range provides more niches for temperature sensitive fungal types than a site with more even temperature. The highest temperature range is at low altitude (Table 2.11). The highest plant diversity was at 250 m altitude, with a decrease both to low altitude sites and to high altitude sites (Fig. 1.6; Fosaa, 2004b). The plant species richness therefore was quite similar for the low altitude sites and the high altitude sites. Several studies, e.g. van der Heijden *et al.* (1998) and Bever *et al.* (2001), have found a link between high plant diversity and high fungal diversity, but as the plant diversity was not significantly different at either of the two altitudes the higher fungal species

richness found to be related to greater temperature range should not be due to plant diversity.

Many habitats typically support at least 10-20 and often as many as 40 distinct fungal types (Fitter *et. al.,* 2004). In total, this study found 20 fungal types, but only 7 of these showed up more than once or twice, and were identified with sequencing. The maximum number of types found on a single site was 7, of which 4 were main types (Table 2.11). Other studies have found 19-30 AM fungal types when analysing around 50 roots (Vandenkoornhuyse *et al.*, 2002; Husband *et al.*, 2002, Öpik *et al.*, 2003). The analysis here is based on only 15 samples, and thus the sampling intensity is low compared with the three studies mentioned above. The sampling intensity is more like that of Helgason *et al.* (1999), which, from a similar number of clones as used in this study, identified 8-10 AM fungal types. This indicates that the Faroese sub-arctic system might support the same number of AM fungal types as do, for example, a typical English site.

Whether cold sites have specialised mycorrhizal fungi cannot be determined by this study. The high altitude sites, which in general are the coldest, do in fact have types that group quite nicely with fungi found on other warmer places, e.g. Type C (Glo 3), Type BL (similar to *Acaulospora capsicula*), Acau5 (distant to *Acaulospora laevis*). On low altitude sites, one type is found that groups into a distinct group, Type D (a Glomus type). It is common to find "new" types in studies analysing nSSU sequences. For example, Vandenkoornhuyse *et al.* (2002) found 16 types unique to a study analysing a temperate grassland, Husband *et al.* (2002), comparing fungal types in a tropical forest with temperate field studies, found 19 unique types, and Öpik *et al.* (2003) found 9 novel AM fungal sequence groups from Estonian grassland and boreal forest sites. These unique types probably only indicate that there are a multitude of AM fungi to be identified, and that with a higher sampling intensity more AM fungal types will be revealed.

In this study the AM fungal types were identified by sequencing after a preliminary identification by RFLP patterns. Such strategies are tedious, expensive and poorly suited to the analysis of multiple samples (Kowalchuk *et al.*, 2002). Although informative, this approach requires an extensive analysis of large numbers of clones (Muyzer *et al.,* 1993), and a higher number of successful clones would have improved the data presented in this study considerable. A method of avoiding bias due to the cloning step could have been to use denaturing gradient gel electrophoresis (DGGE) before the cloning step, eliminating the need of a substantial number of clones, as demonstrated by Muyzer *et al.* (1993). This

approach has also been used within AM research (e.g. Kowalchuck *et al.*, 2002; Öpik *et al.*, 2003; de Souza *et al.*, 2004).

Öpik et al. (2003) detected one to four different DGGE bands from a typical root sample, indicating different mobility. They then extracted PCR-products from the DGGE bands and found, by using cloning and RFLP, that in a single DGGE band there could be multiple RFLP types.

The two plant species differed in mycorrhizal response. While *Agrostis capillaris* responded to nitrogen, *Ranunculus acris* responded to soil phosphate but also to nitrogen (Table 2.8). A fine root system as that of *Agrostis capillaris* might, relative to a coarse root system as that of *Ranunculus acris,* indicate that a plant is less dependent upon mycorrhizal colonisation for acquiring phosphate (Fitter and Moyersoen, 1996; Fitter and Merryweather, 1992). From this it could be the case that *Ranunculus acris* is more dependent upon the mycorrhizal symbiont than *Agrostis capillaris* for nutrient uptake, though *Agrostis capillaris* might gain other benefits from the symbiosis, for example protection against pathogens (Newsham *et al.*, 1995a).

The annual variation might be due to temperature, as there was a 2°C difference in mean summer temperature between the two years. The altitudinal difference might be explained by temperature, but also nutrient availability. The variation found due to aspect is probably a nutrient related response, since there were no significant temperature differences.

Though the temperature data are based upon a mixed dataset of measurements, they are interpolated from data measured on the site. This is not an unusual approach. The literature specifically on interpolation of soil temperature is sparse, though many papers compare methods for estimating temperature and other climatological parameters (Barringer and Lilburne, 2000). Both regional and local effects have an effect on air temperature together with elevation, and Monestiez *et al.* (2001) constructed a model where they were able to quite accurately to predict the temperature on a site; this model was based on data from weather stations in combination with categorical environmental classes. Fu and Rich (2002) compared results from an insolation-modified temperature model with field measurements of soil temperature. Also they had problems with sensors dug up by animals or bad connections, and therefore they had fewer functioning sensors than usually considered as a statistically sufficient samle size. Fu and Rich (2002) formulated regressions to predict soil temperature as a function of elevation and insolation. Accuracy of climatic data is important (Monestiez *et al.,* 2001), but there is no one best method, and the choice of interpolation method is very data dependent (Barringer and Lilburne, 2000).

On its own, the mean temperature explained 8.5% of the variation in the %RLC in *Agrostis capillaris* (Fig. 2.5). In combination with soil nutrients, however, 23% was explained in *Agrostis capillaris.* The important nutrients seem to be nitrogen and carbon, and could therefore indicate that it is the mineralisation rate that determines the dependency of the mycorrhizal symbiosis. The soil content of nitrogen and carbon (Table 2.5) might appear high, but they were within the same range as found in alpine grasslands (Körner, 1999, p. 151).

It is known some ecto- and ericoid mycorrhizal fungi are able to degrade organic compounds and hence provide the host with nutrients (Read and Perez-Moreno, 2003), by using extracellular acid proteinase to extract amino acids from proteins and then use the amono acids as nitrogen sources (Smith and Read, 1997, p. 259-275). As there was a good correlation between nitrogen and loss of ignition this could be one source of nutrients in these sub-arctic soils, and indeed a few studies have suggested that some AM fungi appear to be able to acquire otherwise unavailable nutrients from organic compounds (Hodge *et al.*, 2001; Koide and Kabir, 2000). However, as no saprotrophic capabilites have been reported for AM fungi, it seems unlikely that the fungus itself was involved directly in organic matter decomposition (Hodge, 2006). Hodge (2003a) found that plant nitrogen capture from organic material was not related to either internal or external mycorrhizal parameters, and that the response of roots was more important in nitrogen capture from the organic material than that of the fungal partner.

The uptake by AM fungal hyphae of soil organic nitrogen is probably in inorganic form (Jin *et al.*, 2005; Govindarajulu *et al.*, 2005) and therefore AM fungal hyphae, plant roots and the rest of the microbial community compete for the available nitrogen (Hodge, 2003b). Under such circumstances the mycorrhizal plants might have an extra advantage in the competition as they can take nutrients up both directly and by acquiring nutrients from the symbiotic fungi. Some studies have indicated that some plants are able to take up nitrogen from organic sources (Lipson and Monson, 1998; Streeter *et al.*, 2000). In a study testing for direct uptake of glycine by dominant grasses Streeter *et al.* (2000) used dual labelled glycine (^{13}C-^{15}N) and found ^{13}C in shoot material indicating that a quantity of the added glycine had bypassed microbial mineralisation.

Dark septate fungal endophytes (DSE), which often are found to colonise roots from alpine and arctic sites (Read and Haselwandter, 1981), might also influence nutrient capture. DSE were found at all sites and altitudes in this study, colonising the same roots as the AM fungi (data not shown). Several different enzymatic activities have been detected in DSE, and studies suggest that some

strains of DSE may be involved in host nutrient acquisition (Jumpponen and Trappe, 1998); in particular DSE have been shown to be capable of increasing host P concentration under some experimental conditions (Jumpponen, 2001).

In a study accessing the impact of temperature on the uptake and assimilation of nitrogen, the grass *Lolium perenne* was found to take up relatively more ammonium than nitrate or glycine, in particular when grown at low temperature (11°C) rather than high temperature (21°C). Further, that if both nitrate, ammonium and glycine were available, then the relative uptake of ammonium was still the highest, while the proportions of nitrate and glycine were more alike (Thornton and Robinson, 2005). Commonly ammonium nitrogen dominates the soil solution in organic and acid alpine soils, but evidence is accumulating that soluble organic nitrogen is potentially even more important in cold soils than either ammonium or nitrate (Körner, 1999, p. 152).

It is increasingly acknowledged that the AM symbiosis is responsive to both phosphate and nitrogen. If both phosphate and nitrogen are sufficient in the medium, AM root colonisation is suppressed, but no suppression occurs if either of these two nutrients is limited (Blanke *et al.*, 2005; Corkidi *et al.*, 2002; Sylvia and Neal, 1990).

Thus findings from the literature might suggest that increased nitrogen uptake might create an increased demand for more phosphate and hence an increased AM root colonisation. Further it is clear that the disolved organic nitrogen is an important part of most soils, although direct proof that plant nitrogen uptake from organic nitrogen constitutes a major ecosystem flux is still lacking (Jones *et al.*, 2005).

The data support the hypothesis that plants from a single habitat that differ in mycorrhizal dependency would be colonised by distinct fungal communities. The data shown in Fig. 2.8 and Table 2.10 indicate that some fungal types might have preferences towards one plant species, while other fungal types might be sensitive to altitude. This was supported by the model suggested by loglinear analysis: Removing the plant species from the model generated a much worse fit, though a good fit also required a factor representing the habitat. This finding is another indication that the earlier view that any AM fungus would colonise any plant needs to be reconsidered, and that the fungal community probably is a reflection of plant community, nutrient availability, temperature, as well as other biotic and abiotic factors.

To conclude, the communities of AM fungi found in thermally distinct environments in the Faroe Islands are different, especially at high altitude (cold

sites) few fungal types were found. The diversity of the AM fungal community was greatest where the thermal heterogeneity of the habitat was greatest, which was in the lowland. Plants from a single habitat that differ in mycorrhizal dependency were colonised by distinct fungal communities, because the benefits they acquire from colonisation differ.

Chapter 3

The International Tundra Experiment – the arbuscular mycorrhizal component

Abstract

- A high altitude experiment was set up in 2001 to test whether it was possible to measure a direct temperature impact on the fungi in the field using open-top chambers (OTC). The experiment was set up inside an enclosure, which introduced an additional factor: sheep were excluded from the fenced areas, although other herbivores, such as hares, did have access. There were, therefore, three treatments: grazed plots outside the fence; ungrazed plots inside the fence; and the open top chambers inside the fence.
- Roots from *Agrostis capillaris* were examined for arbuscular mycorrhizal (AM) colonisation. Although there was a limited response in percent root length colonisation (%RLC) to the OTCs, they had a visible impact on plant growth, as the plants increased in size. Temperature logging carried out during one year indicated that the soil temperature was not significantly affected by the OTC.
- The Shannon-Weiner AM fungal diversity, estimated from T-RFLP profiles, was highest in grazed plots and lowest in the OTCs. No clear pattern in the T-RFLP profiles was found due to either treatment or seasonal changes.
- The fraction of fine roots (%FR) was negatively correlated with the minimum temperature measured 7-14 days prior to sampling as was the %RLC to a lesser extent. There was a significant positive correlation for %RLC and for %FR. This could mean that the response observed in the %RLC for temperature is due to the response of fine roots rather than a fungal response.

Introduction

Atmospheric CO_2 concentration has increased by 31% since 1750 (Houghton *et al.*, 2001). One consequence has been an increase in global temperature, which, over the 20th century, has increased by about 0.6°C. This temperature increase is expected to continue. Some have predicted an increase from 1.4°C to 5.8°C over the period 1990 to 2100 (Houghton *et al.,* 2001). Both CO_2 and temperature can impact plant growth. Elevated CO_2 can make plants grow faster and make them change the allocation of carbon (Staddon *et al.*, 2002). Elevated temperature can not only directly affect the plant by altering root growth, morphology and nutrient uptake kinetics, but also indirectly by altering the rates of decomposition and nutrient mineralisation (Pregitzer and King, 2005). Here the main focus is not on plant responses to climate change, but rather on the impact of climate change on symbiotic arbuscular mycorrhizal (AM) fungi. All manipulations or changes that affect one part of the symbiosis are likely to also affect the other part. Thus, it can be difficult to distinguish between whether an effect observed on AM fungi might be due to a plant, rather than a fungal, response. For example, altered plant growth might change the plant nutrient demand, which in turn can affect mycorrhizal dependency.

All organisms have temperature optima for growth, often due to enzymatic activity, and therefore temperature will have direct effects on mycorrhizal fungi. However, Staddon *et al*. (2002) suggested, with reference to the impact of elevated CO_2 on the mycorrhizal fungi, that we may find the effect of temperature on mycorrhizas in natural ecosystems to be, in the main, due to temperature induced changes to plant communities rather than a direct AM fungal response to temperature.

There have been several attempts to estimate the impact of changed temperature on AM fungi, mainly in laboratory experiments (Hayman, 1974; Schenck and Smith, 1982; Borges and Caney, 1989; Staddon *et al*., 2004; Heinemeyer and Fitter, 2004; Gavito *et al*., 2003 and 2005). Most of the laboratory experiments used cultured fungal types, known to be able to grow at room temperature (20-25°C).

To assess the effect of temperature change on natural fungal communities other approaches have been used, for example, the natural change in temperature using altitudinal gradients (Väre *et al*., 1997; Ruotsalainen *et al.*, 2004). Mycorrhizal researchers seem not to have used transplant experiments along altitudinal gradients, but some have brought field samples into a greenhouse or growth chamber (Monz *et al.*, 1994). Another approach is artificially to warm the soil or site.

Rillig *et al.* (2002b) used heaters, warming both the canopy and the soil surface by 1-2°C. In comparison, Heinemeyer *et al*. (2003) and Edwards *et al.* (2004) used a heated grid to artificially change the soil temperature alone by a constant 2.7°C above ambient at 2 cm depth with no temperature effect on the canopy. Staddon *et al.* (2003b) also used heating cables, elevating the winter temperature only by 3°C above ambient.

All these active methods of warming soil in the field require electricity and would therefore be difficult to use at a high altitude site with extreme wind conditions. For the purpose of this study, a passive open-top chamber (OTC) is almost the only option, although it has disadvantages: little or no temperature control, altered wind and humidity, and a small treatment area (Shaver *et al*., 2000).

The Faroe Islands participate in the International Tundra Experiment (ITEX). ITEX measures the responses of major circumpolar vascular plant species to climate variations and environmental manipulations, using standard protocols. The manipulations involve passive warming of tundra plots above the average growing season temperature by 1-3°C in open-top chambers (OTC) (Marion *et al.*, 1997), and the purpose is to increase the temperature of both air and soil. The Faroese ITEX site was therefore chosen as a means of testing the response of AM fungi to elevated temperature in an extreme environment.

By choosing a high altitude site, the hypothesis that in cold environments fungi are adapted to low temperatures could be tested. The object was to determine whether i) the fungal community changed in warmed plots, and ii) the decline in root colonisation observed in the fieldwork at warmer sites was also found in warmed plots (Chapter 2). Since the OTCs were protected from sheep within a fenced enclosure, two controls were needed: (i) ungrazed, unwarmed sites and (ii) grazed, unwarmed sites.

However, the successful temperature logging from 2003 revealed that the gain in soil temperature obtained by the OTC warming treatment in the Faroes was more or less negligible, even though a seasonal difference in temperature was observed. Further, there was a very profound effect from the fencing, in addition to the shelter that the OTC obviously provided for the plants, as they grew bigger than those plants outside the fencing.

These factors or observations give the opportunity to test the following questions:

- Are %RLC responses to temperature due to plant responses to temperature or does temperature affect the fungal community and the colonisation pattern?

- Is it the minimum, maximum or mean temperature or the range of temperature that is important, and what time interval before sampling is important?
- Does grazing by sheep change the fungal community and the colonisation pattern?

Materials and Methods

Description of ITEX

The Faroese ITEX station was established in the autumn of 2001 and is placed inside an enclosure that was established in 2000. The enclosure is required to resist the combination of extreme winds and sheep. Sheep are curious animals and would damage the wires needed for the ITEX hexagons to withstand the extreme winds. The ITEX station is located at 600 m a.s.l. In 2000 another enclosure was established at 70 m a.s.l., but without OTCs,

Ten hexagons were constructed according to the ITEX manual (Molau and Mølgaard, 1996), and located together with eight control plots inside the enclosure (Fig. 3.1). The vegetation plots were 50x50 cm.

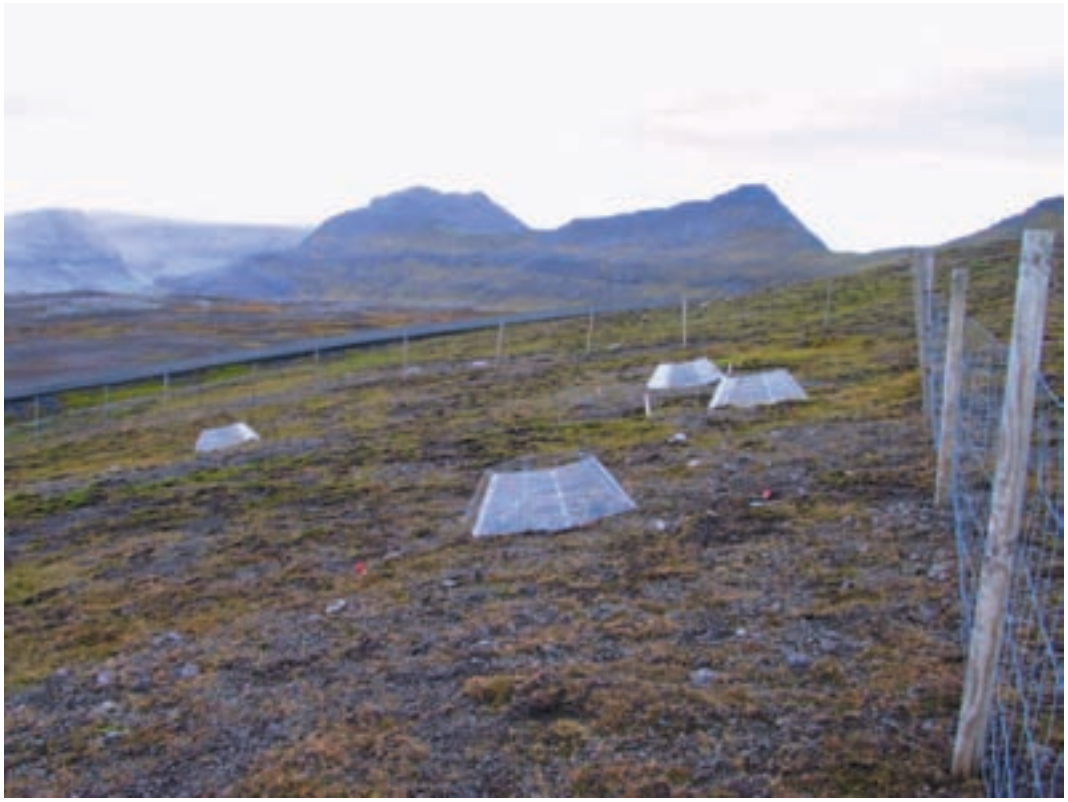


Fig. 3.1. The ITEX hexagons. The design of the site was so that there were 3 treatments: Grazing – outside the fence; Enclosure – inside the fence; OTC – inside the hexagons.

Original in colour

Light-intensity, CO_2 and ITEX

Some studies have found that temperature-enhancing systems decrease light quantity and quality, and thus reduce photosynthesis (Debevec and MacLean, 1993; Havström *et al.*, 1993; Wookey *et al.*, 1993). These studies have mainly measured the PAR in plastic tents and greenhouses. However, according to Marion *et al.* (1997), the OTC allow more direct solar radiation to the plants than complete enclosures, and should not be as adversely affected by altered light.

The CO_2 content is also potentially affected by the degree of enclosure. However, Marion *et al.* (1997) claimed that the evidence to date indicated no significant difference in CO_2 concentrations between controls and chambers.

On the other hand relative air humidity is typically lower inside chambers compared with ambient air, but the magnitude of this effect suggests that the decline is largely caused by increased temperature (Marion *et al.,* 1997). The humidity values mentioned by Marion *et al.* (1997) are in the range of 53-84%. Typical Faroese values are from 80-98%.

Precipitation, sunshine and temperature in 2002 and 2003

According to measurements from the Danish Meteorological Institute measured in Tórshavn (15 km south from the ITEX site, at 50 m a.s.l.), the precipitation in July/August for the years 2002 and 2003 was quite normal, with 2002 a little drier than 2003. The accumulated monthly hours of sunshine in Tórshavn was higher in 2002, but the mean annual temperature was around 0.5°C higher in 2003. The mean temperature in July and August was around 2°C higher in 2003 than in 2002 (Cappelen and Jørgensen, 2003 and 2004).

Soil available phosphate and humidity

Soil cores (2.5 cm diameter, 5 cm deep) were on 17 May 2004 taken pairwise inside and outside the OTCs (total 18 samples), and pairwise inside and outside the fence (total 12 samples). Water soluble phosphate was extracted by shaking 1 g soil in 20 ml deionised water for 30 min., then assayed by the molybdenum blue method (Allen, 1974). Soil moisture content was calculated as the mean of three measurements of the volumetric moisture content measured with a ThetaMeter type HH1 and ThetaProbe Type ML2X from Delta-T Devices Ltd., Cambridge, UK.

Temperature logging

Data loggers from Tinytags (Tinytags *Plus* from Gemini Data Loggers, Chichester, UK) measured the soil temperature 1 cm below the surface every hour from

late May 2003 to the middle of March 2004. The final temperature measurements consist of the mean of 3 loggers inside the OTCs, and the mean of 2 loggers outside the OTCs. (The starting point was 10 loggers arranged pairwise outside and 10 inside, but not all measurements were available during the summer period). Measurements outside the OTC and outside the enclosure were assumed to be similar, but there might have been a slight difference, as the vegetation cover in 2005 was found to have increased by 14% in the enclosure compared with the year 2001 when the experiment was established. In the OTCs, the vegetation cover had increased by 28% (Anna Maria Fosaa, unpublished data).

The temperature-logging data for the summer period 2002 are very incomplete, as all the flexes were cut, probably by hares. Therefore, they are not included in the analysis.

Sampling method

Three small soil cores with *Agrostis capillaris* were sampled randomly from each treatment: Grazing (outside the fence), Enclosure (inside the fence), OTC (inside the open-top chambers), in total 9 samples from each sampling date. There were three sampling dates each year (2002: July 1st, July 22nd and Aug. 11th; 2003: July 21st, Aug. 7th and Aug. 24th); the total number of samples per year was 27.

The roots were washed with tap water and then divided in two: one-half was used to measure the %RLC, as described in the previous chapter, while the other half was used for molecular work. The latter was dried and stored at room temperature (2002) or frozen (2003) until extraction. In 2002, samples for molecular analysis were only taken on July 22nd and Aug. 11th.

Percent root length colonisation

The roots were cleared, stained and the root colonisation recorded following exactly the same methods as in Chapter 2.

Molecular analyses

DNA extraction: The roots were ground to fine powder in liquid nitrogen. The DNA extracts from 2002 were obtained by the CTAB method (Gardes and Bruns, 1993), while the DNA from the samples from 2003 was extracted using the DNeasy Plant Mini Kit (Qiagen, Valentia, California, US) according to the manufacturer's protocol. The three root samples from each treatment from 2002 were pooled for each sampling day, providing only one DNA extract per treatment per sampling day, in total 6 DNA extracts from 2002. The DNA from the roots from 2003 were extracted individually, providing 27 DNA extracts.

PCR: The genes amplified with PCR were those that define the nSSU ribosomal RNA (rRNA). The samples were amplified using the Multiplex PCR Kit (Qiagen, Valentia, California, US), 5 pmol of unlabelled primer AM1 (Helgason *et al.*, 1998) and 5 pmol of labelled NS31 (Simon *et al.*, 1992). Q-solution (Qiagen, Valentia, California, US), a reagent modifying the melting behaviour of DNA, was added according to the manufacturer's protocol. Amplification on a PTC-200 (the DNA engine cycler developed by MJ Research, now Bio-Rad Laboratories, Hercules, California, US), 95°C for 15 min., 40 cycles with 94°C for 30 sec., 58°C for 90 sec. and 72°C for 90 sec. The final extension was 72°C for 10 min. All 6 DNA extracts from 2002 were successfully amplified, while of the 27 DNA extracts from 2003, only 25 extracts were successfully amplified.

T-RFLP: Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis is a quantitative molecular technique assessing microbial community diversity (Liu *et al.,* 1997). Fluorescently end-labelled PCR product were digested with *Hin*fI and then separated by gel electrophoresis and detected on a CEQ8000 automated sequence analyser (Beckman Coulter, Fullterton, California, US) at the Technology Facility at the University of York. The fragment length was analysed with Beckman Coulter CEQ8000 software. Though the peak area in theory should be an estimate of the relative abundance of fungal types with a restriction site providing fragments with corresponding length to where the peak is located, other studies have shown that these values can be variable among replicates (Vandenkoornhuyse *et al.*, 2003; Johnson *et al.*, 2003). Therefore, Vandenkoornhuyse *et al.* (2003) and Johnson *et al.* (2003) used presence/absence values for the fungal abundance. It also turned out that the peak site was not absolute, but could differ with a few base pairs within analysis in a single run, and with several (up to 5-6) base pairs between different runs, making it difficult to compare between samples. This is a known phenomenon using this technique, and makes it quite impossible to attribute a particular fragment to a taxon (Thorunn Helgason, pers. comm.). Therefore, the analysis of the T-RFLP-fragments has focused on statistical analysis allowing usage of presence/absence values, such as Sørensen's similarity indices, Shannon-Weiner diversity indices based on presence/absence values per treatment and year, and hierarchical cluster analysis.

Statistics

All statistical analyses were carried out using SPSS version 11.03 for Mac OS X. Pearson's correlations were used when not otherwise stated. To determine

which temperatures were the most significant, stepwise regression was used. The entry/removal criteria for stepwise regression was the probability of F (enter 0.05, removal 0.1). All R^2 values are adjusted. For post-hoc tests the Bonferroni test was used.

The sampling period in the statistical analysis was divided into weeks (1-52 per year).

Diversity tests used were Shannon-Weiner and species richness. Shannon-Weiner was calculated by the formula:

$$H_0 = -\Sigma p_i \ln p_i$$

where p_i in this case was used as the frequency of the different T-RFLP peaks per treatment.

The input data for the hierarchical cluster analysis was the presence/absence values of the T-RFLP peaks from 2003 only. The samples from 2002 differed considerably from 2003, but that might be an artefact as the T-RFLP results stem from two different runs. As only 6 samples were available for 2002, and no on-site temperature data as well, the 2002 data were omitted from the final analysis. The cluster method was Ward's, using binary squared Euclidean distance measurements. To find the parameters that explained the clusters, categorical regression was used. Categorical regression extends the approach of standard linear regression by simultaneously scaling nominal, ordinal and numerical variables. The procedure quantifies categorical variables such that the quantifications reflect characteristics of the original categories (Meulman and Heiser, 2001). The hierarchical cluster analysis used only the T-RFLP profiles for the dendrogram, so the cluster analysis and the categorical regression are independent of each other.

Results

Soil temperature, soil phosphate, soil humidity, and plant responses

Temperature logging from June 2003 to February 2004 showed that the difference produced by the OTC was small but consistent, between 0.2 and 0.3°C (Fig. 3.2). In high summer (July – August) the difference was marginally significant ($F_{1,1486}$ = 3.572; 0.059). The most profound effect of the OTC is a decrease in the soil temperature range in July 2003.

However, the mean monthly temperature variation during June-August 2003 was nearly 3°C (Fig. 3.2a), and the differences between maximum and minimum temperature was greatest in June-July (Fig. 3.2b). The difference in mean monthly temperature per year was around 2°C (Cappelen and Jørgensen,

2003 and 2004). Thus, the seasonal temperature variation is 10 times that achieved using the OTC, and in comparison the warming effect of the OTC is negligible. The monthly temperature range in June and July 2003 was higher outside the OTC; this might indicate a neutralising effect of the hexagons.

The temperature probes were located 1 cm below the surface, while the root material sampled was mainly between the surface and to a depth of 5 cm. No temperature measurements were taken from a depth of 5 cm at this site, but from other sites in the Faroes there are indications that the temperature in general is 0.5-0.8°C warmer at a depth of 5 cm than at 1 cm and that the temperature range is greater at 1 cm than at 5 cm below the surface (E. Olsen, unpublished data).

Although no vegetational data were analysed, the plants were visibly larger inside the hexagons than outside, providing more photosynthetic area, but also requiring more nutrients, an effect that might have been due to an increase in air temperature or directly to sheltering from the wind.

Water-soluble phosphate and soil moisture content were measured in May 2004. There was no significant response, however, to either grazing or OTCs, and there was no change in soil moisture by the treatments or any correlation between phosphate and moisture content. The mean water-soluble phosphate concentration was 0.8 µg g^{-1} dried soil, while the mean soil moisture that particular day was 31% (vol).

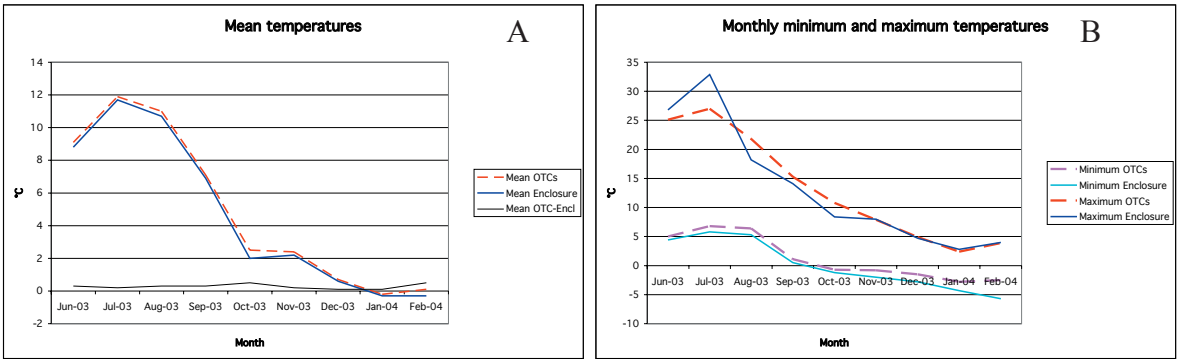


Fig. 3.2. Mean monthly temperature values based upon hourly measurements from June 2003 to Feb 2004. All measurements are taken 1 cm below the vegetation. Fig. 3.2a show mean monthly temperatures, Fig. 3.2b show the minimum and maximum temperatures per month. Mean OTC = mean monthly temperature inside the OTC. Mean Enclosure = mean monthly temperature outside the OTC. Mean OTC-Encl = the mean temperature in the OTC minus the mean temperature measured outside the OTC. Minimum (OTCs or Enclosure): the lowest temperature measured within the month either inside the OTCs or in the Enclosure. Maximum (OTCs or Enclosure): the highest temperature measured within the month either inside the OTCs or in the Enclosure. All temperature measurements are in °C.

%RLC and treatments

The design of the experiment allows several combinations of treatments to be considered, with the reference plots for both the grazed plots and for the OTCs in the enclosure. Comparing year-on-year, there was no significant response to treatments in 2002, but in 2003 the grazed plots had a higher %RLC than the plots in the OTCs ($F_{2,18} = 3.836$; $p = 0.041$). Relative to the reference plots in the enclosure, no significant effect of either grazing or warming was found (Fig. 3.3). The seasonal changes in %RLC were marginally significant in 2003 ($F_{2,18} = 3.485$; $p = 0.053$). Both the response to treatment and the seasonal variations disappeared when using the fractions of fine roots (%FR) as a covariate (not shown), indicating that the change in %RLC could be an indirect response to changes in %FR, rather than a direct response to the treatments.

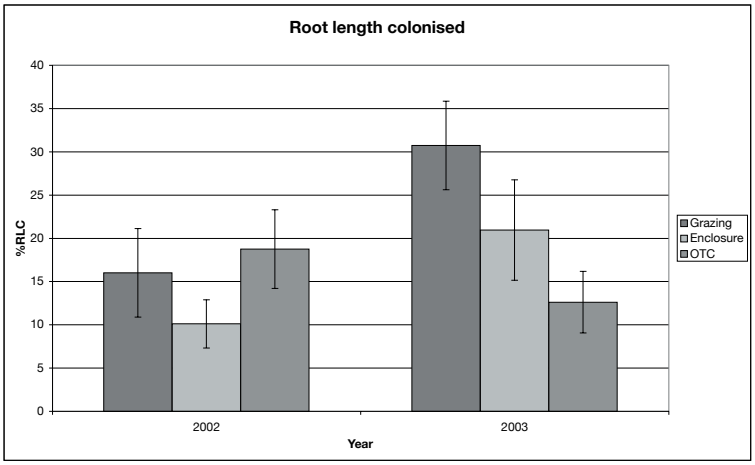


Fig. 3.3. The mean %RLC: treatments vs. years. Values are means ±SE, n = 54. There was no significant difference between the two years or between the three treatments.

%RLC, fine roots and grazing

The roots were finer in grazed plots than in ungrazed plots, but there was no significant difference in the fraction of fine roots between the enclosure and the OTCs (Fig. 3.4).

The %RLC did not have a significant difference when analysing all treatments and both years (Fig. 3.3). However, when only taking into the account the grazed plots and the enclosure, the difference between years was significantly greater in 2003 ($F_{1,32} = 8.334$; $p = 0.007$), and the grazed plots had a marginally significant higher colonisation ($F_{1,32} = 3.126$; $p = 0.087$).

%RLC was positively correlated with the proportion of fine roots ($r = 0.467$; $p < 0.001$; $n = 54$), regardless of treatment (Fig. 3.5a). Furthermore, the fraction of fine roots in root samples was higher outside the fence where grazing was permitted. The %RLC and %FR responded very similarly to the different

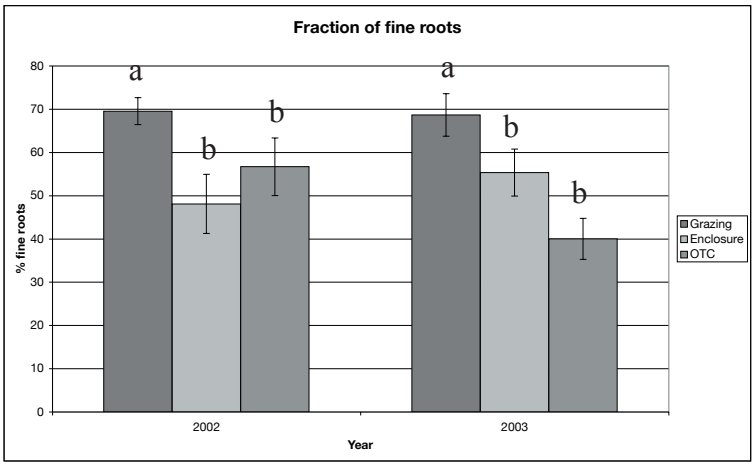


Fig. 3.4. The mean %FR: treatments vs. years. Values are means ±SE, n = 54. There was no significant difference between the two years, but grazing was significantly different from the two other treatments ($F_{2,48}$ = 8.384; p = 0.001)

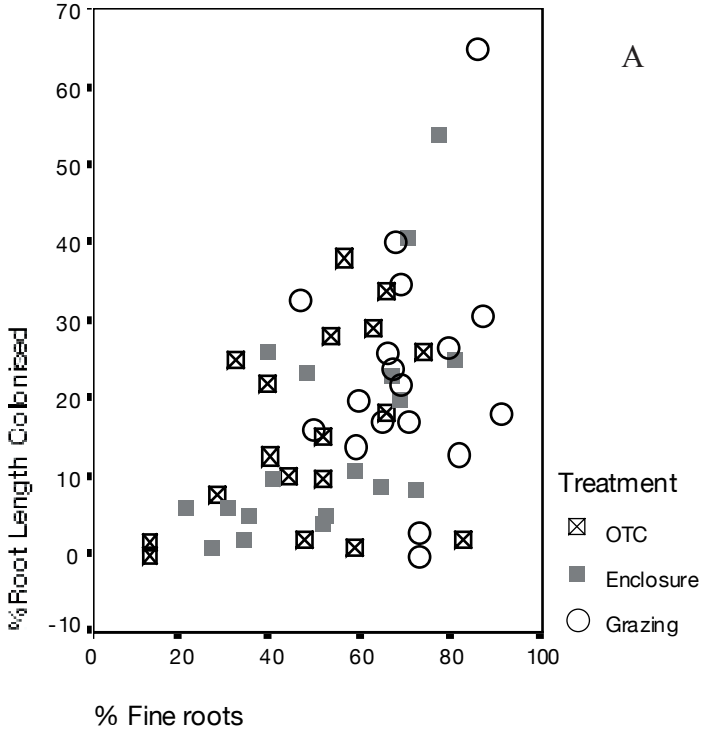
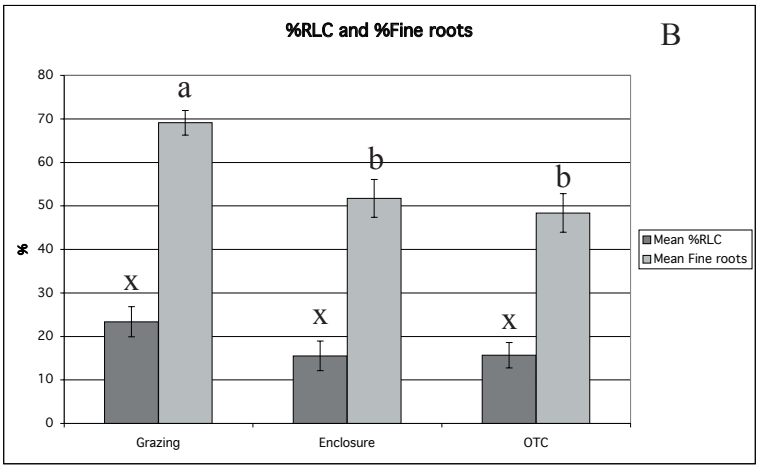


Fig. 3.5. The correlation between %RLC and %FR (a) and the response of %RLC and %FR at the three treatments (b). In (a) r = 0.467; p < 0.001, n = 54. In (b) values are means ± SE, n = 54. Different letters indicate significantly different means. ANOVA indicated the treatment had a significant effect ($F_{2,47}$ = 0.8008; p < 0.001) for %FR, while year or week had no influence.



treatments. The fraction of fine roots and the %RLC are both higher in grazed plots, while there was no difference between enclosure and OTC (Fig. 3.5b). The %FR had a significant response to grazing compared with the reference plots in the enclosure ($F_{1,49} = 5.276$; $p = 0.026$).

%RLC and temperature

To test whether the temperature prior to the sampling day affected the %RLC, the correlation between colonisation and temperature was measured in selected periods. Most of the significant correlations were negative except for those the week immediately prior to sampling and the key variable was the mean minimum temperature (Table 3.1). Stepwise regression entered the minimum temperature measured during the week starting two weeks prior to sampling (Day 7-14) first and no subsequent variables were added (Stepwise regression: $F_{1,25} = 13.034$; $p = 0.001$, $R^2 = 0.316$), revealing a strong negative relationship (Fig. 3.6). The minimum temperature did not change linearly through the season, but the %RLC did decrease progressively in the OTC plots (Fig. 3.7).

The responses of %FR to temperature were very similar to the %RLC; for example, the best correlation between %FR and temperature was negative and with the same minimum temperature measurement as with %RLC. %FR was always more closely correlated to all temperature measurements than %RLC (not shown).

Days before sampling day	Mean temperature	Maximum temperature	Minimum temperature	Temperature range
Sampling day	n.s.	r = 0.479 p = 0.012	n.s.	r = 0.475 p = 0.012
Day 1-7 (days)	r = 0.452 p = 0.018	r = 0.428 p = 0.026	n.s.	r = 0.447 p = 0.019
Day 1-14 (14 days)	n.s.	n.s.	r = -0.540 p = 0.004	n.s.
Day 3-10 (7 days)	n.s.	n.s.	r = -0.577 p = 0.001	n.s.
Day 7-14 (7 days)	r = -0.455 p = 0.017	n.s.	r = -0.585 p = 0.001	n.s.
Day 1-30 (30 days)	r = -0.471 p = 0.013	n.s.	r= -0.474 p = 0.013	n.s.

Table 3.1. Correlation coefficients and probabilities between %RLC and different temperature measurements (n = 27).

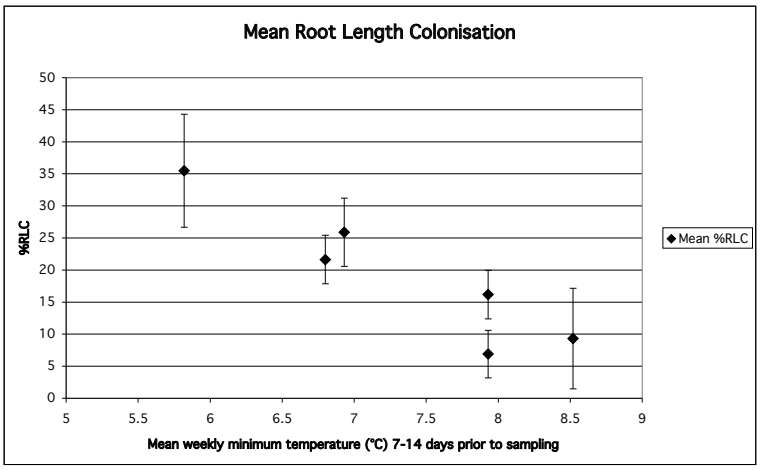


Fig. 3.6. Mean %RLC explained by mean minimum temperature (°C) measured 7-14 days prior to sampling. Values are means ± SE, n = 27.

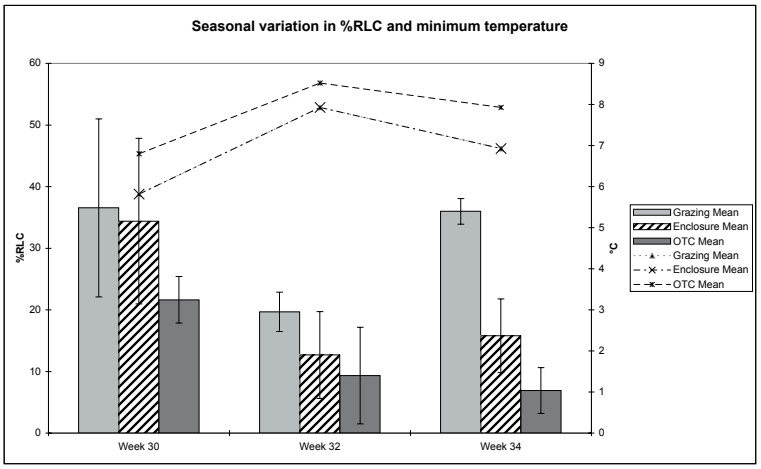


Fig. 3.7. Seasonal variation in mean %RLC (colums, left axis) and minimum temperature (°C) measured during the period 7-14 days prior to sampling (lines, right axis). Values are means ± SE, n = 27. Each column is the mean of 3 samples.

According to post-hoc tests the mean %RLC in week 30 was significantly different from week 32 ($F_{2,18} = 3.836$; $p = 0.041$, as well as the grazed plots differed marginally significantly from the plots in the OTCs ($F_{2,18} = 3.485$; $p = 0.053$). The minimum temperature measured in the OTC was around 1°C higher than in the enclosure; the temperature in the grazed plots is assumed the same as in the enclosure.

Fungal diversity

The fungal diversity was estimated by using the T-RFLP peaks obtained from each root as a profile. Only presence/absence values were used, and therefore the Shannon-Weiner diversity indices are higher than if relative abundance had been used.

The estimated Shannon-Wiener diversity suggests higher diversity in the grazed plots, compared with the plots inside the fence, regardless of whether the diversity is estimated from a Shannon-Wiener diversity index, or just by the number of different peaks (Table 3.2).

	Grazing	Enclosure	OTC
Shannon-Weiner diversity index	3.02	2.82	2.73
Number of peaks	17	14	12

Table 3.2. Diversity data of the AM fungal T-RFLP profiles. Only samples from 2003 are included.

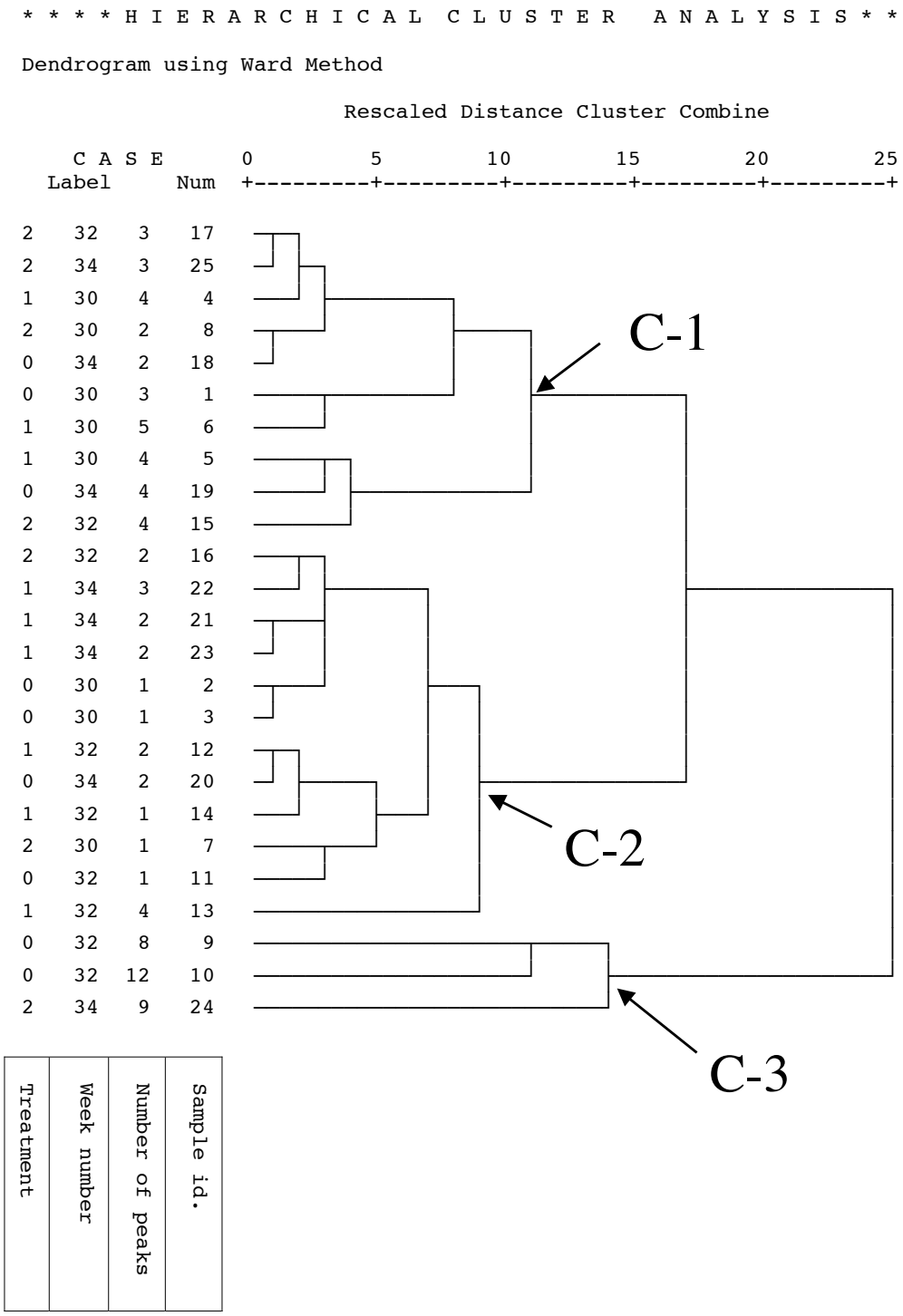


Fig. 3.8. Hierarchical cluster analysis using the T-RFLP profiles as presence/absence values with the Ward's method and binary squared Euclidean distance measurements. The corresponding treatment, week-number and number of peaks are presented in the columns to the left together with the Sample ID. Three main clusters were identified: C-1, C-2 and C-3. Only samples from 2003 were used in the analysis. Treatments are indicated by numbers -- 0 stands for grazing, 1 for enclosure, 2 for OTC.

Hierarchical cluster analysis using the T-RFLP profiles as presence/absence values suggested three main clusters, C-1, C-2 and C-3 (Fig. 3.8). To analyse the differences among groups in the dendrogram (Fig. 3.8), categorical regression was used, using the three clusters as the dependent. From Fig. 3.8 Cluster 3 contains the samples with the most peaks, and Cluster 2 those with fewer peaks. Therefore, the number of peaks was used as an independent in the categorical regression together with treatment and week number (Table 3.3). Table 3.3 indicates that the number of peaks was the main predictor of the clusters. To test whether it is the treatment or the week that determines the number of peaks an ANOVA test was carried out with the number of peaks as the dependent; this test suggested neither treatment nor week was significant, but there was a marginally significant interaction effect ($F_{4,16} = 2.901$; $p = 0.056$), suggesting that the number of peaks responded differently to treatment depending on the time of sampling.

Clusters explained	R^2	d.f.	F	P	Coeff.	F	P
1, 2 and 3	0.858	5,19	30	< 0.001	# peaks	139.2	< 0.001
					treatment	4.2	0.035
					week	0.2	0.858

Table 3.3. Categorical regression analysis of the main clusters found in Fig. 3.8. Cluster and treatments are entered as nominal values, week as ordinal and number of peaks as numerical value.

Discussion

The OTCs had a very small and marginally significant effect on mean temperature, and the percent root length colonised (%RLC) had a similar limited response to the OTC treatment. However, during this experiment it was also possible to measure both the impact of OTCs on temperature as well as follow the seasonal temperature fluctuations measured on the site. These direct temperature measurements showed that there was greater %RLC at low temperature (Fig. 3.6), which is what the results from the altitudinal study (Chapter 2) also indicated. Moreover, more than 30% of the variation of %RLC could be explained by the minimum temperature.

The %RLC was positively correlated with the fraction of fine roots and both %RLC and %FR were negatively correlated to temperature. The apparent temperature dependency could also be a seasonal effect, but using week as a covariate did not change the response of %RLC at the treatments significantly.

Colonisation was higher in grazed plots than in the OTC in 2003; however, this significance disappeared when using %FR as a covariate. The observed differences in mycorrhizal colonisation in response to grazing and OTCs may have been due to changes in the fraction of fine roots in response to temperature and grazing, rather than a direct response of the mycorrhizal fungi themselves.

The good correlation between %FR and %RLC might suggest that greater colonisation would be expected if fine root production increased. This is in contrast to the expectation that a plant's mycorrhizal dependency can be predicted from the root thickness. Sanders (1991) found that the colonisation density was a function of root diameter, with a positive correlation, meaning that the %RLC increases with increasing root diameter. Sanders results are for mean diameter per plant species, and this is in accordance with the results obtained in the fieldwork (Chapter 2), as *Ranunculus acris* has a coarser root system than *Agrostis acris*, and a higher %RLC. Fitter and Merryweather (1992) presented some unpublished data from G. Grimm, who examined the colonisation density in the ground flora in a group of deciduous woodland species. At the within-plant level they found a negative relationship: the coarsest roots of individual plants were the least infected. This finding is also in accordance with the results obtained here, as the higher the fraction of fine roots, the higher the %RLC.

However, according to Smith and Read (1997, p. 72) it is very difficult to determine direct effects of environmental variables on fungal colonisation using %RLC, unless it has been ascertained that there are no environmental effects on root growth itself. Moreover, differences in %RLC of different species or different genotypes of the same species of plant may be controlled not by actual susceptibility of the root systems but by differences in the rates of root growth.

Grazing affected the ratio of fine roots, so the fraction of fine roots was higher in grazed plots; this is consistent with what others have found. For example, Frank *et al*. (2002) found that grazers stimulated both aboveground and belowground productivity by 21% and 35%, respectively. Pucheta *et al*. (2004) found that productivity of very fine roots was almost twice as high in a grazed site as in an ungrazed site, despite the fact that both sites had similar total live biomass, and root turnover rate was twofold greater at the grazed site. Derner *et al.* (2006) found that the relative proportion of fine root biomass was greater at grazed sites.

The enclosure might also have had an impact on the available nutrients. The plants are protected from herbivory by sheep, but at the same time there is no input of new nutrients from sheep urine and sheep faeces. A study using *Agrostis*

capillaris to examine the impact of defoliation on the distribution of nitrogen between shoot, roots and soil nitrogen pool found that on its own, defoliation did not change the microbial biomass nitrogen. On the other hand, root biomass was decreased by regular defoliation and this coincided with greater amounts of nitrogen stemming from sheep urine (in this case synthetic sheep urine) (Williams *et al.*, 2003). There is no indication of whether the fraction of fine roots was affected by defoliation or sheep urine in this study (Williams *et al.,* 2003), but if the ratio between weight and root length behaved as those presented in Chapter 4, then a larger biomass should indicate a greater fraction of fine roots.

Studies examining the effect of defoliation on root carbon do however indicate that the outcome may be difficult to predict; for example a study using ^{14}C found significant positive relationships between herbivory and carbon allocated to roots, roots exudates and root and soil respiration (Holland *et al.*, 1996). Also Bazot *et al.* (2005) found that defoliation increased root soluble carbon concentration, but these changes did not influence carbon availability or soil microbial growth, and Mikola and Kytöviita (2002) did not find evidence for increased photosynthate to soil decomposer food webs following defoliation.

Studies examining the effect of defoliation on AM fungi have shown both positive, negative and neutral responses. Daft and El-Giahmi (1978), Bethlenfalvay and Dakessian (1984), Bethlenfalvay *et al.* (1985), Gehring and Witham (review article, 1994) all found a decrease in colonisation due to herbivory. However, more recent studies have suggested an increase in colonisation due to herbivory (Eom *et al.*, 2001, Gehring *et al.,* 2002, Kula *et al.*, 2005). Bentivenga and Hetrick (1992) and Lugo *et al.* (2003) found no change in colonisation. Hokka *et al.* (2004) found that the effects of grazing depended on the plant species, as they found a decrease in some grasses, and increase in others. Hokka *et al.* (2004) concluded that the interaction between mycorrhizal colonisation and defoliation is likely to be extremely complex.

The response of *Agrostis capillaris* to grazing is variable. Some studies have found the abundance of *Agrostis capillaris* to decrease when grazed (Magnússon and Magnússon, 1990; Crawley, 1990) According to Magnússon and Magnússon (1990) the abundance and cover of *Agrostis capillaris* decreases with increasing grazing intensity. This study from Iceland might be the best predictor to what to expect in the Faroe Islands, as the climatic conditions are quite similar, though the Faroes are more oceanic than Iceland.

Other studies have found the abundance of *Agrostis capillaris* to decrease when the grazers were removed (Hartley and Mitchell, 2005; Hulme *et al.*,

1999). The decline in *Agrostis capillaris* when grazers were removed have been explained by a change in the plant composition to more grazing-intolerant plant species such as *Deschampsia flexuosa* and *Molinia caerulea* (Hulme *et al.*, 1999).

In the Faroes *Agrostis capillari*s was more abundant in the enclosures than in plots outside the enclosure, which corresponds to the Icelandic findings. The lower quantity of fine roots in the enclosure could be an indication of increased competition with species that are less grazing-tolerant than *Agrostis capillaris*.

In general, *Agrostis capillaris* is considered as a grazing-tolerant plant species (Murray *et al.,* 2004; Matejkova *et al.*, 2003), as shown by a study where plants of *Agrostis capillaris* were subjected to defoliation, and then measured for carbon exudation using stable isotopes (^{13}C) (Murray *et al.*, 2004). There was no significant difference in overall carbon exudation from the plants as a result of defoliation but defoliation significantly increased exudation of recent photosynthate. The latter could indicate that the metabolic rate of the grazed plant is higher than of the ungrazed, and thus there are more sugars available for the AM fungus in an grazed plant than in an ungrazed.

Grazing turned out to be the most important factor in this experiment. Therefore an additional treatment with simulated grazing, i.e. clipping, might have clarified the results, providing a more direct comparision to the grazed plots. It was, however, not anticipated that grazing would be such an important factor. Further, clipping would have interfered with the other experiments going on in the OTCs.

The plots in the enclosure were the appropriate controls for the OTCs, and the lack of any differences between the enclosure and the OTCs in %RLC probably reflects the very limited effect the OTC had on soil temperature. However, the lack of soil temperature enhancement does not necessarily mean that there was no impact stemming from the treatment. Marion *et al.* (1997) give an example of one site where the soil temperatures were depressed, but the air temperatures were enhanced. Klein *et al.* (2005) concluded that the effect of the OTCs on soil temperature depends on whether the temperature gradient is strong enough to produce a significant heat flux into the soil, especially in the presence of high biomass or litter cover. Thus the larger plants inside the OTCs can prevent soil warming by the OTCs. The constant decrease in %RLC during the season particularly in the OTC could be an indicator of an insulating effect from the plant cover (Fig. 3.7).

The finding that the plants grew larger inside the OTCs is in accordance with what is found at other ITEX sites (Walker *et al.*, 2006). For example

Hollister *et al.* (2005) found that the net result of warming was an increase in canopy height and canopy closure because of an increase in size of individual plants. This study (Hollister *et al.,* 2005) was from Alaska, and the OTCs warmed air temperatures near the ground by on average 0.6-2.2°C throughout the growing season. The change in soil temperatures due to the OTC in Alaska varied between –0.8 and 0.7°C (Hollister *et al.,* 2006). In a study from Iceland (Jónsdóttir *et al.,* 2005) the air temperatures measured were 0.7-2°C higher in the OTCs than outside, but in contrast to the air temperatures, experimental climate warming was not accompanied by an increase in soil temperatures. The work of Jónsdóttir *et al.* (2005) did not support the hypothesis that an increase in leaf area index can explain the discrepancy between air and soil temperatures, as in their study the discrepancy was greatest where leaf area was low. Thus the main effect of the OTCs in general seems to be an increase in air temperatures, while the change in soil temperatures is more complex, depending on micro-climate, plant community, and chamber artefacts (Jónsdóttir *et al.*, 2005).

The larger shoots might have had another effect, namely that the observed increased leaf area inside the OTCs (Anna Maria Fosaa, unpublished data) also provide a larger photosynthetic area, with possibilities for increased carbohydrates in the plants and thereby also for the mycorrhizal fungi acting as a carbohydrate sink. Increased carbon flow to the root would, however, be expected to be reflected in increased mycorrhizal colonisation, but no significant increase was observed in the OTCs

Another reason for the decrease in %RLC in the OTC could be due to shading by the OTCs rather than to temperature. Shading can affect the root-turnover, here in terms of a lower %FR, and as a consequence the %RLC, as demonstrated by Heinemeyer *et al.* (2003). If it is so, then any observed changes in the fungal community might also be due to shading rather than soil temperature.

The temperature, sunshine and precipitation all differed from year to year. The summer of 2002 was the first growing season after the hexagons were set up, and the decreased number of fungal types inside the hexagons might have been due to a short-term flush of nutrients due to increased mineralisation. As 2003 was considerable warmer than 2002, then, if the decline was due to temperature, the observed change inside the OTC in 2002 should have been seen in the enclosure in 2003. However, if the vegetational cover was higher in 2003, due to increased air temperature, then the soil might have received less of the heat flux than soil under lower vegetational cover, especially inside the OTCs. Further to this, increased precipitation might act as a cooling factor. Thus the increased number

of types and Shannon-Wiener diversity in 2003 may stem from the facts that i) the soil temperature was unaffected by temperature in 2003, but not in 2002; ii) the effect observed was transient, triggered by a change, but that the system had found a new balance in 2003.

One consequence of climate change is elevated temperatures – these may have consequences for nutrient cycling, simply by increased mineralisation due to a more efficient microbial community, of which the AM fungi play a significant part. An increased mineralisation can be short-lived (Giardina and Ryan, 2000) until a new balance is found, but while it lasts it can lessen the colonisation in less mycorrhiza dependent plants, as they absorb their nutrients directly from the soil when they are available.

If there were any changes to the AM fungal community due to the treatments, they were limited. The Shannon-Weiner diversity, as well as the number of peaks, increased in the grazed plots, but decreased in the OTCs relative to the plots in the enclosure. According to the hierarchical cluster analysis, however, the number of peaks was the main pattern found from the profiles, and this was not linked to a particular treatment or week.

To conclude: the percentage root length colonisation was greatest at low temperature, but as the %RLC was correlated with the fraction of fine roots, which also was correlated to low temperature, the %RLC response to temperature might not have been a fungal response to temperature, but one mediated by a root response. Further, a higher mycorrhizal colonisation was found in grazed samples than in samples from the OTCs. The Shannon-Weiner AM fungal diversity, based on T-RFLP profiles, was higher in the grazed plots, but lower in the warmed plots, compared with the ungrazed reference plots.

Chapter 4

Growth and survival of arbuscular mycorrhizal fungi at low temperatures

Abstract

- Arbuscular mycorrhizal (AM) fungi from cold sites were tested for their ability to maintain growth at low soil temperature and whether their growth was affected by prolonged periods of elevated temperature.
- *Agrostis capillaris* from three locations, two Faroese and one Scottish, colonised with native AM fungal communities, were grown at two temperatures, 10°C and 25°C. From one of the Faroese sites, additional plants were first grown at 25°C for either 2 or 4 weeks, and then for 25-27 weeks at 10°C.
- Of the two Faroese sites, roots from the high altitude site had a higher root length colonisation than roots from the low altitude Faroese site. The site with the lowest temperature was the high altitude Faroese site. The highest root colonisation at low temperature, however, was found in roots from the high altitude Scottish site.
- Arbuscular mycorrhizal diversity was estimated by terminal restriction fragment length polymorphism (T-RFLP). The plant roots from the coldest site had a higher estimated AM fungal diversity than those from the other two sites.
- Generally plants responded to warming by producing more fine roots and root length at 25°C. The plants from the coldest site (the high altitude Faroese site) had the largest response to high temperature. Also these samples showed the highest percentage of low diameter roots (<0.5 mm).
- After 25-27 weeks, the effects of the pre-warming were still profound on both the amount of root colonisation and on the composition of the fungal community.

Introduction

Most experiments examining the impact of temperature on arbuscular mycorrhizal (AM) fungi hitherto have used lab cultures and have shown that colonisation increases with increasing temperature up to approximately 30°C (Smith and Read, 1997, p. 75). However, there are exceptions: for example, Monz *et al.* (1994) transferred large intact soil cores from the field to controlled environment chambers and found a decrease in colonisation with increasing temperature. With current techniques, the majority of types within the phylum Glomeromycota are unculturable, and those that can be cultured might be an ecologically distinct (and unrepresentative) sub-sample (Fitter, 2005). AM fungi able to grow under lab conditions might be more weedy members of the phylum, and thus able to cope with a wider range of host species and a variety of abiotic factors, but less able to cope with, for example, competition from other fungal species. Therefore, it might be that many AM fungi are more temperature sensitive than the commonly cultured types, and only grow within a limited temperature range.

The two previous chapters have dealt with samples collected in the field. The results thus obtained may be ecologically realistic, but can be difficult to interpret, as several factors can influence the outcome. These factors range from abiotic, such as soil, light, moisture and temperature, to biotic, including competition from other plants and herbivory (of both leaves and roots).

From field material collected during two growth seasons, it was evident that the percentage root length colonised (%RLC) in *Agrostis capillaris* increased with altitude. Altitudinal gradients were used as surrogates for changes in temperature (Chapter 2), with a difference in temperature of approximately 4°C from low altitude to 600 m altitude. The arbuscular mycorrhizal colonisation increased at high altitude, but this might have been a response to either temperature, or to other factors such as less available phosphorus at high altitude, or genetic differentiation of the host plant *Agrostis capillaris*. However, the ITEX data (Chapter 3) also suggested that the %RLC is negatively correlated to temperature, possibly as a result of a more direct effect of temperature on the percentage fraction of fine roots (%FR), which in turn influences the %RLC.

Mycorrhizal fungi differ in their response to temperature. Sánchez *et al.* (2001) measured the growth of eight species of ectomycorrhizal (EM) fungi at five temperatures from 5-30°C. All of the fungi grew best at 23°C and all were able to grow at 5°C, but only 2 managed to grow at 30°C. Tibbett *et al.* (1998) showed that many EM fungi are still able to grow at low temperatures (ca. 0-1°C).

Merryweather and Fitter (1998b) found that the roots of the winter active

geophyte *Hyacinthoides non-scripta* were colonised by different fungi in winter and in summer, which implies that there are AM fungi that are adapted to growing at low soil temperatures in the field. Similarly Heinemeyer *et al.* (2003) found seasonal variation in fungal types when studying root colonisation and fungal types during a year in an experimental garden. This study suggested that soil temperature has little impact on mycorrhizal colonisation. Heinemeyer and Fitter (2004) in a pot study found that both internal and external mycelium increased when the temperature was increased by 8°C from 12 to 20°C.

Gavito *et al.* (2003) state that AMF development and function is more likely influenced by the temperature component of climate change than by any change in CO_2 concentration, and that external mycelium is more sensitive to low temperatures than the internal mycelium. The conclusion from this study with subterranean clover colonised with either *Glomus caledonium* or native Danish AM fungi from field soil is that external mycelium does not develop at 10°C, but that both internal colonisation and plant roots grow at low temperature. If this were the case, then survival and development of AM fungi during cold periods and at cold sites would rely completely on internal colonisation. Alternatively, there might be winter-active fungal types, or cold-tolerant types, that are able to develop external mycelium at low temperatures.

Gavito *et al.* (2005) examined the growth ability of three different isolates of AMF and found that although the optimum temperature for the growth of external mycelium was around 24°C, there was some growth even at 6°C for some AM fungal types. They also measured the total root length and the colonised root length. The total root length did not always show the same temperature optimum as the external hyphae, but root colonisation had a very similar response to temperature as the external hyphae. Total root length increased with temperature, but also responded to the type of AM inoculum.

These examples from studies with mycorrhizal fungi examining external hyphae, root colonisation and root length suggest that some mycorrhizal fungi are able to function at low temperatures. The external mycelium in general, however, might be more limited at low temperatures than the internal colonisation.

If the higher levels of colonisation found at high altitude in the Faroes were due to low temperature, it might be that fungal growth in general is less restricted by low temperature than is root growth, or that distinct types of AM fungi present at high altitude are able to grow at low temperatures.

The Faroese average temperature at sea level in summer is around 10°C, with a mean minimum temperature of around 5°C and a mean maximum

temperature of around 12°C (Climatological norms from Tórshavn June-August 1961-1990; Cappelen and Laursen, 1998). The annual mean temperature for the high altitude Faroese site, Sornfelli, is 2°C (Christiansen and Mortensen, 2002), and for the Faroese low altitude site, Velbastad, 6°C (Cappelen and Laursen, 1998). For the Scottish site, Sourhope, the annual mean temperature is 7°C (ECN database). Because of the extreme oceanic nature of the climate, extreme temperatures are very rare and native AM fungi from the Faroes will rarely experience temperatures above 20°C. The question posed here is whether the fungi are able to grow at temperatures outside this range for prolonged periods.

The main object of this experiment was to determine whether fungi from colder sites have a greater ability to maintain growth at low soil temperature. The second objective was to test whether the growth of fungi from cold sites is affected by prolonged periods of elevated temperature.

Material and Methods

All the samples used in this experiment were from cold sites. Sornfelli (Faroe Islands, 600 m a.s.l.), Velbastad (Faroe Islands, 200 m a.s.l.) and Sourhope (Scotland, 500 m a.s.l.). For details about the Faroe sites, see Fig. 2.1. The Scottish site is located at 55°28'N, 02°14'W. All sites are natural grasslands, grazed by herbivores. A total of 56 soil cores (5 cm diameter, 10 cm deep) were collected from under *Agrostis capillaris*. The sampling date in the Faroes was on 29 Aug 2003, and in Scotland on 29 Oct 2003. The cores from each site were transported in cardboard boxes in a cooled box to keep the samples below 10°C. They were stored overnight in a refrigerator at 7°C. The next day the cores were wrapped into nylon fabric with mesh sufficiently wide to allow plant roots, as well as fungal hyphae, to grow through.

The soil cores, together with the layer of nylon mesh, were planted in a 10 cm diameter pots. The medium surrounding the wrapped cores was 60% terragreen, 40% sand (vol/vol), with 0.25 g of bone meal per litre medium. The bone meal was the only nutrient added through the whole experiment. The pots were watered daily with tap water.

Eight pots each from Sornfelli and Sourhope, and four from Velbastad were placed in a growth chamber at 25°C. At the same time the same number of pots were grown at 10-12°C, in total 40 pots. As no growth chamber that could maintain a temperature of 10°C at high PAR flux was available, plants were grown in a chilled display counter (Trimco "Nice" Mobile Display Counter NF100CA,

Stockport, UK, Deck Size 0.52 m^2), with a set temperature of 10-12°C. The entire counter was placed in a greenhouse.

To check whether a temperature of 25°C stimulated, inactivated or eradicated the fungi, a pre-warming treatment was administered. A total of 16 pots with Faroese high altitude samples (Sornfelli) were grown at 25°C for 2 weeks ("pre-warm 2"; 8 samples) and 4 weeks ("pre-warm 4"; 8 samples) with the same medium and in the same growth chamber as the other pots growing at 25°C, and transferred to the chilled display counter for the rest of the time.

Roots that had grown out of the soil core into the surrounding medium were harvested 13, 19 and 29 weeks after the first sampling date. The first two harvests provided no results (no visible colonisation [under microscope] or convincing PCR-products). All samples intended for examination for arbuscular fungal colonisation and fungal growth were harvested from outside the nylon mesh. The function of the nylon mesh was to ensure that the roots examined had grown under the new conditions (i.e., new roots), and that the fungi that were colonising them had therefore grown under a known temperature. At the final harvest, roots from the original soil core were also sampled (old roots).

Samples for %RLC and for diversity analysis (T-RFLP) were taken from the periphery outside the membrane separating the old and new roots. From each pot, two sub-samples of new roots were taken from opposite sides; the sub-samples were mixed and used for %RLC examination and DNA extraction. The sample for %RLC was put directly into test tubes with 10% KOH while ca. 1 ml of washed fresh root material was frozen in Eppendorf tubes, ready for DNA extraction. The staining method was as described in Chapter 2. Root length colonisation was measured following McGonigle *et al.* (1990).

The DNA extraction, digestion and diversity examination (T-RFLP) used the same protocol as ITEX-samples from 2003 (Chapter 3), and the analysis of the T-RFLP fragments followed the same principles and restrictions as mentioned in Chapter 3. Shannon-Weiner indices, based on the presence/absence of T-RFLP peaks, were used to estimate the fungal diversity; the Sørensen similarity coefficient was calculated to estimate the similarity between samples. Hierarchical cluster analysis was used to look for patterns in the T-RFLP peaks.

At the final harvest, the whole, new-grown root system was scanned and then the root length and diameter was determined with WinRHIZO Pro 4.1c from Regent Instruments, Inc., Quebec, Canada. Total root length per pot was measured (several scans); root diameter was grouped into 10 categories, from <0.5 mm to thicker than 4.5 mm, and root length in cm within each category was calculated.

Subsequently, the roots were dried at 70°C for one week and the dry weight measured. The shoots were not taken for dry weight measurement.

Statistics

All statistics were carried out in SPSS 11.03 for Mac OS X. The samples were tested for normal distribution with the One-Sample Kolmogorov-Smirnov test. Parameters that failed the normal distribution test were normalised by using the square root of the sample or taking the (log + 1). All bars in graphs are ±SE. When testing for variance of mean Univariate Analysis of Variance (ANOVA) was used. As the %RLC data were not normally distributed, they have been square-root transformed. Post-hoc tests used were Bonferroni.

Diversity was estimated by Shannon-Weiner indices. Shannon-Weiner was calculated by the formula:

$$H_0 = -\Sigma p_i \ln p_i$$

where p_i in this case was used as the summarised presence/absence values of T-RFLP peaks within a group.

Similarity tests were calculated by using the formula:

$$S = 2N_{ab}/(N_a + N_b)$$

This is a pairwise test known as the Sørensen similarity coefficient, estimating the similarity between two samples based on presence/absence values. N_{ab} is the number of clone types that are shared between the two samples, while N_a and N_b is the total number of clone types in each sample. This is a simple test that can be done in a spreadsheet, but as the number of calculations quickly adds up with increasing numbers of samples, EstimateS software was used (Colwell, 2005). The values range from 0 to 1, where 1 indicates a complete match and zero indicates that there are no types in common.

The hierarchical cluster analysis used Ward's method, using binary squared Euclidean distance measurements. To find the parameters that explained the clusters, categorical regression was used, with all category indicator variables entered on the nominal scaling level. Categorical regression extends the approach of standard linear regression by simultaneously scaling nominal, ordinal and numerical variables. The procedure quantifies categorical variables such that the quantifications reflect characteristics of the original categories (Meulman and Heiser, 2001).

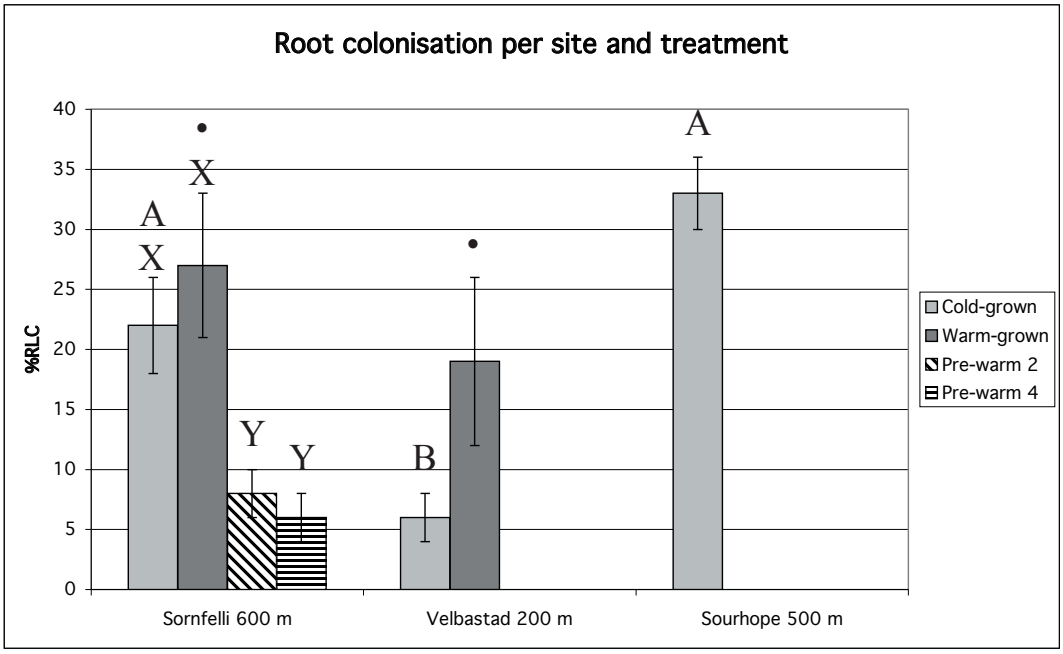


Fig. 4.1. %RLC in plants from the three locations in response to temperature. Different letters indicate a significantly different level of colonisation according to post-hoc test. A-B combinations are used for the cold-grown plants. X-Y combinations are used for the four treatments from Sornfelli. As the %RLC was not normally distributed, %RLC data were square-root transformed for statistical analysis. See text for further explanation.

Results

Root length colonisation

The %RLC from field samples was greater at low temperature, but AM colonisation in roots from Sornfelli and Velbastad when grown in laboratory was not lower at high temperature (Fig. 4.1). Colonisation data at 25°C for Sourhope are missing because the samples were lost. The cold-grown pots from Velbastad differed significantly from the two others ($F_{2,37} = 11.968$; $p < 0.001$), while there was no significant difference between the colonisation in warm-grown pots in relation to site. When looking at Sornfelli only across all four treatments, both the warm-grown and the cold-grown pots had a significantly greater colonisation than found in pots from the two pre-warmed treatments ($F_{3,60} = 6.527$; $p = 0.001$). As warming did not reduce the colonisation in plant roots from Sornfelli it was unexpected that the %RLC data indicated that a pre-warming treatment affected the ability of the fungi to colonise plant roots. The %RLC in the pre-warmed plants was much lower than for the cold-grown plants from Sornfelli, but not much different from the cold-grown plants from Velbastad.

Molecular analyses

The amplification success rate was low, especially for the new roots (Table 4.1). Two of the treatments, "Pre-warm 4" and "Velbastad cold" only contributed one sample each for the diversity analysis. Therefore, the two pre-warming treatments were pooled, hereinafter named "pre-warmed".

	Sornfelli		Velbastad		Sourhope	
	New roots	Old roots	New roots	Old roots	New roots	Old roots
Cold-grown	4 (8)	4 (8)	1 (4)	4 (4)	3 (8)	8 (8)
Warm-grown	6 (8)	5 (8)	2 (4)	4 (4)	6 (8)	7 (8)
Pre-warm 2	2 (8)	8 (8)				
Pre-warm 4	1 (8)	8 (8)				

Table 4.1. PCR amplification success rate. Number of samples that amplified (total number of pots in brackets) according to treatment, site, and new or old roots.

To guard against artefacts, such as peaks due to partial digestion, Vandenkoornhuyse *et al.* (2003) amplified two independent PCRs, two independent digestions and duplicated all T-RFLP gels from each sample. Johnson *et al.* (2003) duplicated the T-RFLP gels. These replicates showed that the peak area from the T-RFLP varied between the replicates, and thus these studies only used presence/absence for the diversity analysis. Vandenkoornhuyse *et al.* (2003) omitted samples where the peaks in replicate T-RFLPs did not display the same qualitative diversity signature. In the present study, there was no replicate of the T-RFLP gels, and hence the "identity" of peaks as well as the peak height must be considered with care. This lack of replicates meant that it was not possible to leave out samples that, for example, had more peaks and / or otherwise unusual peaks even though their overall accuracy might be questionable. This is especially true for one sample from Sornfelli with 34 peaks and one from Sourhope with 21 peaks, both cold-grown, old roots. Therefore, all samples were maintained in the analysis.

The number of peaks found for each treatment and site is presented in Table 4.2. In this study, a total of 71 different T-RFLP peaks were found, of which one was found only once, while the majority occurred between 2 and 9 times (61 peaks). Nine peaks occurred between 10 and 71 times. The total number of samples was 73.

	Sornfelli (38 samples)	Velbastad (11 samples)	Sourhope (24 samples)
Total number	69	24	47
Cold-grown	52	23	40
Warm-grown	35	4	26
Pre-warmed	47		

Table 4.2. The number of T-RFLP peaks found in roots from the three sites and treatments.

The Shannon-Weiner diversity indices were calculated from summarised T-RFLP presence/absence values per site and treatment (Table 4.3). The diversity differs for both site and treatment. A general trend is that the cold-grown plants have the highest T-RFLP diversity, followed by the plants grown with a pre-warmed temperature. The lowest diversity was found in roots from warm-grown plants. The highest diversity is found for Sornfelli, while the lowest diversity is found for Velbastad. The Shannon-Weiner values are high; they would have been lower if relative abundance had been taken into account.

	Sornfelli	Velbastad	Sourhope
Total Shannon-Weiner value	3.794	2.876	3.325
Cold	3.716	2.975	3.366
Warm	3.259	1.197	2.777
Pre-warmed	3.378		

Table 4.3. Shannon-Weiner diversity indices based on summarised presence/absence values for the three sites and treatments.

To assess the similarities both within groups and between groups, Sørensen's similarity coefficients were calculated (Table 4.4). The highest mean similarity values were found in the warm treatment. Both Velbastad and Sourhope were most similar to themselves, but that was not the case for Sornfelli (see values in bold in Table 4.4). The T-RFLP peaks from cold-grown roots had higher similarity with the T-RFLP peaks from the pre-warmed treatment.

	Sornfelli	**Velba-stad**	**Sour-hope**		**Cold**	**Warm**	**Pre-warmed**
Sornfelli	0.31 ±0.007	**0.34** ±0.010	0.29 ±0.007	**Cold**	0.31 ±0.010	0.29 ±0.007	**0.33** ±0.009
Velbastad		**0.41** ±0.033	0.40 ±0.016	**Warm**		**0.49** ±0.014	0.26 ±0.006
Sourhope			**0.41** ±0.014	**Pre-warmed**			**0.39** ±0.016

Table 4.4. Sørensen's similarity coefficients calculated for three sites (left) and treatments (right). Pre-warmed treatment contains both pre-warming treatments. The values are means, ± standard error. The Sornfelli data include all treatments, both cold, warm and pre-warmed. See text for explanation of values in boldface.

Pre-warming reduced %RLC, compared with the cold-grown plants from Sornfelli (Fig. 4.1), and did so to a level similar to that of cold-grown plants from the low altitude Velbastad-site. Since this effect might have been due to a community change, Sørensen's similarity coefficients were calculated for treatment*site combinations (Table 4.5). Within-combination similarity was greatest in all cases, but the pre-warmed treatment was otherwise most similar with Velbastad cold-grown and more so than to the other treatments with material from Sornfelli (see values in bold in Table 4.5).

		Sornfelli			Sourhope		Velbastad	
		Cold	Warm	Pre-warmed	Cold	Warm	Cold	Warm
Sornfelli	Cold	0.36	0.22	**0.33**	0.26	0.27	0.35	0.22
	Warm		0.37	**0.25**		0.43		0.51
	Pre-warmed			**0.38**	0.31	0.23	**0.35**	0.33
Sourhope	Cold	.	.	.	0.34	0.38	0.27	0.34
	Warm	.	.	.	.	0.50		0.59
Velbastad	Cold	.	.	.	.	.	0.40	0.25
	Warm	.	.	.	.	.		0.73

Table 4.5. Sørensen's similarity values for all site and treatment combinations. Values are means. See text for explanation of values in boldface.

Hierarchical cluster analysis suggested three main clusters (C-1, C-2 and C-3 in Fig. 4.2). A Sørensen's similarity analysis was run looking at the

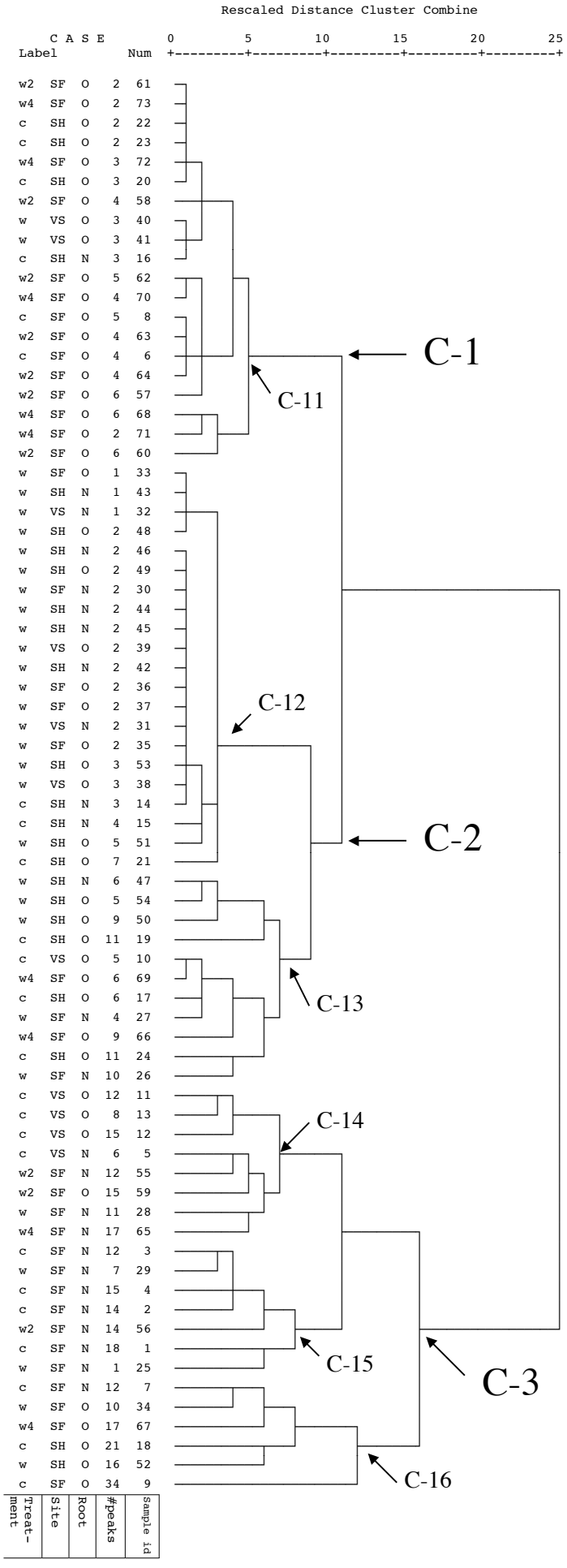


Fig. 4.2. Dendrogram from a hierarchical cluster analysis suggesting three main clusters (C-1, C-2 and C-3), and 6 sub-clusters (C-11 to C-16). Columns to the left are: treatment (c = cold, w = warm, w2 & w4 = pre-warming treatments); site (SF = Sornfelli, VS = Velbastad, SH = Sourhope); root (N = new, O = old); number of peaks, and lastly, sample id.

	Cluster 1	Cluster 2	Cluster 3
Cold	25	29	46
Warm	7	77	17
Pre-warmed	63	11	26

Table 4.6. The percentage distribution per treatment of T-RFLP-profiles according to clusters suggested by hierarchical cluster analysis.

similarities between the clusters. All were most similar to themselves.

The distribution of treatments and sites within the clusters is presented in Table 4.6 and 4.7. The two pre-warming treatments are the dominant treatment in Cluster 1, warming treatment in Cluster 2, while the cold treatment is the most prominent in Cluster 3 (Table 4.6). The two Faroese sites are distributed rather evenly through the three clusters, while Sourhope is predominantly found in Cluster 2 (Table 4.7).

To analyse the differences among groups of the dendrogram (Fig. 4.2), categorical regression was used (Table 4.8). The clusters that were suggested by the hierarchical cluster analysis were used as the dependent variable and the categorical indicator variables (treatment, site, and new or old roots) were used as the independent variables. The hierarchical cluster analysis used only the T-RFLP profiles for the dendrogram, so the cluster analysis and the categorical regression are independent of each other.

Table 4.8 shows that there was a significant difference between the three clusters. The test determines if omission of a predictor variable with all other predictors present significantly worsens the predictive capabilities of the model (Meulman and Heiser, 2001). In this case, for the test of the differences between the three main clusters, the treatment and site were both significant for the cluster-design. When only examining the difference between Clusters 1 and 2, removing treatment significantly worsens the model. For Clusters 2 and 3, both treatment and site are important, while for the differences between Clusters 1 and 3 treatment and new or old roots determines the clusters.

	Cluster 1	Cluster 2	Cluster 3
Sornfelli	37	24	39
Velbastad	18	47	36
Sourhope	17	75	8

Table 4.7. The percentage distribution per site of T-RFLP-profiles according to clusters suggested by hierarchical cluster analysis.

Clusters explained	R^2	d.f.	F	P	Coeff.	F	P
1, 2 and 3	0.394	6,66	8.786	<0.001	Treatment	26.271	<0.001
					Site	11.780	<0.001
					Root	0.327	0.569
1 and 2	0.442	6,45	7.746	<0.001	Treatment	29.687	<0.001
					Site	2.272	0.115
					Root	0.873	0.355
2 and 3	0.364	6,46	5.966	<0.001	Treatment	16.151	<0.001
					Site	15.355	<0.001
					Root	0.458	0.502
1 and 3	0.273	6,34	3.501	0.008	Treatment	5.506	0.003
					Site	2.361	0.110
					Root	9.162	0.005

Table 4.8. Categorical regression analysis of the main clusters found in Fig. 4.2. All variables are categorical, scaling level on nominal. First column indicates which clusters are explained.

Root morphology

The warm-grown pots had a larger root system than the cold-grown pots (Table 4.9). The total root length was highest in the warm-grown Sornfelli pots. The two pre-warming treatments also showed slight increases in length – with four weeks pre-warming producing slightly more than two weeks pre-warming. The warm-grown pots had a significantly higher root length than the other treatments. The fraction of the finest category (%<0.5 mm) showed that the samples from Sornfelli had a considerably higher "%<0.5 mm" fraction than the two other, both at low and high temperatures (Table 4.9).

The time the plants had to grow new roots was different for the two Faroese sites (29 weeks) and for Sourhope (21 weeks). The Sourhope plants managed to grow more roots after 21 weeks at low temperature than the Sornfelli plants did after 29 weeks. Plants from Sourhope did not produce as extensive a root length at high temperature as plants from Sornfelli. The total root length in the plants from Sornfelli was six times longer than in cold-grown plants from Sornfelli, in plants from Velbastad the warm-grown had 3 times longer roots, and plants from Sourhope had four times longer roots than the cold-grown (Table 4.9).

Variable	Treatment	Sornfelli mean	Velbastad mean	Sourhope mean
%<0.5 mm TRL cm	**Cold-grown**	75 ±1.4 3879 ±338	66 ±4.6 5221 ±1387	68 ±2.2 4174 ±751
%<0.5 mm TRL cm	**Warm-grown**	81 ±0.6 23217 ±2653	74 ±4.1 13482 ±4559	71 ±2.6 16588 ±1814
%<0.5 mm TRL cm	**Pre-warm 2**	74 ±2.0 4713 ±710		
%<0.5 mm TRL cm	**Pre-warm 4**	72 ±1.8 4911 ±495		
Overall statistics	ANOVA %<0.5 mm Site $F_{2,48} = 8.069$; $p = 0.001$ Treatment $F_{3,48} = 4.154$; $p = 0.011$ Post hoc: Sornfelli differ from the other two sites and cold-grown differ from warm-grown. TRL Treatment $F_{3,48} = 40.770$; $p < 0.000$ Post hoc: Warm-grown differ from all other treatments			

Table 4.9. Mean root measurement ±SE according to treatment, site and tests of variance. As the root parameters were not normally distributed, the parameters were transformed by using (log +1). Abbr. (units in brackets): %<0.5 mm = Percental fraction of roots with diameter <0.5 mm (% of TRL); TRL = Total Root Length (cm per pot). Only significant F-values are shown.

Discussion

One of the questions this experiment intended to answer is whether fungi from colder sites have a greater ability to maintain growth at low soil temperature. The findings presented in Chapter 2 indicated that the %RLC in plants from high altitude was greater than from low altitude. The site from where the samples from Sornfelli stem is at 600 m a.s.l. and thus higher than the two other sites. In addition Sornfelli is the coldest site of the three used here. From this it would be expected that the cold-grown Sornfelli plant roots would have a greater AM colonisation than the roots from the warmer sites. This assumption is only partially supported by the results presented here. Cold-grown pots from Velbastad have a lower AM colonisation than Sornfelli. At the same time, AM colonisation in cold-grown roots from Sourhope was greater than in the roots from Sornfelli (Fig. 4.1). In addition, the %RLC in the roots maintained at 25°C from Sornfelli was higher than in the cold-grown roots, in contrast to the data from the field (Chapter 2). However, only one fungal type is needed to yield a high %RLC, as demonstrated by a multitude of experiments using single-spore cultures.

Thus, a high %RLC might not be an indicator of a thriving fungal community adapted to a new temperature, but rather an individual response of fungal types. In the warm-grown samples from Velbastad with a high %RLC, only four peaks were found, in contrast to 23 found in cold-grown samples from the same site (Table 4.2). Given that the number of T-RFLP peaks does not correspond to the amount of colonisation, the results might indicate that only some AM fungi form an extensive visible colonisation. This is consistent with the findings of Merryweather and Fitter (1998b) when they examined the %RLC of different fungal morphotypes colonising roots of *Hyacinthoides non-scripta,* and with differences between spore data and root PCR data (e.g. Clapp *et al.*, 1995).

Warming decreased both the Shannon-Weiner diversity and the number of peaks. However, in the pre-warming treatments both the Shannon-Weiner diversity indices and the number of peaks resembled more the data from the cold-grown pots. Therefore, one of the more surprising findings was that the fungal colonisation in the pre-warmed plant roots did not recover during the 25-27 weeks at low temperature. One reason for the discrepancy in the %RLC for the different treatments and sites could be due to different fungal communities. For example, it might be that in the pre-warming treatment some AM fungi that are able to form an extensive colonisation at low temperature are eradicated. However, as the treatments used different environments – a chilled counter versus a growth chamber – other factors than temperature might have influenced the result. Shading, for example, has been demonstrated to have the ability to decrease the %RLC, as well as change the fungal community (Heinemeyer *et al.*, 2003). Thus, the lower %RLC in cold-grown roots from Sornfelli, compared with the warm-grown, might have been due to lower PAR flux densities in the chilled counter.

As the pots were grown at different temperatures it is likely that the soil moisture was affected. Several approaches to diminish this impact have been used. For example Heinemeyer and Fitter (2004) watered their pots three times per day in an attempt to limit any moisture difference. Other have tried to adjust the soil water content by weighing the pots (van der Heijden *et al.*, 2006). Though plant response to colonisation by AM fungi can be affected by the available water content, the response depends on the severity and periodicity of drought or flooding, and several studies have shown that plant moisture deficits of -1.5 to -2.0 MPa did not affect mycorrhizal colonisation (Entry *et al.*, 2002, and references therein). Other have found that moisture deficits increased %RLC (Simpson and Daft, 1990), and as the water might have evaporated to a greater extent at 25°C than at 12°C, and the substrate therefore been drier, this might have contributed to

the observed greater %RLC in the warm-grown plant roots.

According to Ellenberg's original model, *Agrostis capillaris* should be rather indifferent to soil moisture. However, a study from the Faroe Islands, using Huisman-Olff-Fresco models (Huisman *et al.*, 1993) of species abundance with respect to predicted community indicator values suggested calibrated Ellenberg species indicator values for the Faroe Islands, and *Agrostis capillaris* got an F (soil moisture) value of 6 on a scale from 1 to 10 (Lawesson *et al.*, 2003). Another study has dealt with Ellenberg's values in combination with AM fungi; they found that fungal biodiversity decreased with light intensity and soil moisture, but increased with soil pH and nitrogen availability (Mulder *et al.*, 2003). If this was the case for the present study, and the warm-grown pots were drier than the cold-grown, the AM fungal diversity should be lower in the cold-grown pots, which was not the case. On the other hand the light intensity probably was higher in the growth-chamber (25°C) than in the counter, and the lower light in the counter might have contributed to the observed greater AM fungal biodiversity in the cold-grown plant roots.

In the experiment here, the added nutrients in terms of bonemeal were very low. The idea was to keep it low to stimulate fungal hyphal external growth. However, the added level of nutrients might have been too low. The amount of bonemeal was similar to what used by Staddon *et al.* (1999) (0.20 g per litre compared with 0.25 g in this study). However, they used bonemeal only as a supply of phosphorus, and added nutrients (without phosphorus) later in the experiment. No additional nutrients were added in this present study, but the plants did not look nutrient deficient. Bearing in mind that the soil cores stem from cold and wet soils, with a high organic nitrogen content, the soil-cores might have supplied the plants with the needed nitrogen. As organic nitrogen has to be degraded by microorganisms, and these often are more efficient at high temperatures, more ammonia and nitrates might have been released in the warm grown pots. Nitrates are mobile and therefore prone to leach; therefore it is likely that these moved into the sand/terragreen substrate more readily in the warm-grown pots. On the other hand, the initial microorganisms in the soil cores should be well adapted to function at 12°C, and the cold-grown pots should also have experienced some nitrogen fluxes due to microbial degradation.

The fungal T-RFLP profiles from cold-grown roots from Sornfelli were, according to Sørensen's similarity indices, more similar to Velbastad than to Sourhope (Table 4.4) though the similarity was not particularly high. Also, according to the cluster analysis, the fungal community at Sourhope appeared to

be distinct from those at the two Faroese sites, as most of the Sourhope samples clustered in Cluster 2, which is the same cluster as most of the Faroese warm-grown samples were grouped into, but not the Faroese cold-grown or pre-heated (Table 4.6 and 4.7, Fig. 4.2).

The highest mean similarity values were found within the warm treatment, which also manifested the lowest Shannon-Weiner diversity indices. The similarity coefficients obtained in this study ranged from 0.31 to 0.49 (Table 4.4) and were considerable lower than those obtained by Johnson *et al.* (2003), where the similarity values within a group ranged from 0.59-0.79, though the samples from the warm treatments in this present study are nearer to Johnson's *et al.* (2003) values, namely from 0.37 to 0.73 (Table 4.5).

The field work (Chapter 2) indicated that the fraction of fine roots may play an important role in determining colonisation. In this experiment there was also a positive correlation between the fraction of fine roots and the %RLC. As with the %RLC, the fine root fraction was highest at high altitude, where it also is the coldest. Often plants develop coarser roots at low temperatures. This is a general trend found in many pot-studies (e.g., Heinemeyer and Fitter, 2004; King *et al.*, 1999; Gavito *et al.*, 2003 and 2005). Other studies using field-material have found finer and longer roots at low temperatures (Rillig *et al.*, 2002b; Edwards *et al.*, 2004). The total root length increased at higher temperature, as did the fine root fraction. This indicates that root diameter is finer at higher temperature, and therefore that low temperature is not likely to be the explanation for the finer roots found at high altitude in the fieldwork.

The treatments had a more profound effect on the plants from Sornfelli than on those from either Velbastad or Sourhope, indicating that cold-adapted Faroese strains might have a greater response to high temperature than strains from lower altitudes or latitudes.

The contradictory results with higher %RLC in roots grown at 25°C, but lower %RLC after pre-warming might be similar to the findings from other studies (Table 1.1). Those that used a fixed temperature (Hayman, 1974; Schenck and Smith, 1982; Borges and Caney, 1989; Gavito *et al.*, 2003) all found an increase in root colonisation at higher temperatures. The field studies (Rabatin, 1979; Black and Tinker, 1979) and a study that simulated field temperatures (Monz *et al.*, 1994) all found lower root colonisation at higher temperatures. None of these studies used an intentional pre-warming, but field temperatures in most locations should be sufficiently variable to provide a natural pre-warming. This needs, however, further studies before any conclusions can be drawn.

The results from the pre-warming treatments can be compared with a plant study by Marchand *et al.* (2006), where arctic plant species were exposed to two consecutive heat waves (+6°C) of 10 days each, with a 5 days recovery period in between. During the first heat wave, most of the plant species were functioning better. However, after the second heat wave there were indications that the plants had lost their resistance to low temperature. Marchand *et al.* (2006) found that the plant performance deteriorated substantially in the aftermath of the heat waves; thus the observed lack of fungal growth in this study, measured as %RLC, could be a fungal response to a malfunctioning plant, if for example it did not produce enough photosynthate to feed the fungal symbiont.

To conclude: there was a difference in the response in root colonisation in plant roots from different sites. Both root morphology and the fungal communities (estimated by T-RFLP profiles) responded to both temperature and site. The most surprising result was that a pre-warming had an opposite effect to warming on root colonisation.

Chapter 5

The interaction of sugar and phosphate content in *Plantago lanceolata* and the symbiotic fungus *Glomus hoi* in response to growth temperature and phosphate supply

Abstract

- The benefits for the two symbionts in the arbuscular mycorrhizal (AM) symbiosis were tested in relation to soil temperature and soil phosphate.
- Specimens of *Plantago lanceolata* inoculated with *Glomus hoi* were grown at 25°C and received nutrient solution at full strength or 10% strength phosphate. Three weeks before harvest half of the pots from each phosphate treatment were transferred to 15°C.
- Percentage root length colonisation (%RLC), and phosphate and carbohydrate concentrations in plant roots and internal fungal mycelium were also measured.
- Fungal phosphate concentration was greater at high temperature, while the arbuscular colonisation was dependent on both phosphate and temperature treatment.
- Plant phosphate concentration was positively correlated with the number of vesicles, as was fungal sugar concentration with the number of arbuscules.
- The plant carbohydrate concentration was estimated to be 10 times higher than in the fungus, and conversely the fungal phosphate concentration may have been 10 times higher than in the plant. Due to these concentration differences, both symbionts could act as major sinks driving the reciprocal flux of phosphate and carbohydrates.

Introduction

The abundance of arbuscular mycorrhizal (AM) fungi is traditionally quantified by measuring how widespread their colonisation is in roots. Thus, several methods can be found where the mycorrhizal colonisation is measured as the fraction of the total length of a root sample in which hyphae are observed, regardless of the intensity of the colonisation, e.g. "the magnified intersection method" by McGonigle *et al.* (1990) and "the gridline intersection method" by Brundrett *et al.* (1994). Root length colonisation (RLC) data from field experiments (Chapter 2) with *Agrostis capillaris* could indicate that increased temperature results in reduced colonisation, whereas this effect was not seen in a glasshouse experiment (Chapter 4).

In the fieldwork (Chapter 2), the frequency of arbuscules was found to show little variation among species or altitudes, but to be negatively correlated to the mean temperature, while vesicles were more abundant in roots of *Ranunculus acris* (coarse roots), in particular in roots from low altitude, and were positively correlated to soil phosphate. This might indicate that the development of these structures responds to temperature and phosphorus availability.

Field studies are often hard to interpret, since there are so many uncontrolled variables. However, by examining the field data more closely, especially the effect of the aspect on soil phosphate availability (Chapter 2), the hypothesis arose that a combination of phosphate and temperature might explain the observed reduction of colonisation at warmer sites. The higher colonisation at low temperature could be explained if at low temperature there is less available phosphorus and therefore the plants are more dependent on the mycorrhiza. Most mycorrhizal studies have dealt with phosphorus-limited conditions. For example, Koide (1991) found that when phosphorus is limited, the maximum extent to which mycorrhizal colonisation can improve plant performance is predicted to be a function of the phosphorus deficit of the plant, i.e., the difference between phosphorus demand and phosphorus supply. Thus, a plant with a low phosphate concentration in its tissues should be more responsive to mycorrhizal colonisation. A few studies have worked with excessive phosphorus; for example, Mosse (1973) found that mycorrhizal plants given excessive phosphorus grew worse than plants grown in phosphate-deficient soils, compared with non-mycorrhizal plants. Mosse's study suggested that the plants are able to block new colonisation, but not to control colonisation already present, and that the growth of fungi in a root system of a plant that is not limited by phosphorus might resemble growth of a root pathogen.

Fungal biomass associated with well-colonised roots has been estimated at between 3% and 20% of root weight (Smith and Read, 1997, p. 108). As obligate symbionts, the only means for the fungi of gaining carbohydrates is from the plant, and the fungal carbohydrates are used for metabolism as well as for growth. It is known that in the AM symbiosis there is an exchange of phosphate to the plant and carbohydrates to the fungus. The mechanisms behind this exchange, however, are matters for debate. According to Smith and Smith (1996), the phosphorus transfer is controlled by the plant, probably actively, at the arbuscular interface, while the fungi probably take up their carbohydrates through the hyphal interface.

It might not be so important exactly where the uptake occurs, but if the exchange is active, then the symbionts might behave as parasites, unless active defences are used to control each other. If on the other hand, the carbohydrates and phosphates are transferred to the symbiont by a concentration gradient, then the symbiont might be restricted by a low concentration difference, but stimulated when the concentration in the symbiont is high. The latter would ensure that the symbionts normally would not act as parasites, though apparently parasitic associations can occur at particular stages in the development, for example, in the first few weeks following germination (Johnson *et al.*, 1997).

This experiment was set up to verify 1) whether the carbohydrate and phosphate content of both symbionts could explain some of the responses found in the fieldwork (Chapter 2) to temperature, and 2) whether soil phosphate in combination with temperature can affect the %RLC. The aim was to test the following hypotheses and questions:

- The benefits for the two symbionts in the AM symbiosis are dependent on both soil temperature and soil phosphate.
- The observed decrease in %RLC in the field at low altitude is due to reduced fungal growth at elevated temperature.
- %RLC might be an indicator of the benefits the plant achieves from the symbiosis, but is it suitable as a direct estimate of fungal biomass?
- The mycorrhizal fungus increases vesicle production at high soil phosphate, but arbuscule production at low temperature.

Material and methods

Plant and fungal material

Seeds of *Plantago lanceolata* (Ribwort plantain) were germinated on wet filter paper in a Petri dish. When the seeds germinated, the seedlings were transferred to a tray with a substrate consisting of a mixture of sand and terragreen (ratio 5:4 terragreen:sand by vol). The substrate was rinsed twice to remove any soluble phosphate.

After one week 61 seedlings were transferred into 10 cm pots, one in each pot. Each pot was inoculated with mixed substrate/root material of *Plantago lanceolata* with *Glomus hoi* (BEG 101, UY1416) placed around the roots of the seedling (approximately 20 ml).

Experimental design and adjustments

The experiment had two initial treatments: low and high phosphate. In the last three weeks of the experiment, the plants were further divided into two temperature treatments: 15°C and 25°C.

Originally, it was assumed that it would be possible to get enough external mycelium to measure fungal phosphate and carbohydrate, but that it would be difficult to be sure that all the fungal hyphae were AM. Therefore, the idea was to use a non-mycorrhizal plant species, *Sinapis alba* L., as a control, extracting external hyphae from there as well (expecting that the amount would be negligible) as described in Staddon *et al.* (2003a). Unfortunately, external mycelium growth was negligible in all pots. In addition, *S. alba* L. grew very poorly in the growth-chamber and started flowering immediately. For the *S. alba* plants, root and shoot dry weight were measured, as was the %RLC. However, the amount of root of *S. alba* (on average 4.7 g dry weight) combined with a very low colonisation (always below 5%) did not provide enough material for fungal phosphate and sugar analysis, and so these were not measured.

The pots were planted and received their first nutrients at the same time (24 March 2004). Rorison's nutrient solution (Hendry and Grime, 1993) was used: high phosphate pots received unmodified solution (100% phosphate), and the low-phosphate pots received a modified solution with 10% phosphate,- but all other nutrients were at full strength. The pots were placed in a growth chamber (Conviron Model E15, Winnipeg, Canada, temperature 25 °C, 60–80% RH) for 9 weeks, and then separated into two temperature treatment groups, but also continued with the phosphate treatments. The pots were fed twice a week, 20 ml per pot, for the first 6 weeks, then three times a week for another 6 weeks until

harvest on 20 June 2004. For the last 3 weeks of the experiment half of the pots from each treatment were transferred to a 15°C growth chamber (Conviron Model PGR15, Winnipeg, Canada).

The light conditions were changed during the growth period. During the main period, the plants were grown at 330 μmols $m^{-2}s^{-1}$ for 16 hours per day, but this was changed to 200 μmols $m^{-2}s^{-1}$ one week before half of the plants were transferred, so the PAR should be the same in both growth chambers.

Flower buds were removed when noticed in an attempt to delay flowering.

Methods

At harvest, the number of flower stems and leaves (alive and dead) were counted and then each plant was separated into shoot and root. The shoot was dried in a drying oven at 65°C for 6 days. Two small sub-samples were taken from the root (from opposite sides of the pot) for measurement of root length colonisation, then the roots were frozen in liquid nitrogen and stored at –20°C until they could be freeze-dried.

Root length colonisation was determined by the method of McGonigle *et al.* (1990). Frequencies of arbuscules, vesicles, and hyphae connected to either arbuscules or vesicles were recorded.

A preliminary test, where fungal material was extracted from plant roots, used root material from 8 pots; it was not possible to measure the total root biomass from these pots in the same way as for the rest of the pots, therefore the total plant biomass is not available for these pots. From two of these pots there was no root material left for %RLC examination.

For biochemical analysis, 6 replicates of each treatment (24 pots in total) were chosen by selecting plants with minimal contamination by non-mycorrhizal fungi observed during measurement of root length colonisation.

The roots from the 24 selected plants were then separated into two parts, one half (approximately 5 g fresh weight) was frozen for extraction of fungal material, while the rest was freeze-dried for 5 days, then ground to a fine powder. Plant carbohydrate and plant phosphate were measured from the freeze-dried plant root powder. The freeze-dried roots were weighed both fresh and dried, and the water-content determined. The total root dry-mass was a calculated value, where the dry mass of the plant material that was used for fungal extraction was estimated by using the % dry matter in the freeze-dried half, and added to the value of the other half.

The extraction of internal fungal material from mycorrhizal roots was done

according to the method initially described in Saito (1995), with adjustments in Solaiman and Saito (1997) and Solaiman and Saito (2001).

Approximately 5 g of frozen roots (not dried) were allowed to thaw in 0.005 M CaSO4 for a couple of minutes. Then they were cut with scissors into 5 mm long pieces and 50 ml of digestion solution was added. The digestion solution consisted of 0.3 M mannitol, 0.05 M DDT, 0.01 M MES and adjusted to pH 5.5 with NaOH (10 M) and cellulase (5.2 g per liter; Cellulysin from Calbiochem, Darmstadt, Germany, CAS number 9012-54-8, Activity more than 10,000 units / g dry weight), 0.9 g of Pectinase (BioChemika from Fluka, Pectinase from *Rhizopus* sp., no 76285, 1g, 5.3 U per gram), 150 µl of BSA (Promega, Madison, Wisconsin, US). The samples were allowed to digest for 3 hours at 37°C in a shaker, and then left overnight in a fridge.

Next day the samples were poured into a sieve, washed with distilled water, and twice rinsed in a beaker of distilled water before they were transferred to another beaker, 40 ml of distilled water added, and the solution homogenised with a blender (Multiquick MR 400, 250 watt, from Braun GmbH, Kronberg, Germany) for 1.5 min. The solution was filtered through cheesecloth into a new beaker. The root mass on the cheesecloth was put into the blender twice more with 40 ml of distilled water each time.

Of the total volume of 120 ml, 10 ml were filtered onto a 0.45 µm cellulose nitrate filter, stained with acid fuchsin, to check for fungal and plant structures. Hyphae and vesicles could be found, as well as some very fine structures, which might have been either very fine hyphae or arbuscules. Very few plant parts were observed.

The rest of the extract was poured into 4 centrifuge tubes and centrifuged for 10 min at 3500 rpm. Empty Eppendorf tubes were weighed on a microbalance (mean of 3 weighings). Most of the supernatant was then discarded, the pellet re-suspended in approximately 1 ml of the supernatant, and transferred into four 1.5 ml Eppendorf tubes (of known weight). These Eppendorf tubes were then centrifuged for 30 min. at 14000 rpm and 4°C, and the water removed with pipettors. The tubes were then reweighed (mean of 2 weighings), and the fresh weight calculated. The tubes were stored at -20°C for sugar- and phosphate analysis.

To measure both the phosphate content of the root and of the internal hyphae, the molybdenum blue method (Allen, 1974) was used.

The carbohydrate extraction was as follows: Five µg of freeze-dried plant root powder (for plant analysis), or approximately 20 µg fresh-weight fungal

material (for fungal analysis), was placed in a 1.5 ml Eppendorf tube; 500 µl of 80% ethanol was added and then the solution was heated to 80°C for 10 min. The tube was then centrifuged at 12000 rpm and the supernatant consisting of ethanol with soluble carbohydrates was collected into another Eppendorf tube. These steps – but without heating – were repeated twice, so the total amount of supernatant was 1.5 ml. The supernatant was let to evaporate overnight at 50°C.

For two of the plant samples there was some spillage and they were therefore omitted from the analysis for quantitative sugar analysis. These samples belonged to separate groups, so the number of samples in the analysis was 5 for two of the groups, and 6 in the other two groups.

Two fungal samples were omitted from the dataset, that were also from two separate groups. They were omitted because they were from a preliminary run with 6 samples to test the method. Two of the 6 samples in the test run were in the group that later was chosen for the final analyses, and these two samples were therefore not in the final run. However, it turned out that the results differed significantly from the new run.

Soluble oligosaccahrides were analysed on a Dionex BioLC system (Sunnyvale, California, US) by the Technology Facilities at York University, UK, with the column PA-10. The samples were run for 20 min. The sugars that were used as known standards were arabinose, arabitol, cellobiose, dulcitol, erythritol, fructose, galactose, glucose, glycerol, isomaltose, maltitol, maltose, mannitol, mannose, melizitose, psicose, raffinose, ribitol, ribose, sorbitol, stachyose, sucrose, trehalose, and xylose from Sigma-Aldrich (St. Louis, Missouri, US). Unknown sugars were quantified by using the response factor for sucrose. A peak that eluted at the same retention time as a known sugar was considered as that specific sugar. The retention times were expected to vary 1-2% (D. Ashford, pers. comm), which might influence how reliable the separation of for example mannitol and trehalose was, as the retention times for these two sugar-alcohols were only 0.02 min apart.

Statistics

All statistics were carried out in SPSS 11.03 for Mac OS X. The samples were tested for normal distribution with the One-Sample Kolmogorov-Smirnov test. Pearson's correlation was used. When testing for variance of mean Univariate Analysis of Variance (ANOVA) was used.

Results

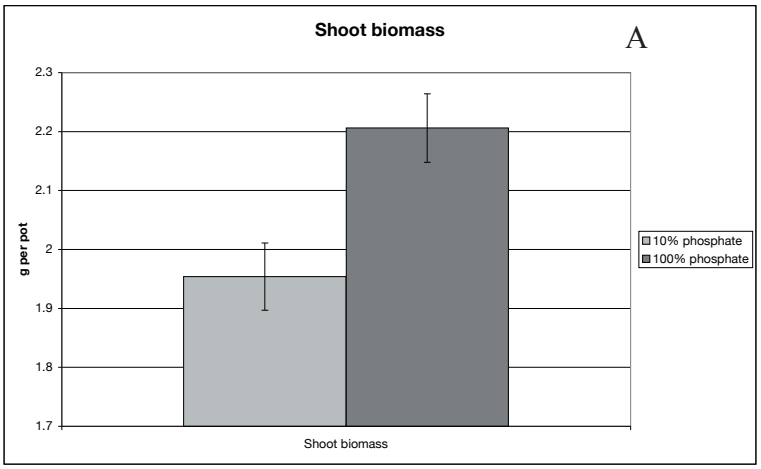
Most of the variables measured displayed no response to the treatments and so the mean values are presented in Table 5.1. Significant direct responses to treatments were seen only in fungal phosphate concentration, arbuscular colonisation (%RLCarb) and plant shoot biomass (Table 5.2). Shoot biomass was higher at full strength phosphate treatment (Fig. 5.1a), and the fungal tissues contained more phosphate when growing at high temperature (Fig. 5.1b). The percentage of root length colonised by arbuscules was greatest in the combination of high temperature, low phosphate treatment (Fig. 5.1c), but responded to both treatments (Table 5.2). The %RLC was dominated by vesicles, 72% of the variation of %RLC could be explained by the %RLCves (Linear regression, $F_{1,57} = 153$; $p < 0.001$).

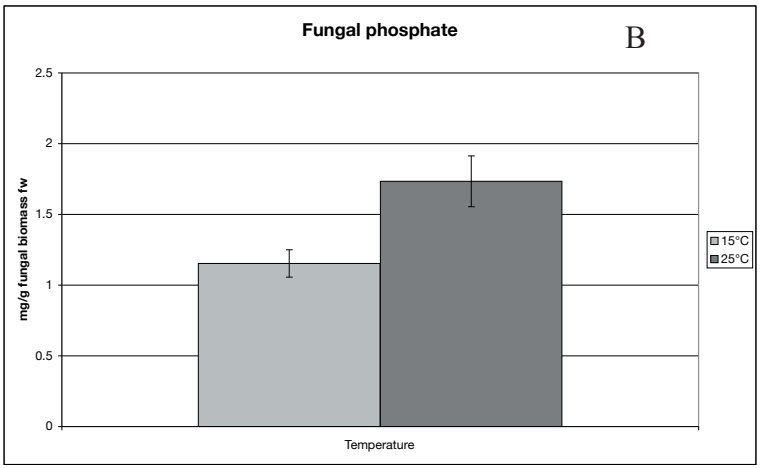
Variable	Units	Mean	SE	N
Fungal biomass	mg fw per g plant root dry weight	13.9	0.6	20
Fungal biomass / pot	mg fw per pot	128.9	5.4	22
Estimated fungal dw in % of root dw	% (estimated 15% dw)	0.2	0.01	20
Plant biomass	g dw per pot	11.43	0.2	53
Fungal sugars	pmol per mg fungal biomass fw	77.9	5.5	22
Plant root sugars	pmol per mg root dw	5516	119	22
Plant root phosphate	mg/g root dw	1.5	0.1	24
Fungal phosphate	mg/g fungal fw	1.6	0.1	21
%RLC	percentage root length colonised	25.8	1.5	59
%RLCarb	percentage root length colonised by arbuscules or hyphae with arbuscules	4.5	0.9	59
%RLCves	percentage root length colonised by vesicles or hyphae with vesicles	24.2	1.3	59
Number vesicles	number of vesicles per 100 intersections	19.6	1.5	59

Table 5.1. Means of biomass, sugar, phosphate and root colonisation measurements. SE = Standard Error of Mean. N = number of samples. Abbreviations: fw = fresh weight, dw = dry weight.

	Phosphate	Temperature	Phosphate*Temp.
Shoot biomass	$F_{1,57} = 9.723$; $p = 0.003$	n.s.	n.s.
Fungal phosphate	n.s.	$F_{1,17} = 15.666$; $p = 0.001$	n.s.
%RLCarb	$F_{1,55} = 11.291$; $p = 0.001$	$F_{1,55} = 7.092$; $p = 0.010$	$F_{1,55} = 6.110$; $p = 0.017$

Table 5.2. The variables with a significant response to treatments tested with univariate analysis of variance.





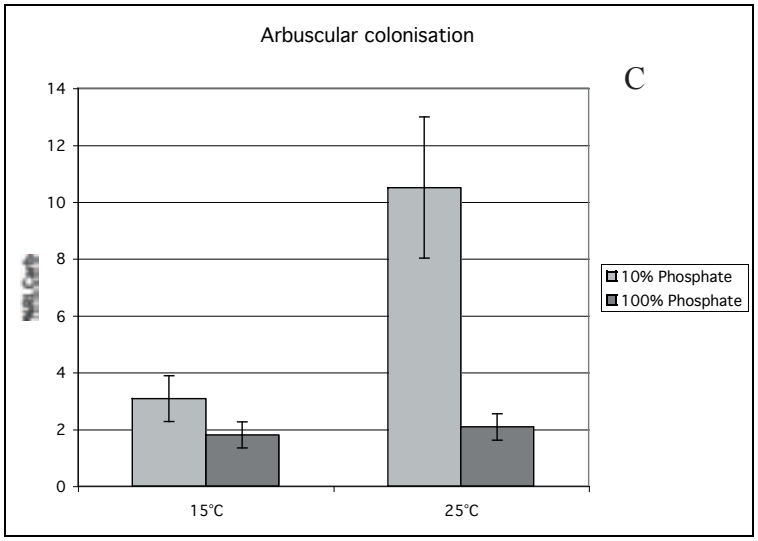


Fig. 5.1 (a) Mean shoot biomass with phosphate provided at either 10% strength or full strength, **(b)** Mean fungal phosphate concentration with growth temperature either 15°C or 25°C, **(c)** Mean arbuscular colonisation, with phosphate provided at either 10% strength or full strength and growth temperature either 15 or 25°C. Bars indicate ±SE.

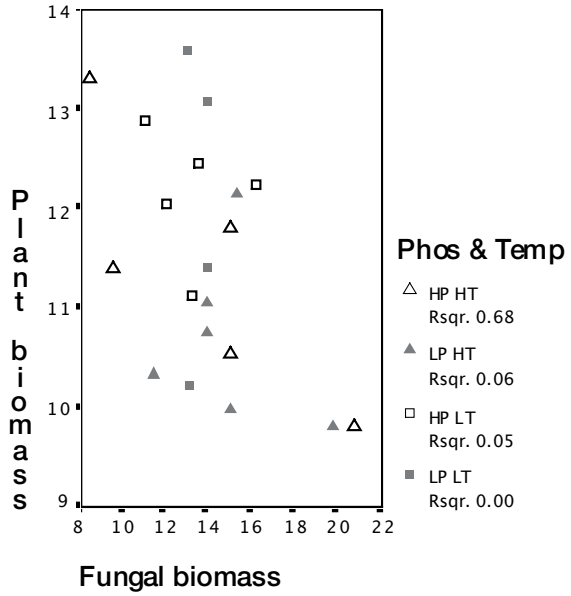
In addition to these significant responses to treatments, there were some correlations between variables (Table 5.3). The negative correlation between plant and fungal biomass implies that increased fungal biomass may have negatively affected plant growth. Within treatments, this negative correlation was restricted to high phosphate treatment and high temperature treatment (Fig. 5.2). Abundance of arbuscules and vesicles were correlated to different variables. Plant phosphate was higher in samples with many vesicles under most conditions, the relationship was less profound when both phosphate and temperature were low (Fig. 5.3). Fungal sugars increased with arbuscular colonisation, and did so under most conditions, apart when both phosphate and temperature were high (Fig. 5.4).

The number of vesicles was marginally significantly higher in the high phosphate treatment, but only when plant phosphate was used as a covariate ($F_{1,21} = 3.650$; $p = 0.070$); on the other hand analysing the response of %RLCves (instead of the number of vesicles) to phosphate treatments with plant phosphate as covariate indicated that the root length with vesicles was greater at high phosphate treatment, but the %RLCves was also influenced by the plant phosphate content ($F_{1,21} = 5.021$; $p = 0.036$. Covariate $F_{1,21} = 5.751$; $p = 0.026$).

	All samples		LP		HP		LT		HT	
	r	p	r	p	r	p	r	p	r	p
Biomass Plant/Fungi	-0.510	0.022			-0.702	0.024			-0.656	0.028
Plant P/ number vesicles	0.536	0.007			0.753	0.005	0.511	0.090	0.636	0.026
FuSugars/ %RLCarb	0.654	0.001	0.835	0.001			0.552	0.098	0.745	0.005

Table 5.3. Pearson's correlations of selected variables. Only significant correlations are shown. Biomass Plant/fungi: Plant biomass (g dw pot) correlated with fungal biomass (mg fw /mg plant root dw). Plant P/no vesicles: Plant phosphate (mg/g dw) correlated with the frequency of vesicles per 100 intersections. FuSugars/%RLCarb: Fungal sugars (pmol/mg fw) correlated with the percentage root length colonised with arbuscules.

There was insufficient fungal material left for estimation of the water content of *Glomus hoi* mycelium. Harley (1971) estimated hyphal water content to be 90% of fresh weight. For fungal mycelium in general a common estimate is 80%, as used by Solaiman and Saito (2001). *Glomus intraradices* from monoxenic



5.2. The correlation between plant biomass and fungal biomass. Open triangles: High phosphate treatment in combination with high temperature. Gray triangles: Low phosphate treatment in combination with high temperature. Open squares: High phosphate treatment in combination with low temperature. Gray squares: Low phosphate treatment in combination with low temperature. The R-squared values are unadjusted.

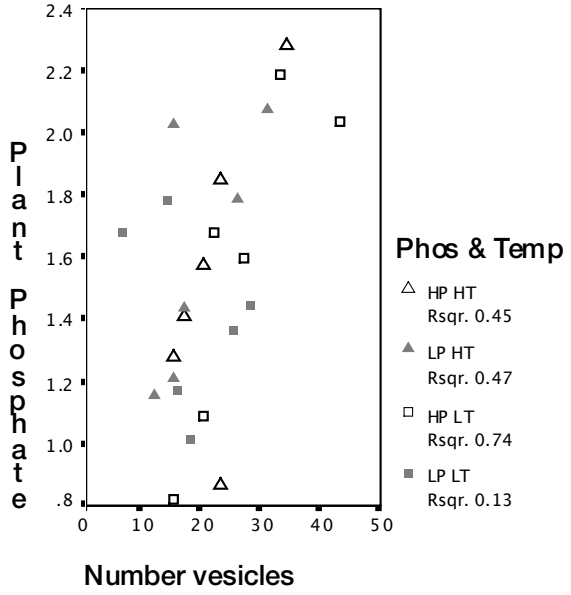


Fig. 5.3. The positive correlation between the number of vesicles per 100 intersections and plant phosphate concentration. Symbols as in Fig. 5.2. The R-squared values are unadjusted.

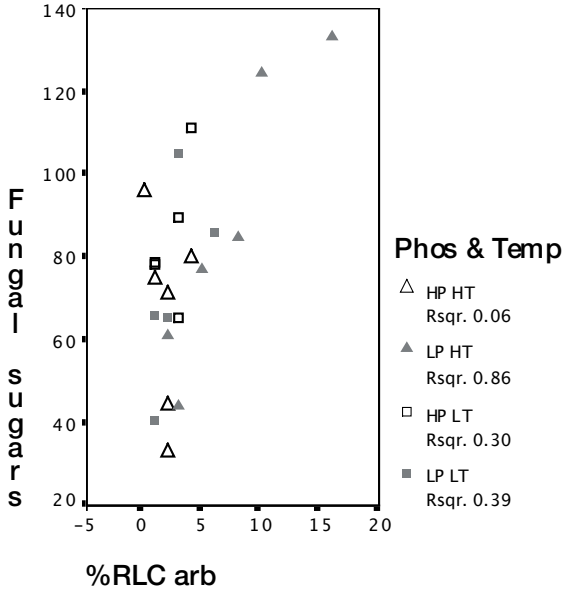


Fig. 5.4. The positive correlation between fungal sugars and the arbuscular colonisation (%RLCarb). Symbols as in Fig. 5.2. The R-squared values are unadjusted.

cultures is found to have 86% water content (Pål-Axel Olsson, pers. comm.). Therefore, the water content of the fungal biomass here was assumed to be 85%, which implies that the fungal sugar and phosphate values could be 8.5 times higher if the dry weight were used.

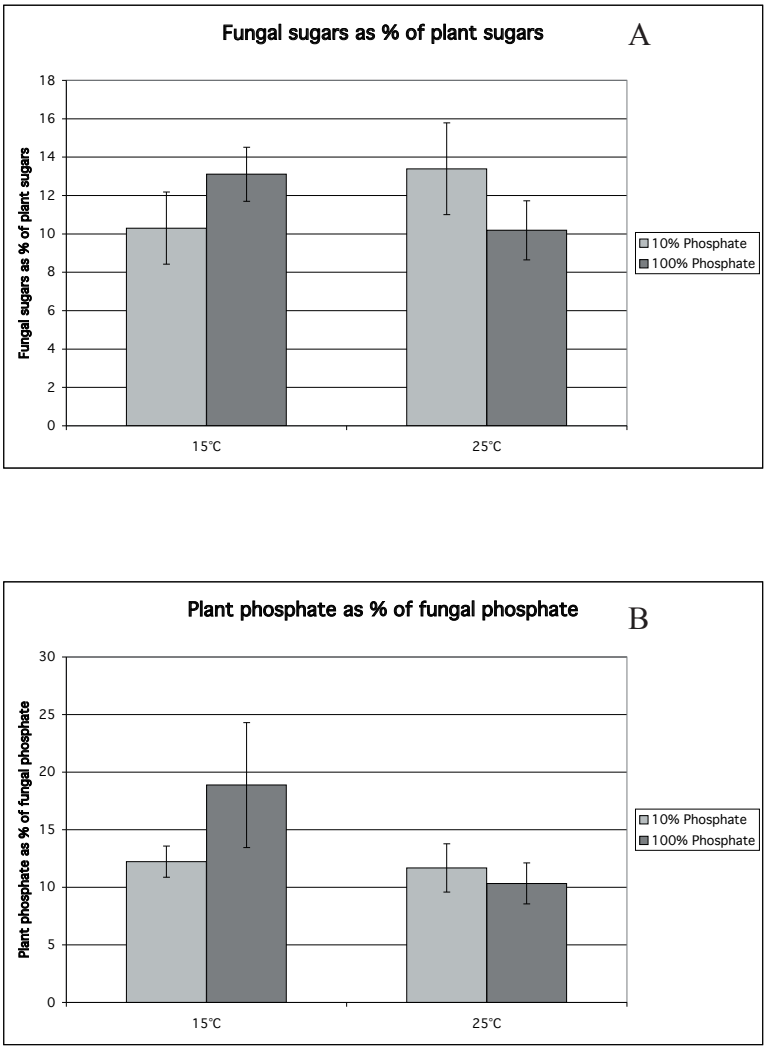


Fig. 5.5 (a) Mean fungal sugars as % of plant sugars and **(b)** Mean plant phosphate concentration as % of fungal phosphate concentration (see units in Table 5.1), in each case with phosphate provided at either 10% strength or full strength and growth temperature either 15 or 25°C. Fungal water content is estimated to be 85%. Bars indicate ±SE.

Fig. 5.5 shows estimated relative plant and fungal sugar and phosphate concentrations; it appears that fungal sugar concentrations were very low relative to plant sugars, but that fungal phosphate concentrations were high relative to plant phosphate concentrations.

In the sugar analysis peaks corresponding to 31 plant sugars and 16 fungal sugars were found: 13 of the plant sugars and 11 of the fungal sugars had a retention time similar to those used as known sugar standards (Table 5.4). The main soluble sugar in the plant root material was unidentified (retention time 11.37-11.52), and might consist of a complex of different oligosaccharides, as the peak is rather wide (Fig. 5.6). The peak 11.37-11.52 accounted for ca. 30% of the total plant sugars. Isomaltose, glucose and mannitol accounted for around 10% each. Glucose was the main fungal sugar, accounting for ca. 40% of the total amount, while mannitol, trehalose, cellobiose and an unknown sugar (retention time 6.22) accounted for upwards of 10% (Table 5.4).

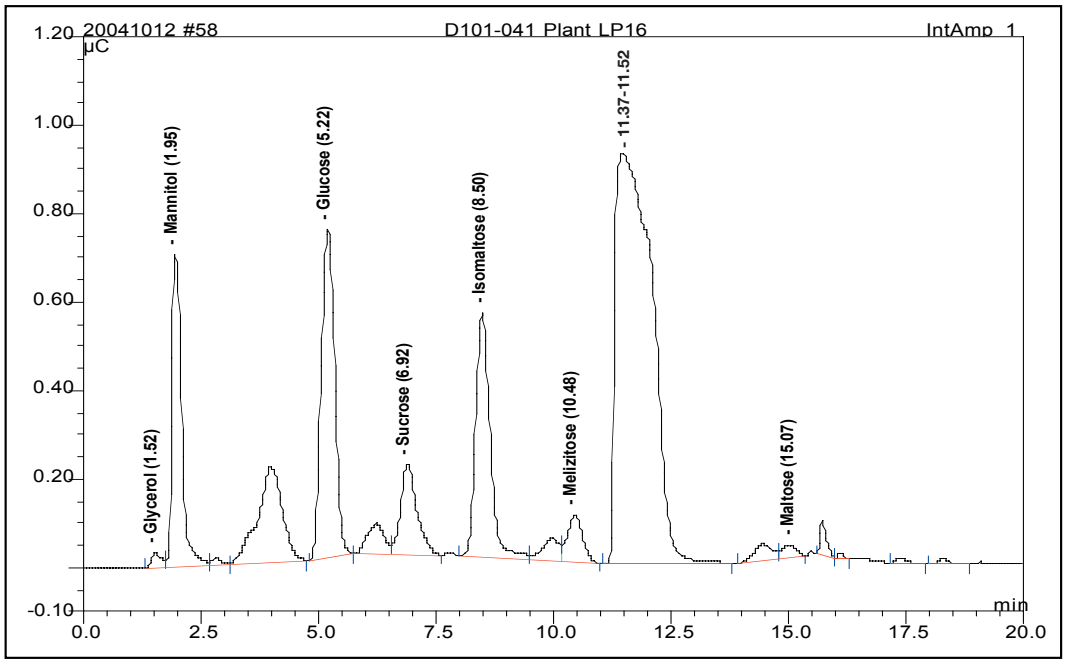


Fig. 5.6. An example of the output from the analysis of oligosaccharides showing a typical plant sample. Several unidentified sugars were found. The largest peak, between melizitose and maltose is referred to as "11.37-11.52". The number in brackets after the sugar name indicate the retention time.

Two fungal sugars had a significant response to treatments: mannitol and trehalose both responded to phosphate, but in opposite directions (Fig. 5.7, Table 5.5). Several plant sugars had a significant response to treatments; all of them responded significantly to temperature, and some of them also to phosphate (Fig. 5.8 and 5.9, Table 5.5).

Plant root oligosaccharides	Mean %	Fungal oligosaccharides	Mean %
%Plant glycerol	0.3	%Fungal glycerol	4.1
%Plant sorbitol	0.4	%Fungal arabitol	0.2
%Plant erythritol	0.1	%Fungal sorbitol	0.3
%Plant mannitol	9.2	%Fungal mannitol	11.1
%Plant 2.80-2.84	0.2	%Fungal trehalose	10.6
%Plant 3.55-3.58	0.5	%Fungal arabinose	3.5
%Plant 3.98-4.06	7.5	%Fungal glucose	41.2
%Plant glucose	11.6	%Fungal 6.22	11.3
%Plant fructose	0.6	%Fungal 7.22	0.3
%Plant 6.22-6.30	1.1	%Fungal isomaltose	1.0
%Plant sucrose	3.7	%Fungal melizitose	0.5
%Plant 7.22	< 0.05	%Fungal cellobiose	13.5
%Plant isomaltose	12.8	%Fungal maltose	1.3
%Plant raffinose	< 0.05	%Fungal 15	0.7
%Plant 9.82-10.03	1.2	%Fungal 16.05-16.18	0.1
%Plant melizitose	1.6	%Fungal 16.6	0.4
%Plant stachyose	9.0		
%Plant 11.37-11.52	30.5		
%Plant cellobiose	3.9		
%Plant 11.95	2.6		
%Plant 13.23-13.28	< 0.05		
%Plant 14.28-15.57	1.0		
%Plant 14.85-14.93	0.2		
%Plant maltose	0.6		
%Plant 15.49-15.55	< 0.05		
%Plant 15.72-15.80	0.7		
%Plant 16.05-16.18	0.1		
%Plant 16.64-16.75	< 0.05		
%Plant 17.32-17.42	0.1		
%Plant 18.34-18.40	0.2		
%Plant 19.02-19.18	< 0.05		

Table 5.4. The mean percent of each sugar-peak of total plant sugars (left) and total fungal sugars (right).

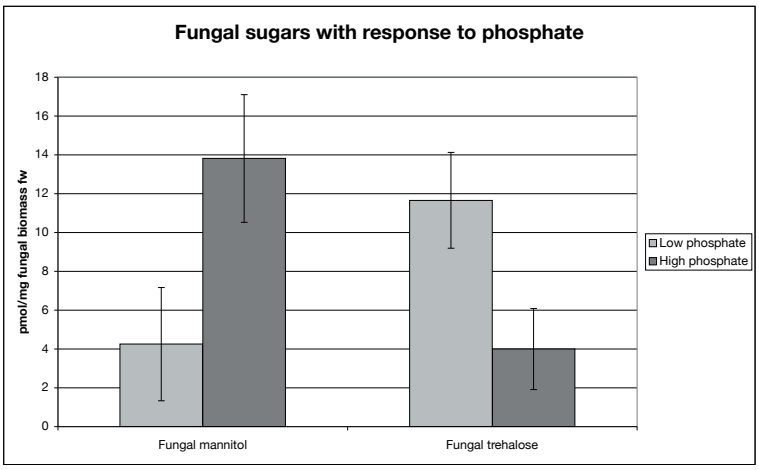
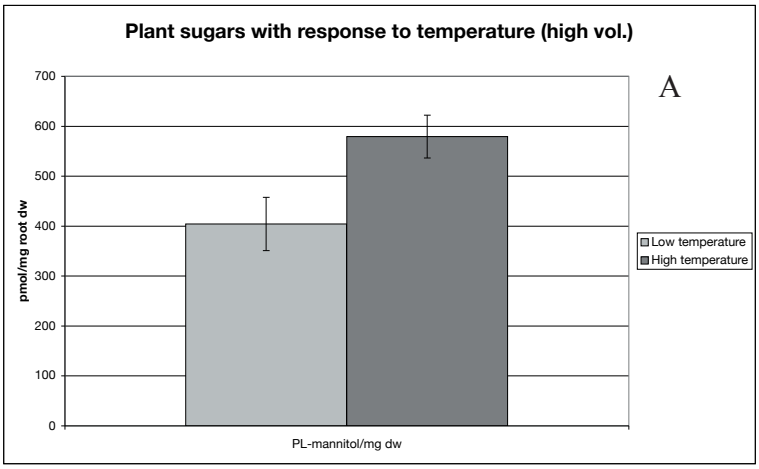


Fig. 5.7. Means of the response of the fungal sugars mannitol and trehalose to phosphate provided at either 10% strength or full strength. Bars indicate ±SE.



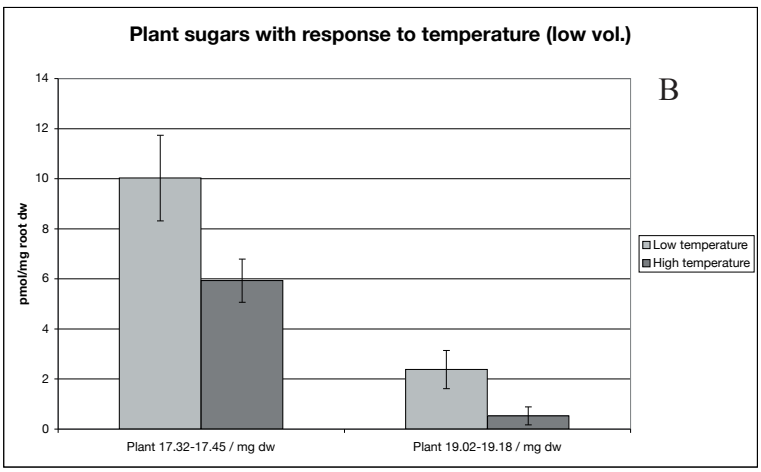


Fig. 5.8. Means of the plant sugars with **(a)** more than 100 pmol/mg plant root and **(b)** less than 100 pmol/mg plant root, which reponded significantly to growth temperature at 15 and 25°C. Bars indicate ±SE.

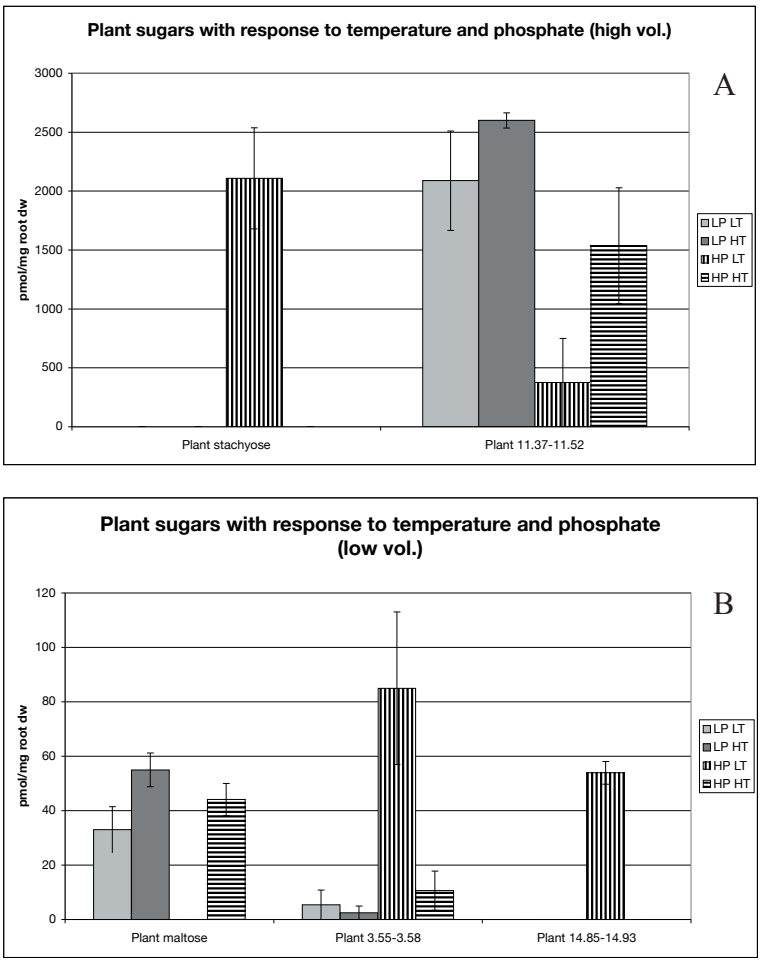


Fig. 5.9. Means of the plant sugars with **(a)** more than 100 pmol/mg plant root and **(b)** less than 100 pmol/mg plant root, which reponded significantly to the factorial design with phosphate provided at either 10% strength or full strength and growth temperature either 15 or 25°C. Bars indicate ±SE. LP LT: low phosphate and low temperature treatment; LP HT; low phosphate and high temperature treatment; HP LT: high phosphate and low temperature treatment; HP HT: high phosphate and high temperature treatment.

	Phosphate	Temperature	Phosphate*Temp.
Fungal mannitol	0.048	n.s.	n.s.
Fungal trehalose	0.039	n.s.	n.s.
Plant maltose	0.002	< 0.001	0.081
Plant mannitol	n.s.	0.023	n.s.
Plant stachyose	< 0.001	< 0.001	< 0.001
Plant 3.55-3.58	0.007	0.016	0.025
Plant 11.37-11.52	0.001	0.038	n.s.
Plant 14.85-14.93	< 0.001	< 0.001	< 0.001
Plant 17.32-17.42	n.s.	0.043	n.s.
Plant 19.02-19.18	n.s.	0.046	n.s.

Table 5.5. Analysis of variance with identified and unidentified plant and fungal sugars, measured as pmol/mg of root dw and fungal biomass fw respectively. Identified sugars are named, unidentified sugars are named after the time in minutes it took them to appear. Only p-values are shown, and only sugars that had a significant response to either treatment are shown.

The ratio of fungal biomass (mg fw per g plant root dw) to fungal phosphate (mg per g fungal fw) was used as an estimator of whether the fungi utilized the phosphate for growth. The mean ratio of fungal biomass/fungal phosphate at low temperature was 12.0, but at high temperature 8.1 (Fig. 5.10), indicating less biomass at high temperature per fungal phosphate ($F_{1,17} = 5.672$; $p = 0.029$).

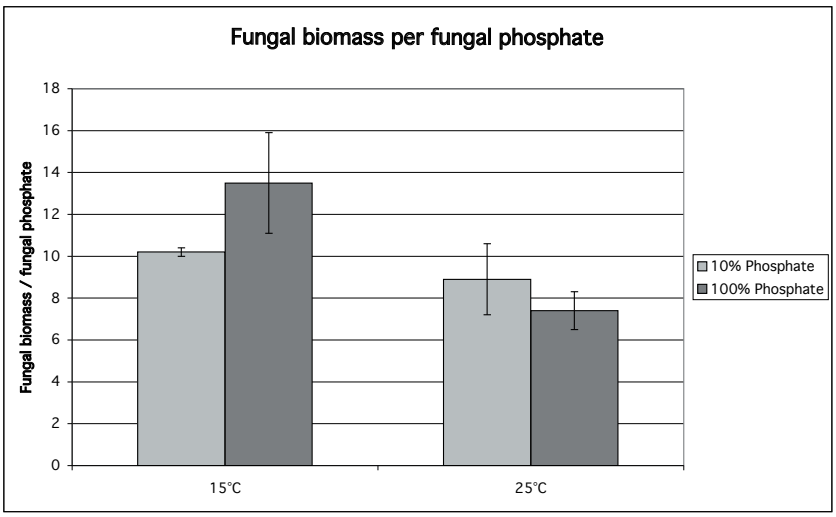


Fig. 5.10. The mean ratio of fungal biomass/fungal phosphate in response to the factorial design with phosphate provided at either 10% strength or full strength and growth temperature at either 15 or 25°C. Bars indicate ±SE. Fungal biomass / fungal phosphate = Fungal biomass (mg fw per g plant root dw) divided by the fungal phosphate (mg per g fungal fw).

It might be expected that the %RLC or number of fungal structures would be positively correlated to the fungal biomass, but such a relationship was not found. At low temperature, the root length colonised with arbuscules was weakly correlated with total fungal biomass per pot ($r = 0.618$, $p = 0.057$, $n = 10$). Fungal biomass (mg fw per g plant root dw) shared no relationship to %RLC.

Discussion

One striking result from this study was that, when allowing for the water content of the fungal biomass, the carbohydrate concentration in the plant root tissue was around 10 times higher than that in the intraradical fungal tissues (Fig. 5.5). The fungus should therefore act as a major sink and receive sugars under all conditions. The same applies to the ratio of fungal phosphate to plant phosphate; here the fungal phosphate concentration is around 10 times that in the plant, and thus the plant should act as a sink and receive phosphates under all conditions. In the calculations the fungal water content was estimated to 85%. Even if the fungal water content were considerably lower, say 50%, with the resultant numbers decreasing three to four-fold, the symbionts would still act as sinks in the same way.

The plant root material consisted of both plant and fungal tissue; however, the estimated fungal percentage of plant root biomass was 0.2%, and therefore the fungal material should not have had any significant impact on the plant measurements. This is further confirmed by the sugar profiles, for example there was no trehalose in the plant measurements, though trehalose accounted for a similar amount as mannitol in the fungal sugar pool. During the extraction of fungal tissue from the plant roots, contamination by plant material might have occurred, and membranes might have been damaged followed by leakage. However, direct plant contamination should then be reflected in the sugar profiles, which it is not. If there was leakage from the cells, the fungal phosphate concentration would have been under-estimated.

The original idea was to measure phosphate and sugars in the external mycelium, but there was too little external mycelium to be able to extract enough fungal material to analyse. Therefore the internal mycelium was used instead. This lack of external fungal growth could be due to the low light intensity unavoidably used during the last weeks of the experiment, as shading decreases growth of the external mycelium (Heinemeyer *et al.*, 2003).

The method of homogenising the material might seem rather severe. Some studies have obtained internal fungal mycelium by using enzymatic digestion only, and then used needles to isolate the fungal material; however, this method proved to be effective for roots of some plant species only (Hepper *et al.*, 1986; Shachar-Hill, Y., pers. comm.). Further to this, in this present study fungal structures were visible, and according to Solaiman and Saito (2001) the structures might also have been functional, as they were able to measure phosphate efflux from intraradical hyphae separated from roots, and their results suggested, that at

the least part of the P efflux was not leakage from damaged tissue.

Solaiman and Saito (2001) also found a high fungal phosphate content compared with the plant phosphate. Their fungi had 30 times more phosphate per mg than the plant, based on fresh weight. Solaiman and Saito (2001) used *Allium cepa* L. roots colonised with *Gigaspora margarita*, so some differences were to be expected due to different plant and fungal species combination, as different AM fungus-plant combinations are functionally diverse both in effectiveness of the fungi as symbionts and in responsiveness of the plants in terms of total P uptake and growth (Smith *et al.*, 2003).

If the fungus and the plant act as sinks in response to carbohydrates and phosphate respectively, then the fungal carbohydrate concentration and the plant phosphate concentration would not be expected to show any direct response to soil temperature or soil phosphate, which is what was found.

Smith *et al.* (2004) found that all AM plants contained some phosphate obtained from the fungus, regardless of responsiveness measured as plant growth and phosphate content. This could be because the plant acts as a sink. They did not find any clear relationship between %RLC and plant growth or P response. In the findings presented in this chapter, there is a relationship between plant phosphate concentration and colonisation, in particular the amount of vesicles (Table 5.3).

There has been some debate whether root length colonisation measurements are an ideal measure of the benefits achieved from the symbiosis. For example, McGonigle (1988) analysed 78 published field trials and found that there was no relationship between the magnitude of the change in colonisation and that of the change in yield, measured as plant biomass. Hart and Reader (2002) found a good correlation between %RLC and the ergosterol content of the roots, though some fungal species had a better fit than others.

According to the findings presented here, an increase in %RLC does not necessarily mean that the fungal biomass is higher. The colonisation was dominated by vesicles (Table 5.1), and the contribution from vesicles to fungal biomass might differ from the contribution from arbuscules. The fungal biomass was measured by fresh weight, and the water content might differ in vesicles and arbuscules, for example vesicles, if they are lipid stores, might contain less water than arbuscules.

The hypothesis that the number of vesicles would increase with increased soil phosphate was only partially supported, as it was only when taking into consideration the plant phosphate content, which also tended to be higher when receiving full strength phosphate nutrient solution. The arbuscules did not have

a temperature response as expected. The response of arbuscular colonisation to phosphate was highly significant (Table 5.2), with more arbuscules at low soil phosphate, which is in accordance with what is found in most studies (Smith and Read, 1997, p. 77). However, the number of arbuscules increased at high temperature (Fig. 5.1c), instead of the expected decrease.

The fungus responded to high temperature by accumulating phosphate in the tissue. This could be explained if their uptake mechanism is limited by low temperature, or if the plant is more efficient in acquiring phosphate from the fungi at low temperature. These explanations, however, would likely be reflected in the plant phosphate, and there was no evidence for that. Therefore, another explanation might be that the fungus for some reason was not able to increase its biomass consistent with the phosphate concentration at high temperature, and therefore accumulated phosphate. If fungal growth is inhibited by high temperature, this could be a partial explanation for the decrease in %RLC observed at warmer sites in the fieldwork.

Under warm and phosphate rich conditions there was a negative correlation between plant and fungal biomass (Fig. 5.2). This treatment combination in the study by Mosse (1973) resulted in worse plant growth for mycorrhizal plants given excessive phosphorus. In the same treatment combination the fungi might have been able to take up sugars without producing arbuscules, as there was no relationship between fungal sugars and arbuscular colonisation for this particular treatment combination (Fig. 5.4) and the arbuscular colonisation was low (Fig. 5.1c). Thus at high phosphate and high temperature treatment the fungus produced few arbuscules, but received sugars and delivered no phosphate in return, but instead accumulated phosphate. This implies that the phosphate exchange mainly occurs in the arbuscules as suggested by several studies (e.g. Smith and Smith, 1996; Jakobsen *et al.,* 2002).

According to Solaiman and Saito (1997) glucose is the most likely substrate for AM fungi. Shachar-Hill *et al.* (1995) demonstrated that *Glomus etunicatum* incorporates glucose into trehalose, mannitol and glycogen. In the host the glucose was incorporated into sucrose, but to a lesser extent in mycorrhizal roots than non-mycorrhizal. The findings from this study show that the fungal glucose is the most abundant sugar in the internal mycelium, and that both trehalose and mannitol are present as well. Glycogen was not measured. It can not be ruled out that the high content of glucose (Table 5.4) stems from glycogen degradation during the 3 hours enzymatic digestion. Another indication that this could be the case is that glucose showed no response to treatments.

The plant sugars have not been through any digestion and might therefore show a more realistic picture. In contrast to the fungal sugars, where only mannitol and trehalose showed a significant response to the phosphate treatment, and none fungal sugars responded to the temperature treatment, several plant sugars responded to both treatments, implying that even though the total sugar pool as a whole did not respond to the treatments, the individual sugars did. To analyse the individual sugar pools further studies are required.

To conclude, the benefits for the plants are in terms of increased phosphate obtained from the fungal phosphate pool through the arbuscules. The fungal phosphate concentration was dependent on temperature, while the arbuscules were dependent on both substrate phosphate and temperature. Further there was a relationship between fungal sugars and arbuscular colonisation which implies that with more arbuscules the plant should receive more phosphate, and the fungus more sugars. The arbuscules are probably the main site for nutrient exchange, though it is likely that exchange also occurs at other interfaces due to the large concentration differences in the two symbionts.

Chapter 6

General discussion

General discussion

The unique position of the Faroe Islands in the midst of the North Atlantic Current makes them a relevant place to look for potential effects of climate change, because any changes in the surface water is likely to have a significant and immediate impact on the local Faroese climate. Vegetational studies have suggested that alpine plant zones have moved to a lower altitude in a cold period. However, no studies have examined the biotic part of Faroese soils in response to climate change. This project was on arbuscular mycorrhiza (AM), and the first step forward was to characterise the AM fungal communities of the Faroes (Chapter 2). In a continuation of the field work, Open Top Chambers (OTC) placed on one high altitude site as part of the International Tundra Experiment (ITEX) were used to examine the impact of passive warming (Chapter 3), and from the same site samples were taken for a controlled warming experiment (Chapter 4), looking at differences in root colonisation and fungal communities at a temperature close to the mean summer temperature in the Faroes (11°C) and one temperature within the upper limit of what is measured in the Faroes (25°). All the initial experiments used field material with unidentified fungi. The final experiment, however, used a typical laboratory species of both plant and AM fungi, examining the impact of temperature on symbiotic function (Chapter 5).

The main finding from this project is that arbuscular mycorrhizal (AM) fungi from the Faroe Islands respond to temperature, and do so in the same way as other field studies have shown: less root colonisation at elevated temperatures. However, there was evidence that there may be cold-adapted AM fungal taxa in this cool, wet environment, and this finding has large implications for the response of biological communities to climate change. The response also varied with plant species, root morphology and nutrient availability.

The field survey found that root length colonisation (%RLC) was greater at high altitude in *Agrostis capillaris* but not in *Ranunculus acris.* Moreover, in *Agrostis capillaris* the colonisation was greater in a colder year; this suggests that temperature may play a role. The %RLC from the OTC experiment also showed a higher colonisation at low temperature. When field samples were treated with controlled temperatures in a laboratory, the results were not so conclusive, as there were considerable differences in colonisation responses from the different sites. However, pre-warming followed by low temperatures reduced colonisation, indicating that, after a fortnight with high temperatures, the fungi were not able to respond once low temperatures were restored.

Root morphology also had a profound response to temperature: high colonisation was associated with a large fraction of fine roots in both plant species, and the latter variable may have been the one that responded directly to temperature. The field responses were different from those in the laboratory: in the field the fraction of fine roots was greatest at high altitude (in *Agrostis capillaris*) and in the ITEX experiment the fraction of fine roots had a better correlation than %RLC to the minimum temperature. However, when brought into laboratory, the fraction of fine roots was much greater in warm-grown pots than in cold grown, which probably was responsible for the greater colonisation at high temperature. A greater fraction of fine roots at high temperature is what usually is found in controlled experiments, but one important and often overlooked aspect of soil temperature is short-term variability, and the multiple soil factors that covary as soil temperaturer increase (Pregitzer *et al.*, 2000).

Even though effects on total colonisation were slight, there was evidence for a temperature-related change in the composition of the fungal assemblage in roots. Some of the fungal types were only found at low altitude, where both temperature and temperature range are higher. Moreover, species richness of the fungi was a function of the temperature range, which is consistent with the idea that fungal taxa differentiate with respect to temperature, and that a variable environment allows more taxa to co-exist. The same phenomenon might explain the lower fungal diversity in the OTCs, as the temperature range particularly during the summer was reduced.

The fungal diversity was clearly affected by laboratory-based temperature manipulations, with the highest diversity in cold-grown roots, and lowest in the warm-grown. In the pre-warmed roots the fungal diversity was higher than in the warm-grown, but lower than in the cold-grown, suggesting that some fungal taxa adapted to the cold were eliminated, while other cold-adapted fungal taxa were able to recover once low temperatures were restored after a warm period.

The impact of temperature on symbiotic function was examined in controlled conditions with a cultured fungal isolate. The main effect was that temperature increased fungal phosphate concentration, while arbuscule frequency was dependent on both substrate phosphate and temperature. Further there was a relationship between fungal sugars and arbuscular colonisation which implies that with more arbuscules the plant should receive more phosphate, and the fungus more sugars. The experiment used a fungus isolated from a warm soil (York, UK) and found more arbuscular colonisation at high temperature, if the substrate phosphate was low. In the field more arbuscular colonisation was found at high

altitude, and it would be important to know how a high altitude Faroese fungus would behave in an experiment as that in Chapter 5.

There are several issues not dealt with in this study, including the mechanistic processes of transporting sugars and ions through membranes. Several studies have dealt for example with root nitrogen acquisition and assimilation (Miller and Cramer, 2004). However, though different transporter and assimilatory genes able to provide access to different pools of nitrogen, such as ammonium, nitrate and amino acids, have been identified, the role of each in plant nitrogen nutrition has still to be determined, and Miller and Cramer (2004) questioned the role of AM fungi in acquiring nitrogen, although the AM fungi may facilitate mineralisation of organic nitrogen.

Little is known about the mechanisms of nitrogen uptake by AM fungi (Hawkins *et al.*, 2000), though some hypotheses have been presented. For example Jin *et al.* (2005) suggested that AM fungi take up inorganic nitrogen and are able to transfer the nitrogen to the host after several steps which include amino acids and co-transport with polyphosphate.

Polyphosphate is accumulated in the external hyphae, and the accumulation is rapid: according to Ezawa *et al.* (2003) it takes less than three hours after phosphate application for the concentration of polyphosphate to reach a maximum. Polyphosphate has been recognized as the primary phosphorus compound transferred from external hyphae to internal and arbuscular hyphae (Saito, 2000), and the function may be to reduce the osmotic stress at high internal phosphate concentrations (Bücking and Shachar-Hill, 2005). The relative amount of phosphate is higher in the internal hyphae than in the external (Solaiman and Saito, 2001); on the other hand the polyphosphate chains are shorter in the internal hyphae than in the external hyphae (Solaiman *et al.*, 1999).

Radiotracer studies have demonstrated that phosphate moves from the soil via the fungal hyphae to the plant, but relatively little is known about the phosphate transport proteins involved (Harrison *et al.*, 2002). Several phosphate transporters have been isolated from plant material (Rausch and Bucher, 2002), and it is demonstrated that plant phosphate transporters are co-localised with the arbuscules (Harrison *et al.*, 2002). The uptake of phosphate occurs through high-affinity phosphate transporters in the soil interface, while low-affinity phosphate transporter work at the symbiotic interface inside the root (Ayling *et al.*, 1997). Phosphate transporters are found at three interfaces of a mycorrhiza: at the external hyphae, the root epidermis, and cells containing arbuscules or hyphae (Karandashov and Bucher, 2004). Some high-affinity phosphate transporters only

expressed in the external mycelium, have been cloned from AM fungi (Harrison and van Buuren, 1995; Maldonado-Mendoza *et al.*, 2001), but AM phosphate transporters are also found in internal structures, which might imply that the fungus has some control over the amount of phosphate delivered to the plant (Benedetto *et al.*, 2005).

The use of radioactive isotopes as tracer agents is a useful tool to follow nutrient translocation from source to sink (Smith and Read, 1997, p. 384). Another technique is nuclear magnetic resonance (NMR) spectroscopy, a technique based on the differences in the magnetic properties of the nucleus of different isotopes; this technique can be used to find out which starting atom ends up where after a reaction, because NMR detects not only isotopic differences, but also gives an indication of the position of the atom. Mass spectrometry (MS) can also be used to follow a nutrient, since mass spectra recorded with sufficiently high resolution can distinguish among isotopes based on the different masses resulting from the different number of neutrons.

Different isotopes have been used for different purposes. Heinemeyer *et al.* (2006) used ^{13}C to follow labelled carbon from the shoot to the external mycelium, with the aim to find the rate of carbon allocation, respiration and acclimation in external mycelium in response to temperature. Govindarajulo *et al.* (2005) and Jin *et al.* (2005) supplied external mycelium with ^{15}N and ^{13}C and were then able to analyse the amino acid labelling. To trace phosphorus both ^{32}P and ^{33}P have been used. Smith *et al.* (2004) used ^{33}P to measure the fungal contribution to plant phosphate uptake, while Thingstrup *et al.* (2000) used ^{32}P to measure the transport capacity in AM hyphae. Staddon *et al.* (2003a) used ^{14}C to find the turnover rate of mycorrhizal hyphae, and found the turnover time to be 5-6 days, though the hyphae can be relatively persistent after death. Therefore Staddon *et al.* (2004) drew attention to the necessity of vital staining of mycorrhizal structures.

Only a relatively small portion of AM fungal hyphae are metabolically active according to studies with viable staining methods examining enzymatic activity of AM hyphae (Hamel *et al.*, 1990, Saito *et al.*, 1993, Addy *et al.*,1998, Miller *et al.*, 2002). Other approaches to stain only vital tissue are for example confocal microscopy in combination with specific pH sensitive dyes (Ayling *et al.*, 1997) or in combination with immunolocalization using antibodies that correspond to a specific protein fragments (Harrison *et al.*, 2002).

In the following sections some main topics are discussed in more details.

- **AM fungi growing under field conditions – i.e. temperature fluctuations – have less colonisation at high temperature.**

The findings from this project showed the same trend as found in the literature from other studies: the root colonisation in field samples decreased with increased temperature (Chapter 2 and 3), in contrast to the general finding from laboratory studies that the root colonisation increases with temperature (Smith and Read, 1997, p. 75). This might not be contradictory. Most laboratory studies used constant temperatures, while the field studies had temperature fluctuations. Constant temperatures might give some particular fungi with their temperature optimum at the set temperature the option to proliferate during the whole period, while temperature fluctuations might allow a range of fungi to proliferate for short periods at each respective temperature. The finding in field studies from cold sites that there was greater colonisation at low temperature might just reflect the fact that the temperatures in general are low, and the local fungi are adapted to low temperatures.

- **AM fungal communities are more diverse if growing in a wide temperature zone rather than in a more narrow one.**

Fieldwork (Chapter 2) showed lower AM fungal diversity at high altitude, where the temperature is lower, and greater diversity at low altitude, where the temperature is higher, but where also the temperature range is greater. As the temperature range seems to be important for diversity, it would be interesting to work with different simulated temperature ranges, constant temperatures, a range of 5°C, 10°C, 15°C, 20°C etc. in order to see whether diversity increases with the span of the temperature. An increase in the temperature range should according to a model suggested by Schwilk and Ackerly (2005) provide more temperature optimum niches and therefore it would be likely to find more types when using greater temperature range.

- **Cold-adapted AM fungal communities are more diverse if growing at a constant low temperature rather than a constant high temperature.**

Fewer fungal types were able to grow at high temperatures than at low temperatures; however the relatively high fungal diversity in the pre-warmed roots suggest that high temperature did eradicate some, but not all, of the fungal types (Chapter 4). It can, however, not be concluded from this experiment whether cold-adapted AM fungi are eliminated or lose their ability to grow after a pre-warming.

- **The nutrient exchange in the symbiosis might be determined by differences in carbon and phosphate concentration in plant and fungus.**

The site of the nutrient exchange has been discussed without any conclusion as yet, for example Smith and Smith (1996) suggested three models for transfer of phosphate and carbohydrate across fungus/plant interfaces. a) Traditional model, where the exchange solely occurs in the arbuscules, 2) Spatial separation, with phosphate at the arbuscular and carbohydrates at the hyphal interfaces, and 3) Hybrid/uniform transfer model, where the transfer can occur at all interfaces. The spatial separation model might be the model that has had most support recently. For example, Fitter *et al.* (2000) suggested that, as hyphae can colonise roots effectively without producing arbuscules, this indicates that the intercellular hyphae at least in part is the location for carbon exchange, though the site of P transfer from fungus to plant probably is the arbuscule.

The findings from this study, showing sugar concentration in plant roots 10 times higher than in fungal tissue and phosphate concentration in fungal tissue 10 times higher than in plant root tissue (Chapter 5), suggest that the exchange might occur at all interfaces. According to Saito (2000) phosphate release from active arbuscular or intracellular hyphae to the host cell may not occur via leakiness of the fungal cell membrane, but rather through ion transport channels. Arbuscules have a large surface area and therefore much of the exchange is likely to occur at the arbuscular interface, as indicated with the positive correlation between arbuscules and fungal sugar concentration.

- **AM fungi and climate change: the warming scenario**

The temperature in the Faroe Islands is closely linked to the ocean temperature, which is the reason for the relative cold summers and the relative warm winters (Cappelen and Laursen, 1998). In oceanic areas, the annual temperature range is small (Crawford, 1997). The Intergovernmental Panel on Climate Change states that it is very likely that nearly all land areas will warm more rapidly than the global average, particularly those at northern high altitudes in the cold season (Houghton *et al.,* 2001). Warm winters pose special threats to plants in cold regions by disturbing their inactive period (Crawford, 2000). General climate changes are also expected to affect plant communities. For example sub-alpine plants are expected to invade altitudes previously out of limits for them, and the dominance of existing species will be changed (Henry and Molan, 1997). The effects of warming can be upwards migration of plant species and if the mountains are not high enough to allow the species to migrate to a higher altitude, species

can disappear (Grabherr *et al.*, 1995). Plants and fungi are intimately linked, so the effect on plant growth would be expected to affect fungal growth (Fitter, 2001).

The findings from the warming experiment (Chapter 4) suggest that a general warming would also be expected to have a direct impact on the fungi themselves both in terms of abilities to grow, and in terms of survival, which in turn would impact the plant communities (e.g. van der Heijden *et al.*, 1998).

In a climate change context, fungal diversity may be very vulnerable, and locally adapted ecotypes could become extinct (Fitter *et al.*, 2004). Both the findings from the field and from the warming experiment indicate that there are cold-adapted types in the AM fungal flora of the Faroes, and that these might be affected by climate change, especially in terms of fungal communities, as the fungal diversity decreased as a result of warming. In the warming experiment, it was only the fungal diversity in *Agrostis capillaris* that was estimated, and as a consequence one cannot infer that plant diversity in general will be affected. However, a high fungal diversity might very well be crucial for plant survival in a changing environment.

- **AM fungi and climate change: the cooling scenario**

In contrast to a warming scenario, in an oceanic climate lower winter temperatures are generally expected to affect plant species positively, because lower winter temperatures reduce disturbance of plants in winter dormancy (Crawford, 2000). Lower summer temperature would be expected to produce a serious reduction in the temperate vegetation zone (Fig. 1.5). The effects of cooling have received only little attention, they are likely to lead to a downward migration (Fosaa *et al.*, 2004). In this mycorrhizal project, no cooling experiment was conducted. To be able to make any prediction regarding what would happen to the fungal community in a cooling scenario, a similar experiment to the warming experiment (Chapter 4) would have to be carried out.

- **AM fungi and climate change: the pre-warming scenario**

Constant temperatures, as utilised in the warming experiment, are not a natural phenomenon. Normally, there are temperature fluctuations. However, two weeks of around 20°C can occur, though this is an extremely rare event in the Faroes.

The decrease in root colonisation found in the OTC in 2003, but not in 2002, might have been due to the heat wave that occurred throughout the whole Europe in 2003. Also the Faroes experienced their hitherto highest temperatures

in 2003, with the highest temperature ever recorded on 17 July (26.3°C at 85 m a.s.l.; Cappelen and Jørgensen, 2004). This weather pattern might have influenced the fungi, in particular those in the OTCs (Chapter 3).

The surprising result that pre-warmed roots had less colonisation even though they grew for nearly 30 weeks under the same conditions as cold-grown might indicate that a prolonged period at 25°C changes the growth abilities of the AM fungus (Chapter 4). The fungal diversity in the pre-warmed roots was found to be higher than in the warm-grown roots, but lower than in the cold-grown, indicating that the AM fungi were not all eradicated by the pre-warming. The question is whether the loss of growth ability is reversible, or if, for example, a prolonged cooling is needed, i.e. a cold winter. This should be easy to test, and is important that it be done, especially with respect to climate change.

In the experiments dealt with here, the responses of the AM fungal communities are in the main represented by the AM fungal communities in *Agrostis capillaris*. The colonisation in *Ranunculus acris* showed a different pattern than in *Agrostis capillaris* both in respect of %RLC and fungal communities, and the colonisation in the two plant species had different responses to temperature and soil nutrients. However, if it is true that prolonged warming also changes the AM fungal community in other plant species, then a change in the plant composition might be expected due to changes in the mycorrhizal community. If the planet only experiences an increased warming due to global climate change, the ecosystem will probably adjust to that reality, shifting the plant community at high altitude towards a more lowland one. There will be more serious consequences if the Gulf Stream is impacted, whereby we first experience a prolonged increase in temperature, and then a cooling. If the local mycorrhizal fungi are not able to compensate or adapt to the changed environment, increased erosion could be one of the results, because mycorrhizal fungi produce external mycelium that binds soil particles together to aggregates. AM hyphae contain glomalin which is thought to play a significant role in reducing water- and wind-erosion (Rillig *et al.,* 2002a). Due to glomalin, and other microbial products, soil aggregates are formed into crumbs, and thus exert a stabilising influence, which is important for the extent of erosion damage (Brady, 1984, p. 57, 542).

The findings in this project could indicate that there already has been a pre-warming following by cold period, or at the least they can illustrate what might occur: all but one of the fungal types found at high altitude were also found at low altitude, while several fungal types were found at low altitude but not at high altitude (Table 2.10). This distribution could have been caused by the

increase in temperature in the period 1930-1960 (Fig. 1.4), causing the vegetation and the AM fungi to migrate upwards. The fungal types that were most sensitive to high temperatures might have disappeared during this period, as the mountains were not high enough to provide a refuge at greater elevations. During the cold period (1965-1995) there should have been sufficient time to migrate downwards, but there might not have been many fungi left.

Conclusions

The following hypotheses and questions were confirmed by this project:

- The communities of AM fungi found in thermally distinct environments in the Faroe Islands are different.
- The diversity of the AM fungal community was greatest where the thermal heterogeneity of the habitat was greatest.
- Plants from a single habitat that differ in mycorrhizal dependency were colonised by distinct fungal communities, possibly because the benefits they acquire from colonisation differ.
- Open Top Chambers have an impact on AM fungi, but the increase in soil temperature is not in the scale of the expected warming from global climate change.
- Fungi from colder sites have a greater ability to maintain growth at low soil temperature – at least when comparing Faroese sites.
- The growth of fungi from cold sites was affected by prolonged periods of elevated temperature.
- The benefits for the two symbionts in the AM symbiosis are dependent on both soil temperature and soil phosphate.

Appendices

Appendix 1

Temperature measurements used in Chapter 2.

		Tórs-havn	Gráfelli				Mosarøkur				Sornfelli				Ørvisfelli			
			Lapse rate: 0.4°C/100 m				Lapse rate: 0.6°C/100 m				Lapse rate: 0.4°C/100 m				Lapse rate: 0.8°C/100 m			
		Alti-tude	Altitude				Altitude				Altitude				Altitude			
Year	Month	50	70	350	600		150	350	600		150	350	600		50	350	600	
2000	1	4.1	1.8	0.7	0.1		2.0	1.1	0.2		2	0.7	0.4		2.7	0.7	-0.6	
2000	2	3.3	1.3	0.2	-0.8		1.2	0.3	-0.9		1.3	0.2	0.1		1.9	-0.1	-0.6	
2000	3	3.5	1.6	0.5	-0.6		2.1	1.1	-0.6		2.3	0.5	-0.1		2.5	0.5	-0.6	
2000	4	3.3	1.6	0.5	-0.8		2.6	1.5	-0.9		3.3	0.5	-0.2		2.8	0.8	-0.6	
2000	5	7.4	6.5	5.4	4.1		8.6	7.6	4.6		10.7	5.4	5.4		9.2	7.2	2.9	
2000	6	7.8	8.5	7.4	4.6		10.1	8.3	5.1		11.7	7.4	6.6		10.3	8.3	5	
2000	7	10	11.6	10.5	7.3		13.3	11.5	8.1		10.7	10.5	9.2		13.1	11.1	7.7	
2000	8	10.9	11.7	10.6	8.4		12.3	11.3	9.7		11.9	10.6	9.3		13.3	11.3	8.1	
2000	9	10.2	9.8	8.7	7.5		10.1	9.1	7.6		11.0	8.7	7.5		10.5	8.5	6.4	
2000	10	8.5	7.1	6	5.5		6.9	6.1	4.5		8.6	7.3	5.1		7.6	5.6	4	
2000	11	6.1	3.9	2.8	2.6		3.5	2.7	1.1		5.4	2.8	1.5		4.4	2.4	0.9	
2000	12	4.2	3.3	2.2	0.3		2.7	2.0	1.1		2.8	2.4	1.3		3.8	1.8	0.7	
2001	1	5.4	2.4	1.3	1.7		2.2	1.2	-0.1		4.4	1.2	0.1		3.0	1.0	-0.6	
2001	2	2.4	2.1	1	-1.9		1.7	1.0	0.0		0.3	0.9	-0.2		2.6	0.6	-0.5	
2001	3	2.9	1.0	-0.1	-1.3		1.3	-0.2	-0.3		1.0	-0.2	-0.8		1.4	-0.6	-0.9	
2001	4	4.6	3.2	2.1	0.7		4.4	2.4	-0.1		3.3	2	-0.3		3.6	1.6	-0.6	
2001	5	7.6	8.1	7	4.4		9.1	7.6	6.5		7.4	7.2	5.6		8.0	6.0	3.9	
2001	6	8	9.3	8.2	4.9		10.2	8.7	7.5		8.0	8.5	6.6		8.6	6.6	4.3	
2001	7	10.1	11.8	10.7	7.4		12.1	10.6	9.7		10.8	10.2	8.3		11.4	9.4	6.6	
2001	8	11.2	12.0	10.9	8.8		11.8	8.3	8.7		12.3	10.3	8.8		12.9	10.9	7.8	
2001	9	9.5	5.0	3.9	6.6		9.1	5.9	6.1		10.0	8	6.3		10.6	8.6	6.0	
2001	10	9.3	4.4	3.3	5.9		8.6	5.4	5.7		9.7	7.1	5.9		10.3	8.3	5.8	
2001	11	5	1.8	0.7	1		3.6	0.7	0.9		3.9	2.6	1.3		4.5	2.5	1.0	
2001	12	4.4	2.4	1.3	0.6		2.8	-0.3	0.4		3.0	2.0	1.1		3.7	1.7	0.4	
2002	1	5	1.8	0.7	0.6		2.8	0.2	0.6		3.9	2.7	1.8		4.5	2.5	1.0	
2002	2	2.6	1.6	0.5	-0.5		1.1	-0.9	-0.5		0.6	-0.4	-1.0		1.3	-0.7	-1.6	
2002	3	3.5	5.4	4.3	-0.7		1.3	-1.1	-0.8		1.8	0.8	0.0		2.5	0.5	-0.6	
2002	4	6.4	7.9	6.8	1.8		5.5	1.8	1.8		5.8	4.6	3.5		6.4	4.4	2.6	
2002	5	7.7	11.2	10.1	4.7		7.7	4.1	4.5		7.5	6.2	5.0		8.2	6.2	4.0	
2002	6	10.2	11.6	10.5	8.7		10.9	8.1	8.8		11.0	9.5	8.0		11.5	9.5	6.7	
2002	7	10.9	15.5	14.4	8.5		11.5	7.5	8.4		11.9	10.4	8.8		12.5	10.5	7.5	
2002	8	11.7	12.7	11.6	9.4		13.1	12.1	10.3		13.0	11.4	9.7		13.6	11.6	8.4	
2002	9	11.1	11.9	10.8	8.6		12.3	10.4	9.5		12.2	10.7	9.0		12.7	10.7	7.7	
2002	10	7	6.6	5.4	3.7		6.7	5.0	4.1		6.6	5.3	4.2		7.2	5.2	3.2	
Temperature range			**14.5**	**14.5**	**11.3**		**12.2**	**13.2**	**11.2**		**12.7**	**11.8**	**10.7**		**12.3**	**12.3**	**10.0**	

Mean montly temperatures. Numbers with green background are monthly means measured on the site. Numbers with blue background are estimated from values obtained from the metereological institute in Tórshavn by a regression with measurements previously measured on the site. Numbers with gray background are estimated from a nearby site, corrected for lapse-rate. Numbers with pink background are based on regressions between measurements from a nearby site corrected for lapse rate and measurements from Tórshavn. All field values are measured with Tiny-Tags *Plus* dataloggers (Gemini Data Loggers, Chichester, UK), with the probe 1 cm under the vegetation.

APPENDIX 2

The unpublished primer AM2 that was used to amplify partial small-subunit ribosomal DNA fragments of about 360 base pairs is as follows:

5’-TAG TAT TCA ATT GTC AGA GG-3’

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