Doctoral Thesis

Effects of elevated atmospheric CO₂ on arbuscular mycorrhizal fungi in an agricultural model grassland

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EFFECTS OF ELEVATED ATMOSPHERIC CO$_2$ ON ARBUSCULAR
MYCORRHIZAL FUNGI IN AN AGRICULTURAL MODEL GRASSLAND

A dissertation submitted to the
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presented by

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SUMMARY

The exceptionally fast rise of atmospheric carbon dioxide partial pressure (pCO$_2$), a key component of global environmental change, may have profound implications for the functioning of ecosystems in the future. Increased photosynthetic production and plant growth stimulated by the rise of pCO$_2$ may lead to an accumulation of non-structural carbohydrates (C) and intensify the mineral nutrient demands of plants. As a consequence, the interaction between plants and their symbiotic soil fungal partners may be altered. This may particularly apply to the arbuscular mycorrhizal fungi (AMF, Glomeromycota), because AMF rely exclusively on C supplied by living plants and are strongly implicated in plant mineral nutrition. AMF growth rates, the structure of AMF populations and communities and the physiological functioning of mycorrhizal symbioses may change.

Effects of seven to ten years free-air CO$_2$ enrichment in permanent, agricultural monocultures of *Trifolium repens* (white clover), a legume, and *Lolium perenne* (perennial ryegrass), a grass, were studied. This included: (1) A field study, based on traditional microscopical techniques, (2) an inoculation experiment with *T. repens* and pure fungal cultures isolated from the experimental field site, and (3) a molecular ecological field study, using PCR assay and DNA sequencing.

**1** In the first field study, root colonisation levels by AMF and spore densities were microscopically assessed in permanent monoculture plots of *T. repens* and *L. perenne*. Generally, abundance of AMF in roots and soils from plots exposed to elevated atmospheric pCO$_2$ conditions for seven years was higher. In particular, AMF associated with roots of *L. perenne*, a plant known to experience severe N-deficiency, produced more hyphae, arbuscules, vesicles, and spores. This suggests that AMF profited from an increased availability of C in the roots of plants grown under elevated atmospheric pCO$_2$. Moreover, there were significant interactions among nitrogen (N) fertilisation, host plant species and levels of AMF root colonisation with hyphae and vesicles. N-fertilisation reduced the levels of AMF root colonisation in *L. perenne*, but did not alter them in *T. repens*, a finding best explained by differences in host plant N-metabolisms with the legume having access to additional N provided by nodule symbiosis.

**2** In the pot experiment conducted under controlled conditions using nine *T. repens* genets and fourteen single spore isolates of AMF, we found that eight years of elevated atmospheric pCO$_2$ had selected for more beneficial (symbiotically effective) AMF strains of *Glomus claroideum* and *G. intraradices*. The fungal strains originating from plots which had been exposed to elevated atmospheric pCO$_2$ increased foliar N-concentration of *T. repens* host plants by simultaneously stimulating biological N$_2$ fixation in the nodule symbiosis and increasing the fraction of N taken up from the potting substrate. This finding is contrary to initial expectations that assumed that fungal strains with a history of exposure to elevated atmospheric pCO$_2$ should be more C-costly and thus depress host growth under non-limiting phosphorus conditions. Furthermore, host plants of *T. repens* inoculated with fungal strains isolated from *T. repens* monoculture plots showed lower root colonisation and increased
biological N$_2$ fixation, compared to plants inoculated with AMF strains originating from mixed culture plots planted with *T. repens* and *L. perenne*. Moreover, symbiotic functioning, measured by physiological indicators, was strongly influenced by the particular combinations of plant genet (nine) and fungal isolate (fourteen).

(3) In the molecular ecological field survey on AMF we used newly designed morphospecies-discriminating PCR primers for fungal identification within roots of *T. repens*. The population genetic structure of an AMF species (*G. mosseae*) was affected by ten years of CO$_2$ fumigation, but not the community composition of six AMF species, located within the same roots. This population-level change indicates that AMF may adapt to new conditions under elevated atmospheric pCO$_2$, even if shifts in the community structure (species level) are lacking. Adaptations through changes in the relative frequency of AMF strains could also explain the difference in symbiotic functioning that was detected in regard to host N-nutrition. Additional findings were a surprisingly uniform, overall relative abundance of the six detected AMF species, although the communities in the six sampled plots were (marginally) significantly structured spatially.

Overall, results of this PhD thesis contribute to a better understanding of the ecology of AMF under high input agricultural conditions and reveal possible responses of mycorrhizal fungi to rising atmospheric pCO$_2$. Changes in the structure of AMF populations and altered symbiotic functioning of AMF strains seem likely under prolonged elevated atmospheric pCO$_2$. Findings made here highlight the key role AMF will play for future ecosystems functioning under a scenario of global environmental change.
ZUSAMMENFASSUNG

Der ausserordentlich schnelle Anstieg des atmosphärischen Kohlendioxidpartialdrucks (pCO\textsubscript{2}), einer Schlüsselkomponente der globalen Umweltveränderung, dürfte tiefgreifende Auswirkungen auf das Funktionieren zukünftiger Ökosysteme haben. Eine durch den pCO\textsubscript{2}-Anstieg angekurbelte Photosyntheseproduktion und ein gesteigertes Pflanzenwachstum könnten zur Akkumulation von nicht-strukturellen Kohlenhydraten (C) und zu einem erhöhten Bedarf an mineralischen Nährstoffen führen. Als Konsequenz davon könnte sich die Wechselbeziehung zwischen den Pflanzen und ihren symbiotischen Bodenpilzpartnern verändern. Dies könnte besonders für die arbuskulären Mykorrhizapilze (engl.: Arbuscular Mycorrhizal Fungi, AMF; Glomeromyceten) zutreffen, da die AMF ausschliesslich vom C aus lebenden Pflanzen abhängig sind und weil sie zu einem bedeutenden Masse an der mineralischen Pflanzenernährung beteiligt sind. Die Wachstumsraten der AMF, die Struktur der AMF Populationen und der AMF Gemeinschaften und das physiologische Funktionieren der Mykorrhizasymbiose könnten sich verändern.

Die Auswirkungen von sieben bis zehn Jahren atmosphärischer Freiland-CO\textsubscript{2}-Anreicherung in landwirtschaftlichen Dauermonokulturen von *Trifolium repens* (Weissklee), einer Leguminose, und von *Lolium perenne* (Englisch Raygras), einem Gras, wurden studiert. Folgende Untersuchungen wurden gemacht: (1) eine auf traditionellen mikroskopischen Methoden beruhende Feldstudie, (2) ein Animpfungsexperiment mit *T. repens* und mit vom Feldexperiment isolierten pilzlichen Reinkulturen und (3) eine molekular-ökologische Feldstudie mittels eines PCR Nachweisverfahrens und DNA-Sequenzierens.


(2) Im Topfexperiment unter kontrollierten Bedingungen mit neun *T. repens* Genets und vierzehn AMF Einzelsporenlösisolaten, zeigte sich, dass acht Jahre erhöhter atmosphärischer pCO\textsubscript{2} zuträglichere (symbiotisch effektivere) AMF Stämme von *Glomus claroideum* und *G. intraradices* gefördert hatte. Die Pilzstämmle aus Versuchsflächen, welche erhöhtem atmosphärischem pCO\textsubscript{2} ausgesetzt gewesen waren, erhöhten die N-Konzentration in den


1 General Introduction

1.1 Rising Atmospheric pCO₂ – a Key Component of Global Environmental Change

Rising atmospheric carbon dioxide partial pressure (pCO₂) is one of the fundamental factors of global environmental change (Bazzaz, 1990; Keeling et al., 1995; Falkowski et al., 2000; Körner, 2000). In particular, the rapid anthropogenically fuelled increase of atmospheric pCO₂ due to fossil fuel combustion and land use change since the industrial revolution, has caused an increase of more than one fifth of pre-industrial levels (see Petit et al., 1999; Watson et al., 2000; and Grace, 2004 for a historical outline). These extremely fast changes in pCO₂ may have profound effects on terrestrial ecosystems. However, relatively little is known about the consequences of rising atmospheric pCO₂ on belowground ecosystems, which could encompass changes in biomass, activity and taxonomic or genetic composition of soil biota (Sadowsky & Schortemeyer, 1997; Lal, 2003). Elevated atmospheric pCO₂ may stimulate plant growth (Kimball, 1983; Bazzaz, 1990), but reduce evapotranspiration (i.e. increase the water-use efficiency) of plants and lower the mineral nutrient content of plant litter, thereby leading to an increased immobilisation of soil nutrients (Norby & Luo, 2004; Pendall et al., 2004a). These alterations in soil conditions represent important secondary factors of elevated atmospheric pCO₂, besides stimulation of photosynthesis and global warming (i.e. the greenhouse effect), which could affect the above and below-ground biota. In addition, atmospheric pCO₂ may interact with other global change factors, such as anthropogenic N deposition and soil warming, which only recently began to be investigated (Egerton-Warburton & Allen, 2000; Heinemeyer et al., 2004).

1.2 Significance of Grasslands for the Carbon Cycle

Globally, grassland ecosystems cover more than 20% of land surface (Parton et al., 1995) and harbour more than 10% of soil C (Eswaran et al., 1993; Stocker et al., 1997). Among the agriculturally used land surfaces, managed grasslands account for 20-30% in Europe and for 70% in Switzerland. In Switzerland, 28% of the arable land (about 118’000 ha) are high input and frequently cut grasslands, which are often part of crop rotations. These botanically impoverished, but highly productive, forage grasslands are sawn stands of plant cultivars. Two typical perennial plant species of temperate grasslands and pastures are the stoloniferous legume, Trifolium repens L. (white clover), and the cool-season C3 caespitose grass, Lolium perenne L. (perennial ryegrass). These two species show different strategies of nitrogen (N) assimilation. L. perenne takes up mineral N from soil, whereas T. repens supplements its N-requirements, depending on the level of soil N availability, by assimilating atmospheric N₂ in symbiosis with the bacterium Rhizobium leguminosarum biovar trifolii.

The global C cycle, influenced by biogeochemical and climatological processes, has attracted considerable scientific attention, due to the growing concern about the potential consequences of rising atmospheric pCO₂, caused by human activities (Falkowski et al., 2000).
Identifying the main sources of CO$_2$ (i.e. sites of emission) and the sinks (i.e. reservoirs) has become an important research objective in the endeavour to mitigate the anthropogenic contribution to the rise in atmospheric pCO$_2$ (Friedlingstein et al., 1995; Schimel, 1995; Houghton et al., 1998; Schindler, 1999). The main terrestrial C sinks were found to be represented by growing forests, permanent grasslands and their soils, which store about three times the amount of C present in the atmosphere. The significance of soils for the terrestrial and global C cycle becomes apparent when it is acknowledged that soils account for four times the amount of C stored by above-ground vegetation (Watson et al., 2000). The large reservoirs of terrestrial C in grasslands are the reason why they are considered important to relieve the anthropogenic effect of rising atmospheric pCO$_2$ on earth’s climate (Watson et al., 2000; BUWAL, 2004). In particular, estimates for C sinks in Switzerland identified a large potential for C sequestration in the expansion of permanent grasslands through conversion of arable land (Leitfeld et al., 2003).

### 1.3 Role of Arbuscular Mycorrhizas in Plant Physiology and Ecology

Mycorrhizas are associations of plant roots with soil fungi (gr. mykes = fungus, rhiza = root; de Bary, 1879; Frank, 1885), an example of a symbiosis in which autotrophs live together with heterotrophs. The fungal partners of mycorrhizas, together with the fungal partners of lichens (symbiotic associations of algae and fungi), account for 30% of all fungal species which live in association with photoautotrophs (Carroll, 1992). Mycorrhizal fungal partners are considered the symbionts (or microsymbionts) and the plants are regarded as hosts (macrosymbionts). In general, the fungal symbionts scavenge for poorly available soil nutrients which are then exchanged for carbohydrates provided by the host plant (exceptions are found e.g. in the mycorrhizas of mycoheterotrophic plants; Leake, 2004). The term mycorrhiza covers heterogeneous symbiotic associations, differing in respect to taxonomy of the partners as well as their evolutionary age, ecological function, and abundance of occurrence in particular biomes (Read, 1991). Six to seven classes of mycorrhizas are commonly distinguished, of which arbuscular mycorrhiza is one (Smith & Read, 1997; Read, 2002).

Historically, research on mycorrhizas, particularly on the endotrophic type of mycorrhiza formed by arbuscular mycorrhizal fungi (AMF), was driven by the effort to improve plant production in horticulture and agriculture (Koide & Mosse, 2004). Today, sustainability in agricultural production is thought to depend to a large extent on the integrity of arbuscular mycorrhizas (Mosse, 1973; Doran et al., 1994; Gianinazzi & Schüepp, 1994; Douds & Millner, 1999; Gianinazzi et al., 2002; Mäder et al., 2002). It is only within the past decade that the broader ecological significance of arbuscular mycorrhizas for the functioning of natural or seminatural ecosystems has become evident (Read, 1991; van der Heijden & Sanders, 2002; Hart et al., 2003). Many studies have shown that arbuscular mycorrhizas influence the species composition and productivity of herbaceous plant communities (Grime et al., 1987; van der Heijden et al., 1998; Hartnett & Wilson, 1999; Klironomos et al., 2000; O’Connor et al., 2002).
It seems that the degree of mycorrhizal dependency of the key players in plant communities (mainly the dominant species), determines whether plant species diversity may increase or decrease (Hart et al., 2003; Wardle et al., 2004).

The mechanisms for these overall ecological effects on plant communities are likely to differ in each plant / fungal species pair, due to individual differences in symbiotic benefits (Newsham et al., 1995). Biofertilising, bioprotecting, stress alleviating and soil-bioengineering functions, and in particular, improvements in plant mineral nutrition with phosphorus (P) are the main benefits plants receive from an association with AMF (Mosse, 1973; Smith & Read, 1997). Hyphal networks around the roots of host plants extend into minute soil pores and far beyond the nutrient depletion zones. These fungal networks greatly enlarge the exploitable soil volume of mycorrhizal plants in comparison to non-mycorrhizal plants (Read, 1984). Up to ten times higher total surfaces allow absorption of poorly soluble and poorly mobile nutrient elements (mainly P, zinc and copper) which often limit plant growth. Under poor soil conditions, improved P-nutrition is commonly demonstrated or hypothesised to be the ultimate cause for growth promotion in mycorrhizal host plants (Mosse, 1973; Smith & Read, 1997). Increased tolerance or resistance against pathogens and herbivores and improved tolerance against drought and salt stress are further benefits for mycorrhizal host plants (Newsham et al., 1995; Goverde et al., 2000; Augé, 2001; Gange, 2001).

Most changes in plant communities induced by AMF are likely to originate from altered competitive relations. Alterations in intra- and interspecific plant competition have been demonstrated to occur through differential access to soil resources and through intra- and interspecific hyphal transfer of mineral and possibly also organic nutrients and signalling molecules among host plants (Francis & Read, 1984; Frey & Schüepp, 1992; Moora & Zobel, 1996; West 1996; Robinson & Fitter, 1999; Yao et al., 2003; Carey et al., 2004). Below-ground plant interconnections by a common mycorrhizal fungal mycelium have the potential to alter dominance of members of plant communities via changes in the distribution of resources (Grime et al., 1987; Read, 1998; Giovannetti et al., 2004). Moreover, different vegetative and reproductive growth patterns (e.g. Koide et al., 1988; Streitwolf-Engel et al., 1997; Poulton et al., 2001; Streitwolf-Engel et al., 2001), depending on the level of mycorrhizal dependency (Plenchette et al., 1983; van der Heijden, 2002), may lead to changes in the composition of plant communities (Hart et al., 2003). Different integration of seedlings in an existing soil hyphal network may favour the establishment of certain plant species over others (Moora & Zobel, 1998; van der Heijden, 2004) and thereby alter the plant community composition.

1.4 ALTERATIONS IN ARBUSCULAR MYCORRHIZAL SYMBIOSES DUE TO RISING ATMOSPHERIC pCO₂

1.4.1 IMPLICATIONS OF FUNGAL DEPENDENCY ON THE CARBOHYDRATES FROM HOST PLANTS

Fungal partners in all types of mycorrhiza rely at least in part on carbohydrates (C) provided by their host plants, while some (particularly the AMF) depend completely on the C delivered from
living plants. Usually, 5-20% of plant net-photosynthetic yield are allocated to AMF (Jakobsen & Rosendahl, 1990; Peng et al., 1993; Tinker et al., 1994). This dependency of AMF on C from host plants implies that any factor affecting host-plant photosynthesis or host-plant C allocation, such as shading, herbivory, atmospheric CO$_2$ concentration, and mineral fertilisation, may ultimately affect the growth of AMF and likely their taxonomic and / or genetic composition. Therefore, research on the effects of global environmental change on arbuscular mycorrhizas has concentrated on elevated atmospheric pCO$_2$ (reviewed in: Staddon & Fitter, 1998; Rillig & Allen, 1999; Fitter et al., 2000; Treseder & Allen, 2000; Rillig et al., 2002; Staddon et al., 2002; Treseder, 2004).

In general, elevated atmospheric pCO$_2$ enhances below-ground C allocation of plants (Rogers et al., 1994; Pendall et al., 2004b), particularly under conditions of mineral nutrient limitation (Fischer et al., 1997; Suter et al., 2002). One could assume that additionally fixed C transferred to plant roots should benefit AMF. Thus, growth of AMF within roots and soils may be enhanced when photosynthesis of host plants is stimulated under elevated atmospheric pCO$_2$ (Fitter et al., 2000; Treseder & Allen, 2000; BassiriRad et al., 2001; Rillig et al., 2002). The pot experiments by Klironomos et al. (1998) and Sanders et al. (1998) clearly demonstrated increased C-allocation to AMF by host plants grown under elevated atmospheric pCO$_2$, although there were differences in the response of AMF species. In all studies conducted in the field, elevated atmospheric pCO$_2$ consistently increased C-allocation to AMF (CHAPTER 2, Rillig et al., 2000; Staddon et al., 2004; Treseder, 2004), whereas in many pot experiments, both AMF and plants, equally responded in their growth to contrasting atmospheric pCO$_2$ levels (Staddon & Fitter, 1998; Staddon et al., 1998; Staddon et al., 1999). Findings of these pot experiments raised some doubts whether AMF actually do profit disproportionately compared to plants.

Over the long term, elevated atmospheric pCO$_2$ may lead to changes in the composition of natural AMF communities and / or populations. This may take place through differential response of AMF taxa and / or strains (Klironomos et al., 1998; Rillig et al., 1999a; Treseder & Allen, 2000; Wolf et al., 2003), which in turn may reflect differences in fungal C-requirements or altered competitive relations among fungal strains. Different AMF strains co-inhabiting the same root habitat may use additional C resources differently. Moreover, host plants could mediate shifts in the composition of AMF strains through preference of strains which confer high symbiotic benefits and impose low symbiotic costs. This is of particular importance since elevated atmospheric pCO$_2$ alters the whole balance of plant nutrition, including the metabolisms of C and mineral nutrients. The mineral nutritional benefits conferred by AMF to their host plants may be as important as the fungal C-costs, since they alleviate nutritional growth limitation (C sink limitation) that occurs in plants under elevated atmospheric pCO$_2$ (Fischer et al., 1997; Suter et al., 2002). The importance of the plant mineral nutrition status for the investment in mycorrhizal fungi is formulated in the plant investment hypothesis of Treseder (2004). This plant-centric hypothesis predicts that mycorrhizal host plants should allocate more C to their mycorrhizal fungi when soil nutrients are limiting which is either
the case under lacking or low P- and N-fertilisation or under elevated atmospheric pCO$_2$. Conversely, from the fungal point of view it may be hypothesised that prolonged growth under elevated atmospheric pCO$_2$ either favours more C-demanding (C-costly) and thus plant growth depressing AMF strains, or more efficient AMF strains which benefit plant mineral nutrition. In both cases, better adapted fungal ecotypes should become more frequent, resulting in changes in AMF assemblages over time.

**FIGURE 1.1** A multitude of system intrinsic and extrinsic factors affect the symbiotic nature of mycorrhizas of which the most important are the stage of development, the environmental conditions and the genotypic constitution of symbiotic partners. The whole suit of these features determines the plant, fungal and mycorrhizal phenotypes that are expressed by their genotypes. The mycorrhizal phenotype dynamically changes in response to ontogenetic development of both symbiotic partners and with the age of symbiosis itself. Alterations in the environment may cause shifts in the populations and communities of both symbionts, which may consequently alter ecological functioning of mycorrhizal symbiosis in the long term (modified from Johnson *et al.*, 1997).

1.4.2 **RELEVANCE OF MYCORRHIZAL FEATURES FOR THE ECOLOGY AND EVOLUTION OF MYCORRHIZAS**

At ecologically short time scales mycorrhizal interactions are thought to function along a symbiotic continuum from mutualism to parasitism (Johnson *et al.*, 1997). Environmental conditions and the ontogeny and the genotypic identity of partners are considered the main determinants of where along this continuum the symbiosis is operating (**Figure** 1.1 & 1.2). More precisely, the symbiotic outcome of a particular mycorrhizal association is determined by the sum of reciprocal costs and benefits for the partners. For example, conditions under elevated atmospheric pCO$_2$ are thought to favour AMF strains that require increased amounts of C resources. When such fungal ecotypes are examined under uniform, ambient atmospheric pCO$_2$ conditions they would be expected to be relatively more C-costly and thus less beneficial for their host plants than ecotypes originating from ambient atmospheric pCO$_2$ conditions (**Figure** 1.2). Likewise, conditions under elevated atmospheric pCO$_2$ may favour AMF strains that are more efficient in mineral nutrient uptake, translocation or transfer to host plants. However, in this case, symbiotic costs and benefits would be reversed (see **Chapter** 3).

At evolutionary time scales the benefit conferred by multiple fungal partners to host plants may be the key to understanding mycorrhizal stability and the simultaneous coexistence of fungal strains with various symbiotic abilities (Herre *et al.*, 1999; Wilkinson & Sherratt, 2001). Mycorrhizal fungal assemblages in the roots of plants are known to change seasonally (Giovannetti, 1985; Sanders & Fitter, 1992; Merryweather & Fitter, 1998; Daniell *et al.*, 2001; Heinemeyer *et al.*, 2004) and are acquired *de novo* by each plant generation (van der Heijden, 2004). The absence of vertical transmission of fungal symbionts and the simultaneous presence
of several mycorrhizal fungal partners in the same plant roots conflicts with the view that common interests of the partners should confer evolutionary stability of symbiotic associations (Ewald, 1987; Frank, 1996; Wilkinson, 2001). However, theoretical models for symbioses with horizontal transmission of symbionts has shown that temporal fluctuations and spatial heterogeneities grant evolutionary stability (Doebeli & Knowlton, 1998; Genkai-Kato & Yamamura, 1999; Wilkinson & Sherratt, 2001; Palmer et al., 2003; Yamamura et al., 2004). Conditions of a heterogeneous soil environment and a spatially fluctuating distribution of plant and fungal structures prevail for many mycorrhizas. Thus, horizontal transmission of symbionts and the lack of strict plant-fungal specificities may maintain both more and less beneficial (symbiotically effective) fungal strains in the same habitat and in association with the same host plant (Graham et al., 1996; Graham & Abbott, 2000).

**FIGURE 1.2** A simplified scheme of how more and less beneficial strains of arbuscular mycorrhizal fungi (AMF) may dynamically affect host plant development under the concept of a mutualism-parasitism continuum (Johnson et al., 1997). Phases of net-benefit and net-cost imposed by fungal symbionts on their host plants may change over time. AMF strains designated as more or less beneficial may have experienced selective histories under different pCO₂ conditions.

Mathematical modelling, using biological market models, has shown that mycorrhizal associations become stable when the plants specialise on C and the fungi on P acquisition, which are indeed the main functions ascribed to mycorrhizal partners (Smith & Read, 1997; Schwartz & Hoeksema, 1998). Root colonisation by multiple AMF partners may broaden the spectrum of potential plant benefits and out weight the advantages from strict association of plants with a single mycorrhizal fungal partner. Moreover, flexibility in the choice of fungal partners may allow mycorrhizal host plants to cope with temporally and spatially changing environments (Law, 1985; Rowan et al., 1997; Herre et al., 1999; Baker, 2003). Experimental evidence and arguments presented above indicate that growth of plants under altered environmental conditions such as elevated atmospheric pCO₂ will occur in an ecological context of a great diversity of different AMF strains, preserved at a small spatial scale. Preferential growth of a
subset of AMF strains that match new environmental conditions and the different host plant requirements that are associated with such changes, may lead to shifts in AMF assemblages and alter physiological functioning of mycorrhizas.

1.4.3 Relations of Mycorrhizas with the Global C Cycle and Their Implications for Global Environmental Change

Arbuscular mycorrhizal fungi represent an ubiquitous and evolutionarily ancient group of symbiotic soil fungi. Fossils from the Devonian and Ordovician and molecular clock estimates suggest that AMF existed at least for 460 million years (Pirozynski & Malloch, 1975; Simon et al., 1993; Remy et al., 1994; Redecker et al., 2000a). Mycorrhizas formed by this fungal group are thought to have allowed the first land plants to colonise terrestrial ecosystems by assisting in mineral nutrient and water uptake (Pirozynski & Malloch, 1975; Selosse & Le Tacon, 1998). In these old ages when AMF first appeared, the atmospheric CO$_2$ concentration was at least ten times higher than today (Berner, 1997; Redecker et al., 2000a) and over the subsequent geological time until the present it fluctuated considerably (Petit et al., 1999). Repeated fluctuations in atmospheric pCO$_2$ may have also left their mark in the genome of AMF. It is possible that traits adapted to conditions of elevated atmospheric pCO$_2$ or its fluctuations have been retained in the genomes of AMF and may regain importance under the current rise of atmospheric pCO$_2$.

Regarding the global C cycle it is important to know that mycorrhizal fungi are closely involved in the flow of the main plant nutritional elements, in particular C, N, and P (Aerts, 2002; Simard et al., 2002; Read & Perez-Moreno, 2003). Fungi account for a major part of soil microbial biomass and thus of soil C (Parkinson & Coleman, 1991; Meeting, 1992). AMF are estimated to make up 25% of total soil biomass (Hamel & Smith, 1991) and are thought to play crucial roles in the C cycle (Zhu & Miller, 2003) and in the formation of soil structure (Miller & Jastrow, 1990; Rillig et al., 1999b). Therefore, AMF could contribute to increased soil C stocking and improved soil aggregation as atmospheric pCO$_2$ increases in the future (Treseder & Allen, 2000; Treseder, 2004). It has been shown that AMF produce glomalin, a recalcitrant glycoprotein, deposited in soil. This glomalin accounts for considerable fractions of soil C and thus C sequestration (in the range of thousandth parts of total C transferred to AMF and one tenth of above-ground primary production; Wright & Upadhyaya, 1996; Rillig et al., 1999b; Rillig et al., 2001; Lovelock et al., 2004). However, a considerable part of the C delivered from plants to AMF is rapidly turned over in fungal respiration and rapid hyphal degradation (Staddon et al., 2003a; Staddon et al., 2003b).

1.5 Arbuscular Mycorrhizal Fungi

1.5.1 Uncertainties in Taxonomy and Species Diversity

Arbuscular mycorrhizal fungi (AMF) are members of the phylum Glomeromycota, a sister clade to the other major fungal phyla, Asco- and Basidiomycota, and are thought to be of monophyletic origin (Schüssler et al., 2001). Previously, AMF were classified in the order
Glomales in the phylum Zygomycota (Morton & Benny, 1990; Walker & Trappe, 1993). Eight families in four orders are currently recognised within the Glomeromycota that are phylogenetically sound and mostly supported by distinct morphological characters (Schüssler et al., 2001; Walker et al., 2004). More than 160 species are formally described, based mainly on morphological characters of the large, soil-born fungal spores (Schenck & Pérez, 1990; Walker, 1992; Morton & Bentivenga, 1994; http://invam.caf.wvu.edu; http://www.tu-darmstadt.de/fb/bio/bot/schuessler/amphylo/amphylogeny.html).

Molecular ecological field studies and studies on the phylogeny of AMF using various regions of nuclear ribosomal DNA have revealed an impressive genetic diversity (Redecker et al., 2000b). The finding of new ribotypes not yet related to any formally described morpho-species suggests that AMF diversity may be far greater than that inferred from sporal morphology (e.g. Helgason et al., 1998; Vandenkoonhuyse et al., 2002; Wirsel, 2004). The authors who report such findings hypothesise that the most prominent AMF species in natural ecosystems, may not correspond with those kept in living pot-culture collections [INVAM, International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi, http://invam.caf.wvu.edu; BEG, La Banque Européenne des Glomales, now international Bank of Glomeromycota, (http://www.kent.ac.uk/bio/beg/index.htm)]. Therefore, knowledge from experiments with isolates of culturable species may have little predictive value for the understanding of AMF in natural ecosystems which comprise a far greater number of taxa.

Reasons for the underestimated taxonomic diversity based on morphological characters may be that certain AMF taxa rarely sporulate in nature or in pot cultures. Spores and ideally one or several pure, living cultures are needed for a sound formal description of a new species. Living cultures are necessary, because only large numbers of spores reveal the entire variation in spore morphology, and characters may change during spore development. New descriptions of AMF species should take a multidisciplinary approach aiming at species descriptions which are biologically and evolutionary meaningful. Morphotaxonomists and scientists working on molecular phylogenetics of AMF should collaborate to find the best characters to describe new species. Taxonomic uncertainties and difficulties in reasonably defining AMF species has become apparent from studies that reveal potential ‘species continua’ consisting of isolates of different morpho-species which share nrDNA sequence haplotypes (Clapp et al., 2001; Rodriguez et al., 2004). The complexity in defining AMF morpho-species also became obvious by thorough morpho-taxonomic revisions that found several, synonymous species (Walker & Vestberg, 1998; Walker et al., 2004).

1.5.2 PECULIARITIES OF AMF BIOLOGY AND GENETICS

The mycelia of AMF are coenocytic and thus multinucleate, as are their large soil-born spores, which are formed either singly, in clusters, or in sporocarps. There is no mode of sexual reproduction known for AMF and in the asexual propagation cycle of AMF, which include hyphal and sporal propagation, a mononucleic cell stage is lacking. Ploidy has only been
determined for the model AMF species, *Glomus intraradices* Schenck & Smith, and found to be haploid (Hijri & Sanders, 2004). Exact chromosome numbers remain unknown for all AMF species.

AMF have a peculiar genetic organisation with a genome consisting of hundreds of nuclei simultaneously present within one spore (e.g. Viera & Glenn, 1990). Assuming these nuclei harbour considerable genetic redundancy (Kuhn *et al*., 2001; Pawlowska & Taylor, 2004), questions about the inheritance of nuclei and the evolutionary role of such a population of nuclei have been raised. The multinucleate organisation has been proposed to be of potential significance for the evolution of plant-fungal specificity, which is hypothesised to take place through changes in the distribution of different types of nuclei within an AMF genome (Sanders, 2002a; Sanders, 2002b). Moreover, adaptation to the environmental conditions and to host plants could be promoted through nuclear drift that may occur during sporulation and hyphal propagation leading to random assortment of nuclei and thus genetic differentiation (Bever & Morton, 1999; Sanders, 2002b).

The status of AMF as the oldest clade of putative ancient asexual eucaryots raises the question of how they overcome the negative effects associated with lack of sexuality, such as slower genetic adaptation to changing environments and a more rapid accumulation of deleterious mutations (Judson & Normark, 1996; Butlin, 2002; Gandolfi *et al*., 2003). Several mechanisms, either observed or suspected in AMF, might mitigate the drawbacks of asexual propagation. Giovannetti *et al*. (1999; 2001) observed hyphal anastomoses between closely related coenocytic mycelia that seem to provide opportunities for nuclear exchange and thus the remixing of nuclei among different mycelia. Evidence for genetic recombination (e.g. by gene conversion or mitotic recombination) that would enhance the chances to bring beneficial mutations together was found in a nrDNA sequence study (Gandolfi *et al*., 2003). The accumulation of deleterious mutations may be slowed down via bottlenecks during sporulation, which may purge individuals from nuclei with major genetic loads, as indicated in small fragmented plant populations (Paland & Schmid, 2003). Alternatively, coexistence of multiple nuclei may allow them to share gene functions, which were lost on certain nuclei due to accumulation of deleterious mutations.
1.6 Research questions and thesis outline

The aim of this project was to investigate the effects of long term (seven to ten years) Free-Air CO\textsubscript{2} Enrichment (FACE) on the symbiosis and on fungal partners of arbuscular mycorrhiza in a temperate, agricultural model grassland. Investigations were focused on the following main questions:

- Does long term (seven years) atmospheric CO\textsubscript{2} fumigation affect AMF colonisation patterns in roots of \textit{L. perenne} and \textit{T. repens}, and sporulation of AMF in the surrounding soil? (field study: Chapter 2)

- Does a history of elevated pCO\textsubscript{2} (eight years) favour AMF strains in the field that have different symbiotic abilities when tested in artificially established mycorrhizas? (climate chamber experiment: Chapter 3)

- Does ten years of elevated pCO\textsubscript{2} affect the structure of AMF communities and populations in roots of \textit{T. repens} from permanent field monocultures? (field study: Chapter 4)

The studies of this PhD project were conducted either directly in the model grassland plots of the Swiss FACE-experiment (Chapter 2 & 4) or in climate chambers using single spore isolates of AMF originating from these plots (Chapter 3). Mycorrhizal host plants in the FACE experiment were two typical perennial forage species of temperate agricultural grasslands, \textit{T. repens}, a herbal legume, and \textit{L. perenne}, a C3 cool-season grass. These plants associated with their native AMF communities have been grown in permanent monocultures for seven to ten years under ambient or elevated atmospheric pCO\textsubscript{2} prior to investigation. The plant species differ in their strategies of N assimilation, which means that patterns of N-deficiency under FACE were different in the two species. The originally fertile soil of the field experiment was well fertilised with phosphorus and potassium so that only N was expected to limit plant growth.

Since the studies of this PhD thesis were the first conducted in the present FACE experiment on mycorrhiza, the root colonisation levels and spore densities in the field were surveyed. The prominent and sporulating AMF species were then determined using the trap culturing technique. Spores from trap cultures were chosen to establish single spore pot cultures that served to characterise the native AMF species and strains based on nrDNA sequence data. From these pure fungal cultures, inocula were produced and used in pot experiments to test their effects on the growth of host plants (symbiotic efficiency). Molecular tools, developed on the basis of the propagated fungal material were finally applied on DNA extracts of roots from the field to identify the colonising AMF taxa. With these materials and tools in hand, it was possible to investigate three aspects of the effects of CO\textsubscript{2} fumigation on AMF in this long-term FACE experiment: (1) fungal growth response in the field, (2) alteration in symbiotic efficiency of mycorrhizas formed by individual fungal strains in a pot experiment and (3) taxonomic and genetic changes in the composition of AMF assemblages in roots in the field.
Hypotheses resulting from the first research question (Chapter 2) were that levels of root colonisation by different types of AMF structures would either remain the same or increase under elevated atmospheric pCO$_2$ compared to ambient pCO$_2$. Finding the first response pattern would imply that AMF adapted their growth proportionately with the growth of plants and/or that AMF strain composition remained the same under different atmospheric pCO$_2$ conditions. Alternatively, finding increased root colonisation would suggest that AMF profited from increased C-allocation by their host plants and/or that the AMF strain composition changed under elevated atmospheric pCO$_2$ to more heavily colonising individuals. Similarly, changes in the spore densities in the soil could be signs of increased C-allocation or changes in the composition of AMF strains having different growth strategies.

Expectations from the second research question (Chapter 3) were that a history of growth under elevated atmospheric pCO$_2$ would have favoured more C-demanding and/or more heavily colonising AMF strains or strains more efficient in mineral nutrient uptake from soil. This is because conditions under elevated atmospheric pCO$_2$ are known to stimulate the rate of photosynthesis in plants and thereby should increase the availability of non-structural C and mineral nutrient deficiencies in plants. The hypothesis is proposed that altered nutrient resource conditions, lasting over eight years, have acted selectively and affected the assemblage of different AMF strains. This in turn would have made it more likely that fungal strains better adapted to the new conditions were preferably isolated from the experimental field plots. If two sets of such isolated AMF strains were adapted to contrasting CO$_2$ histories and tested under uniform host plant and environmental conditions, they are expected to differ in their C-requirements and ability to supply host plants with mineral nutrients.

Regarding the third research question (Chapter 4) it was hypothesised that altered plant nutritional conditions and thus changed cost/benefit relations in the mycorrhizal symbiosis over ten years of CO$_2$ fumigation could have acted as forces of selection on AMF. Shifts in the composition and structure of AMF assemblages colonising plant roots could have taken place at the level of communities and/or populations. To detect the AMF that colonise the roots of T. repens in our experimental field site, DNA-identification tools were developed consisting of species-discriminating PCR primers (Appendix). Using these specific PCR primers the presence/absence of six AMF species was determined for a community study and DNA-sequences of one of these species were produced for a population study (Chapter 4).
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2  **Arbuscular mycorrhizal fungi benefit from seven years of free-air CO\textsubscript{2} enrichment in well-fertilised grass and legume monocultures**

**Published as:**


**2.1 Abstract**

Rising atmospheric carbon dioxide partial pressure (pCO\textsubscript{2}) and nitrogen (N) deposition are important components of global environmental change. In the Swiss Free-Air Carbon dioxide Enrichment (FACE) experiment the effect of altered atmospheric pCO\textsubscript{2} (35 Pa versus 60 Pa) and the influence of two different N-fertilisation regimes (14 versus 56 g N m\textsuperscript{-2} a\textsuperscript{-1}) on root colonisation by arbuscular mycorrhizal (AM)- and other fungi (non-AMF) of *Lolium perenne* and *Trifolium repens* were studied. Plants were grown in permanent monoculture plots, and fumigated during the growth period for seven years. At elevated pCO\textsubscript{2} AMF and non-AMF root colonisation was generally increased in both plant species, with significant effects on colonisation intensity and on hyphal and non-AMF colonisation. The CO\textsubscript{2} effect on arbuscules was marginally significant (P = 0.076). Moreover, the number of small AMF spores (≤ 100 µm) in the soils of monocultures (at low N-fertilisation) of both plant species was significantly increased, whereas that of large spores (> 100 µm) was increased only in *L. perenne* plots. N-fertilisation resulted in a significant decrease of all measured root colonisation variables in *L. perenne*, but not in *T. repens*. This phenomenon was probably caused by different C-sink limitations of grass and legume. Lacking effects of CO\textsubscript{2}-fumigation on intraradical AMF structures (under high N-fertilisation) and no response to N-fertilisation of arbuscules, vesicles and colonisation intensity suggest that the function of AMF in *T. repens* was non-nutritional. In *L. perenne*, however, AM-symbiosis may have amended N-nutrition, because all root colonisation parameters were significantly increased under low N-fertilisation, whereas under high N-fertilisation only vesicle colonisation was increased. Commonly observed P-nutritional benefits from AMF appeared to be absent under the phosphorus rich soil conditions of our field experiment. We hypothesise that in well-fertilised agricultural ecosystems, grasses benefit from improved N-nutrition and legumes from increased protection against pathogens and / or herbivores. This is different to what is expected in nutritionally limited plant communities.
2.2 Introduction

Rising atmospheric carbon dioxide partial pressure (pCO$_2$) is one of the major factors of global environmental change (Keeling et al., 1995), which is predicted to have considerable influence on plant growth and productivity in high input agricultural grasslands (Kimball, 1983; Rogers et al., 1994). With increased productivity, mineral nutrient demand and carbohydrate status of plants are altered. This may have profound effects on communities of organisms and on processes in the soil (Sadowsky & Schortemeyer, 1997; Marilley et al., 1999; Montealegre et al., 2000). Arbuscular mycorrhizal fungi (AMF), an important group of obligate biotrophic symbionts at the soil-root interface, provide a crucial link for nutrient exchange between plants and soil: carbohydrates flow from the plant to the soil, and mineral nutrients in the reverse direction (Smith & Read, 1997). The presence of a functional mycorrhizal symbiosis should affect carbon allocation between host plants and their root symbionts, AMF or N$_2$-fixing bacteria (Olesniewicz & Thomas, 1999). In addition, AMF are expected to modulate plant response to elevated pCO$_2$, by means of improving mineral nutrition (mainly P, Zn, Cu) and increasing resistance/tolerance of plants against an array of environmental stressors (Smith & Read, 1997). Moreover, alterations of inter- and intraspecific plant competition may occur, which are caused by different dependencies of individual plant species on the mycorrhizal symbiosis (Plenchette et al., 1983; O’Connor et al., 2002; van der Heijden, 2002). Altered competitive abilities of plant species under elevated pCO$_2$ may translate into changes in plant community composition and consequently affect productivity of ecosystems (Bazzaz, 1990; Lüscher et al., 1998). Changes in plant composition and ecosystem productivity are known to originate from differential growth responses on the level of plant functional groups (Clark et al., 1997; Hebeisen et al., 1997). For example, in legumes there is a stronger yield increase at elevated pCO$_2$ compared to grasses, which probably is caused by growth limitation of grasses due to nitrogen (N) deficiency (Soussana et al., 1996; Zanetti et al., 1997; Daepp et al., 2001).

Belowground responses to elevated pCO$_2$ are still poorly understood for both plants and soil organisms (Tiedje et al., 1999). Even less is known on the response of AM symbiosis and AMF communities at elevated pCO$_2$ in well-fertilised grassland ecosystems (Mäder et al., 2000). Significance of AMF colonisation may be very different in high input agricultural grassland with mostly bred forage plants compared to natural, nutritionally (P and N) limited plant communities. Moreover, experimental results on the effect of elevated pCO$_2$ on AM often conflict. In some studies elevated pCO$_2$ have promoted AMF root colonisation, whereas in others there has been no effect or even a decrease in root colonisation [reviewed in Staddon & Fitter (1998) and Rillig & Allen (1999)]. Most of these experiments have been conducted under strictly controlled conditions in growth chambers or greenhouses. Only two prior studies (Rillig et al., 1999a & 2000) have been performed under natural conditions employing long-term CO$_2$ fumigation.

In the present study, we examined effects of elevated pCO$_2$ on AM in two of the most extensively grown cool-season forage plant species, *Lolium perenne* L. cv. Bastion and *Trifolium repens* L. cv. Milkanova. Both were grown for seven years in permanent monoculture
plots under elevated (600 ppm) and ambient (350 ppm) atmospheric CO$_2$ concentration and high (56 g N m$^{-2}$ a$^{-1}$) and low (14 g N m$^{-2}$ a$^{-1}$) N-fertilisation (NH$_4$NO$_3$) in the Swiss Free-Air Carbon dioxide Enrichment (FACE) facility. The following hypotheses were tested: (1) Root colonisation by AMF and by saprobic fungi is increased under elevated pCO$_2$. (2) Effects of elevated pCO$_2$ are more pronounced under high N-fertilisation. (3) N-fertilisation affects AMF colonisation of roots with effects being greater in grasses than in legumes. The amount of arbuscules and vesicles is a measure of symbiotic functioning, physiological status and C-partitioning in the plant-fungus association (Klironomos et al., 1996). Under elevated pCO$_2$ with higher mineral nutrient demands, following increased rates of photosynthesis, we expect an increase of arbuscules (Fitter et al., 2000; BassiriRad et al., 2001; Rillig et al., 2002). These AMF structures are the most important sites for nutrient exchange. Moreover, C-storage relevant vesicle colonisation should increase under elevated pCO$_2$, especially in the N-limited grass. Also, root infection by parasitic or saprobic fungi (non-AMF) should increase because at elevated pCO$_2$ plants provide more carbohydrates than at ambient pCO$_2$ conditions. Under high N-fertilisation, nutritional effects of phosphorus may become relevant and different responses of functional groups (grass versus legume) may be amplified. Our study on mycorrhizal colonisation at elevated CO$_2$ is the first conducted under field conditions in a well-fertilised grassland typical of Central Europe. For artificial grassland, a frequent agricultural practice is to seed a very limited number of forage plants including ryegrass and white clover, similar to conditions applied here. Our findings may also be relevant for more divers agricultural grassland where these two species usually make up a large proportion of the plant community.

2.3 MATERIALS AND METHODS

2.3.1 EXPERIMENTAL FIELD SITE

This study is part of a large field experiment using Free-Air Carbon dioxide Enrichment (FACE) technology since 1993 (Zanetti et al., 1996; Hebeisen et al., 1997; Suter et al., 2002). The facility is located at the experimental station of the Institute of Plant Sciences (ETH Zürich) in Eschikon (550 m a.s.l., 8°41’E, 47°27’N, 20 km north-east of Zürich). The experiment was designed as a split-plot with three blocks, where three rings were CO$_2$ fumigated and three control rings were kept under ambient CO$_2$ conditions. CO$_2$ concentration was the main plot factor, plant species and N-fertilisation were the subplot factors. Treatments were randomised within each ring. There were two levels of atmospheric CO$_2$ partial pressure (pCO$_2$), ambient (35 Pa) and elevated (60 Pa), and two levels of nitrogen, high (56 g N m$^{-2}$ a$^{-1}$) and low (14 g N m$^{-2}$ a$^{-1}$), used. Nitrogen was applied as ammonium nitrate dissolved in water. The amount of N applied per re-growth period was adjusted to the expected seasonal biomass production. N-fertilisation started at the beginning of the growing season (early April) and was repeated immediately after the following five annual cuttings (Zanetti et al., 1996). *Trifolium repens* L. cv. Milkanova (white clover) and *Lolium perenne* L. cv. Bastion (perennial ryegrass) were grown in permanent monoculture swards and in a two species mixture. The FACE facility as well
as edaphic and climatic conditions are described in more detail by Hebeisen et al. (1997), Zanetti et al. (1996) and Suter et al. (2002). Briefly, the soil is a fertile, eutric cambisol (clay loam according to the US classification) with pH (H₂O) values between 6.5 and 7.6. Available phosphorus and potassium (according to Dirks & Scheffer, 1930) ranged from 1.2 to 6.0 mg kg⁻¹ soil and from 18 to 49 mg kg⁻¹ soil, respectively. Annually in spring, P and K fertiliser was applied as 12.5 g m⁻² P₂O₅ and 29 g m⁻² K₂O. Mineral nutrients in these amounts are considered to be non-limiting for forage crops under a temperate climate with wet winters and for this soil type. In addition to several commonly used pesticides, the monoculture plots of *T. repens* were treated with an aqueous suspension (1 g l⁻¹, used as a soil drench) of the systemic thiazol fungicide Benlate [50% benomyl (a.i): methyl 1-[butycarbamoyl]-2-benzimidazole carbamate DuPont; Wilmington, DE, USA] at a concentration of 5 g m⁻² each spring to control fungal disease (particularly *Fusarium* ssp. and *Sclerotinia trifoliorum* J. Eriksson). Plots were weeded manually throughout the years, as necessary.

2.3.2 Determination of AMF root colonisation and spore densities

Only monoculture plots were sampled, because with the small number of replications dictated by constraints of the FACE experiment the expected variance in the two-species mixtures would have obscured potential treatment effects. From each plot of *L. perenne* and *T. repens* (FACE subplot), three soil cores (2 cm diameter x 10 cm depth) were taken in late October 1999 after the fifth annual cutting. Samples of *L. perenne* were taken next to a plant individual and those of *T. repens* below a stolon to account for differences in the structure of the plant root system. Roots were hand picked from soil samples while passing soil through a 5 mm sieve. Then they were washed, cut into about 1 cm pieces, cleared, and stained using a modification of the technique described by Phillips and Hayman (1970), where lactoglycerin is substituted for lactophenol and 0.05% methyl blue is used in addition to trypan blue. Percentage colonisation was determined according to the magnified line intersection method (McGonigle et al., 1990). Briefly, one microscopic slide with 20 root fragments of about 1 cm length were assessed for 100 intersections with the hair line in the ocular (0.04 mm²) using bright field illumination of a compound microscope (Axioplan, Carl Zeiss, Göttingen, Germany) at 200 x magnification. This method, using microscopic slides, was preferred over the grid-line intersect method (Giovannetti & Mosse, 1980) because it allows distinction between AMF and non-AMF [defined according to Rillig et al. (1998)] in the field root samples. At each intersection, (1) the type of fungal root colonisation (AMF versus non-AMF), (2) presence or absence of intraradical hyphae, (3) arbuscules, or (4) vesicles were noted. Percentage colonisation is defined as the ratio of intersects with a specific fungal structure to total intersects multiplied by 100. Colonisation intensity was calculated by ascribing to each intersect score a value of two, if more than half of the root cortex was colonised by AMF structures and a value of one, if less than half of the cortex was colonised. Values of colonisation intensity may therefore range between zero and 200.
Sieved soil samples from the FACE subplots under low-N-fertilisation treatment from which roots had been picked were further used to determine spore abundance. Of each sample 10 grams of fresh soil were shaken in 200 ml water for 15 min. Soil suspensions were then wet sieved (Gerdemann, 1963) using nested sieves of 500 and 40 µm mesh size. The fraction collected on the 40 µm sieve was centrifuged twice in a sucrose gradient (Daniels & Skipper, 1982) to further separate the fraction containing the AMF spores. After thoroughly rinsing with distilled water spores were counted submerged in water in a petri dish under a stereomicroscope (Olympus SZX12, Olympus Optical Co., Nagano, Japan) at 50 times magnification. Spores were assigned to two size classes according to an arbitrarily defined diameter threshold of 100 µm. After determining soil humidity in each soil sample, spore abundance was expressed as spores per gram of dry soil.

2.3.3 Determination of Above Ground Dry Matter Yield and Shoot Nitrogen Concentration

Above ground dry matter yield was measured in an area of one square meter, while plant parts from accompanying species, which occurred despite a strict weeding regime, were excluded from the measurements (Hebeisen et al., 1997). Shoot tissue nitrogen concentrations were determined on ball-mill grounded and incinerated above ground dry matter by means of gas chromatography. These analyses were performed in the stable isotope laboratory of the University of California, Davis (USA).

2.3.4 Statistical Data Analyses

Statistical analyses were based on a randomised three-factorial design including two CO$_2$, two N, and two host plant species treatments with CO$_2$ treatments arranged in three replicate FACE-rings (blocks). For cross-factorial treatment (i.e. subplot), data from three replicate samples were analysed. Since CO$_2$ fumigation was applied to the entire FACE-ring, a split-plot model was used to test for treatment (CO$_2$, N and host plant species) effects on root colonisation parameters with CO$_2$ treatment as the main plot factor and N-fertilisation and host plant species as the subplot (split) factors. The main factor effect, CO$_2$ treatment, was therefore tested against a first order error term, defined as the CO$_2$ x FACE-ring (block) interaction mean square error (d.f. = 4). As split factors, the effect of N-fertilisation and that of host plant species were tested against the residual error (d.f. = 12). Analyses of variance (ANOVA) were performed, using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS) package (v. 6.12, SAS Institute, Cary, NC, USA). Significance of observed differences were determined at the 95% probability level. Data were tested for normality using the Univariate procedure, and homogeneity of variances were tested by residual plots in SAS, prior to statistical analyses. For analysing colonisation intensity, data were arcsin-square root transformed; the fractional vesicle colonisation data could not be transformed to meet the assumption of normality in ANOVA. t-tests for pairwise comparison of means between CO$_2$ treatments and linear regression analyses were performed with the software package JMP version 4.0.3 (SAS Institute Inc. 1989-2000).
2.4 RESULTS

2.4.1 EFFECTS OF CO\(_2\) AND N ON ROOT COLONISATION BY AMF AND NON-AMF

At elevated pCO\(_2\) and under both N-treatments AMF root colonisation of both host plant species was increased (Figure 2.1). Colonisation levels of all three measured intraradical AMF structures (hyphae, arbuscules and vesicles) tended to be higher, although significant differences were only found for hyphal colonisation (P = 0.037) and colonisation intensity (P = 0.046) (Table 2.1 & Figure 2.1). The subplot factor plant species was significant for colonisation by vesicles (P = 0.003), due to higher numbers of vesicles found in L. perenne and lower numbers in T. repens (Figure 2.1). The effect of N-fertilisation was significant for hyphae (P = 0.006), arbuscules (P = 0.037), vesicles (P = 0.002), total colonisation and colonisation intensity (both P = 0.007). The only significant interaction term in the three-way ANOVA were found between N-fertilisation and plant species (Table 2.1). Significance mainly originated from the lack of any mycorrhizal response to increased N-fertilisation in T. repens. Three-way interactions were never significant.

Root colonisation of L. perenne by other endophytes (non-AMF) was increased in the high N-treatment under elevated pCO\(_2\) (P = 0.036). Host plant species clearly differed in the level of colonisation by non-AMF (P < 0.001), with higher levels in L. perenne compared to T. repens. However, elevated pCO\(_2\) generally tended to increase root colonisation by non-AMF (P = 0.082), irrespective of host plant species and N-treatment.

![Figure 2.1](image-url) Percentage field root colonisation by hyphae (H), arbuscules (A), vesicles (V) and non-AMF (O), as well as values for colonisation intensity by AMF (I) in *Lolium perenne* (perennial ryegrass) and *Trifolium repens* (white clover). Data are from the fifth annual harvest in late October 1999. Plants were grown in permanent monoculture plots in the Swiss FACE experiment at ambient (35 Pa) and elevated (60 Pa) atmospheric pCO\(_2\) for seven years. Different plots of each species were subjected to low (14 g N m\(^{-2}\) a\(^{-1}\)) and high (56 g N m\(^{-2}\) a\(^{-1}\)) N-fertilisation (subplot factor). Means and standard errors, calculated from the averages of each of the three root samples per FACE subplot, are displayed (n=3). Note that values for the AMF colonisation intensity may range between 0-200 (see MATERIALS AND METHODS). Composite bars indicate colonisation at ambient pCO\(_2\) (black) and percent increase at elevated pCO\(_2\) (grey). * Pairwise difference (t-test, P ≤ 0.05).
TABLE 2.1 Significance levels of effects of different factors and factor interactions on arbuscular mycorrhizal root colonisation of *Lolium perenne* and *Trifolium repens* based on a split-plot analyses of variance (ANOVA).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>df</th>
<th>Hyphae</th>
<th>Arbuscules</th>
<th>Vesicles</th>
<th>Total colonisation</th>
<th>Colonisation intensity&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Non-AMF</th>
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<td>Error A (block x CO&lt;sub&gt;2&lt;/sub&gt;)</td>
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<td>CO&lt;sub&gt;2&lt;/sub&gt; x N-fertilisation</td>
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<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>N-fertilisation x host plant</td>
<td>1</td>
<td>*</td>
<td>ns</td>
<td>**</td>
<td>*</td>
<td>*</td>
<td>ns</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt; x host plant x N-fertilisation</td>
<td>1</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Error B (residual)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Roots were sampled from permanent monoculture plots in the Swiss FACE experiment in autumn 1999 after seven years of CO<sub>2</sub> fumigation (60 Pa vs. 35 Pa). The block (FACE-ring) effect was not significant and is therefore not shown.

<sup>1</sup> Determined as described under MATERIALS AND METHODS.

Significance levels: ns, P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001

2.4.2 EFFECTS OF CO<sub>2</sub> AND HOST PLANT SPECIES ON AMF SPORE ABUNDANCE IN FIELD SOILS

Spores found in field soils were arbitrarily assigned to two categories, ‘small spores’ (diameter ≤ 100 μm) and ‘large spores’ (>100 μm). At elevated pCO<sub>2</sub> the number of small spores was significantly higher in plots of both host plant species (*L. perenne* and *T. repens*, P = 0.010), whereas the number of large spores was higher only in plots of *L. perenne* (P = 0.044, TABLE 2.2 & FIGURE 2.2). However, the total number of spores was significantly increased in plots of both species (P = 0.010). At elevated pCO<sub>2</sub> (pooled for both species), the increase of small spores was 72%, that of large spores 46%. Across species and treatments, numbers of small spores were always significantly higher compared to numbers of large spores. Comparing the two species, the level of small spores was significantly higher in plots of *L. perenne* (by 33 %) than in plots of *T. repens* at both pCO<sub>2</sub> levels (P = 0.011, FIGURE 2.2).

2.4.3 RELATIONSHIP BETWEEN ROOT COLONISATION INTENSITY AND ABOVE GROUND DRY MATTER YIELD

Above ground dry matter yield from the fifth annual cutting in late October 1999 accounted for 14.2% of the annual yield in *L. perenne* monoculture plots and for 9.7% in *T. repens* plots (M. Schneider; unpublished data). In *L. perenne*, there was a significant negative linear relationship between the AMF root colonisation intensity and the above ground dry matter yield under elevated pCO<sub>2</sub> (P = 0.009), but not at ambient pCO<sub>2</sub> (FIGURE 2.3). Other root colonisation
variables (hyphae, arbuscules and vesicles) were similarly related with above ground dry matter yield. In particular, vesicle numbers were negatively correlated with yield. Moreover, both yield and root colonisation were generally higher under elevated pCO$_2$ compared to ambient pCO$_2$ conditions (Figure 2.3). For T. repens, such a linear relationship was not significant at either CO$_2$ condition (data not shown).

**Figure 2.2** Number of arbuscular mycorrhizal fungal spores in field soil samples from permanent monocultures of *Trifolium repens* and *Lolium perenne* grown at low N-fertilisation (14 g N m$^{-2}$a$^{-1}$) in the Swiss FACE experiment. Soil samples were taken after the fifth annual harvest in late October 1999, after seven years of FACE treatment (60 Pa vs. 35 Pa). Means and standard errors, calculated from the averages of three soil samples per FACE subplot, are given. Composite bars indicate spore densities at ambient pCO$_2$ (black) and increase at elevated pCO$_2$ (grey). * Pairwise difference (t-test, P ≤ 0.05).

2.4.4 Relationship between root colonisation intensity and shoot N and P concentration

Although shoot nitrogen concentration decreased with increasing colonisation intensity in both species, this relationship was significant only in *L. perenne* (P = 0.037, Figure 2.4). For this analysis data from both pCO$_2$ and N-fertilisation treatments were pooled to provide more data points, although CO$_2$ fumigation may dilute and N-fertilisation may enrich the shoot N-concentration (particularly in *L. perenne*). Nitrogen concentrations in above-ground yields

**Table 2.2** Significance levels of effects of CO$_2$ and host plant species and their interaction on arbuscular mycorrhizal spore abundance in soils of *Lolium perenne* and *Trifolium repens* monoculture plots under low N fertilisation (14 g N m$^{-2}$a$^{-1}$) based on a split-plot analysis of variance (ANOVA).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>df</th>
<th>Small spores (≤ 100 μm)</th>
<th>Large spores (&gt; 100 μm)</th>
<th>Total spores</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main plot factor</strong></td>
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<td></td>
</tr>
<tr>
<td>CO$_2$</td>
<td>1</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Error A (block × CO$_2$)</td>
<td>4</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Sub plot factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Host plant species</td>
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<td>*</td>
<td>ns</td>
<td>*</td>
</tr>
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<td>CO$_2$ × host plant</td>
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<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Error B (residual)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Soil samples were taken after the fifth annual harvest in autumn 1999 after seven years of FACE treatment (60 Pa vs. 35 Pa). The block (FACE-ring) effect was not significant and is therefore not shown.

Significance levels: ns, P > 0.05; *, P < 0.05
were consistently higher in *T. repens* than in *L. perenne* (Figure 2.4). Colonisation levels of hyphae and vesicles were similarly correlated with shoot N-concentrations, whereas for arbuscules there was only a weak trend in the same direction (in *L. perenne*) or no correlation at all (in *T. repens*).

No significant relationship between colonisation intensity and phosphorus concentration was found in both host plant species (data not shown).

**Figure 2.3** Above ground dry matter yield of *Lolium perenne* monoculture plots as a function of root colonisation intensity by arbuscular mycorrhizal fungi (for definition see Materials and Methods). Data are from the fifth annual harvest in late October 1999. Plants were grown in permanent monoculture plots in the Swiss FACE-experiment at ambient (35 Pa) and elevated (60 Pa) atmospheric pCO₂ for seven years. Linear regressions were performed on pooled data from both nitrogen fertilisation (14/56 g N m⁻² a⁻¹) treatments. Symbols: triangles: ambient pCO₂ treatment; squares: elevated pCO₂ treatment (open symbols represent data points derived from the low N-fertilisation treatment, closed symbols from the high N-fertilisation treatment).

### 2.5 Discussion

#### 2.5.1 Effects of Elevated pCO₂ on Root Colonisation

In the grassland plant species, *L. perenne* and *T. repens*, field root colonisation by mycorrhizal structures (hyphae, arbuscules, and vesicles) was increased at elevated, compared to ambient pCO₂. However, the effect was only significant for hyphal colonisation and colonisation intensity (Table 2.1 & Figure 2.1). Increased colonisation as a response to elevated pCO₂ was also previously observed in growth chamber experiments (Klironomos et al., 1996; Hartwig et al., 2002). This is concordant with the hypothesis of a higher availability of carbohydrates in roots which may stimulate the symbiosis. However, marginally positive, or no response to elevated pCO₂ have also been reported (Monz et al., 1994; Jongen et al., 1996; Staddon et al., 1998; Rillig et al., 1999a), particularly when CO₂-induced accelerated plant growth was taken into account. Intraradical hyphal growth tends to increase simultaneously with root length resulting in no net response of root colonisation at elevated pCO₂. Unlike our study, the majority of investigations [reviewed by Staddon & Fitter (1998) and Rillig & Allen (1999)] were neither conducted under free-air conditions, nor with long-term adapted plant individuals and AMF communities. This may account for the absence of a CO₂ response of AMF colonisation in those investigations. Interestingly, in our study colonisation intensity increased more (+37%) than the simple total percentage of root colonisation (+28%), an observation already made in another forage grass species, *Lolium multiflorum* Lam. (Rillig et al., 1999a). The increased colonisation levels found under elevated pCO₂ confirmed our expectations. However, it is possible that plants were in different growth stages between ambient and elevated pCO₂ at harvest time (Rey & Jarvis, 1997), which could have affected our observations. At least in *L. perenne*, acceleration of re-growth after cutting was observed at elevated pCO₂ in the Swiss FACE-experiment (Suter, Nösberger et al., 2001).
FIGURE 2.4 Shoot nitrogen concentration as a function of root colonisation intensity by arbuscular mycorrhizal fungi (for definition see MATERIALS AND METHODS) of Trifolium repens and Lolium perenne. Data are from the fifth annual harvest in late October 1999. Plants were grown in permanent monoculture plots in the Swiss-FACE experiment at ambient (35 Pa) and elevated (60 Pa) atmospheric $pCO_2$ for seven years. Linear regressions were performed on pooled data from both $pCO_2$ and nitrogen fertilisation (14/56 g N m$^{-2}$ a$^{-1}$) treatments. Symbols: triangles: $L. perenne$ at ambient $pCO_2$; squares: $L. perenne$ at elevated $pCO_2$; circles: $T. repens$ at ambient $pCO_2$; diamonds: $T. repens$ at elevated $pCO_2$ (open symbols represent data points derived from the low N-fertilisation treatment, closed symbols from the high N-fertilisation treatment).

We also found an increase in non-AMF root colonisation under elevated $pCO_2$, however with considerable difference between host plant species (TABLE 2.1 & FIGURE 2.1). This conflicts with findings by Rillig et al. (1998; 1999a; 1999b), but is in good agreement with those reported by others (Klironomos et al., 1997; Gavito et al., 2002). A possible explanation for these inconsistencies could be the choice of host plants. In our study as well as in that by Gavito et al. (2002) agricultural cultivars were used, whereas Rillig et al. (1998; 1999a; 1999b) studied wild plants.

2.5.2 EFFECT OF N TREATMENT ON ROOT COLONISATION

Root colonisation (including all arbuscular mycorrhizal structures) was negatively affected by N-fertilisation (TABLE 2.1 & FIGURE 2.1). However, only $L. perenne$ responded with a strong reduction in colonisation rates under high N-fertilisation, whereas effects in $T. repens$ were not significant (FIGURE 2.1). The host plant-dependent response may be due to the functional differences between grasses and legumes. Legumes, compared to grasses, depend much less on soil N because of their ability to fix atmospheric $N_2$. Therefore, in our experiment, where P- and K-fertiliser was applied annually to meet the needs of forage crops, $T. repens$ was adequately supplied by all nutrients. However, compared to the latter the grass $L. perenne$ may not have had sufficient N, which could have been supplemented by its uptake and transfer by AMF symbionts (Frey & Schüepp, 1993; Hodge et al., 2001). Alternatively, association with AMF species or strains with different capabilities for N acquisition (Frey & Schüepp, 1993; Hawkins et al., 2000; Azcon et al., 2001) and sensitivity could explain differences in responses of root colonisation. The observed responses to N-fertilisation were more consistent than those to $pCO_2$ treatments, and agreed with most earlier findings (Rillig & Allen, 1998; Cairney & Meharg, 1999; but see Ryan & Ash, 1999). Our results suggest that N-inputs may be relevant for potential changes in AM symbiosis and / or AMF community composition, and underline the significance of anthropogenic atmospheric N deposition. N-inputs into grassland ecosystems may be more important for global environmental change than the rise in $pCO_2$ (Klironomos et al., 1997; Rillig et al., 2002). Our second hypothesis that effects of elevated $pCO_2$ may be
more pronounced under high N-fertilisation was unsupported in our experimental plots with well-fertilised plant monocultures. The CO$_2$ x N-fertilisation interactions were not significant for any of the measured root colonisation parameters (Table 2.1).

Root colonisation by non-AMF increased at high N-fertilisation and elevated pCO$_2$ in *L. perenne*, as expected for saprobic and parasitic fungi, which require N for assimilation of the supplementary carbohydrates. Increased biomass of non-AMF under nutrient addition was also observed in another field experiment on mycorrhizas (Klironomos *et al.*, 1996).

### 2.5.3 Above Ground Biomass and Shoot N Concentration

Based on our limited data, a clear relationship between mycorrhizal colonisation intensity and above ground biomass production across pCO$_2$ treatments and host plant species seems to be missing. Only at elevated pCO$_2$ and only for *L. perenne*, there was a significant negative correlation between root colonisation intensity and shoot biomass (Figure 2.4). At ambient pCO$_2$, there was no obvious relationship between these parameters for *L. perenne*, and at both pCO$_2$ conditions for *T. repens*. These findings conform with a meta-analysis of 78 growth experiments (McGonigle, 1988), which suggest that a general relationship between yields and increased root colonisation levels by AMF is lacking.

Likewise, colonisation intensity and shoot N-concentration were negatively correlated in *L. perenne*, but not in *T. repens* (Figure 2.4). AMF root colonisation of the grass probably deprived N from the above ground parts of the host by retaining it in the fungal structures in roots and in soil. This interpretation is consistent with the higher colonisation level observed under low N-fertilisation (Figure 2.1) and suggests that at least under elevated pCO$_2$ growth of *L. perenne* is more N-limited due to mycorrhizal colonisation (Figure 2.3 & 2.4). Negative or weak relationships between shoot N-concentration and colonisation intensity, and the lack of any relationship between shoot phosphorus concentration and root colonisation (data not shown) does not support the AMF growth model of Treseder and Allen (2002). These authors suggest that fungal growth is controlled by restricted C-supply by the plant, if mineral nutrients (particularly N or P) are adequately supplied. In our study, such limitations could have occurred in *T. repens* showing rather low root colonisation and only a weak increase at elevated pCO$_2$. By contrast, intraradical fungal growth in *L. perenne* did not seem to be limited. For this species with a higher colonisation rate under low N treatment (Figure 2.1 & 2.4), fungal growth response may be host-induced, because increased intraradical fungal surfaces would allow for a better N-flux to the host. In fact, N-deficiency was observed in *L. perenne* plants from the experimental field plots (Zanetti *et al.*, 1997; Daepp *et al.*, 2001).

### 2.5.4 C-Allocation to Fungal Root Colonisation and AMF Spores

The increase of vesicle colonisation at elevated pCO$_2$ in *L. perenne* under both N-fertilisation treatments together with the increase of hyphal colonisation under low N-fertilisation (Figure 2.1) suggest that additional carbohydrates were transferred to fungal structures in
the roots. Increased spore numbers found in plots of low-N-fertilisation at elevated pCO$_2$ (Figure 2.2), further support this interpretation. Sink limitations for assimilated carbohydrates, as reported for *L. perenne* in this FACE experiment (Daepp *et al.*, 2001), may promote C-allocation into AMF structures. However, in *T. repens* this may be the case only in part, because energy of additional carbohydrates may be used for increased N$_2$-fixation (Zanetti *et al.*, 1996; Olesniewicz & Thomas, 1999).

Increased colonisation levels may indicate a change in the mutualism – parasitism – continuum (Johnson *et al.*, 1997). At least for *L. perenne*, smaller above-ground dry-matter yields with increasing colonisation intensity at elevated pCO$_2$ (Figure 2.3) suggest a sub-optimal relationship with AMF. Therefore, the symbiotic status of *L. perenne* in our experiment may be in the upper range of the proposed curvi-linear relationship between root colonisation and mycorrhizal dependency (Gange & Ayres, 1999). A negative relationship between percentage maximum dry weight of shoots and percentage of root length colonised was also found in an experiment involving an unidentified isolate of *Glomus* and *L. rigidum* (Schweiger *et al.*, 1995). Moreover, the pronounced C-sink strength of AMF under elevated pCO$_2$ would be consistent with possible changes in AM-functioning. Rillig *et al.* (1999b) hypothesized that long-term CO$_2$ fumigation may induce physiological adaptations of individual fungi or changes in AMF communities. Such adaptations or changes in our experimental field plots could account for increased root colonisation by vesicles of *L. perenne*.

Possible changes in AMF composition in the Swiss FACE-experiment are suggested by different spore frequencies in soils. Spore densities in plots of low-N-fertilisation were significantly higher at elevated pCO$_2$ compared to ambient pCO$_2$, and were higher in samples from *L. perenne* compared to samples from *T. repens* plots (Figure 2.2). Spore morphotypes matching species descriptions of *Glomus claroideum* Schenck & Smith, *G. constrictum* Trappe, *G. mosseae* (Nicol. & Gerd.) Ger. & Trappe and *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders contributed mostly to the arbitrarily defined size class of ‘large spores’, those matching *G. diaphanum* Morton & Walker to the class of ‘small spores’ (Schenck & Pérez 1990; International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM): http://invam.caf.wvu.edu/fungi/taxonomy/speciesID.htm). Difference in colonisation patterns in *L. perenne* compared with *T. repens* may reflect host preferences for different AMF groups, an explanation also suggested for observed differences in the abundance of vesicles in a fertilisation experiment in a rain forest ecosystem (Treseder & Allen, 2002). Furthermore, monocultures by themselves may selectively induce changes in the fungal community. Zhu *et al.* (2000) using a combined reciprocal trap culture inoculum-spreaders-receiver system, demonstrated quantitative host preferences of AMF communities adapted to the hosts, *L. perenne* and *T. repens*. Moreover, groups of AMF species are known to respond differently to N-fertilisation and to soil tillage (Egerton-Warburton & Allen, 2000; Jansa *et al.*, 2002). Thus, all CO$_2$-, N- and plant species-treatments are likely to have affected AMF communities.
2.5.5 Patterns of root colonisation and spore abundance

Our findings of relatively high levels of AMF root colonisation in L. perenne compared to T. repens were surprising and differed with those of many earlier studies on the same plant species (e.g. Read et al., 1976; Zhu et al., 2000). Possible explanations for this conflict are: First, residues of the fungicide benomyl, which has been regularly applied for disease control in T. repens plots, could have depressed AMF root colonisation and sporulation in T. repens plots or eradicated efficient colonisers and spore producers. However, fungicidal effects of benomyl diminish rapidly with long-term application (Menge, 1982; Merryweather & Fitter, 1996), probably due to stimulation of community growth of degrading bacteria. Second, root colonisation by non-AMF could have obscured assessment of true AMF. Yet, root colonisation levels of both root symbionts were determined simultaneously, which should have precluded possible double counts. Finally, roots of plants other than L. perenne (e.g. weed grasses) may have been erroneously included in the sample. However, root samples were taken and sorted carefully to avoid any weeds. Thus, we are confident that our results are not biased by methodical artefacts.

An alternative non-nutritional explanation for the observed differences in root colonisation levels of grass and legume, is the potential for improved pathogen resistance and / or tolerance of mycorrhizal plants (Gange & Ayres, 1999). This may be particularly important for grasses with finely branched root systems and a large root surface vulnerable to pathogen attack (Newsham et al., 1995; Gange & Ayres, 1999). Such indirect mutualistic AMF effects are suggested to be the most frequent effects on plant community structure and dynamics in other grasslands (Hartnett & Wilson, 2002). However, the proposed relationship between root fibrousness and mycorrhizal protection may be less relevant for highly bred plant cultivars, which were selected for maximum yield and disease resistance (Graham, 2000).

An obvious limitation of our study is that results are based on a single sampling taken at a particular time of the year. In future studies, seasonal variations in root colonisation levels should be considered. Moreover, for evaluating the role of AM, particularly with regard to C-allocation patterns and C-fluxes under a scenario of elevated pCO$_2$, extraradical fungal structures including hyphae and their products (e.g. glomalin) must be taken into account.

2.6 Conclusions

Responses of fungal root colonisation (particularly in L. perenne) and spore abundance in soil after seven years of exposure to elevated pCO$_2$ under field conditions confirm the hypothesis that AMF and non-AMF are capable to absorb an increased supply of carbohydrates. We hypothesize that AMF provide non-P-nutritional benefits under the phosphorus rich soil conditions of our field experiment. Such benefits may include improved N-nutrition and increased protection against pathogens and / or herbivores. Furthermore, results suggest that grasses may benefit more from AM symbiosis than do legumes in well-fertilised agricultural grassland. This is different to what is expected in nutritionally limited plant communities. Moreover, response of
intraradical AMF structures to N-fertilisation was more profound than response to long-term CO₂ fumigation. These findings suggest that effects of anthropogenic N application may be as important for the functioning of AM as effects of increasing pCO₂, and underline the view of global environmental change as a multi-factor phenomenon.

2.7 ACKNOWLEDGEMENTS

We wish to thank Thomas Flura, Werner Wild and Anni Dürsteler for help in the field and laboratory and all the people who have been maintaining the FACE facility over the years – particularly Dr. Herbert Blum for coordination. Comments of Prof. Dr. Christopher L. Schardl helped to improve the manuscript. The Swiss FACE was supported by grants from the Swiss Federal Institute of Technology (ETH), the Swiss National Energy Foundation, the Swiss National Science Foundation, the Swiss Department for Energy, the Swiss Department for Agriculture and the Brookhaven National Laboratory (NY, USA).

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3 MYCORRHIZAS IMPROVE N NUTRITION OF *TRIFOLIUM REPENS* AFTER EIGHT YEARS SELECTION UNDER ELEVATED ATMOSPHERIC pCO$_2$

Submitted as:


3.1 Abstract

Altered environmental conditions may change populations of arbuscular mycorrhizal fungi and thereby affect mycorrhizal functioning. We investigated whether eight years of free-air CO$_2$ enrichment has selected fungi, which differently influence nutrition and growth of host plants. In a controlled pot experiment, two sets of seven randomly picked single spore isolates, originating from field plots of elevated (60 Pa) or ambient pCO$_2$, were inoculated on nodulated *Trifolium repens* (white clover) plants. Fungal isolates belonged to *Glomus claroideum* and *G. intraradices* and host plants were clonal micropropagates derived from nine genets. Total nitrogen concentration and proportion taken up from the potting substrate was increased in leaves of plants inoculated with fungal isolates derived from elevated-pCO$_2$-plots. Fungal isolates varied considerably in their stimulating effect on biological nitrogen fixation and in root colonisation parameters. Physiological preferences of particular fungal isolates for plant genets are suggested. We conclude that rising atmospheric pCO$_2$ may select for fungal strains, which are more efficient in soil-plant nutrient transfer.

3.2 Introduction

The long-term response of fertile grassland ecosystems to rising atmospheric pCO$_2$ may depend heavily on legumes (Soussana & Hartwig, 1996; Zanetti *et al.*, 1997). This is because legumes establish two important symbioses, one with bacteria, collectively known as rhizobia forming the nodule symbiosis, and the other with arbuscular mycorrhizal fungi (AMF, Glomeromycota), an ubiquitous group of soil fungi. The nodule symbiosis provides legumes with biologically fixed nitrogen (N), whereas AMF are considered to mainly improve phosphorus (P) nutrition of the host plant under nutrient poor soil conditions. N and P are the main growth-limiting elements in natural ecosystems and may become even more limiting under rising atmospheric pCO$_2$ (Keeling *et al.*, 1989; Bazzaz, 1990).
AMF may be particularly affected by rising atmospheric pCO$_2$, because they depend exclusively on carbohydrates (C) provided by plants and because they play an important role as mediators in the flow of nutrients between plant and soil (Staddon & Fitter, 1998; Rillig & Allen, 1999; Fitter et al., 2000; Rillig et al., 2002). Stimulation of photosynthesis under elevated pCO$_2$ may supply AMF with additional C, but at the same time host plants may have higher nutrient demands due to increased plant growth (Bazzaz, 1990). Higher nutrient demands of plants could be met by increased extraradical hyphal densities of AMF providing an enlarged nutrient absorption surface (Sanders et al., 1998). Moreover, mineral nutrients are thought to be more limiting for plants than for fungi (Treseder & Allen, 2002), due to a smaller soil volume exploited by plant roots in comparison to fungal hyphae.

Conditions of elevated pCO$_2$ can be simulated by Free-Air CO$_2$ Enrichment (FACE) technology that does not alter microclimate (Hendrey, 1992; Long et al., 2004). Under FACE, the growth of plants has been shown to be limited, because increased nutrient demands could not be readily covered from the soil nutrient pool and cause plants to accumulate the surplus of C primarily in the roots (Rogers et al., 1994; Suter et al., 2002 and references therein). In fact, AMF may take advantage of such favourable conditions as suggested by a significant increase of AMF structures observed in roots of Lolium perenne, a grass grown in monoculture plots of the Swiss FACE-experiment (CHAPTER 2). AMF also responded with an increase in C-allocation to elevated pCO$_2$ from a natural source at a grassland site in New Zealand (Rillig et al., 2000).

Given the dependency of AMF on plant C and their role in plant nutrition, prolonged growth under elevated pCO$_2$ may induce selective changes in AMF populations in different ways. On the one hand, increased C-availability may favour fungal strains that are more C-demanding, colonise roots more heavily and thus are able to out-compete other strains through resource depletion and / or habitat occupancy. On the other hand, nutrient-limitation in soil may select for fungal strains that are more efficient in improving mineral nutrition of host plants. Literature reports support either of these hypotheses. AMF strains prevalent in agricultural soils often differ in their degree of root colonisation and thus impose different C-costs to their hosts (Graham et al., 1996; Graham & Abbott, 2000). Moreover, C-costs imposed by growth and maintenance of fungal structures resulted into negative growth responses of host plants (e.g. Peng et al., 1993), while Gange and Ayres (1999) proposed a curvilinear relationship of root colonisation and symbiotic benefits. Particularly in the tripartite symbiosis of legumes, mycorrhizal C-costs may call for attention (Bethlenfalvay et al., 1985; Murphy, 1986). Up to 20% of net-photosynthetic yield are diverted to mycorrhizas (Jakobsen & Rosendahl, 1990) in addition to the 25% of the shoot C required for nodule symbiosis (Schulze, 2004). Indeed, C-requirements of AMF at this magnitude emphasise the selective potential of an altered plant C-status at elevated pCO$_2$. Supporting the second hypothesis, differences in the acquisition and transfer capabilities to host plants were found among AMF isolates for P (Jakobsen et al., 1992; Smith et al., 2004) and N (Hawkins et al., 2000; Azcon et al., 2001). This is a necessary precondition for potential shifts in the assemblage of fungal strains after nutrient requirements of host plants have changed.
In the present study we investigated whether eight years of FACE in a model grassland has selected for AMF strains that differ in their effects on nutritional status and growth of host plants. In a pot experiment under uniform climate chamber conditions, two sets of AMF strains with histories of exposure to different atmospheric pCO₂ were tested for their symbiotic behaviour in associations with different genets of *Trifolium repens* (white clover). One set consisted of fungal isolates that originated from field plots exposed to elevated atmospheric pCO₂ (60 Pa) for eight years (elevCO₂ isolates), and the other consisted of isolates from field plots with ambient atmospheric pCO₂ conditions (ambCO₂ isolates). Nine different host plant genets were inoculated with fourteen randomly chosen fungal isolates to form two-partner mycorrhizal associations. We hypothesised that elevCO₂ isolates would behave more costly and thus depress plant growth compared to the ambCO₂ isolates, or alternatively would improve plant mineral nutrition resulting in higher leaf and root nutrient contents. Particularly, the N-limitation reported for plants growing in the FACE plots of otherwise well-fertilised grassland was considered as factor that may potentially select for AMF strains that can improve the N status of host plants.

In addition, different N-fertilisation regimes applied in the model swards from where the fungal isolates originated, and different plant cover were evaluated for their effects on the experimental mycorrhizas. The selected single spore isolates belonged to two distinct AMF morpho-species and allowed to assess the effects of species and isolate identity on mycorrhizal symbiosis. Furthermore, potential specificities or preferences of fourteen AMF isolates for nine clonally propagated genets of *T. repens* were analysed based on symbiotic performance of fungal isolate / plant genet associations in an orthogonal design. Non-mycorrhizal controls (NM) grown at different P-fertilisation levels were used to assess the P-growth response of the *T. repens* host genets. Plant and fungal morphometric growth parameters served as indicators of constitution and potential fitness, while the P- and N-concentrations of the leaf and root fraction served as measures of nutritional status of host plants.

To our knowledge, this is the first experiment under controlled conditions and using multiple single spore isolates that assessed the physiological implications of a potential selective effect of an environmental factor on AMF field-assemblages.

### 3.3 Materials and Methods

#### 3.3.1 Origin of Arbuscular Mycorrhizal Fungal Isolates

Isolates of AMF were obtained from permanent plots of the Swiss FACE-experiment, located 20 km NE of Zürich at the field station of the Institute of Plant Sciences in Eschikon (ETH). The FACE experimental setup consisted of six 18 m-diameter rings: three control rings with ambient air and three rings with elevated atmospheric pCO₂ (60 Pa). The CO₂ fumigation took place over eight years (1993 - 2000) during daytime and vegetation period. Experimental monoculture plots of *Lolium perenne* L. cv. Bastion and *Trifolium repens* L. cv. Milkanova and the mixture of both plant species were arranged according to a split-plot design with atmospheric CO₂ concentration as the main plot treatment and N-fertilisation (14 and 56 g m⁻² yr⁻¹) and plant
cover as the subplot treatments. Within each ring the six treatments were randomised among subplots (2.8 x 1.9 m). The soil was a clay loam, classified as fertile, eutric cambisol (FAO Classification System). For further details about the field experiment see Zanetti et al. (1996) or Suter et al. (2002).

3.3.2 AMF INOCULA

Open-pot trapping cultures (Gilmore, 1968) were set up in the greenhouse with soil samples taken in autumn 2000 from the twelve cross-factorial treatments of the Swiss FACE-experiment and pairs of the trap-host plant species listed in Table 1. Pure isolates were established on Plantago lanceolata L. (ribwort plantain) after five months, using single spores from trapping cultures. All successful single spore isolates (approx. 10%) belonged to genus Glomus. Of these, 14 originated from plots subjected to ambient, and 44 originated from plots, subjected to elevated pCO$_2$. From each of these two pools of AMF isolates seven were randomly chosen, irrespective of morpho-species identity and trap-culture origin (Table 3.1). The selected fungal isolates belonged to G. claroidum Schenck & Smith (Gc) and the G. intraradices (Gi) Schenck & Smith species complex. Gi-isolates were initially sub-classified as G. intraradices and G. aggregatum Schenck & Smith which, however, became untenable after single spore and inoculum propagation [for a similar finding see Johnson (1993, p. 752)].

Inocula were produced over a period of 7.5 months on Zea mays L. cv. Corso (maize) and P. lanceolata in potting substrate that consisted of 1:2:2 volumes of pasteurised field soil from the upper horizon [clay loam, pH (H$_2$O): 5.8, available P (Olson): 52 mg P kg$^{-1}$, re-inoculated with a filtrate of the natural microflora], silica sand (0.7-1.2 mm) and an expanded attapulgite clay (Oil Dry Chem-Sorb WR 24/18, Brenntag Mediterrâ¨e Export, Vitrolles Cedex, France), and then were stored in dry condition at 4°C for eight months.

**Table 3.1** Identity, host plants in the field of origin and CO$_2$ and nitrogen (N) history of the fourteen single spore isolates of arbuscular mycorrhizal fungi (AMF) used in this experiment. Fungal isolates belonged to Glomus claroidum (Gc) or the G. intraradices (Gi) species complex. Particularly, inocula of Gi-isolates included root cortices of Zea mays L. cv. Corso (maize) and Plantago lanceolata L. (ribwort plantain) that were densely packed with vesicles, and numerous completely hyaline vesicles. Spores of Gi, released from broken up cortices, formed in certain cases sporae aggregates of up to a macroscopic size. Abbreviations: Df: Dipsacus fullonum L.; La: Leontodon autumnalis L.; Lp: Lolium perenne L.; Ob: Oenothera biennis L.; Pl: P. lanceolata L.; Tp: Tagetes patula L.; Tr: Trifolium repens L.; Zm: Zea mays L. cv. Corso.

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Morpho-species</th>
<th>Field host/s</th>
<th>pCO$_2$ (Pa)</th>
<th>N-fertilisation (g m$^{-2}$ a$^{-1}$)</th>
<th>Trap hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>Gc</td>
<td>Tr</td>
<td>35</td>
<td>56</td>
<td>Df &amp; La</td>
</tr>
<tr>
<td>57</td>
<td>Gi</td>
<td>Tr</td>
<td>35</td>
<td>56</td>
<td>Df &amp; La</td>
</tr>
<tr>
<td>283</td>
<td>Gc</td>
<td>Lp &amp; Tr</td>
<td>60</td>
<td>14</td>
<td>Ob &amp; Tp</td>
</tr>
<tr>
<td>284</td>
<td>Gc</td>
<td>Lp &amp; Tr</td>
<td>60</td>
<td>14</td>
<td>Ob &amp; Tp</td>
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<td>Gi</td>
<td>Lp &amp; Tr</td>
<td>60</td>
<td>56</td>
<td>Pl &amp; Zm</td>
</tr>
<tr>
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<td>Gi</td>
<td>Lp &amp; Tr</td>
<td>60</td>
<td>56</td>
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<td>Tr</td>
<td>35</td>
<td>56</td>
<td>Df &amp; La</td>
</tr>
</tbody>
</table>
3.3.3 PLANT MATERIAL

*T. repens* L. cv. Milkanova (Dansk Planteforaedling, Research Division, Store Heddinge, Denmark), an agronomically important legume that is common in temperate grassland ecosystems world wide, was used as host plant for the experiment.

Clonal material was produced from uniform single node cuttings of different genets of *T. repens*. Leaf nodes were surface disinfected and pre-cultured on cellulose filter paper on top of 1 x Hoagland (Hoagland & Arnon, 1938) nutrition solution agar (0.7%, w/v). After ten days micropropagates were transplanted to fine ground Perlite, and after additional 14 days the nine genets (A, B, F-L) with the most vigorous and uniform plantlets were selected for the current inoculation experiment.

3.3.4 EXPERIMENTAL SET UP AND GROWTH CONDITIONS

The inoculation experiment used nine replicate one-litre pots (14 x 10 cm) for each of the 14 AMF inocula. Replicate pots were planted each with a different micropropagate of the nine *T. repens* genets, resulting in an orthogonal design with each plant genet / AMF isolate combination represented once (126 pots). For control, six additional sets of pots with the nine plant genets were used (54 pots). Inocula were thoroughly mixed into AMF-free potting substrate (composition as described above) in a volumetric ratio of 2:5. Inocula consisted of dried pot culture material that contained spores, colonised root fragments, and extraradical hyphae. Control pots received a mock-inoculum treatment which was AMF-free, but otherwise identical to the inoculated pots (NM-control). Additional NM-control pots, also replicated nine times, were established for five different P-fertilisation treatments. These pots received cumulated amounts of 50, 100, 150, 200 and 250 mg ready available phosphorus (KH$_2$PO$_4$) per kilogramme of dry potting substrate in eight weekly applications (50 ml) from the third week onwards. Substrate surfaces of all pots were covered with a layer of coarse silica sand to prevent formation of adventive roots from leaf nodes of stolons which could have given plants unequal access to soil nutrients.

To balance the non-AMF microbial community qualitatively (Koide & Li, 1989), 50 ml of a suspension of a filtrate prepared from the inocula were applied to each pot. For determining the proportion of biologically fixed N (%bfN$_2$), 10 g Na$_{15}$NO$_3$ (10% enriched) and 5 g Ca($_{15}$NO$_3$)$_2$ (5% enriched) were added as soil $^{15}$N tracer to the same suspension. *L. perenne* cv. Bastion (perennial ryegrass) seedlings were grown as reference plants in two additional pots that received the same amount of $^{15}$N (McAuliffe *et al.*, 1958). To promote nodulation of *T. repens* plants, dense plate-washes of *Rhizobium leguminosarum* bv. *trifolii* (strain RBL 5020, Leiden, The Netherlands) were added.

Two identical climate chambers (Conviron, PGV-36, Winnipeg, Manitoba, Canada) were required to carry all pots. Portions of four and five pots of each AMF isolate or P-fertilisation treatment were randomly chosen and assigned to one of the climate chambers, in which they were positioned randomly. Growth conditions consisted of a light phase of 16 h
(450-550 µmol m⁻² s⁻¹ PPFR at plant height), including dawn phases of 30 min twice a day, a
day/night temperature profile of 23/15°C at plant height and 70-80% relative air humidity. Cool
white fluorescent lamps and incandescent bulbs (Osram Sylvania Inc, St. Marys, PA, USA)
provided the high photosynthetic active irradiance. Automatic drip-irrigation with deionised
water from a tensiometer-type system (Tropf Blumat, Weninger GmbH & Co. KG, Telfs,
Austria) maintained substrate humidity at about 40%, or 50-60% of its water holding capacity,
preventing any leaching from pots. Twenty-four, 32 and 43 days after transplantation 50 ml of
0.1 x Hoagland nutrient solution (Hoagland & Arnon, 1938), lacking P, were applied to each
pot. On day 43 an additional 50 ml of a dense suspension of rhizobia were added to pots.

3.3.5 PLANT GROWTH MEASUREMENTS

During the course of the experiment non-destructive measurements of the following parameters
were made weekly: leaf numbers and whole plant leaf area development between the 12th and
47th day after inoculation, presence of stolons between the 26th and 47th day, and number of
inflorescences with more than half the flowers open between the 40th and 73rd day. To facilitate
recording of leaf and inflorescence numbers, white vanish was used to label petioles and stalks
that were already counted. Whole plant leaf area was estimated by measuring the length of
the midrib of the middle leaflet of each individual trifoliate leaf and applying the equation,
area = a*lengthᵇ, using plant genet specific coefficients which were determined in a separate
experiment (data not shown).

Seventy-three days after inoculation, plants of T. repens were destructively harvested.
Shoots were separated into leaves, petioles, inflorescences (flower heads), stalks and stolons,
while simultaneously counting leaf and inflorescence numbers, and measuring the longest and
total stolon length. Whole plant leaf area was determined by passing all leaf laminae through a
LI-COR 3000A leaf area meter (Li-Cor Inc., Lincoln, NE, USA). Fresh weights and dry weights
of all harvested plant fractions were recorded, the latter after drying to constant weight for at
least 48 h at 65°C. From each washed root system, a random sub-sample (≤ 1 g) was preserved
in 70% ethanol for root length determination using the grid line intersect method (Tennant,
1975) adapted for computer scanner-assisted estimation. Length estimates for the whole root
systems were made based on dry weights of the sub-samples and the whole root systems. The
dry weight of whole root systems was corrected for the two sub-samples, and root/shoot dry
weight ratio, specific leaf area and specific root length were calculated using the dry weight of
the respective plant fractions.

3.3.6 FUNGAL GROWTH MEASUREMENTS

Root colonisation by AMF characteristic fungal structures were determined in root fragments
(~ 1.5 cm in length) that were preserved in 10% potassium hydroxide of a random sub-sample
(≤ 0.3 g) taken from each root system; and hyphal length density in the potting substrate was
calculated for each pot. In addition, 52 days after inoculation (i.e. about three weeks prior to
destructive harvest) intraradical mycorrhizal colonisation was assed in roots of plant genets J
and K, which were sampled from one soil core (Ø 1 cm). These root colonisation measurements provided an estimate of the stage of colonisation of all 14 AMF inocula. At harvest, the root subsamples in which colonisation levels were determined were randomly taken from the middle zone of root systems. Root fragments were cleared and stained, using a modified method of Philips and Hayman (1970) in which lacto-glycerol was substituted for lacto-phenol and methyl blue was used in addition to trypan blue. Proportions (%) of root length colonised by the different AMF and non-AMF structures were determined by enumerating 100 intersections of at least 20 roots per sample, using the magnified intersections method at 200 x magnification (McGonigle et al., 1990). Providing a superior estimate for intraradical fungal biomass, colonisation intensity (I) was recorded following Plenchette and Morel (1996), except that only four instead of five intensity classes (1-25, 25-50, 50-75, 75-100% of root cortex colonised) were scored, using the formula \[ I = \frac{(25x + 50y + 75z + 100t)}{(x + y + z + t)}, \]
where x, y, z, t are the number of intersects in each class.

Density of extraradical hyphal length was estimated for duplicate samples of 5 g of fresh mixed substrate from three cores (Ø 1 cm), taken in-between plant and pot edge. A modified membrane filter technique that applied the formula of Newman (1966) was used (Jakobsen et al., 1992; Sylvia, 1992). After an aqueous extraction, intersections of hyphal fragments were enumerated on 0.45 µm gridded nitrocellulose filters of 25 mm diameter (HAWG Millipore, Bedford, Massachusetts, USA), while calculated length estimates were corrected for the dilution of suspensions and water contents of each individual sample of potting substrate.

The following derived measures were calculated: Root Length Colonised by AMF (RLC, product of the proportion of root length colonised by hyphae and root length) mycorrhizal benefit (MB) based on total plant dry weight and leaf number according to Gange and Ayres (1999) \[ MB = (M / mean(NM) - 1) \times 100 \], and mycorrhizal dependency (MD) based on total plant dry weight according to Plenchette et al. (1983) \[ MD = (mean(M) / mean(NM) - 1) \times 100 \].

### 3.3.7 Nutrient concentrations in plant tissue

Chemical analyses of P and N were carried out on sub-samples of the ground leaves (without petioles) and roots. These plant fractions were chosen, because they represent the physiologically most active organs.

For measuring P-concentrations, approximately 500 mg of dry powder (40°C) was incinerated at 500°C for 6 h, dissolved in 10% HCl, filtered (0.5 µm) and analysed by the method of Murphy and Riley (1962). Dilutions to the range required, were measured in a continuous flow Skalar apparatus (Skalar Analytic GmbH, Erkelenz, Germany) at 880 nm, where the absorbance by the ascorbic acid-reduced antimony-phospho-molybdate complex is maximal.

The ¹⁵N isotope dilution method (McAuliffe et al., 1958) was used to determine partitioning of N in T. repens between symbiotic N₂ fixation and uptake from the potting substrate. Total N and percentage ¹⁵N were determined in 2 ± 0.1 mg dry powder (40°C) of
T. repens (and L. perenne as reference plant) at the Stable Isotope Facility of University of California, Davis, using a continuous flow Isotope Ratio Mass Spectrometer (cf-IRMS, Europa Integra, PDZ-Europa Scientific; Sandbach, UK). Atom percent \(^{15}\)N excess (i.e. \(^{15}\)N enrichment) was calculated and applied to the formula of McAuliffe et al. (1958) to estimate %%bN\(_2\). The complement to 100% represents the proportion of N that was taken up from the potting substrate.

3.3.8 Statistical analyses

The experimental design was cross-factorial, with the treatments AMF isolate (14 levels), P-fertilisation (six levels, only for NM controls), and genet of T. repens (nine levels). Repetition was partial, with each AMF isolate (for inoculation treatments) or P-fertilisation level (for NM controls) being replicated by the nine genets of T. repens. Accordingly, genets of T. repens were represented only once in combination with a particular AMF isolate (for inoculation treatments) or only once in combination with a particular P-fertilisation level (for NM controls; further details are described above under EXPERIMENTAL SET UP). This experimental design provided control over the variability within AMF inocula and among genets of T. repens. It allowed to detect effects of individual AMF isolates and genets of T. repens and the overall interaction, but not the directions of AMF isolate x plant genet interactions. In addition, the effect of the factors CO\(_2\), N, field host history, AMF species identity and the technical factor climate chamber (each with two levels) were analysed.

Analyses were made using linear mixed ANOVA models for harvest data, or repeated measure-mixed ANOVA (ANOVAR) models for non-destructive measurements over time (SPSS v.11.0 for MacOSX). AMF isolate was treated as random factor, since single spore isolates were randomly selected from two pools of isolates available for each CO\(_2\) history. All other experimental factors were defined as fixed factors, since they had non-exchangeable and predetermined levels. CO\(_2\), N, field host and AMF species identity were used nested within AMF isolates. NM-control treatments were excluded in ANOVA, because we were interested in effects caused by different AMF isolates and not by colonisation status of plants.

Statistical models that included factor interactions were simplified by stepwise backward elimination of least non-significant interaction terms, using maximum likelihood (ML) variance estimation and Schwarz’s Bayesian Information Criterion (BIC) to control for increase in model goodness. Final significances of main effects and retained interaction terms were determined via restricted maximum likelihood (REML) variance estimation.

Effects of AMF species identity and sampling time on root colonisation parameters in genets J and K of T. repens were tested using two-way ANOVA. Likewise, P-fertilisation and plant genet effects on plant parameters of NM controls were analysed by two-way ANOVAR and ANOVA.

In cases where data needed to be transformed to meet assumptions for ANOVA the guidelines in Zar (1999) were used. When necessary, weights and concentrations were
Ln (x+1), counts were $\sqrt{x+3/8}$ and percentage ratios arcsin$\sqrt{x}$-transformed. The $\alpha = 0.05$ level of significance was kept for all statistical tests and data were presented as means and standard errors of the mean.

For multiple pairwise comparisons of means of factor levels (i.e. AMF isolate and P-fertilisation effects) Tukey-Kramer’s HSD (honestly significant difference) test was used, as implemented in JMP v.5, after confirming that the investigated effect was significant in the ANOVA. Even pairwise comparison of levels of the factor AMF isolate, which was initially defined as random factor for analyses on the effects of isolate histories, were performed to highlight inter-isolate variability which is of special biological interest. Pearson pairwise correlation coefficients were calculated and their significance determined in two-tailed t-tests as indicators of the direction of relationship between measured plant and fungal parameters.

For analyses on individual fungal isolate-plant genet associations Friedman’s tests were performed, since our experimental design was orthogonal with the plant genet-AMF isolate combinations replicated once. Such rank sum tests were used for testing for different performance of genets of T. repens upon inoculation with AMF isolates (plant parameters) and different performance of AMF isolates in association with genets of T. repens (fungal parameters).

3.3.9 Preferences among AMF isolates and plant genets

For illustrating the AMF-isolate x plant genet interactions, the Finlay-Wilkinson (F-W) plotting procedure was adopted (Finlay & Wilkinson, 1963). These plots graphically show the degree of preference and the symbiotic outcome relative to the means of AMF isolates and T. repens genets. The F-W plotting procedure is commonly used in plant breeding to investigate relative phenotypic stability and productivity of collections of plant varieties under different environmental conditions (Finlay & Wilkinson, 1963; Brancourt-Hulmel et al., 2003). Here, the mean of a dependant variable (measurement) over all AMF isolates per plant genet is used to estimate plant genotypic variation. This is achieved by first calculating for each AMF isolate an orthogonal regression based on single parameter values and the mean value over all AMF isolates per plant genet. In the F-W plot a single AMF isolate is characterized by the regression coefficient of this fitted line and by the mean parameter value across plant genets. Thus, regression coefficients greater or smaller than one indicate that there is preference of AMF isolates for plant genets, while coefficients of exactly one mean that AMF isolates have equal symbiotic performance on all host plant genets (i.e. they are generalists). With increasing values on the abscissa the symbiotic outcome is more positive (i.e. mutualistic), indicated by e.g. larger host plants or improved nutrient uptake.

Principle components (PC) calculated on the covariances were derived from a set of potentially interdependent plant and fungal parameters, characterising interactions of fungal isolates and plant genets. Selected variables that were significantly affected by the combination of AMF isolate and plant genet identity were standardised ($s = 0$, $sd = 1$) prior to analysis to neutralise the heterogeneity among measures and to eliminate effects of extreme values.
3.4 RESULTS

Identity of genets of *T. repens* had most significant effects on plant and fungal growth patterns, which is well established from many previous studies (e.g. Caradus et al., 1993; Eason et al., 2001 and references therein). Therefore, we present and discuss such effects in general only if they interacted with other factors investigated here.

3.4.1 EFFECTS OF MYCORRHIZAL INOCULATION AND FUNGAL SPECIES ON DEVELOPMENT OF *T. REPENS*

Mycorrhizal inoculation retarded the growth of *T. repens*, which was indicated by fewer leaves, smaller leaf area, less inflorescences and delayed stolon initiation of plants (data not shown). However, genets of *T. repens* differed in their growth response to mycorrhization, as was evident from several significant interactions between AMF inoculation and plant genet (data not shown). Likewise, leaf formation (leaf number and area) of plant genets depended on the AMF species to which fungal isolates belonged, which was apparent from significant interactions between AMF species identity and plant genet (data not shown). In general, plants colonised by isolates assigned to *G. intraradices* (Gi isolates) were more vigorous with increased numbers of leaves, inflorescences and stolons compared to plants colonised by isolates of *G. claroideum* (Gc isolates), at least during the first six weeks after inoculation.

3.4.2 DYNAMICS OF MYCORRHIZAL ROOT COLONISATION

Fifty-two days after inoculation and three weeks prior to harvest, proportions of root length colonised by hyphae, arbuscules, vesicles and root colonisation intensity of two genets of *T. repens* (J and K) were assessed. These histological parameters should provide insight into the developmental stage of root colonisation and potential dynamics, when compared to the colonisation levels at harvest. Only root colonisation intensity changed significantly during the final three weeks of the experiment, with a decrease observed in all colonised plants irrespective of AMF species identity ($F_{1, 51} = 6.60$, $P = 0.013$; data not shown). Although proportions of roots colonised by hyphae and vesicles remained unchanged, they were generally lower in the plants colonised by Gc isolates compared to those colonised by Gi isolates ($F_{1, 52} = 8.19$, $P = 0.006$ and $F_{1, 52} = 55.06$, $P \leq 0.001$; data not shown).

None of the NM control plants was colonised by AMF at harvest, 73 days after inoculation. Furthermore, different spore numbers present among inocula did not influence the outcome of the experiment as indicated by statistical analysis with spore number treated as covariate (ANCOVA; data not shown).

3.4.3 EFFECT OF PHOSPHORUS NUTRITION ON GROWTH OF *T. REPENS*

Application of phosphorus to NM control plants in five different amounts neither affected whole plant leaf number and leaf area, nor number of inflorescence or stolon formation (data not shown), although mean leaf and root P-concentrations of plants were significantly increased compared to the mock-inoculated controls which received no P treatment ($F_{5, 52} = 45.40$, 45.40,
P ≤ 0.001; F_{5, 52} = 93.19, P ≤ 0.001; data not shown). P-concentration of fertilized plants was also higher than in plants inoculated with AMF (data not shown).

$N_2$-fixation responded positively to P-fertilisation; %bfN$_2$ in leaves and roots of NM plants was increased (leaves: F$_{5, 52} = 4.70$, P = 0.002; roots: F$_{5, 52} = 5.33$, P ≤ 0.001; data not shown), although levels of %bfN$_2$ were significantly smaller than in mycorrhizal plants (F$_{1, 19} = 48.34$, P ≤ 0.001; F$_{1, 19} = 19.47$, P ≤ 0.001; data not shown).

Among the other morphometric plant parameters (total plant dry weight, whole plant leaf area, inflorescence number, measures of stolon development), root length was the only parameter, which was influenced by P application (F$_{5, 51} = 2.59$, P = 0.041). Values of root length were highest at a cumulated application of 200 mg P per kg potting substrate, which corresponded to the level where also the highest P-concentration in tissues was measured (data not shown). Interestingly, P-concentrations in tissues of mock-inoculated plants did not differ from the concentrations of the AMF inoculated plants (range between 1.64 ± 0.06 and 2.36 ± 0.26 mg g$^{-1}$ for leaves and between 1.41 ± 0.08 and 1.85 ± 0.17 mg g$^{-1}$ for roots).

3.4.4 Correlations in other selected parameters

Root P-concentration was negatively (P < 0.01) correlated with root length ($r^2 = -0.502$), extraradical hyphal length ($r^2 = -0.292$) and RLC ($r^2 = -0.391$). Similarly, root N-concentration was negatively (P < 0.01) correlated with root length ($r^2 = -0.569$), extraradical hyphal length ($r^2 = -0.296$) and RLC ($r^2 = -0.438$). Proportions of root length colonised by hyphae were negatively (P < 0.01) correlated with %bfN$_2$ of leaves ($r^2 = -0.264$) and roots ($r^2 = -0.564$).

When mycorrhizal benefit according to Gange and Ayres (1999) was calculated based on total plant dry weight or leaf number, including all mycorrhizal plants (n = 126), no or only weak indication for an optimal value of root length colonised by arbuscules was found. However, values of hyphal colonisation seemed to be at an optimum at 50-60 % of root length colonised (data not shown), and were reached by some of the isolates of the *G. intraradices*-species complex (Figure 3.3a & 3.4e).

3.4.5 Effect of CO$_2$ history of fungal isolates

Growth conditions of AMF strains under elevated or ambient CO$_2$ for eight years prior to isolation did influence the effects of AMF on the nitrogen concentrations in leaves of experimental *T. repens* hosts (Figure 3.1 & 3.2). Total foliar N-concentration was increased in plants inoculated with elevCO$_2$ isolates, compared to plants inoculated with ambCO$_2$ isolates. This increase was accompanied by a higher proportion of N derived from the potting substrate on the expense of the fraction obtained by N$_2$ fixation (%bfN$_2$), although in absolute terms the amount of N from biological N$_2$ fixation was also higher. Patterns of %bfN$_2$ observed in leaves were similar also in roots (pCO$_2$ history: F$_{1, 11.96} = 5.93$, P = 0.032; AMF isolate: Z = 2.26, P = 0.024; data not shown).
3.4.6 Effect of Nitrogen and Host Plant History of Fungal Isolates

Although fungal isolates were selected for studying the effects of past CO$_2$ fumigation on the experimental mycorrhizal symbioses, information on N-fertilisation and plant cover present in the field plots of origin allowed us to test for selective effects of past N-fertilisation regime and host plants.

The eight single spore isolates from field plots fertilised with the high N level (56 g m$^{-2}$ a$^{-1}$) tended to produce more hyphae (8.57 ± 0.90 vs 5.84 ± 0.44 m g$^{-1}$), and their hosts had higher foliar P-concentrations (2.07 ± 0.07 vs 1.91 ± 0.07 mg g$^{-1}$) than the six isolates from plots with the low N-fertilisation level (14 g m$^{-2}$ a$^{-1}$). Effects on extraradical hyphal length density (F$_{1,11.58}$ = 3.39, P = 0.091) and foliar P-concentration (F$_{1,8.84}$ = 3.53, P = 0.094) were marginally significant.

The four single spore isolates obtained from monoculture plots of *T. repens* (TABLE 3.1) produced significantly less intraradical hyphae and their nodulated host plants fixed significantly

**FIGURE 3.2** Percentage of total foliar nitrogen that was biologically fixed by nodule symbioses (%bfN$_2$) in associations of *Trifolium repens* (white clover) and 14 single spore isolates of arbuscular mycorrhizal fungi (AMF). Fungal isolates had been growing under ambient atmospheric CO$_2$ partial pressure (pCO$_2$) and Free-Air CO$_2$ Enrichment (60 Pa pCO$_2$) for eight years prior to isolation. Bars represent means with standard errors of the mean of non-transformed raw data (n = 9). F and Wald Z (for AMF isolate effect) values were calculated from mixed ANOVA with AMF isolate nested within pCO$_2$ history on angular square root transformed data for pCO$_2$ history (F$_{1,11.85}$ = 7.24, P = 0.020) and AMF isolate (Z = 2.05, P ≤ 0.001) effects. Species identities of fungal isolates are given in brackets after isolate numbers (Gc, *Glomus claroideum*; Gi, *G. intraradices*). Different letters above bars indicate significant differences according to Tukey-Kramer’s HSD test at P ≤ 0.05, performed over isolates per pCO$_2$ history.
FIGURE 3.3 Effect of past host plants of arbuscular mycorrhizal fungi (AMF) on root colonisation parameters and percentage of total root nitrogen (N) that was biologically fixed (%bfN$_2$) in nodule symbioses. (a) Proportions of root length colonised by the fungal structures, hyphae (grey bar segments) and arbuscules (black bar segments), and (b) root %bfN$_2$ for associations of 14 fungal single spore isolates with Trifolium repens (white clover) ten weeks after inoculation / transplantation. Isolates had been grown in monocultures of T. repens and bi-species cultures of T. repens and Lolium perenne (perennial ryegrass) for eight years, prior to isolation. Bars represent means with standard errors of the mean of non-transformed raw data (n = 9). F and Wald Z (for AMF isolate effect) values were calculated from mixed ANOVA with AMF isolate nested within plant cover history on angular square root transformed data for plant cover history (for hyphae: F$_{1, 11.94}$ = 14.52, P = 0.003; for root %bfN$_2$: F$_{1, 12.05}$ = 5.54, P = 0.036) and AMF isolate (for hyphae: Z = 2.20, P = 0.028; for arbuscules: Z = 2.00, P = 0.046; for %bfN$_2$: Z = 2.20, P = 0.028) effects. The mock-inoculation treatment (NM) was excluded from the statistical analysis. Species identities of fungal isolates are given in brackets after isolate numbers (Gc, Glomus claroideum; Gi, G. intraradices). Different letters above bars indicate significant differences according to Tukey-Kramer’s HSD test at P ≤ 0.05, performed over isolates per plant cover history for hyphae and root %bfN$_2$, and over all isolates for arbuscules.

more N$_2$ (FIGURE 3.3), compared to the ten isolates from plots with a mixture of T. repens and L. perenne. Similar to the effects on hyphal root colonisation, host plant history and AMF isolate identity affected the proportions of root length colonised by vesicles (field host history: F$_{1, 11.99}$ = 8.15, P = 0.015, AMF isolate: Z = 2.20, P = 0.018) and root colonisation intensity (field host history: F$_{1, 11.75}$ = 17.88, P = 0.001; AMF isolate: Z = 1.79, P = 0.073). Also, host plant history of the isolates affected root %bfN$_2$ and leaf %bfN$_2$ alike, however, with only marginally significant effects on leaf bfN$_2$ (field host history: F$_{1, 11.81}$ = 3.50, P = 0.087; AMF isolate: Z = 2.05, P = 0.041). Interaction of CO$_2$ and host plant history, which both affected %bfN$_2$, could not be tested, due to loss of degree of freedom in ANOVA.

3.4.7 EFFECTS OF AMF SPECIES IDENTITY AND VARIATION AMONG ISOLATES WITHIN SPECIES

The effects of species identity of single spore isolates on growth parameters of T. repens, initially recorded non-destructively, persisted till harvest. Plants inoculated by isolates of G. claroideum (Gc) were smaller with significantly smaller whole plant leaf area, fewer inflorescences and stolons of less dry weight, compared to plants inoculated with isolates of the G. intraradices-species complex (Gi, FIGURE 3.4a-d). Similar effects of AMF species identity were observed for shoot (F$_{19.09}$ = 12.18, P =0.007) and root (F$_{1, 10.41}$ = 12.17, P = 0.005) dry weights (data not shown), and for leaf number
FIGURE 3.4 Effect of species identity of single spore isolates of arbuscular mycorrhizal fungi (AMF) on seven plant and fungal morphometric parameters ten weeks after inoculation / transplantation. Bars represent means with standard errors of the mean of non-transformed raw data for *Glomus claroideum* (five isolates) and *G. intraradices* (nine isolates), grown in association with *Trifolium repens* (white clover). F values were calculated from mixed ANOVA for the effect of AMF species identity: (a) Whole plant dry weight (g), $F_{1, 9.20} = 13.00$ (P = 0.005); (b) whole plant leaf area (cm$^2$), $F_{1, 10.42} = 5.72$ (P = 0.037); (c) dry weight per stolon (g), $F_{1, 9.97} = 6.29$ (P = 0.031); (d) inflorescence number, $F_{1, 1032.40} = 2.29$ (P = 0.131); (e) proportions of root length colonised by arbuscules (%), $F_{1, 11.40} = 6.13$ (P = 0.030), and by vesicles (%), $F_{1, 11.99} = 14.29$ (P = 0.003); and (f) extraradical hyphal length (m g$^{-1}$), $F_{1, 10.94} = 13.29$ (P = 0.004). Significant differences are indicated (ns, P > 0.05; *, P ≤ 0.05; **, P ≤ 0.01).

(F$_{1, 10.18} = 6.68$, P = 0.027; data not shown). Plant leaf area as shown in FIGURE 3.4b reflects more closely the potential for photosynthetic carbohydrate (C) acquisition and thus is an indicator for prospective plant growth. Proportions of root length colonised by arbuscules were significantly higher in plants colonised by Gc, compared to plants colonised by Gi isolates, whereas proportions of roots colonised by vesicles and extraradical hyphal lengths were significantly lower (FIGURE 3.4e, f). Furthermore, patterns of RLC values (AMF species identity: $F_{1, 11.91} = 6.20$, P = 0.029; AMF isolate: $Z = 2.13$, P = 0.033) were similarly related to species identity and matched with those of roots colonised by vesicles. Likewise, patterns of root length colonised by hyphae ($Z = 2.31$, P = 0.021) and root colonisation intensity ($Z = 2.17$, P = 0.030) were similar.

Single spore isolates of the same species (intraspecific) differed in their effects on initial leaf area development (ANOVAR, AMF isolate: $F_{12, 212.99} = 1.96$, P = 0.029, data not shown) and on the proportion of root length colonised by hyphae and arbuscules (FIGURE 3.3a). These effects, however, were less pronounced than effects explained by AMF species affiliation (FIGURE 3.4a and not shown data). Furthermore, isolates differed in their effects on %bfN$_2$ in leaves (FIGURE 3.2) and roots (FIGURE 3.3b).

All other parameters of *T. repens* plants including specific leaf area, root length, specific root length, root : shoot ratio, leaf and root P-concentrations, and root N-concentration were not affected by fungal species identity, isolate identity or the treatment histories (CO$_2$, N, field host) of AMF isolates (data not shown).
3.4.8 Effect of fungal isolate / plant genet combination

Mycorrhizal symbioses were influenced by particular combinations of single spore isolates of AMF and genets of *T. repens*. Thereby, several host plant parameters were affected, including total dry weight ($X_{13} = 26.27, P = 0.016$), root length ($X_{13} = 38.41, P \leq 0.001$), specific root length ($X_{13} = 33.27, P = 0.002$), root : shoot ratio ($X_{13} = 28.40, P = 0.008$), whole plant leaf number ($X_{13} = 26.948, P = 0.015$), whole plant leaf area ($X_{13} = 26.90, P = 0.013$), leaf %bfN$_2$ ($X_{13} = P \leq 0.001$), root %bfN$_2$ ($X_{13} = 76.48, P \leq 0.001$), and total stolon length ($X_{13} = 25.01, P = 0.023$). Similarly, particular genets of *T. repens* affected colonisation patterns of particular AMF single spore isolates, including proportions of root length colonised by vesicles ($X_{8} = 17.47, P = 0.026$), and RLC ($X_{8} = 29.83, P \leq 0.001$).

To illustrate interactions among particular AMF isolate/plant genet combinations we adopted the F-W plot (Figure 3.5) as described in more detail under Materials and Methods. Principle components (PC) were derived from total dry weight, root length, total leaf number, leaf %bfN$_2$, total stolon length, proportion of root length colonised by vesicles, and RLC. Principle component 1 best explained root length ($r^2 = 0.887, P \leq 0.001$), PC2 best explained leaf %bfN$_2$ ($r^2 = 0.584, P \leq 0.001$), and PC3 best explained proportions of root length colonised by vesicles ($r^2 = 0.545, P \leq 0.001$). All three PC, used in the F-W plots, account together for 87.74% of total variance.

The means of regression coefficients were significantly larger than one in all three F-W plots (Wilcoxon signed rank test: $P \leq 0.031$), which indicates overall preference of AMF isolates for particular genets of *T. repens*. Individual isolates differed in relative physiological preference (indicated on the ordinate) and relative symbiotic benefit (indicated on the abscissa), but there was no pattern related to AMF morpho-species identity or environmental histories of AMF isolates.

3.5 Discussion

With this pot experiment we show that Free-Air CO$_2$ Enrichment (FACE) for eight years, applied in a permanent, agricultural model-grassland, has selected AMF populations with modified capabilities in symbiosis.

3.5.1 Effects of fungal isolate history

3.5.1.1 CO$_2$ Fumigation effect

Nodulated *T. repens* plants inoculated with single spore isolates of AMF previously grown under FACE conditions (elevCO$_2$ isolates) had significantly higher foliar N-concentrations than plants associated with isolates grown under ambient conditions (ambCO$_2$ isolates). They took up nearly twice the amount of total foliar N-concentration from the potting substrate and did not acquire it via increased biological N$_2$ fixation. AMF are known to contribute to plant N-nutrition (Tobar *et al.*, 1994; Hawkins *et al.*, 2000; Azcon *et al.*, 2001), including the uptake of nitrate via extraradical hyphal structures (Bago *et al.*, 2004). Since improved N-nutrition translates into
FIGURE 3.5 Finlay-Wilkinson plots visualizing the fungal isolate x plant genet interaction of fourteen single spore isolates of arbuscular mycorrhizal fungi (AMF) in association with nine genets of *Trifolium repens* (white clover, for isolate numbers see TABLE 3.1). Plots are derived from the principle components (PC) of a factor analysis on covariances of total dry weight, root length, total leaf number, proportion of total leaf nitrogen that was biologically fixed, total stolon length, proportion of root length colonised by vesicles, and root length colonised by AMF, determined ten weeks after inoculation / transplantation. The ordinate shows coefficients from a regression of single PC-values for each AMF isolate on mean PC-values over all AMF isolates per plant genet. These values estimate the relative degree of preference of a particular AMF isolate for the nine genets of *T. repens*, arranged according to the size of the principle component. The horizontal, dashed line separates AMF isolates with preferences for plant genets with higher than mean PC-values (regression coefficient > 1) and those with preferences for plant genets with lower than mean PC-values (regression coefficient < 1). A regression coefficient of one would indicate no preference of a particular AMF isolate for the host plant genets. The vertical, dashed line shows the mean of all AMF isolates. Only values with an orthogonal correlation $r^2 \geq 0.5$ are shown; therefore, certain isolates are absent in the plots. Dots represent isolates of the *Glomus intraradices*-species complex and squares isolates of *G. claroideum*. The means of regression coefficients shown are significantly larger than one (one-tailed Wilcoxon signed rank test: PC1, $z = 18.00$, $P = 0.004$, df = 7; PC2, $z = 7.50$, $P = 0.031$, df = 4; PC3, $z = 7.50$, $P = 0.031$, df = 4). PC1-PC3 explain 87.74% of total variance (PC1: 59.57%, PC2: 14.61%, PC3: 13.57%).

fitness benefits of both mycorrhizal partners, N-uptake capabilities may be particularly prone to environmental selection. The formation of the nodule and mycorrhizal symbioses is controlled by a hormonal autoregulative system (Catford *et al.*, 2003) and possibly by C-demands of both symbionts (Bethlenfalvay *et al.*, 1985 and references therein; Murphy, 1986; but see also Paul & Kucey, 1981). In addition, the legume N-nutritional status at the whole plant level is known to feedback on biological N$_2$ fixation of nodules (Hartwig *et al.*, 1994; Hartwig, 1998; Schulze, 2004). Considering this close interdependence of AMF, rhizobia, and host, our findings may be explained by at least three different hypotheses. First, past conditions of elevated atmospheric pCO$_2$ in the current FACE experiment may have been conditions of N-deficiency for plants (Zanetti *et al.*, 1997; Suter *et al.*, 2002), selecting for fungal strains, more efficient in complementing host plant N-nutrition. The reduced contribution of biological N$_2$ fixation to total N-nutrition could have been due to a feedback regulation mechanism in which the plant N status regulates nodule symbiotic activity (Hartwig, 1998; Schulze, 2004). Second, higher amounts of C available in plants grown under elevated pCO$_2$ may have favoured fungal strains that are more C-costly and are able to out-compete other strains. Higher C-costs of
elevCO$_2$ isolates could have negatively affected the energy demanding biological N$_2$ fixation in nodules, which may explain the reduced contribution of this N acquisition system to total foliar N-concentration. At the same time, higher fungal C-costs, retarding the ontogeny of $T$. repens plants, may have lead to increased total foliar N-concentration. Third, restricted C-availability in plants grown in nutrient rich agricultural soil under ambient pCO$_2$ could have selected fungal strains that are more competitive in C-acquisition from their actual hosts. Such C-competitive isolates may be anticipated to cause a reduction in biological N$_2$-fixation and a retardation of plant development, which would both be accompanied with the N-nutrition pattern found in the experimental plants. The second and third hypothesis, both would predict considerable smaller plant sizes, which were not found. This is why we prefer the first hypothesis suggesting that the elevCO$_2$ isolates increase the N-concentration, at least partly, through improved N-uptake or transfer capabilities. In fact, this assumption is conceivable because difference in total foliar N-concentration between the two groups of mycorrhizal plants was larger than the difference in N derived from the potting substrate (10% vs <1%).

Moreover, we did not find any evidence for a significantly increased fungal biomass in the elevCO$_2$, compared to the ambCO$_2$ isolates, as it was best measured by the colonisation intensity and RLC. This would have been an indication for potential regulation of the plant N-metabolism via C-limitation. However, regulation of biological N$_2$ fixation via C-limitation seemed unlikely, because N$_2$ fixation was not diminished in the smaller plants colonised by Gc isolates, although the AMF species effect might have been a confounding factor here (see below). Nevertheless, C-shortage not translated into significant reductions of plant biomass could have been partially responsible for a concentration effect (Jarrell & Beverly, 1981) that increased foliar N-concentration. This might be because total plant biomass tended to be smaller in the plants colonised by elevCO$_2$ isolates compared to those colonised by ambCO$_2$ isolates, based on an observed trend for smaller shoots.

Our conclusion that a history of growth under elevated pCO$_2$ selected fungal strains with enhanced capabilities to transfer N to $T$. repens hosts challenges other findings from field studies that suggest that AMF communities become less beneficial (in terms of plant growth promotion) upon mineral fertilisation and anthropogenic N-deposition (Johnson, 1993; Egerton-Warburton & Allen, 2000). Increased tissue N-concentrations can be expected to positively influence fitness of plants, and thus the observed services by the elevCO$_2$ isolates have to be considered beneficial.

Negative correlations between extraradical hyphal densities, root lengths and the N-nutritional parameters of $T$. repens suggests that plants colonised by elevCO$_2$ isolates did not improve plant tissue N-concentration via better soil exploitation. Since biological N$_2$ fixation in plants colonised by elevCO$_2$ isolates also accounted for less plant N, the elevCO$_2$ isolates obviously appear to be responsible for more efficient N-uptake or transfer. In fact, improved scavenging by increased investment into higher extraradical hyphal densities would not seem economic, because N is mobile and thus more or less equally distributed in soil.
None of the measured plant or fungal morphological parameters was significantly affected by the CO$_2$ history of AMF isolates. In particular, elevCO$_2$ isolates neither produced more biomass, nor otherwise depressed plant growth as initially expected from one of the complementary hypotheses. However, growth of *T. repens* could have been not responsive enough to mycorrhizal colonisation for revealing such an effect (van der Heijden, 2002), since *T. repens* is known to fully compensate the AMF’s C-demand via photosynthetic adjustment (Wright *et al.*, 1998). Alternatively, the duration of FACE could have been too short to act selectively. AMF are slow-growing and asexual and may possess a particularly high physiological plasticity due to their coenocytic organisation (Sanders, 2002), which are features that all may slow down selective changes.

### 3.5.1.2 N Fertilisation effect

Contrasting N-fertilisation regimes for eight years did not significantly affect any measured plant or fungal parameter, although the AMF isolates from high N-fertilisation plots produced (marginally significantly) more extraradical hyphae and tended to increase foliar P-concentrations. This trend for increased extraradical hyphal growth under higher N-supply may point either to N-limitation of fungal growth (Bago *et al.*, 2004) or increased P-requirements. The trend for an improved P-nutritional status of *T. repens* plants, colonised by fungal isolates that originated from high N-fertilisation plots, indicates that also the higher N : P ratio in N-fertilised soils might have selected for AMF strains that improve host mineral nutrition.

### 3.5.1.3 Host plant effect

An interesting finding rarely reported for individual AMF isolates was that previous association with particular host plants had lasting effects on root colonisation. Proportions of root lengths colonised by hyphae and vesicles, and colonisation intensity were lower in roots of *T. repens* colonised by isolates with a history of growth in monocultures of the same *T. repens* variety (cv. Milkanova), compared to isolates grown in mixed *T. repens/L. perenne* plots. Previous host plants of AMF isolates also affected biological N$_2$ fixation. Association with only one host variety over eight years resulted in an increased %bfN$_2$ of experimental plants. AMF appear to have been differently affected by either pure *T. repens* stands or mixture plots with *L. perenne*. N-limitation of *L. perenne* and thus amplified C-allocation to roots may have favoured fungal strains that more heavily colonise roots.

### 3.5.2 Effects of AMF species identity

Growth responses of mycorrhizal hosts can be explained by the relative importance of C-cost and nutritional benefit, which are known to depend on plant and fungal identity (Jakobsen & Rosendahl, 1990; Lerat *et al.*, 2003; Smith *et al.*, 2004). In this experiment AMF species identity explained differences in growth and development of *T. repens*. The plants colonised by Gc isolates were retarded in growth, compared to those colonised by Gi isolates. Moreover,
plants colonised by Gc isolates had a significantly smaller proportion of root length colonised by vesicles, a smaller RLC, and a lower extraradical hyphal density, but a higher proportion of root length colonised by arbuscules, compared to plants colonised by Gi isolates. These fungal features appear to be characteristic for the two AMF species and match with previous findings (e.g. Graham et al., 1996). Besides host growth, Gc isolates severely reduced other surrogates of host plant fitness, such as dry weight per stolon, number of leaf nodes (corresponding to sites for adventive roots and inflorescences) and number of inflorescences, when compared to Gi isolates.

Hart and Klironomos (2002) reported on the variation of plant growth responses to mycorrhizas at different levels of fungal, biological integration. They found that variation in host growth was of comparable size for AMF species and isolates, which originated from a single field site. By contrast, our findings suggest that taxonomic affiliation of AMF may in fact bear relevance for mycorrhizal symbioses. This seems not only true at the level of genera (Jakobsen et al., 1992; Hart & Reader, 2002), but also at the level of morpho-species and even individual strains. Intraspecific variability (i.e. variation among isolates) of effects on host plant morphometric and physiological parameters was consistently lower than the variation between species (data not shown). Also, variation in most root colonisation parameters was in general lower within species (as evident from the level of significance, Figure 3.3a and data not shown). This overriding effect of AMF species identity, compared to the effect of isolate identity found in this experiment, modifies the picture from the compilation by Hart and Klironomos (2002).

3.5.3 Effects of mycorrhizal inoculation and P-fertilisation on growth of T. repens

Plants inoculated with AMF consistently formed fewer leaves, smaller leaf area, fewer inflorescences and stolons later, compared to NM plants, which is consistent with an overall retardation of plant development upon AMF colonisation. This may suggest that AMF were in general costly for the T. repens hosts, at least over the course of the experiment and under the conditions used. At harvest, root colonisation appeared to be well-developed as was evident from a change over the last three weeks of the experiment in only the colonisation intensity which decreased, but not in the other root colonisation parameters (hyphae, arbuscules and vesicles), which would have indicated further maturation of root colonisation. This suggests that costs for maintenance of fungal biomass has played already a considerable role for the host plants.

P-fertilisation at any level applied to NM plants of T. repens neither accelerated development nor promoted size of plants, nor decreased root : shoot dry weight ratio and specific root length, although P-concentrations of leaves and roots were increased. This lack of symptoms of mineral nutrient deficiency and the lack of growth response to P-fertilisation clearly indicate that P was available for T. repens in sufficient quantity in our pot experiment. Stimulation of biological N\textsubscript{2} fixation by AMF inoculation was much stronger than stimulation by P-fertilisation in NM plants. Therefore, findings from NM control plants with P-addition
suggest that the P-nutritional function of AMF, which could have otherwise also been met by the plants themselves (Smith et al., 2004), was indirect and at best very low. Conditions of sufficient P-supply were also what the AMF have experienced in the field and may be an important precondition for the full expression of C-costs imposed by AMF (Peng et al., 1993; Graham et al., 1996). Moreover, this finding confirms that the choice of our potting substrate was adequate to address questions of potential mycorrhizal C-costs, because it precluded interference with a mycorrhizal P-nutritional benefit for plants.

3.5.4 PREFERENCES AMONG AMF ISOLATES AND GENETS OF T. REPENS

Individual AMF isolate / plant genet associations varied in most of the measured plant and fungal parameters as shown by significant Friedman’s tests. Moreover, F-W plots produced from principle components that simultaneously accounted for several plant and fungal parameters clearly indicate that performance of mycorrhizal associations depended on the particular combination of fungal isolate and plant genet. This finding suggests that physiological preferences among fungal isolates and plant genets exist. However, F-W plotting could not resolve the AMF species differences that were detected by ANOVA, probably because few Gc isolates showed linear correlation ($r^2 > 0.5$) between symbiotic performance and host plant genet in different associations. Despite this limitation, we consider the F-W plotting procedure potentially helpful also for screening of large collections of AMF isolates for the consistency of plant growth responses under different environmental conditions. Eason et al. (2001) have reported that different lines and varieties of T. repens responded quite differently when inoculated with a single AMF isolate. Our inoculation study using multiple single spore isolates and different plant genets points to potential intraspecific preferences or specificities among plant hosts and AMF strains, which may affect the morphology and physiology of mycorrhizas.

3.6 CONCLUSIONS

Knowledge about the impact of rising atmospheric pCO$_2$ on mycorrhizal symbioses is crucial to predict potential changes in ecosystems. We show that elevated atmospheric pCO$_2$ for only eight years in model grasslands has selected AMF strains that can improve N-nutrition of host plants. N-nutritional benefit from the mycorrhizal association was at least partly due to more N derived from the potting substrate but not only by stimulation of biological N$_2$ fixation in the nodule symbiosis. This finding may be ecologically relevant, because particularly non-legumes are known to experience increased N-growth limitation under elevated atmospheric pCO$_2$, which would apparently be alleviated by AMF strains more efficient in soil-plant nutrient transfer.

Intraspecific variability observed in G. claroideum and G. intraradices, and physiological preferences found among particular AMF strains and T. repens genets suggest that future inoculation experiments should, whenever possible, consider this level of intraspecific variation. Furthermore, our findings that previous host plants of AMF isolates had an effect
on root colonisation and N\textsubscript{2} fixation in the nodule symbiosis of experimental *T. repens* plants suggest that host plants too, may act selectively on AMF. This is the first experiment, conducted under controlled conditions, which investigated consequences for the mycorrhizal symbiosis of a potential shift in AMF composition due to altered environmental conditions.

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### 3.8 References


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4 EFFECTS OF TEN YEARS OF EXPOSURE TO ELEVATED ATMOSPHERIC pCO\textsubscript{2} ON A COMMUNITY AND POPULATION OF ARBUSCULAR MYCORRHIZAL FUNGI

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H. GAMPER, I. R. SANDERS, A. LEUCHTMANN (SUBMITTED) EFFECTS OF TEN YEARS OF EXPOSURE TO ELEVATED ATMOSPHERIC pCO\textsubscript{2} ON A COMMUNITY AND A POPULATION OF ARBUSCULAR MYCORRHIZAL FUNGI. MOLECULAR ECOLOGY

4.1 ABSTRACT
Rising atmospheric pCO\textsubscript{2} has the potential to change arbuscular mycorrhizal fungal (AMF) communities and populations, due to alterations in the nutrient balance of host plants. Hundred and sixty-eight root samples of *Trifolium repens* from six plots, half exposed to ambient and half exposed to elevated atmospheric pCO\textsubscript{2} for ten years, were sampled. The presence/absence of six AMF species was determined, using a nested PCR assay with specific PCR primers, targeted to the 28S nrDNA gene. Sixty-four DNA amplicons of *Glomus mosseae* were cloned and from these 29 different sequences were obtained, each of which were found more than once. Exposure to elevated pCO\textsubscript{2} did not affect the partial AMF community except for presence of some less frequent species. All six AMF species were remarkably abundant and evenly distributed over the whole field experiment, although the communities in the six experimental plots were (marginally) significantly structured spatially. Rapid adaptation of AMF populations to altered atmospheric pCO\textsubscript{2} may explain the lack of change in the community. However, an AMOVA on the sequence data set for *G. mosseae* showed that 13.8% of total molecular variance was explained by the pCO\textsubscript{2} history and that 8.1% was due to among plot variation. This shows that only 10 years of exposure to elevated pCO\textsubscript{2} can act as a selection pressure altering the genetic structure of an AMF population. A parsimony network, accounting for frequency and origin of sequence haplotypes, highlighted this CO\textsubscript{2} effect on the genetic population structure of *G. mosseae*. Given the changes in the molecular population structure of an AMF species, it is important to understand whether symbiotic efficiency is also altered.

4.2 INTRODUCTION
Rising atmospheric CO\textsubscript{2} partial pressure (pCO\textsubscript{2}) is one aspect of global environmental change that could affect below ground microbiota (Sadowsky & Schortemeyer, 1997) and in turn may trigger an ecosystem response to elevated pCO\textsubscript{2}. Of the soil microbiota the arbuscular
mycorrhizal fungi (AMF, Glomeromycota; Schüssler et al., 2001) are an obvious group that may be affected (and also mediate ecosystem responses) by this environmental change. AMF are obligate symbiotic soil fungi that colonise the roots of most plants and improve plant growth through their effects on plant nutrient acquisition (Smith & Read, 1997). They play a key role in both natural and agricultural ecosystems (Smith & Read, 1997) because of their multi-functionality in mycorrhizal symbioses (Newsham et al., 1995). AMF are expected being particularly affected by elevated atmospheric pCO$_2$ due to their complete dependence on plant carbohydrates; a resource whose availability is changed when plants are exposed to elevated pCO$_2$ (Suter et al., 2002).

Because AMF mediate the flow of nutrients between plants and soils their response to rising atmospheric pCO$_2$ has been studied (Diaz, 1996; Staddon & Fitter, 1998; Rillig & Allen, 1999; Fitter et al., 2000; Rillig et al., 2000; Johnson et al., 2003). These studies generally indicate an increased growth of AMF under elevated atmospheric pCO$_2$, consistent with expectations of increased C-allocation to AMF and a balance between resources and investments. Rillig et al. (2000) found increased C-allocation to intra- and extraradical AMF hyphae and the soil glycoprotein glomalin, derived from AMF under field conditions. Investments into AMF are one way for the plants to get access to soil nutrients that become more growth limiting under elevated pCO$_2$ (Treseder, 2004). The finding of preferential growth of extraradical hyphae of AMF which are involved in nutrient absorption, support this idea (e.g. Klironomos et al., 1998, Sanders et al., 1998). However, results from pot experiments of other studies also revealed inconsistencies in the response of AMF to CO$_2$ (Staddon & Fitter, 1998; Rillig & Allen, 1999). One of the reasons for the inconsistencies in these studies may be that the responses of AMF may be plant or AMF species specific (Klironomos et al., 1998); a subject that has received little attention. Given that plant growth is differentially affected by different AMF species and vice versa (Chapter 2; van der Heijden et al., 1998; Bever, 2002; Johnson et al., 2004), AMF-specific effects of elevated pCO$_2$ could be ecologically important. Changes in either the AMF community or in the AMF populations as a result of elevated pCO$_2$ is, however, not easy to address because of the difficulties of identification of AMF in plant roots (Clapp et al., 2002; Redecker et al., 2003).

If elevated pCO$_2$ alters resource availability for AMF, then it could act as a strong selection pressure on AMF. If this is the case, then it may also be possible to detect genetic changes in a population of one AMF, even after a few years exposure to elevated pCO$_2$. This is an important question to address, because such changes would mean that AMF species have the potential to evolve to rapidly changing levels of atmospheric pCO$_2$. The effects of elevated pCO$_2$ on the genetic structure of an AMF population have not previously been addressed.

Molecular methods, based on polymerase chain reaction (PCR), show high sensitivity and specificity regarding AMF species detection and identification. They overcome the limited resolution of AMF identification and community reconstruction based on the morphology of spores occurring in the soil; a method that has been shown to produce data that bear little
resemblance to the actual AMF communities that are present in plant roots (Clapp et al., 1995). In fact, DNA-based molecular tools were already successfully applied in other studies on the structure of communities and populations of AMF (Helgason et al., 1998; Vandenkoornhuyse et al., 2003; Koch et al., 2004).

The objective of this study was to investigate whether ten years of Free-Air CO$_2$ Enrichment (FACE) in permanent monocultures of Trifolium repens (white clover) has affected the AMF community structure and the genetic structure of a population of one AMF species, Glomus mosseae. To do this, we have used a PCR-based identification method that targets the 5' end of the nuclear ribosomal large subunit RNA gene (LSU, 28S) that is part of the multi-copy ribosomal gene cluster. This method allowed us to look at the presence and absence of six AMF species in the plant roots under different pCO$_2$ treatments. Additionally, by cloning and sequencing the amplified DNA fragments of G. mosseae, we were able to test whether exposure to elevated pCO$_2$ has resulted in a change in the genetic structure of the population of this fungus. Reasons for choosing this particular DNA region are its relative ease of amplification and the availability of reference sequence information in public data bases. Both are crucial, since template limitation and taxonomic complexity of crude root-DNA extracts are major obstacles to molecular ecological studies on AMF (Clapp et al., 2002).

### 4.3 Materials and Methods

#### 4.3.1 Field Experiment and Root Sampling

The experiment was conducted in field plots of the Swiss Free-Air CO$_2$ Enrichment (FACE) experiment, located 20 km north-east of Zürich (550 m a.s.l., 8°41’E, 47°27’N, experimental station of the Institute of Plant Sciences at ETH). Fumigation has been applied during the vegetation period for ten years. The experiment was designed as a split-plot of three pairs of FACE-rings that were treated as blocks (C: control / ambient; F: fumigated), based on the pre-experimental crop rotation in the plots. The rings were 85 m to 450 m apart from each other. The experimental plot factor was the CO$_2$ fumigation treatment (35 vs. 60 Pa) and the subplot factors, which were randomised within each ring, were a nitrogen-fertilisation treatment (14 vs. 56 g N m$^{-2}$ a$^{-1}$) and a planting treatment [permanent monocultures of white clover (Trifolium repens L. cv. Milkanova), perennial ryegrass (Lolium perenne L. cv. Bastion), and the mixture of both species]. The soil is a fertile, eutric cambisol (clay loam) of neutral pH. Except for nitrogen, soil mineral nutrients were considered non-limiting for the two forage crops [for further details about the experiment see e.g. Zanetti et al. (1996) or Suter et al. (2002)].

Roots of T. repens were sampled from the FACE experiment in early summer when new roots are formed, to avoid potential PCR inhibition due to secondary metabolites that are often built in older roots. Restricting the investigation to only one seasonal time point allowed a more comprehensive sampling, with 28 DNA extracts for studying the AMF community, and 12 vector-cloned specific PCR-amplification products per FACE ring for studying the Glomus mosseae Nicol. & Gerd., Gerd. & Trappe population.
The sampling strategy was hierarchical, with two FACE treatments (ambient vs. elevated pCO₂), six FACE rings (representing three blocks), seven patches of closely intermingled *T. repens* stolons with auxillary roots, and four subdivided root fractions (Figure 4.1). This resulted in a total of 168 root-fractions. *T. repens* roots were sampled from 0.9 x 1.1 m low-N-fertilised monocultures to a maximal depth of 10 cm, in early June 2002, immediately after the second annual cutting. The seven *T. repens* patches with an attached clump of roots of about 10 cm diameter were taken in distances of at least 15 cm from each other. Roots of each *T. repens* patch were washed thoroughly under tap water. The thickest roots and large nodules were excised before a final wash under de-ionised water and subdivision into four equally sized fractions.

One week prior to the sampling for molecular analyses, colonisation of the roots by AMF was assessed histologically following McGonigle *et al.* (1990). Five patches of *T. repens* plants were assessed per FACE ring.

Figure 4.1 The sampling design involved two Free-Air CO₂ Enrichment (FACE) treatments (ambient vs. elevated pCO₂; 60Pa), three FACE rings, seven *Trifolium repens* (white clover) patches and four root fractions per patch for total genomic DNA extraction. These DNA extracts were PCR amplified to detect the presence/absence of six arbuscular mycorrhizal fungal species, using species-discriminating oligonucleotide primers targeted to the 5’ end of the large subunit (LSU, 28S) gene of nrDNA. Out of the positive amplification products of *Glomus mosseae* (Gm), twelve were randomly chosen from each FACE ring for further vector-cloning. Subsequently, the inserts of ten randomly chosen recombinant clones were sequenced, giving a possible total of 120 sequences per FACE ring.

4.3.2 DNA EXTRACTION, PCR AMPLIFICATION, VECTOR-CLONING AND SEQUENCING

Crude DNA extracts of total genomic root-DNA were prepared from up to 20 mg of freeze-dried, pulverised roots, using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). To check for approximately equal total amounts of DNA, the extracts were run on 1% (w/v) 1 x TBE-agarose gels and visualised by UV-transillumination after ethidium bromide (0.01% v/v) staining (Sambrook *et al.*, 1989).
Detection of presence or absence of DNA from six AMF species (G. mosseae, G. claroideum Schenck & Smith, G. intraradices-like Schenck & Smith, G. eburneum-like Kennedy, Stutz & Morton, Acaulospora paulinae Blaszkowski, Scutellospora pellucida (Nicol. & Schenck) Walker & Sanders) in the root extracts was carried out by morphospecies-discriminating nested polymerase chain reaction (PCR) amplification (Appendix). The morphospecies-specific PCR primers, had been adjusted to the local fungal assemblage and were designed based on information of partial sequences from the 5' end of the nuclear ribosomal large subunit gene (LSU, 28S) that were derived from vector-cloned single-spore amplicons. These six pairs of primers (Table 4.1) should be considered as sequence-group specific, because they covered the most frequent, but not all, of the sequence types that were recovered from the given spore morphotypes. For sake of simplicity, we hereafter refer to them as being species-specific. To account for taxonomic uncertainties we called morpho-species and their respective, specific primer pairs G. eburneum- or G. intraradices-like (see Appendix).

We chose to study one AMF species, G. mosseae, in more detail to see whether elevated pCO₂ has resulted in a change in the distribution and abundance of rDNA sequences of this fungus. G. mosseae was chosen, because of its known abundance in agricultural field sites (e.g. Helgason et al., 1998), and because of its high spore number in soil sievings from the studied FACE experiment (H. Gamper pers. observation). For this population study we randomly chose 12 of the 28 theoretically available specific PCR products. For two FACE rings (C301 and F306) there were less than 12 products available, 6 and 9, respectively. Amplification products were purified and vector-cloned (pGEM-T™ vector system, Promega Co., Madison, WI, USA) and the plasmid-inserts were sequenced (for details see Appendix).

Nucleotide sequences were checked manually, using Navigator PPC (version 1.9.2b3, Applied Biosystems Inc., Forster City, CA, USA), compared against available sequence database entries (http://www.ncbi.nlm.nih.gov/BLAST), aligned in Clustal X (version 1.83; Table 4.1 Six pairs of morphospecies-discriminating oligonucleotide primers with annealing temperatures used in polymerase chain reactions (PCR), performed numbers of PCR cycles and lengths of amplified DNA-fragments.

<table>
<thead>
<tr>
<th>Arbuscular mycorrhizal fungal species</th>
<th>Primer pair</th>
<th>Primer sequence (5’-3’)</th>
<th>annealing temperature (°C)</th>
<th>Number of cycles</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomus mosseae</td>
<td>f1/r1</td>
<td>GAAGAAACGTTTTCTGCATCCAG</td>
<td>61</td>
<td>30</td>
<td>221</td>
</tr>
<tr>
<td>G. claroideum</td>
<td>f2/r1</td>
<td>AATCTGCTTCTGTGTTTAACGCAG</td>
<td>56</td>
<td>35</td>
<td>253-257</td>
</tr>
<tr>
<td>G. intraradices-like</td>
<td>f3/r1</td>
<td>GTTTTTCTCGGAAATTGGTTTAGTCC</td>
<td>57</td>
<td>35</td>
<td>207-209</td>
</tr>
<tr>
<td>G. eburneum-like</td>
<td>f4/r2</td>
<td>TAAATCTACCTGGTTTCCAGGTC</td>
<td>65</td>
<td>35</td>
<td>357-359</td>
</tr>
<tr>
<td>Scutellospora pellucida</td>
<td>f5/r3</td>
<td>TCTTTTGAGATGATACCTACGAG</td>
<td>55</td>
<td>30</td>
<td>320-322</td>
</tr>
<tr>
<td>Acaulospora paulinae</td>
<td>f6/r1</td>
<td>TAAATCTCGAGGGTTTCTTGCC</td>
<td>55</td>
<td>30</td>
<td>255</td>
</tr>
</tbody>
</table>
Thompson et al., 1997) and further analysed in MacClade (version 4, Sinauer Associates, Inc., Sunderland, MA, USA) and PAUP PPC (version 4.0.0b10, Florida State University; Swofford, 2003). The sequences of the haplotypes of G. mosseae that were found more than once (those used for analyses) were submitted to GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA, http://www.ncbi.nlm.nih.gov/) under the accession numbers AY649955-AY649983. Additionally, a multiple sequence alignment of all these haplotypes is available from TreeBASE (http://www.treebase.org/treebase/) under the accession number SN1962. Values of sequence identity (ratio of shared identical nucleotide states and total aligned positions) for the five most abundant haplotypes were calculated in relation to their closest matches available in current data bases.

4.3.3 DATA ANALYSES

4.3.3.1 ROOT COLONISATION LEVEL

Mean percentage of root length colonised by AMF structures (hyphae, arbuscules, and vesicles), mean percentage root colonisation intensity as defined by Plenchette and Morel (1996), and mean percentage of root length colonised by non-AMF per FACE ring, were analysed for a CO₂ fumigation effect by one-way ANOVA on logit transformed data (Crawley, 2003). The following pairs of FACE rings were treated as blocks, C106/F105, C204/F204, and C301/F306 based on the agricultural cropping before FACE was adopted.

4.3.3.2 COMMUNITY OF SIX AMF SPECIES

PCR amplification using species-discriminating primers, provided presence / absence data about the occurrence of six AMF species within the T. repens root systems. Since all fungal species were found in all FACE rings, quantitative techniques were used for community analyses that focused on relative differences in abundance.

The FACE treatment (ambient vs. elevated pCO₂) effect on the relative abundance of individual AMF species in root fractions of the FACE rings was tested, using one-way ANOVA with the three blocks defined above. Differences in relative abundance among the FACE rings and among AMF species were assessed by one-way ANOVA, followed by Tukey-Kramer’s HSD tests. Data were logit-transformed, if applicable, since this transformation best suits for data bound between zero and one (Crawley, 2003), otherwise data were angular-square root transformed as recommended by Anscomb (1984) to meet the normality and equal variance assumptions for statistical analyses. The α = 0.05 level of significance was kept for all statistical tests, including the mean comparison Tukey-Kramer HSD test, which accounted for multiple comparisons (JMP, version 5.0, SAS Institute Inc., Cary, NC, USA).

The Kolmogorov-Smirnov two sample test (Sokal & Rohlf, 1995) was used to assess congruence between patterns of abundance of the AMF communities under ambient and elevated atmospheric pCO₂. Additionally, independence of AMF species assemblage and FACE treatment was tested by 2 x 6 contingency table analysis, using the likelihood ratio X² test.
As measures of similarity between pairs of AMF communities of FACE rings Sørensen quantitative indices, also known as Bray-Curtis or Czekanowski coefficient of similarity (Magurran, 2004), were calculated, using the following formula: \( C_n = \frac{2N_{ab}}{N_a + N_b} \). The sum of positive PCR amplifications is taken as surrogate for species abundance, with \( N_{ab} \) denoting the number of shared positive PCR amplifications (i.e. species occurrence) between the two sampling units A and B, and \( N_a \) and \( N_b \) the number of positive amplifications for the sampling units A and B, respectively. A Sørensen quantitative similarity value of one indicates that abundance and AMF species composition are identical between communities, and a similarity value of zero indicates that no AMF species is shared. Phenograms of pairwise Sørensen indices [and of \( F_{ST} \) population values for \( G. mosseae \) (see below)] among FACE rings were constructed, using UPGMA cluster analysis in PAUP PPC. Mantel tests (Sokal & Rohlf, 1995) were used to estimate the association between the matrices of pairwise Sørensen indices [and pairwise genetic variance ratios, \( F_{ST} \) (see below)] and pairwise geographic distances among FACE rings.

As a measure of relations between the relative abundance of pairs of AMF species Spearman’s rank correlation coefficients were calculated. They were used to indicate the direction rather than the strength of correlations, because they do not account for potential confounding effects of the experimental factors.

4.3.3.3 Population Structure of Glomus mosseae

The sequence data set was reduced to only sequence types of \( G. mosseae \) that were found more than once and also by eliminating any non-\( G. mosseae \) sequence. The remaining haplotype frequencies per FACE ring were determined in MacClade. To estimate how much of the genetic variability within the population could be explained by \( \text{CO}_2 \) treatment and field-plot differences, variance components were estimated for FACE-treatment (ambient vs. elevated \( \text{pCO}_2 \)) and FACE ring (three replicates per FACE treatment) in an analysis of molecular variance (AMOVA), considering nucleotide sites as individual loci and using Kimura-2-parameter model of sequence evolution, using Arlequin version 2.001 (Excoffier et al., 1992; Schneider et al., 2001).

To illustrate sequence haplotype diversity, haplotype frequencies and potential evolutionary relations among haplotypes, an unrooted haplotype network was drawn, using TCS version 1.13 (Templeton et al., 1992; Clement et al., 2001). The implemented network-building algorithm, starts from an absolute distance matrix for the haplotypes and draws the minimum number of mutational changes between them as justified by the probability of parsimony statistics (Templeton et al., 1992). We chose a network approach because it is more appropriate than a bifurcating phylogenetic illustration to assess sequence relations within populations where many different nrRNA sequences can be obtained from one individual AMF. This is particularly likely in sequences from the nrRNA multicy copy gene family in multinucleate fungi (Kuhn et al., 2001; Pawlowska & Taylor, 2004). In such sequences not only point mutations may accumulate, but also recombination may take place between gene copies (Gandolfi et al., 2003).
4.4 RESULTS

Total colonisation of *T. repens* roots by arbuscular mycorrhizal fungi (AMF) was moderate with a mean of 20.4% (± 2.3%, SEM; data not shown). There were neither a significant FACE effect (ambient vs. elevated pCO$_2$; one-way ANOVA with blocking, n = 6), nor significant differences among FACE rings (one-way ANOVA, n = 30) on any measurements of AMF colonisation.

4.4.1 COMMUNITY OF SIX AMF SPECIES

From 139 out of a total of 168 root-DNA extracts (82.7%) an AMF DNA fragment was amplified, using six pairs of species-discriminating primers in nested-PCR. In the first round of amplification with the fungus-specific primer pair LR1/FLR2, 155 of 168 root-DNA extracts (92.3%) yielded visible bands on agarose test-gels. In one case of *G. mosseae*, in nine cases of *G. intraradices*-like, and in three cases of *A. paulinae*, an additional PCR-amplification product of different length was visible on agarose gels, although this should not interfere with our goal to investigate the partial AMF community (Appendix).

The colonisation frequencies of all six AMF species, as detected with PCR, did not differ between the atmospheric pCO$_2$ levels (one-way ANOVA with blocking, n = 6), although they varied among the majority of FACE rings and among each other (Table 4.2). *G. mosseae* was the most common species and also showed the most variable pattern between FACE rings (Table 4.2).

The overall rank abundance pattern of AMF species did not differ significantly between the two FACE treatments (Kolmogorov-Smirnov two sample test), although there were differences in the ranking of less abundant AMF species such as *G. eburneum*-like and *S. pellucida* at elevated and ambient pCO$_2$ (Figure 4.2). A 2 x 6 contingency table analysis for independence of AMF species assemblage and FACE treatment did not reveal a significant effect of ten years of CO$_2$ fumigation on the AMF community either ($\chi^2_5 = 5.52$, ns). Likewise, the pattern of the UPGMA-clustering of the Sørensen quantitative similarity indices for the AMF communities

![Figure 4.2](image.png)

**Figure 4.2** Rank/abundance plot for six species of arbuscular mycorrhizal fungi (AMF) in permanent monocultures of *Trifolium repens*, grown under free-air CO$_2$ enrichment for ten years (elevated pCO$_2$: 60 Pa) and ambient pCO$_2$. The ordinate shows the log10-relative species abundance while the abscissa ranks each species in order from most to least abundant. AMF species corresponding to appropriate ranks are indicated with the following abbreviations: Gi, *Glomus intraradices*-like; Gm, *G. mosseae*; Ap, *Acaulospora paulinae*; Gc, *G. claroideum*; Ge, *G. eburneum*-like; Sp, *Scutellospora pellucida*. Open circles indicate ambient pCO$_2$, closed circles represent elevated pCO$_2$. This graphical representation has the potential to reveal environmental effects on the evenness of species abundances in communities. The abundances of each species under the two FACE treatments is expressed as the proportion of total abundance of all species (Magurran, 2004)
of FACE rings was not consistent with a FACE-treatment effect (data not shown). However, the clustering of these indices tended to reflect the geographic distances among individual FACE rings. A marginally significant Mantel test, based on 99 permutations (\(p = 0.080, n = 6\)), further indicated that pairwise community dissimilarity values (calculated as 1-Sørensen quantitative index) were positively correlated with pairwise geographic distances between FACE rings (\(R^2 = 0.386, P = 0.013\), Figure 4.3).

### Table 4.2

Mean frequency of six arbuscular mycorrhizal fungi (AMF), as detected by PCR, in roots of *Trifolium repens* (white clover), grown in permanent monoculture plots under ambient and under Free-Air CO\(_2\) Enrichment (FACE) for ten years. The mean relative frequencies per FACE ring were calculated based on the frequencies per *T. repens* patch (\(n = 7\)), including all 168 root-DNA extracts.

<table>
<thead>
<tr>
<th>AMF species</th>
<th>Mean frequency (%)</th>
<th>Plots at ambient pCO(_2)</th>
<th>Plots at elevated pCO(_2) (60Pa)</th>
<th>Mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C106</td>
<td>C203</td>
<td>C301</td>
</tr>
<tr>
<td>Glomus mosseae</td>
<td></td>
<td>96.4</td>
<td>A</td>
<td>71.4</td>
</tr>
<tr>
<td>G. claroideum</td>
<td></td>
<td>25.0</td>
<td>B</td>
<td>39.3</td>
</tr>
<tr>
<td>G. intraradices-like</td>
<td></td>
<td>78.6</td>
<td>A</td>
<td>46.4</td>
</tr>
<tr>
<td>G. eburneum-like</td>
<td></td>
<td>28.6</td>
<td>ab</td>
<td>17.9</td>
</tr>
<tr>
<td>Acaulospora paullinae</td>
<td></td>
<td>7.1</td>
<td>b</td>
<td>17.9</td>
</tr>
<tr>
<td>Scutellospora pellucida</td>
<td></td>
<td>0.0</td>
<td>b</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Values followed by different small (for rows) or capital letters (for columns) differ significantly according to Tukey-Kramer's HSD tests at \(P < 0.05\), applied on the transformed data.
4.4.2 Population structure of *Glomus mosseae*

The *G. mosseae*-specific primer pair amplified fragments of 221 bp length. The corresponding sequences largely matched with sequences in public data bases labelled as *G. mosseae* or ascribed to members of the *G. mosseae*-species complex (*Glomus Group A*, Schüssler et al., 2001). Exploratory phylogenetic analyses based on current publicly available AMF sequences of the LSU nrRNA gene confirmed the findings from BLAST searches. After excluding sequences that were obvious contaminants, mainly basidiomycetous yeasts (present in six root-DNA extracts) and AMF sequences with exceptionally high numbers of single base substitutions (33 sequences clustering in three groups within the rest of sequences of *G. mosseae*), the data set consisted of 713 sequences of which 229 were unique. To exclude possible PCR artefacts (Pawlowska & Taylor, 2004), only sequence haplotypes that were found more than once were kept for further analyses. After this pruning procedure, 484 sequences from 64 vector-cloned PCR products were left in the data set with each FACE ring represented by 12 vector-cloned PCR products, except rings C301 (6) and F306 (9).

The pruned data set then comprised 29 distinct sequence haplotypes of which 15 occurred in samples from at least two FACE rings (Table 4.3). The number of haplotypes found within a single FACE ring ranged from eight to 13. Five haplotypes were dominant and accounted for 8 to 186 of the 484 sequences. The most frequent haplotype no. 169.4, was found 186 times among the 484 sequences and showed 100% similarity to GenBank accession AF145735, derived from a single spore of strain BEG 25 (Kjoller & Rosendahl, 2000). Haplotypes no. 169.3 (found 14 times) and no. 362.2 (found 8 times) both showed 99.5% similarity to this sequence. The second most frequent haplotype 251.4 (found 166 times) and the haplotype 172.11 (found 44 times) were 99.5% and 99.1% similar to the accession AF396794 (Jansa et al., 2003), respectively.

This high degree of sequence similarity among the haplotypes mentioned above and the similarity of the remaining haplotypes (listed in Table 4.3) to reference sequences of *G. mosseae* (from Genbank and those derived from spores for primer design; Appendix) suggested that all retained sequences can be assigned to the *G. mosseae* population.

**Figure 4.3** Community quantitative dissimilarity \[1 - (Sørensen quantitative index)\] as a function of geographic distance (m) among the Free-Air CO2 Enrichment plots. Abundance data of six arbuscular mycorrhizal fungi (AMF) were used for index calculations. Presence/absence of fungal species within roots of *Tritium repens* was recorded via species-discriminating PCR amplification. Plants and AMF had been growing in permanent monocultures under CO2 fumigation (60 Pa) in the field for ten years. A Mantel test, using 99 permutations, indicated that there was a marginally significant isolation by distance among AMF communities (P = 0.080, n = 6). The linear regression coefficient was $R^2 = 0.386$ (P = 0.013).
TABLE 4.3 Multiple alignment of variable nucleotide positions in the 221 bp long 5' terminal fragment of the nrRNA LSU gene for the sequence haplotypes of *Glomus mosseae* found more than once in roots of *Trifolium repens* (white clover). Read digits of nucleotide positions top down. The relative frequency of haplotypes are listed for each Free-Air Carbon dioxide Enrichment (FACE) ring separately (total numbers of recovered sequences in parentheses). Plants had been growing in permanent monoculture plots under FACE conditions for ten years. Dots indicate an identical nucleotide state as found in the most frequent haplotype 169.4 (names of haplotypes correspond to the root extract in which they were first detected, point delimited from the PCR clone identifier).

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Nucleotide position</th>
<th>FACE rings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(108)</td>
</tr>
<tr>
<td>169.1</td>
<td>T..........................C.</td>
<td>0.0185</td>
</tr>
<tr>
<td>169.3</td>
<td>T..........................C</td>
<td>0.0370</td>
</tr>
<tr>
<td>169.4</td>
<td>G7GTTGAGACCGGAGTCTGTTAAATTT</td>
<td>0.3519</td>
</tr>
<tr>
<td>171.3</td>
<td>A.................T..........</td>
<td>0.0185</td>
</tr>
<tr>
<td>171.5</td>
<td>T..........................C</td>
<td>0.0278</td>
</tr>
<tr>
<td>172.11</td>
<td>T..........................C</td>
<td>0.0093</td>
</tr>
<tr>
<td>176.12</td>
<td>T..........................C.</td>
<td>0.0278</td>
</tr>
<tr>
<td>180.8</td>
<td>A.................T..........</td>
<td>0.0185</td>
</tr>
<tr>
<td>183.12</td>
<td>T..........................T</td>
<td>0.0185</td>
</tr>
<tr>
<td>183.16</td>
<td>T..........................C</td>
<td>0.0185</td>
</tr>
<tr>
<td>191.3</td>
<td>C.................T..........</td>
<td>0.0370</td>
</tr>
<tr>
<td>192.18</td>
<td>C..........................C</td>
<td>0.0093</td>
</tr>
<tr>
<td>205.19</td>
<td>G..........................G</td>
<td>0</td>
</tr>
<tr>
<td>215.1</td>
<td>A.................A..........</td>
<td>0</td>
</tr>
<tr>
<td>215.7</td>
<td>A..........................G</td>
<td>0</td>
</tr>
<tr>
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<td>G.................T..........</td>
<td>0</td>
</tr>
<tr>
<td>231.2</td>
<td>A.................G..........</td>
<td>0</td>
</tr>
<tr>
<td>231.3</td>
<td>C.................C..........</td>
<td>0</td>
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<td>251.1</td>
<td>T..........................C</td>
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<td>253.5</td>
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<tr>
<td>274.4</td>
<td>G.................A..........</td>
<td>0</td>
</tr>
<tr>
<td>285.11</td>
<td>A.................C..........</td>
<td>0</td>
</tr>
<tr>
<td>287.10</td>
<td>C..........................C</td>
<td>0</td>
</tr>
<tr>
<td>292.11</td>
<td>T.................C..........</td>
<td>0</td>
</tr>
<tr>
<td>326.2</td>
<td>C..........................T</td>
<td>0</td>
</tr>
</tbody>
</table>

Sequence haplotype diversity, relative abundance, field site of occurrence, FACE treatment, and possible evolutionary relations among haplotypes, were highlighted with an unrooted parsimony network (FIGURE 4.4). This network indicated that all haplotypes, except two, were related to each other and that those that were more common also had more neighbouring haplotypes (FIGURE 4.4, TABLE 4.3). The commonest haplotype (no. 169.4) occurred more frequently in rings exposed to elevated pCO$_2$. Several of the closely related haplotypes (nos. 253.5, 274.4, 287.10, 292.11 and 326.2) were found exclusively in rings exposed to elevated pCO$_2$. The second commonest haplotype (no. 251.4) was more frequently found in rings with ambient CO$_2$ concentrations. Several closely related haplotypes (nos 183.16, 218.2 and 191.3) were exclusively found in rings that were maintained at ambient pCO$_2$. 

FIGURE 4.4 Unrooted parsimony network of the sequence haplotypes of a 221 bp fragment from the 5' end of the LSU nrRNA gene of *Glomus mosseae*. The sequences were derived from vector-cloned PCR products that were obtained from root DNA extracts of *Trifolium repens* (only haplotypes found more than once are shown). Connecting lines indicate single mutational changes between haplotypes at probability ≥ 95% under parsimony in a coalescent context. Dots designate hypothetical intermediate haplotypes that were not found. Approximate relative abundances of haplotypes from a total of 484 sequences are indicated by the size of the pie graphs. Hatching depicts an origin from CO$_2$ fumigated rings (60 Pa pCO$_2$), unhatched grey an origin from ambient pCO$_2$ rings.

Based on an AMOVA, 13.8% of the total molecular variance were explained by FACE-treatment (ambient vs. elevated pCO$_2$); a considerable amount of genetic variation given that the organism is clonal and that there has only been ten years of exposure to elevated pCO$_2$ (TABLE 4.4).

TABLE 4.4 Variance components estimated from an analysis of molecular variance (AMOVA*) on all sequence haplotypes of *Glomus mosseae* that were found more than once. Sequences were derived from DNA extracts of *Trifolium repens* (white clover) roots, colonised by the field fungal population. *T. repens* had been growing in permanent field plots under Free-Air CO$_2$ Enrichment (FACE) conditions for ten years. Calculated fixation indices are $F_{st} = 0.0585$, $F_{st} = 0.0954$, $F_{st} = 0.0391$.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>Percentage of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among FACE treatments (CO$_2$)</td>
<td>1</td>
<td>38.667</td>
<td>0.130</td>
<td>13.8</td>
</tr>
<tr>
<td>Among FACE rings within FACE treatments</td>
<td>4</td>
<td>26.780</td>
<td>0.076</td>
<td>8.1</td>
</tr>
<tr>
<td>Within FACE rings</td>
<td>478</td>
<td>351.118</td>
<td>0.735</td>
<td>78.1</td>
</tr>
<tr>
<td>Total</td>
<td>483</td>
<td>416.566</td>
<td>0.941</td>
<td>100</td>
</tr>
</tbody>
</table>

* performed in Arlequin version 2.001, using the Kimura-2-parameter model of sequence evolution and considering nucleotide sites as individual loci.
Variance among replicate FACE rings was 8.1%. The rest (78.1%) was contained within the FACE rings (Table 4.4). Pairwise genetic variance ratios ($F_{ST}$) among the FACE rings did not cluster according to FACE treatment, nor was there a pattern of isolation by distances among the FACE rings according to a Mantel test (data not shown).

4.5 DISCUSSION

The present study is the first reporting on the effects of ten years of elevated pCO$_2$ on the occurrences of AMF species in roots from the field and the first investigation on pCO$_2$ effects on an AMF population.

For the following discussion it is important to bear in mind some of the biological particularities of AMF. These fungal species can neither be microscopically distinguished within roots, nor grown without roots. Their coenocytic organisation and clonal propagation blur distinction between individuals and make definition of populations difficult (Sanders, 2002). From a genetic point of view the coenocytic organisation of AMF adds an additional level of biological integration that makes it appropriate to think of nuclei as populations of individuals, although with regard to fungal physiology and ecology, distinct mycelia may be considered as individuals.

4.5.1 CO$_2$ EFFECTS ON THE AMF COMMUNITY

The six AMF species studied, may only represent up to one half of the entire AMF community, based on estimates of species numbers from nrDNA-phylotyping studies in other habitats (Helgason et al., 1998; Vandenkoornhuyse et al., 2002), although obviously they are all common fungi in this community (Table 4.2).

The similarity of the rank / abundance curves for the community at ambient compared to that at elevated pCO$_2$ (Figure 4.2) and the lack of a clustering pattern among the FACE ring communities, using Sørensen quantitative similarities, suggest that there was no detectable FACE-treatment effect on the quantitative composition of AMF communities. The lack of a response to ten years of CO$_2$ fumigation in the present investigation contrasts with evidence for changes in AMF communities that was observed in a previous study based on morphological data of the root colonisation pattern and spore abundance in soils of this field experiment (Chapter 2) or at other field sites (Rillig et al., 1999; Rillig et al., 2000; Wolf et al., 2003). The actual community of AMF in roots can be quite different from the community reconstruction based on abundances of the spores of different AMF species (Clapp et al., 1995), because elevated pCO$_2$ effects on plants may result in changes in sporulation patterns of the fungal species but not their colonization of the roots. Furthermore, because we have only sampled some species in the AMF community, there could be effects of elevated pCO$_2$ on the abundance of other AMF species in the community that we have not studied. Our result is, however, consistent with studies conducted in the glasshouse, where three different co-occurring species of AMF all responded in the same way to exposure to elevated pCO$_2$ (Sanders et al., 1998).
4.5.2 NEW INFORMATION ABOUT AMF COMMUNITY STRUCTURE

Although the study did not reveal major effects of elevated pCO$_2$ on the AMF community structure it did reveal interesting features of AMF community structure, a subject that is still in its infancy. The six selected AMF species showed a relatively high frequency of occurrence and a remarkable evenness of abundance. Only *G. mosseae* showed a tendency for negative association with *A. paulinae* and *S. pellucida*. There were a high number of root systems in which several AMF species were present simultaneously. This contrasts with the ecological host preferences (Vandenkoornhuyse *et al.*, 2002; Vandenkoornhuyse *et al.*, 2003) between plants and AMF in natural habitats. However, recent findings from a natural monoculture of a grass species (Wirsel, 2004) also show considerable AMF species diversity in the root systems of that single host.

The rank / abundance distribution of the AMF species corresponded best to the broken stick model, which describes a very uniform species abundance (Magurran, 2004). It differs with that found by Helgason *et al.* (1999) for an AMF community in roots of a woodland spring-geophyte. The more even distribution pattern of species abundance found in *T. repens* roots may have originated from their common host plant, an important ecological factor that was obviously shared among species under both FACE treatments.

The marginally significant positive correlation between quantitative community differences and geographic distances among FACE rings (Figure 4.3) suggested that site differences in communities of AMF species may be explained by different spacing among study sites at local scale (85-450 m). Little is currently known about the distribution of different AMF species but one recent detailed sampling study of AMF in a grassland in Denmark indicates high patchiness for some AMF species, meaning that the sampling strategy for studying AMF community structure must be very carefully designed (Rosendahl & Stuckenbock, 2004).

A shortcoming of our partial AMF community study may be the single sampling at only one seasonal time point and the single host plant species, considered. We may have missed the critical season in which effects of the past CO$_2$ fumigation were most pronounced or may have missed the plant species with the most eligible AMF association. Composition of AMF communities is known to vary with host plant species, age of the host, season and ecosystem (Husband *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002; Vandenkoornhuyse *et al.*, 2003).

4.5.3 CO$_2$ EFFECTS ON THE GENETIC STRUCTURE OF THE *GLOMUS MOSSEAE* POPULATION

This study is the first study, to our knowledge, that shows that a rapidly changing environmental factor, namely elevated pCO$_2$, acts as a selection pressure to alter the genetic structure of an AMF population. Very little is known about the genetic structure of AMF populations. Only two other studies exist of environmental effects on both AMF populations and communities at the same field site (Jansa *et al.*, 2003; Koch *et al.*, 2004). In those investigations, approximately 12 years of soil tillage significantly changed the abundance of some AMF species and, thus, altered the AMF community structure (Jansa *et al.*, 2003). However, in the same field, there was
no effect of soil tillage on the genetic structure of a population of *G. intraradices* (Koch *et al.*, 2004). In our study, elevated pCO₂ acted differently, having only minor effects on the AMF community, but clear effects on the genetic structure of the population of *G. mosseae*.

The present population study relied on 484 sequences of the 5’ end of the nrRNA LSU gene. Most of the molecular variance contained in this data set originated from within the FACE rings and about one tenth of total variance could be explained by the FACE treatment (ambient vs. elevated pCO₂). A small portion was due to FACE ring replicates within FACE treatments. The high variation within each ring was expected given that previous studies of AMF population structure have revealed both high qualitative and quantitative genetic variation (Bever & Morton, 1999; Pringle *et al.*, 2000; Koch *et al.*, 2004). Despite the small proportion of variance, explained by the FACE treatment, the pattern of how variance was partitioned revealed that the CO₂ fumigation history had a considerable effect on the population structure of *G. mosseae* (Table 4.4). This is remarkable for a slow growing, coenocytic, and clonal organism, considering the relatively short time of only ten years of CO₂ fumigation and the well known heterogeneous soil environment. Moreover, the component of total genetic variance explained by the FACE treatment is unlikely to stem from an effect of geographic distances among the FACE rings, since FACE rings with elevated pCO₂ surrounded those with ambient conditions. Furthermore, no clustering of pairwise F⁻⁻ values corresponding to ring locations was found and no relationship to pairwise geographic distances between FACE rings existed (Mantel test, data not shown).

A shortcoming of this population study, the first on an AMF species in the field, is the single sequence marker used targeting a multi-copy gene. Nuclear ribosomal DNA sequences are known to evolve in complex ways and to show polymorphisms within individuals (Gandolfi *et al.*, 2003). This, however, may be outweighed by the fact that many root-DNA extracts from several experimental plots were used and that it did not rely disproportionately on sequences from a few vector-cloned PCR products.

### 4.5.4 Molecular Approach

Among the different methods that are currently available for population studies on AMF, we consider specific PCR primers most appropriate to work on environmental samples. AFLP analysis is not a viable alternative, since it needs pure DNA templates of the target species in a sufficiently high concentration. Single spores derived from field soil do not contain enough DNA and multi-spore extracts are prone to contamination. Isolation-cultivation via trapping in pot cultures and sterile propagation in split-plate *in vitro* root-organ cultures would have been the only procedure to obtain pure fungal material in adequate quantities for AFLP analysis (Koch *et al.*, 2004). However, drawbacks of this procedure are bias due to preferential growth of different fungal strains under the artificial condition and the considerable labour and time costs. We therefore expected the use of specific PCR primers to be the most appropriate for investigating the impact of ten years of elevated pCO₂ on AMF in *T. repens* roots.
4.5.5 Are CO$_2$ responses of AMF buffered by their plant host?

In the current investigation we detected no major effects of ten years of CO$_2$ fumigation on a selected part of the AMF community. Ten years of CO$_2$ fumigation may have been too short to exhibit a more pronounced effect on community or populations of such slow growing and clonal organisms (Sanders, 2002). Effects of elevated pCO$_2$ on AMF are mainly host plant-mediated and, thus, indirect (Fitter et al., 2000). This may further allow for some kind of buffering via physiological adjustments in host plants. However, to have seen an effect on the genetic structure of an AMF population in such a short time (a response which is also presumably plant-mediated), indeed, suggests that 10 years could be long enough to detect possible changes in the AMF community.

Legumes in the association with N$_2$-fixing rhizobia may not divert enough carbohydrates to their root symbionts since they suffer less from an imbalance between carbohydrates and mineral nutrients under elevated pCO$_2$. Moreover, *T. repens* is known for its capacity to conditionally adjust its photosynthesis (Wright et al., 1998). Such buffering capacity of *T. repens* against CO$_2$ fumigation effects on its microbial symbionts (AMF and rhizobia) would concur with findings in the same FACE experiment on rhizobial populations that did not differ after ten years of CO$_2$ fumigation (M. Richter personal communication). This buffering hypothesis seems indeed conceivable since both regular micro-symbionts of *T. repens* are closely involved in plant mineral nutrition (phosphorus and nitrogen) and both derive their C-resources from the same pool of photosynthates.

4.6 Conclusions

In summary, ten years of elevated atmospheric pCO$_2$ had no detectable effect on the studied partial AMF community but had an effect on the genetic structure of a *G. mosseae* population in *T. repens* roots. Our findings contribute to a better understanding of this fungal group under high input agricultural conditions. Given the effects of CO$_2$ on *G. mosseae* that we have observed, it is now important to understand whether AMF population genetic structure is altered in other AMF species by elevated pCO$_2$ and whether this response allows AMF species to adapt to the changing environment. The rapid adaptation of AMF populations to elevated pCO$_2$ could well be the reason why no overall effects are seen on the relative abundance of each AMF species. Furthermore, assuming that elevated pCO$_2$ acts as a selection pressure on the genetic structure of AMF populations, it is important to know whether these genetic alterations in the population might affect the mycorrhizal efficiency of these significant symbiotic fungi.

4.7 Acknowledgements

The authors would like to thank Ionut Popa for technical assistance. We gratefully acknowledge Dr. Alex Widmer’s effort to keep our molecular lab up-to-date with latest equipment. A research grant from the Federal Institute of Technology (ETH) Zürich provided financial support for this research. Ian Sanders was supported by a Swiss National Science Foundation professorial fellowship (no. 631-058108.99).
4.8 REFERENCES


5 General Discussion

5.1 Complexity of mycorrhizas

The effects of the rapid rise in atmospheric pCO$_2$ on below-ground ecosystems are not well investigated (Falkowski et al., 2000; Lal, 2003). In particular, little is known about the consequences of elevated atmospheric pCO$_2$ on micro-organisms in soils (Sadowsky & Schortemeyer, 1997). The aim of this PhD thesis was, first, to investigate the growth response of arbuscular mycorrhizal fungi (AMF) in roots and soil after seven years of Free-Air CO$_2$ Enrichment (FACE). Second, it was examined whether the composition of the assemblages of AMF strains changed due to eight years of FACE in such a way as to have consequences for the physiological functioning of mycorrhizal symbioses. This was tested by assessing mycorrhizal symbioses established between Trifolium repens host plants and two sets of AMF strains that had previously experienced conditions of ambient or elevated atmospheric pCO$_2$, respectively. Third, using molecular genetic tools, it was investigated whether the community structure of AMF and the population structure of one AMF species, Glomus mosseae, were altered in roots of T. repens due to ten years of FACE. Special features of these studies were that all the study material originated from a single long term FACE experiment with permanent model grasslands and that the effects of altered atmospheric pCO$_2$ on growth and assemblages of AMF were assessed directly in the field. Working under field conditions implied that the studied mycorrhizas were part of the complex biotic relations in soil, characterised by small-scale physicochemical and biological heterogeneity (Meeting, 1992; Tiedje et al., 1999; Wardle et al., 2004). Moreover, the investigated mycorrhizas may have been in competition with C-heterotrophic bacteria, fungi, and protozoa for plant derived C-resources (Bonkowski, 2004).

In the field study (Chapter 2), root colonisation and spore density in root-surrounding soils were assessed in permanent FACE monoculture swards of Lolium perenne and T. repens. Monocultures of plants reduced the complexity of the experimental conditions and made it possible to focus on the identity of host plant species as one potential factor that could have affected C-allocation to AMF. Our results showed that root colonisation and spore density of AMF were higher in autumn of the seventh years of FACE. This suggests that AMF successfully competed for the increased amount of available C under elevated atmospheric pCO$_2$. Elevated atmospheric pCO$_2$ has previously been reported to benefit AMF in another grassland ecosystem (Rillig et al., 2000), where intra- and extraradical hyphal biomass and the soil glomalin content were found to be increased. The present findings were confirmed by an independent study, also conducted in the Swiss-FACE, but for the vegetation period of the tenth years of CO$_2$ fumigation (Staddon et al., 2004). Moreover, a recent meta-analysis on the results of fourteen independent mycorrhizal studies showed that the increase in mycorrhizal abundance was a consistent finding for mycorrhizal responses to elevated atmospheric pCO$_2$ in field ecosystems (Treseder, 2004).
Thanks to the block factorial design of the Swiss-FACE experiment, host plants could be shown to represent mediating factors for the effects of N-fertilisation on colonisation of roots by AMF. Results demonstrated a correlation between high N-fertilisation and lower root colonisation levels by AMF in *L. perenne*, but not in *T. repens*. This species effect might be explained by host plant differences in N-metabolism and consequently also in C-metabolism. Also, differences in root colonisation by vesicles and differences in the number of small spores could be attributed to the effects of host plant species. In the Swiss-FACE experiment severe C-sink limitation in *L. perenne* was found to be an effect of growth-limitation by N-deficiency, which causes an accumulation of non-structural C (Fischer *et al.*, 1997; Suter *et al.*, 2002). These findings are in accordance with the higher biomass allocation into vesicles and spores (organs rich in storage lipids) found in roots of *L. perenne*, but not in roots of *T. repens* in our study. *Trifolium repens*, in symbiosis with rhizobial bacteria, is able to fix atmospheric N\textsubscript{2} releasing this plant from N-deficiencies and thus preventing C-accumulation. Alternatively (or additionally), differences in the abundance of vesicles and spores may point to differences in the strain composition of AMF in the two host plant species.

In the molecular field survey (Chapter 4), the focus was on a six-species AMF community and on the population of *G. mosseae* present in *T. repens* roots in early summer after ten years growth under elevated atmospheric pCO\textsubscript{2}. Difference among the AMF communities of six different plots of the Swiss-FACE experiment was related to geographic distance and not to the CO\textsubscript{2} fumigation treatment, indicating the importance of soil environment. By contrast, the population structure of *G. mosseae* was influenced by CO\textsubscript{2} fumigation, although it is not known whether the effects were direct or indirect. Alterations in host-plant C-availability, in host-plant demands for mineral nutrients, in soil moisture, or in the composition and activity of other soil biota could all have been responsible for the observed population changes. A tremendous sequence diversity was recorded at small spatial scales in the six plots which may have originated from the unusual genetic organisation of AMF (Kuhn *et al.*, 2001; Pawlowska & Taylor, 2004). Different gene copies that are present within a single fungal individual (intra- and / or internuclear polymorphism) were likely responsible for a considerable part of this sequence diversity.

In the inoculation experiment (Chapter 3), which tested two sets of AMF single spore isolates with contrasting, past CO\textsubscript{2} histories on their physiological effects in mycorrhizas, a reductionistic approach was chosen by strictly controlling for homogeneous environmental conditions and the identity of the symbionts. Mycorrhizal associations were assessed that were composed of fourteen single spore AMF isolates which were separately inoculated on nine distinct genets of *T. repens* nodulated by a single rhizobial strain. Despite these efforts of experimental control, results remained phenomenological and mechanistically inconclusive. The regulation mechanisms of the two nutritional root symbioses and the C- and N-metabolism of the host legume *T. repens* are complex and not yet explicable by a unifying, mechanistic regulation-model (Paul & Kucey, 1981; Catford *et al.*, 2003; Schulze, 2004). Several plausible
hypotheses could explain the finding that colonisation by AMF isolates originating from plots fumigated with CO\textsubscript{2} for eight years improved the N nutrition of \textit{T. repens} hosts (see \textsc{Chapter} 3). The fact that the proportion of foliar N taken up from the potting substrate was increased suggests that the AMF isolates with a history of elevated atmospheric pCO\textsubscript{2} may be more effective in N-uptake in comparison to other isolates. However, potential effects of signalling compounds on the plant’s N-uptake system cannot be ruled out. A signalling mechanism measured by means of isotopic tracers applied in a hyphal compartment was recently reported for the P-uptake system of plants (Smith \textit{et al.}, 2004). Synergistic effects of AMF and nodule symbiosis on the N status of the \textit{T. repens} host plants were certainly also present and indicate that the tripartite symbiosis as a whole has been affected by the mycorrhizal fungal strains and their pre-experimental CO\textsubscript{2} history.

Intraspecific variation in AMF that is responsible for differences in mycorrhizal phenotypes has been poorly appreciated in the literature (e.g. Hung \textit{et al.}, 1990; Koch \textit{et al.}, 2004), whereas differences among plant varieties or genets are well documented (e.g. Krishna \textit{et al.}, 1985; Lackie \textit{et al.}, 1988; Eason \textit{et al.}, 2001). In the present inoculation experiment (\textsc{Chapter} 3), the identities of the plant and fungal mycorrhizal partners explained significant parts of variation in many growth and physiological parameters, confirming the importance of intraspecific variability among host plants and fungal symbionts for mycorrhizal functioning. Among individual AMF isolates, there was considerable diversity in the pattern of fungal structures found within roots and in the effects on the N-concentration of host leaves, acquired via biological N\textsubscript{2} fixation. However, the effects of AMF species identity on the growth of mycorrhizal host plants were considerable: isolates of \textit{G. claroideum} consistently suppressed plant growth more than the \textit{G. intraradices}-like isolates. Indications for differential physiological functioning of particular combinations of the nine \textit{T. repens} genets and fourteen AMF isolates in our experiment showed that not only fungal species differences and intraspecific variation in plants and fungi are responsible for complex patterns of mycorrhizal responses, but also the markedly different interactions among mycorrhizal partners. The differences in performance of the individual mycorrhizal associations point to a level of specialisation within mycorrhizas that has relevance for symbiotic, physiological functioning.

Overall, the studies of this PhD project conducted in the field or under controlled pot conditions encountered system complexity at different levels of biological integration. For example, elements of this complexity include the ecosystem with numerous factors, the individual with its own particular properties, the physiological unit (metabolism) with its interacting regulation mechanisms, and the fungal genotype with its complex genomic organisation. Symbiotic relations are \textit{per se} complex, but in mycorrhizas multiple fungal symbionts and the habitat at the interface of plant and soil add many more factors influencing the symbiotic system.
5.2 Sensitivity and Dynamics of Mycorrhizas

The sensitivity of mycorrhizal symbioses to intrinsic (plant and fungal genotype) and extrinsic (ecological disturbance) factors is manifested in the context dependent growth responses of mycorrhizal plants (Jonsson et al., 2001; Cavagnaro et al., 2004). Mycorrhizal functioning can be seen to operate along a mutualism-parasitism continuum (Johnson et al., 1997) with the main determinants being environment, and ontogeny and genotype of the respective partners (Figure 1.1).

Being aware of the labile nature of mycorrhizas, the pot experiment (Chapter 3) aimed at comprehensively understanding the growth responses of host plants. Accordingly, both the intra- and extraradical phase of AMF were assessed, the development of T. repens hosts observed by several morphometric measures, and the plant physiological status at harvest. In a side-experiment with non-mycorrhizal T. repens plants, the importance of P-availability from the growth substrate was analysed by measuring the effect of five-levels of P-addition on plant growth and P-concentrations in the leaves and roots. Results of this experiment allowed us to exclude major P nutritional benefits as a confounding factor in the main experiment.

Measurements on growth and total tissue concentrations of P and N of the mycorrhizal host plants at harvest, however, provided only a crude and integrated impression of net-symbiotic outcome (i.e. benefits and costs). The interplay of environmental conditions, ontogenetic stage of the partners, and identity of symbionts themselves, involves complex, dynamic physiological interactions. Most of these dynamic processes could not be followed with the methods used in the pot experiment, and therefore, a cause-effect understanding about the symbiotic costs and benefits was not obtained. Such a mechanistic understanding for symbiotic functioning would have been particularly helpful in the poorly growth-responsive T. repens (van der Heijden, 2002). More comprehensive experiments with isotopic labelling are needed to obtain precise insight into the relative symbiotic costs and benefits for each mycorrhizal partner (Paul & Kucey, 1981; Tinker et al., 1994).

The current research project relied on an environmentally induced and host plant-mediated disturbance of cost / benefit-relations among the mycorrhizal symbionts. It was based on the assumption of a labile balance between symbiotic services and demands. However, the significance of results may be interpreted with caution, since they were based on assessments at a single time point in the vegetation period of a field study where many uncontrolled factors operate. Therefore, the findings from the two reported studies (Chapter 2 & 4) should be viewed as patterns present at a particular time point in the vegetation cycle of this particular field experiment. The lack of benchmark-information about the environmental conditions in the studied field experiment was partially compensated by a recent mycorrhizal study, which included measurements on soil moisture and root length densities (Staddon et al., 2004). Results from this study and from studies on plant physiological responses, which were all conducted in the same field experiment (Fischer et al., 1997; Isopp et al., 2000) corroborate the findings reported in Chapter 2.
5.3 Rising atmospheric pCO₂ and arbuscular mycorrhizas

Elevated atmospheric pCO₂ alters the nutrient balance in plants and particularly the allocation of photosynthetically fixed C to roots (Fischer et al., 1997; Rogers et al., 1994; Pendall et al., 2004). This is why it can be assumed that AMF benefit from increased C availability, and host plants stimulated in their growth by elevated atmospheric pCO₂ should favour mycorrhizal fungal partners which accommodate their enhanced demands for mineral nutrients (Fitter et al., 2000; Treseder, 2004).

Indeed, root colonisation in plants grown under elevated, compared to ambient atmospheric pCO₂ were found to be higher at a single time point in the seventh year of FACE, as were the spore densities in the surrounding soils. These findings strongly indicate that part of the additionally fixed C was allocated into AMF biomass (Chapter 1). Recently, data on root colonisation and extraradical hyphal length density, collected at three sampling dates in the tenth year of CO₂ fumigation, confirmed our findings, although responses of AMF to the CO₂ treatment were dependant on the level of N-fertilisation (Staddon et al., 2004). Rillig et al. (2000) found more intense root colonisation, higher extraradical hyphal length densities and higher glomalin concentrations for another grassland under the influence of a natural source of CO₂, corroborating the present results of increased biomass allocation to AMF.

Generally, effects of increased C-availability on AMF under elevated atmospheric pCO₂ seem to be less pronounced in pot experiments with inoculated host plants (reviewed in: Staddon & Fitter, 1998; Rillig & Allen, 1999; Fitter et al., 2000) than in field experiments (Chapter 2, Rillig et al., 2000; Staddon et al., 2004; Treseder, 2004). This might be due to a more pronounced acclimation of photosynthesis of plants in pots, compared to plants in the field (Hendrey, 1992; Ainsworth et al., 2003; Long et al., 2004), which results in more C available to AMF in field-grown plants.

Photosynthesis runs seldom at maximum rate and depends strongly on the source / sink relations within plants (Herold, 1980). Accordingly, the growth of mycorrhizal host plants might not be affected by fungal C-costs which would be cancelled out by adjustments in the rate of photosynthesis (Wright et al., 1998a,b). Findings that the C-costs imposed by AMF on the host plants translated into growth depression only under low light conditions (Jensen, 1984; Tester et al., 1986), under C-drain to herbivores (Klironomos et al., 2004), or when other symbionts are present (Bethlenfalvay et al., 1985; Reinhard et al., 1993), may, indeed, support this notion. Our findings did not completely support any theoretical concept of mycorrhizal induced C-costs, since we found an overall depression of plant development upon mycorrhizal inoculation and more severe growth reductions in plants inoculated with isolates of G. claroideum, compared to those inoculated with G. intraradices-like isolates, but no differences in host plants inoculated with the two sets of AMF inocula originating from contrasting atmospheric pCO₂ conditions. The conflicting literature reports on the effect of C-costs induced by AMF on the growth of different plant species may be due to different photosynthetic responses of individual plant species to higher fungal C-requirements (Schulze et al., 1994).
Symbiotic functioning in nature, where whole communities of AMF are associated with a single host, differs markedly from the artificial two-partner symbioses found in pot experiments. This could be a further reason why effects of elevated atmospheric pCO$_2$ on AMF growth are often contradictory under pot and field conditions. The effects of interactions among different AMF species are poorly known but might be critical for root colonisation patterns under natural conditions. Moreover, evolutionary changes in AMF assemblages are likely to occur after prolonged growth under elevated atmospheric pCO$_2$, leading to differences in root colonisation by adapted and non-adapted AMF communities.

The molecular ecological survey, described in Chapter 4, is the first that investigated the responses of an AMF community and of a population of an AMF species in roots to elevated atmospheric pCO$_2$. Atmospheric CO$_2$ fumigation during ten years changed the genetic population structure of *G. mosseae*, but not the composition of an AMF community composed of six species located in the same roots of *T. repens*. It was argued that adaptive changes below the community level, i.e. alterations in the relative abundance of different AMF strains in the population, could explain why the relative frequencies of the six AMF species remained the same. Moreover, such intraspecific dynamics in response to long term CO$_2$ fumigation could account for the differences in the physiological functioning of mycorrhizal symbioses, determined in Chapter 3. However, the lack of any change in the structure of the partial AMF community in response to an altered atmospheric pCO$_2$ observed in our study conflicts with findings reported by others (Rillig *et al.*, 1999a; Wolf *et al.*, 2003). These studies were based on morphological assessments of root colonisation patterns and densities of different spore types in the soil and might thus have concentrated at markedly different members of the AMF community. Moreover, Wolf *et al.* (2003) found that the spore density of only one out of 11 AMF species changed, which was a species not considered in our study.

The well known functions of AMF in plant P nutrition (Smith & Read, 1997) should give them an important role in predictive models of future natural ecosystems. This is because the anthropogenic increases in atmospheric pCO$_2$ and N-deposition reinforce plant P-deficiency in soils, which show an inherently low P availability. The findings that AMF strains with a history of growth under elevated atmospheric pCO$_2$, compared to strains grown under ambient pCO$_2$, improved N-nutrition of a common grassland legume under P sufficient soil conditions (Chapter 3) emphasises the importance of AMF also in future high input, agricultural soil systems. In fertile soils, rising atmospheric pCO$_2$ decreases the relative availability of N, which is usually the second main nutrition element (after P) which limits crop production. Overall, the role of AMF in plant mineral nutrition may become more important as atmospheric pCO$_2$ rises and plant growth generally becomes more limited by mineral deficiencies.
5.4 Significance of Findings

Rising atmospheric pCO$_2$ is a key factor of global environmental change. The studies conducted in the course of this PhD project showed that long term (and immediate) atmospheric CO$_2$ fumigation had effects on the growth of AMF, the symbiotic functioning of AMF in terms of host-plant N-nutrition, and the population structure of one AMF species in the Swiss FACE-experiment. Since AMF have a prominent position at the interface of above- and below-ground systems and they are involved in the flow of nutrients and feedback processes between plant and soil, the present findings suggest that AMF may play a key role in mediating ecosystem responses to elevated atmospheric pCO$_2$.

Nonetheless, rising atmospheric pCO$_2$ is not the sole component of global environmental change with potential effects on AMF and plant communities. In the field survey described in Chapter 2 it was found that N-fertilisation had pronounced effects on the colonisation of roots mediated strongly by the identity of host plant species. Therefore, the findings of the present investigation may stress the need for studies that assess interactions among components of global environmental change, such as anthropogenic N-inputs and temperature changes, but which also do not neglect the interaction of these factors with the effects of mycorrhizal host plants. In our study the identity of host plant species in the field was responsible for significant differences in the root colonisation and sporulation of AMF. Moreover, in our inoculation experiment host plant genets interacting with different AMF isolates had strong effects on several mycorrhizal parameters. All together, these findings highlight the importance of variations in host plant and in fungal strain for the symbiotic outcome of mycorrhizal associations.
6 Perspectives for future research

6.1 Mycorrhizal responses to elevated atmospheric pCO₂

The basic hypothesis underlying this PhD project was that plants in an atmosphere enriched with CO₂ would allocate additional C to their mycorrhizal fungi and thus stimulate growth and / or alter AMF strain composition. However, direct biochemical evidence is lacking that AMF actually profit from a better C-supply and indirect supporting evidence from inoculation experiments has often been equivocal (reviewed in: Staddon & Fitter, 1998; Rillig & Allen, 1999; Fitter et al., 2000). Many previous pot experiments using elevated atmospheric pCO₂ have found that growth of AMF is proportionally adjusted to enhanced growth of plants and that, therefore, the ratio of fungal to plant biomass did not increase.

Two accepted indicators of AMF physiology might help to investigate whether this hypothesis for C-nutritional benefits under elevated pCO₂ applies to AMF: the ratio between phospholipid fatty acids (PLFA) and neutral lipid fatty acids (NLFA) and the concentration of glomalin, an AMF derived glycoprotein. The ratio between the fractions of AMF specific PLFA and NLFA (16:1ω5) could serve as an indicator for the fungal nutritional status, since it measures the ratio between the amounts of structural lipids, i.e. membranes, and storage lipids (essentially oil globules) (Olsson et al., 1995; Olsson et al., 1997; Baath, 2003). Applying such measurements on fungal material present in soils and roots from ambient and elevated atmospheric pCO₂ conditions, and on fungal material of in vitro root-organ cultures, grown on media with different amounts of sugar (Fortin et al., 2002; Jolicoeur et al., 2002) could provide valuable information about the C-allocation by host plants to AMF.

Glomalin, an AMF derived fraction of recalcitrant soil C (Wright & Upadhyaya, 1996; Rillig et al., 1999b), could be used as an indicator to detect consistent long-term changes in the biomass of AMF communities, caused by altered plant C-allocation under elevated atmospheric pCO₂. This is because accumulation of glomalin would integrate a biomass increase of AMF over several years, thanks to its slow turnover rate.

The measurements on the PLFA / NLFA ratio in roots and soil and on the quantity of glomalin in soil are just two simple biochemical tools that would allow an assessment of whether more C is allocated to AMF when host plants are accommodated with ample C. Results from such a study would more definitively answer the hypothesis approached in this PhD project. If the measurements were applied to field material they could also confirm whether AMF successfully competed with other members of the soil community which live on plant-derived C (Bonkowski, 2004). Moreover, it would be important to find out whether AMF growth is truly C limited under current ambient pCO₂ conditions if one wants to predict potential selective effects of elevated atmospheric pCO₂ due to increased C-availability on AMF.

Future ecological research on the interaction of elevated atmospheric pCO₂ with other concomitant climate change factors will be critical, if we are to develop a predictive understanding of the impact of global environmental change on AMF. Interactions among factors explaining ecophysiological patterns could provide indications about underlying mechanisms. Gavito
et. al. (2003) and Heinemeyer et al. (2004) found that higher soil temperatures or increased soil moisture affected both the intra- and extraradical growth of AMF. Nonetheless, the mechanisms of how elevated atmospheric pCO$_2$ actually affects AMF and the mechanisms of how AMF responses may mediate the impact of rising atmospheric pCO$_2$ on ecosystems remain largely unclear, even though the importance of AMF in the global C cycle and for the composition of plant communities is indisputable (Körner, 2000; Treseder & Allen, 2000; Rillig et al., 2002; Hart et al., 2003; Zhu & Miller, 2003).

6.2 **MYCORRHIZAL PHYSIOLOGY**

The physiological functioning of mycorrhizas will be more thoroughly elucidated with the aid of isotopic tracers, ideally in combination with molecular identification and quantification tools. Isotopic tracers of C, N, and P have the potential to elucidate physiological processes that may explain plant growth responses to mycorrhizal colonisation observed in the current ecophysiological study (CHAPTER 3). Quantitative PCR is a new molecular tool that could be used to determine the relative proportions and the dynamics of coexisting AMF strains in roots and soils (Alkan et al., 2004), although when used to compare different fungal strains the gene copy numbers of each individual AMF strain should be determined. Moreover, advanced PCR based techniques (e.g. RT-PCR) may help to distinguish between the physiologically active and non-active proportion of fungal biomass or between active and dormant AMF taxa within the same roots. In addition, if experimental results are to gain more ecological relevance, temporal studies should be extended to the phase of well-developed mycorrhizas and not only be restricted to their establishment phase. However, for all these advanced research questions previous knowledge about basic features of the particular soil-plant-fungal system in use will remain crucial for any successful experimentation on mycorrhizas.

6.3 **RESEARCH RELEVANT FOR NATURAL CONDITIONS**

To increase the ecological relevance of manipulative experiments on arbuscular mycorrhizas, future studies should attempt to account for the ecological lability and complexity of mycorrhizas. This would best be achieved by including both the ontogenetic development of mycorrhizas and multiple AMF partners in experiments conducted in growth substrates that are as similar to natural soils as possible. Special efforts should be made to track the dynamics of individual strains that colonise the same root system. Read (2002a, 2002b) highlighted this by calling for more direct consideration of the natural conditions in future research on the ecology of mycorrhizas. Overly reductionistic approaches should be replaced in favour of more holistic analyses, and long-term investigations should be prioritised over short-term experiments. Attempts to correlate the structural diversity of soil communities, including that of AMF, with the above-ground ecosystem functioning (Wardle et al., 2004) will be vital future research projects in ecology. Model examples for studies that revealed feedback regulations between soil biota, including AMF and plants are those by Bever (2002), Klironomos (2002), and Callaway et al. (2004).
6.4 Molecular ecological studies

Molecular ecological field studies on AMF communities in roots and soils have been successfully conducted using PCR based techniques, coupled with vector-cloning and sequencing (Clapp et al., 2002). Patterns emerging from such culture-independent, environmental studies may increasingly identify the factors that shape the composition and structure of AMF communities. However, direct community assessment in roots and soils, via the application of specific PCR primers (e.g. Helgason et al., 1998; Redecker, 2000) and via terminal restriction length polymorphism (T-RFLP) analysis (e.g. Vandenkoonhuyse et al., 2003; Johnson et al., 2004) are still restricted to qualitative detection of the presence / absence pattern of particular taxa within individual samples. Moreover, these methods are prone to methodological biases related with PCR (e.g. poor primability, PCR selection, drift, and inhibition, chimera formation), which escape any rigorous control (Wagner et al., 1994; von Wintzingerode et al., 1997; Speksnijder et al., 2001). The array of potentially applicable methods on AMF is somewhat limited. The direct use of fingerprinting methods is precluded by the small amount of fungal material, which is often also associated with material from other organisms. To retrieve AMF material in sufficient quantities for common multilocus fingerprinting (e.g. AFLP) the fungi need prior in vitro isolation and propagation (Koch et al., 2004). Results from this kind of studies have a great potential to reveal urgently needed information about the biology, genetics, and ecology of AMF and should not be put off by the initial, considerable effort for isolation and cultivation (Koch et al., 2004). Basic knowledge on the biology of AMF and in particular on their genetic organisation is needed to be able to better interpret results from molecular ecological surveys. As for ecology of mycorrhizas, interdisciplinary research would stimulate theoretical and methodological development. Special relevance for advancements in the field of genetic organisation of AMF might have related research in the field of polyploid plants (Rauscher et al., 2002; Alvarez & Wendel, 2003; Rauscher et al., 2004). All in all, there is a broad array of unexplored research topics in the highly prospering field of physiology, ecology and genetics of arbuscular mycorrhizas of which I were to mention just a few.
6.5 REFERENCES


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APPENDIX

A  **SPECIFIC PCR PRIMERS TO DETECT SIX MORPHO-SPECIES OF ARBUSCULAR MYCORRHIZAL FUNGI WITHIN WHITE CLOVER ROOTS FROM TEMPERATE AGRICULTURAL GRASSLAND**

**SUBMITTED AS:**

H. GAMPER, A. LEUCHTMANN (SUBMITTED) **SPECIFIC PCR PRIMERS TO DETECT SIX MORPHO-SPECIES OF ARBUSCULAR MYCORRHIZAL FUNGI WITHIN WHITE CLOVER ROOTS FROM TEMPERATE AGRICULTURAL GRASSLAND.** MYCORRHiza.

**A.1 ABSTRACT**

Microscopical community and population studies on arbuscular mycorrhizal fungi (AMF, Glomeromycota) within plant roots are hindered by the lack of taxon-discriminating features. For a molecular approach, pairs of specific PCR primers were designed to detect *Glomus mosseae*, *G. claroideum*, *G. intraradices*-like, *G. eburneum*-like, *Acaulospora paulinae*, and *Scutellospora pellucida* within roots of *Trifolium repens*, grown in agricultural grassland. The primers were designed on the basis of sequences from the 5’ end of the nuclear ribosomal large subunit RNA gene (LSU, 28S). Sequences were obtained by performing PCR on single spores that were derived from trap and single-spore cultures. The amplified DNA fragments covered the variable domain 1. Amplicons differed in a size range that was optimal for either fragment length or single strand conformation polymorphism analyses. The species-discriminating primers were successfully applied on environmental genomic root DNA extracts, using a nested PCR approach. *Acaulospora paulinae*, a species that forms small hyaline spores and a *Glomus eburneum*-like species, whose intraradical hyphae could not be stained with several standard techniques, were detected within roots using two specific primer pairs. Comparison of previously published specific PCR primers with our single spore sequence data sets showed considerable nucleotide polymorphism within the priming sites. We conclude that the most frequent sequence types can be different depending on particular AMF populations and suggest that primer sequences should be adjusted to the AMF present at the actual study site.
A.2 INTRODUCTION

The structures of arbuscular mycorrhizal fungi (AMF, Glomeromycota) formed within roots are most directly involved in interactions with host plants, and thus are of particular ecological importance. However, AMF structures within roots lack morphological features which allow to discriminate species, and for some taxa structures cannot even be stained (Abbott & Robson, 1979; Merryweather & Fitter, 1998; Redecker et al., 2000). Morphological species identification of AMF relies on characters of the soil-born spores that often are difficult to discern, because of developmental variation or because they are parasitised. In addition, fungal sporulation may be controlled by several undefined factors and may be different among taxa. Therefore, the identification of AMF in community studies based on spores only may not be reliable (Clapp et al., 1995; Kowalchuk et al., 2002). In order to overcome the problems of species identification, molecular tools, particularly those based on DNA sequence information, have been developed for AMF (Clapp et al., 2002).

However, ecological studies on the obligately biotrophic AMF using molecular tools are also not free of handicaps. First, the limited amount of fungal tissue in the presence of other organisms often hinders straightforward application of established molecular techniques. Second, AMF are only culturable in association with plant roots and therefore, complete separation of AMF tissue from that of other organisms is almost impossible. This is especially true when cultivation is based on a soil system. The only alternative, currently available, is to grow pure fungal material in split-Petri dish root-organ cultures (Fortin et al., 2002; Koch et al., 2004), where fungus and root grow on one side of the dish while the fungus alone grows on the other side. However, this procedure has the disadvantages of laborious isolation, time-intensive cultivation, and growth preference of fungal strains. Therefore, an approach, directly applicable to field root samples is to detect the fungi via polymerase chain reaction (PCR), using specific primers.

Out of a range of PCR approaches established for AMF (Clapp et al., 2002), nested PCR appears to be the most suitable molecular tool to study root colonisation pattern of inoculated plants (van Tuinen et al., 1998a) or plants harbouring the natural fungal communities (Jacquot et al., 2000; Turnau et al., 2001; Jansa et al., 2003). Nested PCR is highly sensitive even in the presence of only low amounts of fungal DNA and an overwhelming background of non-target DNA from plant roots (van Tuinen et al., 1998a). Its detection sensitivity is increased by a pre-amplification of fungal DNA from crude root-DNA extracts using a first pair of fungus specific PCR primers. The second set of primers is then nested within the first amplicons and should specifically amplify the sequences of different AMF species. Besides increasing the sensitivity, this two-step procedure dilutes plant or fungal PCR inhibitors, present in total root-DNA extracts. Nested primers have to be particularly stringent, because multiple fungal species may simultaneously colonise the same roots (this includes members of Glomero-, Basidio-, Asco-, and Zygomycota) (Clapp et al., 2002, Kowalchuk et al., 2002, Renker et al., 2004). In addition, it has been suggested that PCR products should subsequently be vector-cloned to
separate different amplified DNA sequence types which arise from the complex intraspecific genetic organisation of AMF (Kuhn et al., 2001; Pawlowska & Taylor, 2004).

Genetic markers used for the purpose of AMF detection within roots have mainly targeted regions of the nuclear ribosomal RNA gene cistron (Clapp et al., 2002). nrRNA gene clusters have been reported to occur in multiple repeated copies within nuclei (Kuhn et al., 2001; Pawlowska & Taylor, 2004). Depending on the aim of the study, i.e. whether phylogenetic and reference sequence information are needed, and depending on the phylogenetic resolution required, different regions of the cistron are used which are known to evolve at different speed. The nuclear ribosomal small subunit was targeted in many previous studies using primers specific at different taxonomic levels (SSU, 18S; Millner et al., 1998; Simon et al., 1992). Studies that were aimed to differentiate at the level of genera and species often used the nuclear ribosomal large subunit (LSU, 28S; van Tuinen et al., 1998a; Kjoller & Rosendahl, 2000; Turnau et al., 2001; Jansa et al., 2003; Geue & Hock, 2004), while studies at levels below species used the internal transcribed spacer (ITS) region (Redecker, 2000; Renker et al., 2003).

The objective of this study was to develop specific primers for direct detection of six different AMF morpho-species within field-grown roots, namely *Glomus mossae*, *G. claroideum*, *G. intraradices*-like, *G. eburneum*-like, *Acaulospora paulinae*, *Scutellospora pellucida*. We describe the design of primers, the specificity tests using recombinant plasmids of single spore PCR and multisporous DNA extracts of pure cultures, and the application of the primers on environmental root-DNA extracts. The design of primers relied on sequence data sets, generated from single-spore PCR of spore material obtained from single spore and trap cultures. Target of the new specific primers are regions at the 5’ end of the nrRNA LSU gene (mainly the variable domain 1, D1). They amplify different sized DNA fragments and may thus be employed in PCR-assays to detect the six fungal species in field-root samples without the need for sequencing. Sequence polymorphism of DNA fragments within species was analysed further by using single-strand conformation polymorphism analysis.

### A.3 MATERIALS AND METHODS

#### A.3.1 ARBUSCULAR MYCORRHIZAL FUNGAL MATERIAL

Trap cultures (Gilmore, 1968; Brundrett et al., 1999) were established from soil samples of plots of the Swiss free-air CO₂ enrichment experiment [see Zanetti et al. (1996) for a detailed description of the field experiment]. As trap plants the following species pairs were used: *Dipsacus fullonum* L. and *Leontodon autumnalis* L., *Oenothera biennis* L. and *Tagetes patula* L., *Plantago lanceolata* L. and *Zeas mays* L. cv. Corso, *Lolium perenne* L. and *Trifolium repens* L., *Allium porrum* L. cv. Dubouchet and *Glycine max* L. cv. Merrill. The growth substrate consisted of a 1:2:2 (v/v/v) mixture of pasteurised field soil [clay loam, pH (H₂O): 5.8, available P (Olson): 52 mg P kg⁻¹, re-inoculated with a filtrate of the natural microflora], silica sand (0.7-1.2 mm) and an expanded, attapulgite clay (Oil Dry Chem-Sorb WR 24/18, Brenntag Mediterranée Export, Vitrolles Cedex, France).
From the fourth to approximately the ninth month after culture establishment, spores were recovered by wet-sieving, decanting (Gerdemann, 1963) and sugar density-gradient centrifugation (Daniels & Skipper, 1982). Single spore cultures of all *Glomus* species were established on *P. lanceolata*. Newly formed spores of these cultures then provided fungal material for DNA extraction.

The following six AMF morpho-species were identified and genetically analysed (number of spores analysed): *Acaulospora* sp. (4), *Entrophospora infrequens* (Hall) Ames & Schneider (5, young / healthy), *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (12), *G. claroideum* Schenck & Smith (12), *G. intraradices* Schenck & Smith (13), *G. eburneum* Kennedy, Stutz & Morton (7), *A. paulinae* Blaszkowski (2) and *Scutellospora pellucida* (Nicol. & Schenck) Walker & Sanders (14) (Schenck & Pérez, 1990). Identification of *G. intraradices* and *G. aggregatum* Schenck & Smith isolates appeared uncertain after single spore and inoculum propagation [see Johnson (1993) p. 752 for a similar finding] and are, therefore, referred as *G. intraradices*-like.

The following monosporic cultures were used in this study: *G. mosseae* (nos 101, 130, 259, 505, 520), *G. claroideum* (nos 181, 178, 282, 412), *G. eburneum*-like (nos 83, 234, 272, 412) and *G. intraradices*-like (nos 107, 128, 488, 495, 589). Inocula of all these single spore cultures were stored in a 4°C room at the Institute of Plant Sciences of ETH in Eschikon (ZH) and representative cultures were sent to INVAM (http://invam.caf.wvu.edu/) and BEG (http://www.kent.ac.uk/bio/beg/index.htm). Two *G. intraradices*-like isolates (*in vitro* 1 and 2) were propagated in split-Petri dishes on Ri-T-DNA transformed root organ cultures of *Daucus carota* L. They were established from surface sterilised *A. porrum* roots of two different trap cultures, following the procedure by Declerck et al. (1998) with slight modifications as used by Koch et al. (2004).

A.3.2 DNA Preparation

Of all monosporic cultures single-spore and multispor DNA extractions were made from clean and healthy spores selected under a compound microscope at 40-100 x magnification. DNA extracts were obtained, using the DNAeasy Plant Mini Kit, according to manufacturer’s instructions (Qiagen GmbH, Hilden, Germany).

Crude DNA extracts of 168 root samples of *T. repens*, collected from permanent monocultures in early June 2002, were made from 20 mg of freeze-dried and ball-milled roots, also using the DNAeasy Plant Mini Kit.

A.3.3 Nested PCR Amplification on Single-Spore DNA Extracts Using Fungal-Specific Primers

Spores were crushed singly with micro-pestles (made from Pasteur pipettes) in 5 µl sterile deionised water supplemented with 5 µl 20% Chelex-100 (Bio-Rad, Laboratories, Hercules, CA, USA). Suspensions were incubated at 95°C for 3 min and kept on ice until used as PCR template.
In a nested PCR procedure, first the eukaryotic primers ITS3 (White et al., 1990) and NDL22 (van Tuinen et al., 1998b), and then the fungal primers LR1 (van Tuinen et al., 1998a) and FLR2 (Trouvelot et al., 1999) were used to obtain DNA fragments of approximately 700-760 bp length. Reactions were performed in final volumes of 25 μl and 60 μl, for the first and second amplification step, respectively. Both PCR mixtures contained 1 x PCR buffer [supplied by the manufacturer, containing 50 mM KCl, 1.5 mM MgCl₂ and 10 mM TrisHCl (pH 9.0)], with additional 1 mM MgCl₂, 0.2 mM of each dNTP, 1 μM of each primer (HPLC purified, Microsynth GmbBH, Balgach, Switzerland), 0.02 U/μl Taq DNA Polymerase [containing 0.2 mM Tris-HCl (pH 7.5), 0.02 mM dithiothreitol, 0.4 μM EDTA (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA)], and 5 μl of single-spore DNA extract or 12 μl of diluted (1:10-1:1000) products from the first PCR. Thermocyclings were carried out on GeneAmp® systems 2700 or 9700 (Applied Biosystems, Forster City, CA, USA) applying an initial denaturation cycle at 94°C (3 min) and 30 cycles with denaturation at 94°C (45 sec), annealing at 60°C (55 sec), and extension at 72°C (90 sec); the last cycle was followed by a final extension at 72°C for 10 min, before cooling down to 4°C.

A.3.4 Vector-cloning of PCR products

PCR-amplification products were purified, using QIAquick PCR purification columns (Qiagen GmbH, Hilden, Germany), according to manufacturer’s instructions, but with an additional guanidinium hydrochloride (35% w/v) cleaning step. PCR products were ligated into pGEM-T™ vectors at 4°C over night, and ligation products were used in a heat shock transformation of JM109 high efficiency competent Escherichia coli cells (Promega Co., Madison, WI, USA). Recombinant E. coli colonies were identified on X-Gal and IPTG amended Luria-Bertani (LB) Petri plates by α-complementation (Sambrook et al., 1989). One to five positive transformants were randomly picked and grown overnight at 37°C in Miniprep liquid-cultures (Sambrook et al., 1989). Plasmids were recovered from the bacterial cells, using the GFX™ Micro Plasmid Prep Kit according to the manufacturer’s instructions (Amersham Pharmacia Biotech Inc, Piscataway NJ, USA).

A.3.5 Sequencing of vector-inserts

Cycle sequencing was performed according to manufacturer’s instructions on a MJ Research PTC-100™ for both strands of the vector inserts using standard M13 forward and reverse primer and ABI Prism® BigDye Terminator Cycle Sequencing chemistry (version 1.1, Applied Biosystems, Forster City, CA, USA). Extension products were purified by ethanol precipitation and analysed on an automated capillary ABI Prism®310 sequencer (Applied Biosystems, Forster City, CA, USA). Electropherograms were checked and ambiguities corrected using Sequence Navigator PPC (version 1.0.2b3, Applied Biosystems Inc., Forster City, CA, USA). All sequences were compared to known sequences, using BLASTN (Altschul et al., 1997) to confirm their glomalean origin.
Primer-specificity was assured by re-amplification of the recombinant *E. coli* colonies that were obtained by cloning the PCR amplified root DNA extracts. PCR contained 1 x PCR buffer (as above), 0.75 mM MgCl₂, 0.12 mM of each dNTP, 0.6 μM of each M13 Primer (desalted, Microsynth GmBH, Balgach, Switzerland) and 0.02 U/μl Taq DNA Polymerase (as above), and were performed using: 3 min preheating at 94°C, 20 cycles with 45 sec at 94°C, 30 sec at 56°C and 70 sec at 72°C, and a final extension at 72°C for 10 min prior to cooling to 4°C. PCR products were purified in MultiScreen-FB filter plates (Millipore, Billerica, MA, USA) and sequencing reactions performed as before but using the nested vector-primer M13f (5’- TAC GAC TCA CTA TAG GGC GA -3’) only. Extension products were purified using MultiScreen®-HV plates (Millipore Co., Bedford, MA, USA) with Sephadex™ G-50 Superfine (Amersham Biosciences AB, Uppsala, Sweden) according to manufacturer’s instructions.

A.3.6 PRIMER DESIGN AND SPECIFICITY TESTS

The sequences obtained by the procedure described above were used to design AMF species-discriminating primers. Sequences were delimited by the LR1/FLR2 primer pair corresponding to the nrRNA LSU gene region from nucleotide position 199 to approximately 997 of the reference organism *Saccharomyces cerevisiae* (European Ribosomal RNA database, Gent, Belgium; http://www.psb.ugent.be/rRNA/primers/NL.html). Specific primers were deduced on paper from multiple alignments (Clustal X, version 1.83; Thompson *et al.*, 1997; MacClade, version 4, Sinauer Associates, Inc., Sunderland, MA, USA) of sequence haplotypes found for the six AMF morpho-species and information about their frequency. The priming sites were chosen within species-distinct regions, which showed 100% similarity among the most frequently detected sequence types. Primability and stability of priming sites were confirmed to be high in PCR simulations using Amplify version 1.2 (Engels, 1993). GC contents of the amplified fragments (only those with complete primer matching) were derived from the outputs of Amplify.

To test amplification ability of primers and to check the specificity in actual PCR a three-step approach was used. First, the recombinant plasmids of the five most common sequence types of each morpho-species was used to adjust the PCR conditions for the pairs of new primers and to test the specificity in presence of a target and non-target template of known sequence. Then, PCR was performed on multispore DNA extracts prepared from single spore cultures, using a nested (see section below) and direct PCR approach. Finally, specificity of the new primers was evaluated on crude root DNA extracts from plants grown in the field. PCR products of these samples were selected based on the results from 2% (w/v) agarose test-gels for vector cloning; these included two samples in cases of single bands, and four samples in cases of single and (unspecific) double bands (two samples each), for each pair of specific primers. Sequences generated from the recombinant plasmids were used to check for the presence of target and non-target sequences in the amplicons. In total, 67 specific PCR amplicons of *G. mosseae* were vector-cloned, and of each at least ten recombinant plasmids sequenced.
A.3.7 Nested PCR Amplification on Root-DNA extracts Using AMF Taxon-Specific Primers

Nested PCR reactions were performed on multi-spore and crude root-DNA extracts. The first PCR with the fungal-specific primer pair LR1/FLR2 was performed in final volumes of 25 μl as described above. Amplification products were separated by agarose gel (1.5%, w/v) electrophoresis in 1 x TBE buffer and visualised after staining with ethidium bromide (0.01%, v/v) (Sambrook et al., 1989). Depending on intensities of the bands, PCR products were diluted 1:10 to 1:10’000 times before use in nested amplification with specific primers. Mixtures of the second PCR each contained 1 x PCR buffer (as specified above), 1 mM MgCl₂, 0.12 mM of each dNTP, 0.6 μM of each specific primer (specified in TABLE A.1; HPLC purified, Microsynth GmbH, Balgach, Switzerland), 0.02 U μl⁻¹ Taq DNA Polymerase (as specified above) and 2 μl of the dilution from the first PCR product. For amplifications with the f1/r1 primer pair (specific for G. mosseae) the MgCl₂ concentration was reduced to 0.75 mM. Annealing temperatures and cycle numbers were as specified in TABLE A.1; all other thermocycling parameters were as above, except that time for annealing was 40 sec and time for extension 70 sec.

A.3.8 PCR-SSCP Analysis

PCR-Single Strand Conformation Polymorphism (SSCP) analysis was performed to screen for different clones in vector clone libraries and to display intraspecific sequence variation. PCR-SSCP analysis is a sensitive, cheap and fast analytical method to detect sequence diversity among short DNA fragments (Clapp, 1998; Schwieger & Tebbe, 1998; Clapp et al., 2001). It allows separation of PCR products of the same size but of different sequence, due to different electrophoretic mobility upon intra-strand folding of short single DNA strands (Orita et al., 1989; Hayashi, 1991; Yap & McGee, 1993).

### Table A.1

| Morphospecies-specific oligonucleotide primer sequences, annealing temperatures, numbers of PCR cycles performed in polymerase chain reactions (PCR), lengths and GC-contents of amplified DNA-fragments. |
|---|---|---|---|---|---|---|
| **GC content** (%) | **Fragment length (bp)** | **Number of cycles** | **Annealing temperature (°C)** |
| 42.4-44.6 | 221 | 30 | 61 |
| 47.4-49.5 | 253-257 | 35 | 56 |
| 42.3-44.2 | 207-209 | 35 | 57 |
| 40.4-41.4 | 357-359 | 3.5 | 65 |
| 41.0-42.6 | 320-322 | 3.5 | 55 |
| 38.9-42.7 | 255 | 3.5 | 55 |
| **Primers (5'-3')** | **Primer pair** |
| f1 | f1/r1 |
| f2 | f2/r1 |
| f3 | f3/r1 |
| f4 | f4/r1 |
| f5 | f5/r1 |
| f6 | f6/r1 |
| **Arbuscular mycorrhizal fungal species** |
| Glomus mosseae |
| G. claroideum |
| G. intraradices-like |
| G. eburneum-like |
| Scutellospora pellucida |
| Acaulospora paulinae |
SSCP analysis was carried out on PCR amplified vector inserts as described in Clapp et al. (2001) with minor modifications. Two microlitres of PCR product from nested PCR-amplification were mixed with 8 μl of alkaline-denaturing loading buffer, denatured at 94°C for 2 min and immediately snap-cooled on wet ice. Four microlitres of each sample were loaded onto non-denaturating 0.75 x MDE™ (mutation detection enhancement) gels (FMC BioProduct, Rockland, ME, USA) of 0.2 mm thickness and furnished with a 64-well shark-tooth comb. Gels were run with 0.6 x TBE buffer (Sambrook et al., 1989) at 5 W for 14.7 kVhr and at 10°C. They were silver stained according to Bassam and Caetanoanolles (1993) and dried at 60°C. Resolved differences in banding patterns were confirmed to have their origin in sequence polymorphisms by sequencing respective vector insert.

A.3.9 GenBank accession numbers

The sequences generated and used during primer design (matching LR1-FLR2 fragments) are deposited in GenBank of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) under the following accession numbers: *G. mosseae*, AY639156-AY639174; *G. claroideum*, AY639175-AY639201; *G. intraradices*-like, AY639202-AY639224; *G. eburneum*-like, AY639225-AY639241; *S. pellucida*, AY639242-AY639262; *A. paulinae*, A639263-AY639265. Additional less frequent sequences derived from single spore PCR amplifications are: *G. mosseae*, AY639266-AY639281; *G. claroideum*, AY639282-AY639293; *G. intraradices*-like, AY639294-AY639305; *G. eburneum*-like, AY639306; *S. pellucida*, AY639307-AY639326; *A. paulinae*, AY639327-AY639328. Sequences of a *G. clarum*-like spore coloniser of *S. pellucida* and of a presumed *G. claroideum*-like coloniser of *E. infrequens* spores are available under AY639329-AY639347. Sequences from spores with an *Acaulospora / Archaeospora* morphotype are available under AY639348-AY639364. Sequences responsible for the six SSCP banding patterns shown in Figure A.5 are available under the accession numbers AY639365-AY639370.

A.4 Results

A.4.1 Sequences used for the alignment

The sequences that were PCR-amplified from single spores of six AMF morpho-species were approximately 700-760 bp long and revealed nucleotide polymorphisms within species and within individual spores. As described by van Tuinen et al. (1998a), sequences of the four *Glomus* species had a characteristic insert of approximately 50 bp while those of *A. paulinae* and *S. pellucida* had a deletion at this position. Based on multiple alignments of these sequences, one specific primer pair was designed for each of the six AMF morpho-species (Table A.1). In Figure A.2, all priming sites are shown aligned to the reference sequence of *S. cerevisiae*.

A.4.2 Primer location

The six pairs of new morphospecies-discriminating primers (Table A.1) target regions at the 5’ end of the variable domain D1 (B13_1 through B14 of *S. cerevisiae*). Annealing
sites of forward primers f2 (G. claroideum), f4 (G. eburneum-like) and f6 (A. paulinae) are positioned directly upstream of D1 and overlap with each other as do those of forward primers f1 (G. mosseae) and f3 (G. intraradices-like) that lie more downstream and within D1 (FIGURE A.1 & A.2). The forward primer f5 (S. pellucida) binds even further downstream, but also within D1. The common reverse primer r1, shared among A. paulinae, G. claroideum, G. mosseae and G. intraradices-like, lies in between D1 and D2 (C1_1 through D8 of S. cerevisiae). The reverse primer r2 (G. eburneum) binds directly upstream, and the reverse primer r3 (S. pellucida) directly downstream of the indel at the 5’ end of D2 that distinguishes the sequences of A. paulinae and S. pellucida from those of Glomus sp. Our new pairs of specific primers span regions of 207-359 bp that contain D1 and the spacing region in between D1 and D2 (TABLE A.1 & Figure A.1 & A.2).

**FIGURE A.1** Schematic representation of the nuclear ribosomal RNA (nrRNA) cistron: positions of priming sites at the 5’ end of the large subunit (LSU, 28S) gene. Forward PCR primers are shown above, reverse primers below the gene boxes; arrows indicate orientation of primers. Primers in bold were used in this study, other primers (van Tuinen et al., 1998a; van Tuinen et al., 1998b; Kjoller & Rosendahl, 2000; Clapp et al., 2001; Jansa et al., 2003; Geue & Hock, 2004) are shown for comparison. Variable domains are indicated in grey and the indel, distinguishing members of Glomerales and Diversisporales is highlighted with black hatching. The diagram is not to scale.

A.4.3 PRIMER MATCHES WITH ENTRIES IN CURRENT PUBLIC SEQUENCE DATABASES

In BLAST searches on the NCBI web site using just the primer sequences, forward primers f1 and f2 (G. mosseae and G. claroideum) showed closest matches with BEG (La Banque Euripéenne des Glomales, http://www.kent.ac.uk/bio/beg) culture sequences of the corresponding species and weaker matches with sequences of other glomalean species of nrRNA SSU gene-phylogroups A and B (Schüssler et al., 2001). Primers f5 and r3 gave closest matches with sequences of Scutellospora ssp. and Gigaspora ssp., while the forward primer f4 (G. eburneum-like) best matched with sequences of Scutellospora ssp. The rest of the primers did not fit with any glomalean sequence available on GenBank.
Primer specificity tests

In cross-amplification tests of the new primers, using recombinant plasmid templates DNA-fragments of the expected size could be resolved on agarose gels, and specificity of the primers, when tested against non-target plasmids, was confirmed, as there was no amplification product visible (data not shown). Furthermore, cross-amplification checks were performed on multisporid- DNA extracts of several single spore cultures, confirming that primers were specific with respect to these particular AMF species (FIGURE A.3). The multiporid DNA-extracts of G. mosseae, G. intraradices-like, G. eburneum-like and S. pellucida yielded strong amplification even with only a single PCR amplification with the specific primers, whereas extracts of G. claroideum produced only very faint or no amplification product (data not shown).

A.4.5 Primer application on DNA extracts from field-grown roots

Primer specificity under field conditions was evaluated in the course of a molecular ecological study, assessing an AMF community that included the morpho-species for which the species-discriminating primers were described. 168 crude root-DNA extracts of T. repens that...
Cross-amplification test: Ethidium bromide stained agarose gels (1.5 % w/v in 1 x TBE) showing the PCR products derived from multispore DNA extracts of single spore cultures of arbuscular mycorrhizal fungi (AMF) after amplifications with (a) the fungal-specific primers LR1/FLR2, and nested, morphospecies-specific primer pairs (b) f1/r1 (c) f2/r1, (d) f4/r2, (e) f3 / r1 and (f) f5/r3. Species names and numbers of single spore cultures are indicated above lanes. All negative PCR controls contained no amplification products (data not shown). Dilution factors of the fungal-specific PCR products, run in (a), which were used for further specific amplifications (b-f) were: *, 1000; +, 100; x, 10; for all others, 10'000. The 100 bp-step sizing ladders on the right were copied to the left. The amplification product from the DNA extract of Scutellospora pellucida (S. pell.) in (e) most likely stems from contaminating spores that were colonised by Glomus intraradices-like, since the spores for this multispore-DNA extract were derived from trap cultures, harbouring AMF communities with multiple species.

Presumably contained part of the complex microflora of field-soil were analysed for the presence or absence of these species. Amplicons produced by the six specific primers indicate that the new primers were able to detect up to five AMF taxa that simultaneously colonised the same root fraction (Figure A.4).

By sequencing multiple recombinant clones per cloned PCR product many different sequence variants for each of the six AMF morpho-species were found, while at least one sequence variant was found several times. The most common sequence variant (GenBank accession AY649957) from the population of *G. mosseae* corresponded with sequences from spores that originated from five different single spore isolates. This finding indicates that sequences found in spores of trap cultures were also present in intraradical hyphae in the field.

In general, presence of environmental material did not interfere with specificity of primers for target species. PCR amplicons from *G. eburneum*-like (f4/r2) and *S. pellucida* (f5 / r3) showed no additional bands, and also no unexpected sequences were found in the five PCR products that were vector-cloned.

Primer application on environmental samples from crude root-DNA extracts of *Trifolium repens*: Ethidium bromide stained agarose gel (2% w/v in 1 x TBE) showing PCR products of nested amplifications by six pairs of species-discriminating primers (indicated above lanes). The first lane represents the products of the initial general fungal specific amplification (LR1/FLR2) from a DNA extract of field-grown roots, of which the inlet shows magnified, multiple DNA fragments that originate from the different species present. Negative controls contained no PCR products. * indicates PCR products derived from the same root DNA extract.
In a few cases involving the other four specific primers non-specific amplification products were detected as additional bands on agarose gels. In 13 cases (7.7%) co-amplified DNA fragments of a different size were resolved; in one case the f1/r1 (*G. mosseae*), in nine cases the f3/r1 (*G. intraradices*-like) and in three cases the f6/r1 (*A. paulinae*) primer pair was involved. Furthermore, after vector-cloning one selected PCR product from the f2/r1 primer pair (*G. claroideum*) contained non-target sequences, but with glomalean affiliation, even though a single band was present on the agarose gel. The proportion of recovered non-specific sequences for the four AMF species was: *G. mosseae* (62/775 = 8.0%, 67 cloned amplicons), *G. claroideum* (4/25 = 16.0%, two cloned amplicons), *G. intraradices*-like (38/42 = 90.5%, 4 cloned amplicons) and *A. paulinae* (18/38 = 47.4%, 4 cloned amplicons). Non-specific sequences had affiliations to basidiomycetous yeasts or other AMF species in BLAST searches. Definition of specific and non-specific sequences for *G. intraradices* was difficult, because many sequences amplified by the f3/r1 primer pair had closest matches to GenBank accessions ascribed to the SSU gene-phylogroup A (Schüssler et al., 2001). If these sequences were also considered as target sequences, the proportion of non-specific sequences would be reduced to 13/42 (31.0%).

A.4.6 PCR-SSCP Analysis

The sizes of the DNA fragments that were amplified with the newly designed primers lie in the optimal range for SSCP analyses (Orita et al., 1989; Hayashi, 1991; Yap & McGee, 1993). We performed PCR-SSCP analysis on vector-cloned nested PCR products, produced from root DNA extracts of corresponding single spore cultures, for *G. mosseae* (Figure A.5), *G. intraradices*-like and *G. eburneum*-like (data not shown). Considerable diversity in banding patterns suggested that nucleotide sequence polymorphism were present within the amplified DNA fragments of these species. Given that multiple sequence haplotypes were used for designing the primers, sequence diversity should be expected and was confirmed by sequencing recombinant plasmids, responsible for different banding patterns (not all data shown). Sequence differences were characterised by

![Figure A.5](https://example.com/image.png) Application of the f1/r1 primer pair to *Glomus mosseae* using PCR-single strand conformation polymorphism (PCR-SSCP) analysis: Silver stained MDE™ gel (0.75 x v/v) showing SSCP of 221 bp PCR fragments from the 5’ end of the nuclear ribosomal large subunit RNA gene. These variable banding patterns of denatured PCR products were taken from a 64 lane-gel. PCR was applied in two rounds using fungal-specific primers (LR1/FLR2) and nesting, specific primers (f1/r1) on genomic root-DNA extracts of *Plantago lanceolata* grown as host of a single spore pot culture (*G. mosseae* isolate no. 130). Amplified DNA fragments were vector-cloned and recombinant plasmids used as templates for the PCR products responsible for the polymorphisms presented in lanes 1-6. The sequence of the DNA fragment resolved in lane 5 differs from all the others in one nucleotide, while sequences of all other pairs of SSCP differ in two nucleotides. Faint bands accompanying the two strong bands may be interpreted as alternative, metastable conformations, probably formed due to overloading. Faster running heteroduplices are not shown.
point mutations in otherwise equally sized fragments. Illustrated in Figure A.5 are results of a single spore isolate of *G. mosseae* (no. 130) with six obviously different banding patterns, caused by nucleotide differences of the 221 bp long DNA fragments.

**A.5 DISCUSSION**

**A.5.1 SIX NEW SPECIES-Discriminating PCR PRIMERS**

In this article we report on six pairs of newly designed PCR primers that specifically target the domain D1 of the nrRNA LSU gene of the mycorrhizal fungal morpho-species *G. mosseae*, *G. claroideum*, *G. intraradices*-like, *G. eburneum*-like, *A. paulinae* and *S. pellucida*. Control PCR reactions showed that no cross amplification occurred in the presence of recombinant plasmids containing sequence types of other morphospecies, nor in the presence of multispore DNA extracts of different species, nor in the presence of crude DNA extracts from field-grown roots where DNA of different AMF species was present.

The variable domain D1 at the 5’ end of the nrRNA LSU gene was already successfully used for the design of primers that discriminate between AMF morpho-species (van Tuinen et al., 1998a; Kjoller & Rosendahl, 2000; Turnau et al., 2001; Jansa et al., 2003). In previous field studies on AMF, three consecutive PCR reactions were performed using primers that were specific first to eukaryotic DNA, then to fungal DNA and finally to DNA of AMF (Turnau et al., 2001; Jansa et al., 2003). This procedure includes a high risk for PCR errors and formation of artefacts that occur particularly when DNA templates consist of mixtures of different sequence types (Wagner et al., 1994; von Wintzingerode et al., 1997; Speksnijder et al., 2001). In our approach only two PCR reactions are required, resulting in a reduced risk for these artefacts.

**A.5.2 IMPLICATIONS OF NUCLEAR RIBOSOMAL SEQUENCE DIVERSITY FOR PRIMER DESIGN**

Ribosomal sequence diversity of AMF has been studied extensively (Sanders et al., 1995; Clapp et al., 2001; Kuhn et al., 2001; Pawlowska & Taylor, 2004; Rodriguez et al., 2004). In all these cited publications it has been shown that nrDNA sequences not only vary among but also within species and even within single spores. Our analysis of the sequences present in spores derived from trap cultures and single spore cultures of a single field site confirms these findings and emphasises that intraspecific genetic diversity has to be taken into account for the design of primers.

We compared the sequences of previously published primers having annealing sites within the region covered by LR1/FLR2 with our collection of sequences. Presently, there are several specific primers available that target the common morpho-species *G. mosseae*, 5.21 / NDL22 (van Tuinen et al., 1998a), Cal1/Cal2 (Jansa et al., 2003), and GlAa85For / GlAa387Rev (Geue & Hock, 2004) (Figure A.1). We found that the forward primer 5.21 of van Tuinen et al. (1998a) for *G. mosseae* matched only with less abundant sequences (6 of 35), whereas the forward primer LSU4f of Kjoller & Rosendahl (2000), specific to the nrRNA SSU gene-phylogroup A (Schüssler et al., 2001), matched to our sequences from *G. mosseae* and
The corresponding reverse primer LSU7r did not match to sequences of *G. mosseae* and *G. intraradices*, nor to any other of our six AMF morpho-species. The forward primer 38.21 of Turnau *et al.* (2001) for *G. claroideum* matched only to sequences of minor abundance (11 of 39). However, we found a match to *G. claroideum*-like sequences (16 of 17) that we obtained from five young single spores of *E. infrequens*. Spores of *E. infrequens* were derived from trap cultures in which assemblages of several AMF species were present. Thus, parasitation of spores by a specific strain of *G. claroideum* may have occurred, which is the most likely explanation for this strange finding. There was no match of the forward primer A2R of Geue and Hock (2004) with sequences of *G. mosseae*, nor with sequences of *G. eburneum*-like or of *S. pellucida*, indicating that this primer may not be suitable as a universal eukaryotic primer.

Our primer design is based on the most abundant sequence types in each of the six AMF morpho-species. Thus, primers can be considered specific to a certain group of sequences, but may not cover the total sequence diversity that is present in a morpho-species. By designing primers based on the most frequent sequence types, we intended to reach high sensitivity and aimed at amplifying the most frequent and thus biologically relevant individuals of morpho-species present at our field site. It is possible, however, that at other field sites populations differ and show other abundant sequence types. Supporting this hypothesis, many previously published taxon-specific primers only matched with rare sequence types of our collection of AMF sequences, emphasizing that specific primers might need to be adjusted in each case to the local AMF populations.

### A.5.3 Application of species-discriminating PCR primers in ecological field studies of AMF

Molecular ecological studies on AMF rely on the identification of individuals of species. Formerly, research in this field has been inhibited because AMF species cannot readily be distinguished within roots using morphological features. With the use of molecular tools this problem could be overcome, although AMF community analysis still remains difficult, due to the great sequence diversity that is present within a species (Kuhn *et al.*, 2001; Pawlowska & Taylor, 2004). Further complication arises from co-occurring and unknown taxa in environmental samples, such as root-DNA extracts. We recorded unintended co-amplification of basidiomycetous yeasts in several of the root samples, a finding which is not unusual in studies on AMF (Renker *et al.*, 2004). However, the rarity of cases in which our primers did not show absolute specificity and in which target sequences were present anyway, suggest that AMF community analysis was not impaired.

The different sizes of the amplified DNA fragments (Table A.1 & Figure A.4) may be used in PCR-assays as markers to detect the six AMF species in field-root samples via fragment length polymorphism analysis. Nucleotide polymorphisms resolved by PCR-SSCP analysis suggest that the new PCR primers could also be useful at the intra-morphospecies level (Figure A.5). Both, fragment length polymorphism and PCR-SSCP analysis, using dye-labelled
primers, are much facilitated by automated capillary electrophoresis (Kozlowski & Krzyzosiak, 2001; Lento et al., 2003). This new technology shows high sensitivity and reproducibility, which could make nested-PCR and vector-cloning superfluous, and thus will reduce time needed and the risk for methodological artefacts. The size range of the DNA fragments amplified by our primers is ideal for both of these applications.

Many AMF species remain to be molecularly analysed, as it is evident from lacking sequence data available in public data bases for more than half of the described AMF morphospecies. Here we were able for the first time to design primers specific to *G. eburneum*-like and *A. paulinae*, two morpho-species that have so far rarely been studied, because it is difficult to detect them, using traditional methods. The intraradical hyphae of *G. eburneum*-like did not stain using standard histological staining techniques (personal observation) and *A. paulinae* forms small hyaline spores.

**A.6 CONCLUSIONS**

We describe the design and specificity of PCR primers which can be used to discriminate among six morpho-species of AMF within roots (*G. mosseae*, *G. claroideum*, *G. intraradices*-like, *G. eburneum*-like, *A. paulinae*, *S. pellucida*). In order to detect the biologically relevant strains of a species with high sensitivity primers were designed for the most abundant sequence types of the nrRNA LSU gene. Differences among AMF populations at different field sites may explain the poor match of previously published primers with our sequences. Therefore, we suggest that in molecular ecological studies primers should be adjusted to the local AMF populations present at a particular field site.

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EXPERIENCE

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       • Introduction of new molecular genetic techniques and new methods in mycorrhizal 
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PUBLICATIONS


BOOK REVIEW


INVITED SEMINARS

2002  Gamper, H. Arbuscular mycorrhizal fungi (AMF) from the Swiss Free-Air CO₂ Enrichment (FACE) experiment: ecological and genetic aspects. Mycological Colloquium, Institute of Microbiology, Swiss Federal Institute of Technology Zürich, Switzerland.

2002  Gamper, H. & Leuchtmann, A. Molecular diversity of some arbuscular mycorrhizal fungi in the FACE experiment and hypotheses and plans. First international FACE workshop on the Swiss FACE-experiment, Institute of Plant Sciences, Swiss Federal Institute of Technology Zürich, Eschikon, Switzerland.

POSTERS PRESENTED AT MEETINGS

2001  Gamper, H. & Leuchtmann, A. Elevated CO₂ and the mycorrhizal symbiosis: A molecular genetic field study and a physiological climate chamber experiment. Symposium of the Plant Science Center, Zürich, Switzerland.

2002  Gamper, H. & Leuchtmann, A. Molecular diversity of arbuscular mycorrhizal fungi from a well fertilized temperate grassland ecosystem. biology02: Annual Symposium of the Swiss Zoological, Botanical and Mycological Societies, Bern, Switzerland.


2003  Gamper, H. & Leuchtmann, A. Do arbuscular mycorrhizal fungi harbour non-functional rDNA units? biology03: Annual Symposium of the Swiss Zoological, Botanical and Mycological Societies, Zürich, Switzerland.