Mycorrhizal symbioses of *Salix repens*: diversity and functional significance.

Promotor: Dr. L. Brussaard
Hoogleraar in de Bodembiologie en Biologische Bodemkwaliteit

Co-promotor: Dr. Th. W. Kuyper
Universitair hoofddocent, sectie Bodemkunde en Plantenvoeding

Elisabeth Wilhelmina van der Heijden

# Mycorrhizal symbioses of *Salix repens*: diversity and functional significance.

Proefschrift

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Voor mijn ouders

## STELLINGEN

1. Het gebruik van nutrient inflow per wortellengte als maat voor efficientie van het wortelsysteem gaat niet alleen voorbij aan de betekenis van fourageren door mycorrhizahyfen, maar vooral aan het feit dat verschillende mycorrhizaschimmels wortellengte op een verschillende manier beïnvloeden. *Dit proefschrift*

2. Het gebruik van bovengrondse plantenbiomassa voor de berekening van 'mycorrhizal responsiveness', miskent de multifunctionaliteit van verschillende mycorrhizaschimmels voor de plant. *Dit proefschrift*

3. Om het effect van mycorrhiza (en combinaties van mycorrhizas) op respons van de plant te kunnen vaststellen dient allereerst de eenheid van mycorrhiza te worden opgehelderd. *Dit proefschrift*

4. De bewering dat ectomycorrhiza arbusculaire mycorrhiza op hetzelfde wortelsysteem wegconcurreert is veel gekopieerd maar nog nooit aangetoond.

5. Voor het vaststellen van een negatieve interactie tussen soorten vormen statische gegevens geen basis.

6. Het ongeloof van mycorrhiza-onderzoekers in een groot effect van lage mycorrhiza-kolonisatie is ongelooflijk.

7. Experimenten waarin effecten van een groot aantal schimmels op de respons van de plant worden getest resulteren in onooglijke figuren. *Dit proefschrift*

8. De afweging tussen konijnenvraat of schade door geïnteresseerde 'collega' biologen in proefterreinen op Terschelling zal veelal resulteren in het niet plaatsen van rasters.

9. Wetenschappelijk onderzoek naar effecten van afplaggen in grove-dennenbossen, resulterend in herstel van de ectomycorrhizaflora, is nodig om oude Drentse volksgebruiken zoals 'het aanharken van dennenbossen om meer Cantharellen te krijgen' in ere te herstellen (sinds 50 jaar verboden en nog steeds het geval). *Timmerman in Drenthe; J. Baar, Ectomycorrhizal fungi of Scots pine as affected by litter and humus, 1995*

10. Promovendi en mycorrhizas vertonen beide conditionaliteit; hun functioneren is afhankelijk van de omgeving waarin zij moeten presteren.

11. Als men onder 'ervaring in het buitenland' verstaat het onderzoek verrichten onder niet Nederlandse omstandigheden, andere taal en gebruiken, kan deze uitstekend worden opgedaan in Drenthe.

12. Als je bereid bent in geval van levensnood een donororgaan te accepteren, moet je zeker bereid zijn om, ook na je eigen overlijden, je organen voor donatie af te staan.

13. Mobiel telefoneren is een vorm van incontinentie. *Midas Dekkers*

14. Juist in de fietsprovincie Drenthe worden de meeste fietskilometers achter op de auto afgelegd.

Stellingen behorende bij het proefschrift
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Liesbeth van der Heijden
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## ABSTRACT

E.W. van der Heijden.

**Mycorrhizal symbioses of *Salix repens*: diversity and functional significance.**

This thesis investigates the significance of different mycorrhizal fungi, belonging to different functional types (arbuscular mycorrhiza-AM and ectomycorrhiza-EcM), in *Salix repens*. A comparison between above-ground and below-ground observations on ectomycorrhizal fungi (EcMF) indicated that neither diversity nor abundance above-ground can be used to estimate below-ground diversity or abundance. In all habitats *S. repens* was highly EcM and slightly AM. Low colonization by arbuscular mycorrhizal fungi (AMF) reflects plant control over mycorrhizal colonization. Arbuscular mycorrhizal colonization was higher in spring than in other seasons, coinciding with higher above-ground P concentration. In sequential inoculations, in the short term AMF interfered with EcMF but the reverse was not observed, whereas in simultaneous inoculations no mutual suppression was observed. Mobilization of plant defence reactions by AMF was transient and led to co-existence between AMF and EcMF. Plant response to AMF, but not of EcMF, depended on internal shoot phosphorus concentration. None of the eleven EcMF investigated exerted the full range of mycorrhizal benefits. Two strategies of EcM were recognized, viz. root manipulation and root replacement. Magnitude of plant response and amount of mycorrhizal colonization were not correlated. Mycorrhizal benefits showed conditionality with regard to pH and nitrogen-to-phosphorus ratio. Genetic variation of *S. repens* had a large effect on the symbiosis; there was also a large plant origin × soil type interaction. Fungal species origin had only a minor effect on symbiotic effectiveness. After waterlogging intensity of AM colonization was increased and EcMF colonization was decreased. Both changes reflect the differential expression of survival strategies. High mycorrhizal fungal diversity, both in taxonomic and in functional terms is both determining and determined by the wide ecological amplitude of *S. repens*.



**KEY WORDS**: ectomycorrhiza, arbuscular mycorrhiza, diversity, mycorrhizal interactions, multifunctionality, conditionality, dune ecosystems, *Salix repens*.

## VOORWOORD

Anderhalf jaar na aanvang van mijn promotieonderzoek had niemand meer gedacht dat het nog iets zou worden, maar zie hier dan toch.... het proefschrift is klaar. De personen die een belangrijke rol in het tot stand komen van dit proefschrift hebben gespeeld wil ik nu graag met name noemen.

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Mam, zoals je ziet, ik ben niet vergeten mijn resultaten ook óp te schrijven!

## CONTENTS

# 1

# GENERAL INTRODUCTION

## MYCORRHIZAL ASSOCIATIONS

### *General definition*

Mycorrhizas are intimate associations between fungi and plant roots. Such an association is generally considered as a mutualistic symbiosis. The benefit for the fungus is the receipt of carbohydrates from the plant, and the fungus increases resource availability and nutrient uptake of the plant in various ways. Besides foraging for nutrients in the soil, several mycorrhizal fungi produce enzymes capable of breaking down organic carbon, nitrogen and phosphorus, and other mycorrhizal fungi are capable of weathering minerals. Mycorrhizal fungi can also alleviate abiotic (heavy metals) and biotic (pathogens) stress. By increased resource availability and alleviation of stress mycorrhizas alter plant growth and fitness. Plant and fungus co-exist over their respective life-cycles as both have no or only limited ability for independent existence under natural conditions (Smith & Read, 1997).

### *Modern insights*

Despite its land-plant life time existence, the term mycorrhiza lacks a clear definition. De Bary (1887) believed that there was some degree of common life, i.e. of the symbiosis, in almost all associations between plants and fungi. The associations are often classified as biotrophic or necrotrophic. There is, however, a great range of behaviour between these two extremes, not only between the different types of association but also at different times or under different environmental conditions in the same association. These variations in function, sometimes apparent in changes in structural relationships between the symbionts, are also seen in mycorrhizal associations. Furthermore, the identity of the symbionts may have major or minor influences on the structure and function of mycorrhizas. One of the important features in current research on mycorrhizas is the recognition of the considerable diversity of structure and function of mycorrhizas. There is an immense diversity of what we call mycorrhizas. The description, in structural,

developmental and physiological terms, of this diversity is likely to be significant for the understanding of its importance in ecosystems.

Assessment of **mycorrhizal benefit** is by no means straightforward. The ultimate criterion should be the increased fitness of the mycorrhizal plant compared to the non-mycorrhizal plant. However, direct assessment of fitness is usually replaced by parameters such as (above-ground) performance or growth. This should be expressed in currencies of either carbon, nitrogen or phosphorus. Further benefits, which may not be expressed as increased growth, may also occur, e.g. changes in root architecture or increased pathogen resistance. Although mycorrhizal benefit or responsiveness (formerly called mycorrhizal dependency, Plenchette *et al.*, 1983) may be expressed as a single value, it should not be forgotten that benefits as expressed in various currencies do not necessarily correlate. Such differential effects gave rise to the concept of **multifunctionality** (Newsham *et al.*, 1995b) of the mycorrhizal symbiosis. Basically, multifunctionality includes three different aspects, viz. (1) different fungi show different benefits to the same plant under the same environmental conditions; (2) the same fungus shows differential benefits to different plants under the same environmental conditions; (3) the same fungus shows differential benefits to the same plant under different environmental conditions (conditionality). These aspects of multifunctionality will be treated separately in this thesis.

The search for general principles in mycorrhizal functioning resulted in the **standard model of mycorrhizal functioning**. This standard model states that (1) all mycorrhizal symbioses function along similar lines; (2) the main effect of the symbiosis is an extension of the root system resulting in increased uptake of immobile ions such as phosphorus; (3) all other benefits from the symbiosis derive from an improved phosphorus-status of the plant (Tinker *et al.*, 1992). This standard model is at variance with the concept of multifunctionality. Under the standard model, mycorrhizal benefit for different plants and **efficiency** of various mycorrhizal fungi can be compared on the basis of phosphorus inflow rates per unit root length. However, under the concept of multifunctionality, changes in root architecture as affected by mycorrhizal fungi should be included in assessment of mycorrhizal efficiency. If two mycorrhizal fungi are compared, of which one increases root length and the other increases uptake without affecting root length, the paradoxical result may occur that mycorrhizal efficiency (P-inflow per unit root length) will be higher for the second fungus, even though P-uptake might be higher for the first fungus. This theme will further be explored in CHAPTERS 5 AND 6 where I compare **mycorrhizal efficiency** (P-inflow per unit root length) with **mycorrhizal effectiveness**

(based on total uptake rates) and moreover, in CHAPTER 6 I use this difference to review strategies of different mycorrhizal fungi.

As this discussion implies, mycorrhizal benefit is usually viewed from a **phytocentric** perspective. As the mycorrhizal symbiosis shows conditionality (relative benefit for both partners depends on the interaction with environmental conditions), the **mycocentric** perspective should not be out of mind. Maximizing plant fitness does not automatically imply maximizing fungal fitness (Johnson *et al.*, 1997 consider this relation along a continuum from mutualism to parasitism), and questions to what extent the amount of fungal colonization is under **plant** or **fungal control** are highly relevant.

## KINDS OF MYCORRHIZAL SYMBIOSES

### *Arbuscular mycorrhiza*

The most widespread type is the arbuscular mycorrhiza (AM), seen in the early land plants, and today occurring in the majority of herbaceous and graminaceous species of temperate and semi-arid grassland ecosystems as well as in many tree species of tropical and subtropical forests. In AM mycorrhizas, an internal mycelial phase with characteristic structures is present. Commonly observed internal structures are: vesicles, arbuscules, hyphal coils, and internal hyphae. There are problems in extrapolating from structure to function in what now appears to be a structurally and possibly also functionally diverse symbiosis. The term AM covers a diversity of mycorrhizal structures. Arbuscular mycorrhizal development differs, not only over time, but also between plant species, especially with respect to the extent of development of vesicles, coils and arbuscules within the cortical cells. The external phase is important as well, made up of branched single hyphae that ramify through the soil, forming anastomosing networks (Read *et al.*, 1985).

Studies of the rhizomes of *Aglaophyton major* of the early Devonian period show that close associations were formed by the earliest land plants, in which vegetative structures similar to those in present day AM mycorrhizas can be seen (Nicolson, 1975). Since roots of these early land plants were lacking or very poorly developed, exploitation of the soil environment would be inefficient and mycelial systems growing from their carbon sources into soil would provide a significant improvement of absorptive efficiency, so providing a truly mutualistic relationship.

AM fungi (AMF) are a monophyletic group of fungi with unique features that create, ecologically, a functional group of organisms. The AM association evolved only

once with several (putative) independent losses in so-called non-mycorrhizal plants. Support for the thesis that the evolution of land plants coincided with the evolution of the AM symbiosis comes from a molecular phylogeny of the Glomales, the fungi forming the AM mycorrhizas (Simon *et al.*, 1993). A molecular clock dates the origin of the Glomales between 350 and 460 million years ago, contemporaneous with the earliest fossils of land plants (435 Million years ago) and only slightly earlier than the oldest AM fossils (395 Million years ago). These fungi appear to have changed little over 400 million years despite the myriad of changes in plants since those that first invaded the land. This type of root-fungus association was clearly favoured by selection since most individuals in present day natural plant communities are mycorrhizal. This means that ancestors of all land plants had the potential to form arbuscular mycorrhizas, and those that do not nowadays have either lost or suppressed the genes involved (Fitter & Moyersoen, 1996). Evidence that the genes are not lost in many cases comes from the occurrence of AMF in plants that are normally non-mycorrhizal (Tester *et al.*, 1987) or supposedly ectomycorrhizal (Cázares & Trappe, 1993; Cázares & Smith, 1996; Moyersoen & Fitter, 1999).

AM involve a very wide range of plants and a small group of fungi in the Glomales (Zygomycotina). Worldwide there are about 150-160 different species of AMF. However, the AMF species diversity might be underestimated as the group is supposed to be truly asexual and only a limited number of spore characteristics are available for species recognition. Some AMF morphospecies may be species complexes that are similar in morphology but dissimilar in physiology (Walker, 1992). Within one species genetic diversity exists, which may be related to physiological diversity (Clapp *et al.* 1995; Sanders *et al.*, 1996).

*Ectomycorrhiza*

In contrast, the dominant trees of boreal and temperate forest zones have distinct ectomycorrhizas (EcM) formed largely by basidiomycetes. Their vegetative mycelium is predominantly external with a sheath around lateral roots. Hyphae also penetrate inwards between the cells of the root to form a complex intercellular system that appears as a network of hyphae in cross section, called a Hartig net. There is little or no intracellular penetration. The EcM root tips frequently have an extensive mycelial phase made up of strands that spread for considerable distances through soil. It is likely that there are several kinds of species-specific and probably ecologically and physiologically important contacts between mantle structures, rhizomorphs and soil. The internal organization of the mantle

structures and rhizomorphs with respect to hyphal differentiation and with respect to their physical relation can reveal some hints for their function (Agerer, 1992).

The functional significance of EcM fungi (EcMF) for the plant is predominantly an increase in both N and P uptake (Read, 1991). However, again the concept of multifunctionality is appropriate as the EcM symbiosis arose repeatedly. Fitter & Moyersoen (1996) stated that the propensity to form the EcM symbiosis evolved at least twice and illustrated a seed plant phylogeny in which EcM evolved at least three times. Modern plant phylogenies (e.g. APG, 1998; Doyle, 1998) and phylogenies of the higher fungi (Hibbett *et al.*, 1997; Bruns *et al.*, 1998) indicate a substantially higher number for the independent origins of the ectomycorrhizal symbiosis; at least 12 times for higher plants and 17 times for fungi. Worldwide 5,400 species of EcMF have been described (Molina *et al.*, 1992) and recent estimates (Brussaard *et al.*, 1997) suggest that there might well be 10,000 species of ectomycorrhizal fungi.

*Dual mycorrhiza*

Plants that show associations with both AMF and EcMF are called dual mycorrhizal plant species. Well documented examples of dual mycorrhizal tree species are *Alnus* (Molina *et al.*, 1994), *Salix* (Fontana, 1962; Lodge, 1989), *Populus* (Vozzo & Hacskaylo, 1974; Lodge, 1989) and *Eucalyptus* (Lapeyrie & Chilvers, 1985). In these dual mycorrhizal (AM/EcM) plants field observations have sometimes shown AM to be dominant, sometimes EcM (Lodge, 1989). But it is not clear which factors favour dominance by AM or EcM. The definition of dual mycorrhiza is based on both AM and EcM **structures** in the root system. However, the interesting question is whether dual mycorrhiza is a **functionally** mutualistic relationship. Does the plant benefit from having both associations or are higher costs involved? This question is connected with the earlier mentioned problem of plant and fungal control over mycorrhizal colonization.

The apparent dual mycorrhizal stage of these plants might be explained by lack-of-resistance (decreased **plant control**). AMF colonize seedlings due to their supposed initial higher inoculum potential (Read *et al.*, 1977; Chilvers *et al.*, 1987). However, they are rapidly replaced by EcMF (Chilvers *et al.*, 1987; Lodge, 1989). Chilvers *et al.* (1987) and Lodge (1989) have suggested that such replacements might result from EcMF preventing colonization of newly-formed roots by AMF. The EcM sheath would provide a barrier to subsequent AM infections. Pines and tropical EcM tree species have shown low AM colonization in the seedling (sapling) stage (Cázares & Smith 1996; Moyersoen & Fitter

1999). This is consistent with the lack-of-resistance hypothesis if EcM plants primitively retain the genes that are involved in the AM symbiosis.

A second explanation is niche differentiation (increased **plant benefit**). Lodge (1989) noted that many dual mycorrhizal plant species typically grow in flood plains, and suggested that plants with the ability to form both types of mycorrhiza might have a selective advantage in such habitats. She observed dominance by AM under dry and wet conditions, while in moist, but well drained conditions EcM dominated.

In dual mycorrhizal plants **interactions** between EcMF and AMF occur on the same root system, as both might compete for space. Such competitive interactions might be especially important if the plant has only limited control over the colonization. Based on observations on field-collected roots, Chilvers *et al.* (1987), Lodge & Wentworth (1990) and Dhillion (1994) concluded that EcMF were competitively superior, possibly because the sheath provides a barrier to AMF. Coexistence between both mycorrhizal types could still be possible, if AMF have a higher colonizing ability of newly formed root (= higher inoculum potential) or if AMF could invade the sheath. However, static data are inadequate to address questions on competitive interactions. Time course studies of simultaneously and sequentially inoculated plants are necessary to understand their interaction (CHAPTER 4).

### MYCORRHIZAL FUNGAL DIVERSITY AND COMMUNITY STRUCTURE

The diversity of mycorrhizal fungi does not follow patterns of plant diversity. In general, AM plant communities tend to be high in plant but low in fungal species richness, while EcM communities are low in plant but high in fungal richness (Connell & Lowman, 1989; Allen *et al.*, 1995). This statement is biased by the fact that only 150 species of AMF have been recognized (Schenk & Perez, 1990) but more than 5,400 species of EcMF (Molina *et al.*, 1992).

The distribution of mycorrhizal types over the various terrestrial biomes is not random. Read (1991) noted a pattern with the natural climax of arctic (and alpine) biomes being dominated by ericoid mycorrhizal plants, boreal and temperate biomes by ectomycorrhizal plants, and (sub)tropical biomes by arbuscular mycorrhizal plants. Within biomes the occurrence of EcM and AM plants is also not random. In the temperate regions EcM trees occur on acidic soils where litter accumulates and a mor humus profile develops, whereas AM trees develop on the most fertile, neutral soils where litter is easily degraded and a mull humus profile develops. Within succession series a replacement of

vegetation types with different mycorrhizal types can occur. This has been described by Read (1989) for dune ecosystems. He mentioned the importance of mycorrhizas in the sand dune ecosystem. In the dry foredunes, where phosphorus is the main growth limiting nutrient, AM is supposed to predominate. In contrast, in the dune slacks, where accumulation of organic matter occurs, nitrogen mineralization is inhibited due to pH reduction, therefore nitrogen becomes the main growth limiting element and EcM prevails, with its ability to take up nitrogen. As the relative availability of nitrogen and phosphorus is changing with succession in the dune ecosystem, the occurrence of both mycorrhizal types can change as well. The different successional stages are, therefore, characterized not only by a typical nutrient status, but also by a dominant mycorrhizal type.

EcMF are relatively selective of host plant species, while AMF tend to be generalists. However, AMF have shown differences in relative abundances in the rhizospheres of different plant species (McGonigle & Fitter, 1990; Johnson *et al.*, 1992; Bever *et al.*, 1996).

For both EcMF and AMF species richness reported depends upon the size of the area sampled, the length of sampling time, season sampled, and yearly variation in precipitation and temperature. Species counts are only a beginning to understanding diversity patterns. An examination of fungal community relationships with the host plant community is necessary to understand how the fungi respond to both the environment and the host plant. The AM community and individual species of AMF can be determined by local environmental conditions (Allen *et al.*, 1995).

Ectomycorrhizal fungal species diversity can be based on resource partitioning, disturbance, competition or interaction with other organisms (Bruns, 1995). Mycorrhizal fungi compete for two general classes of resources: host-derived carbon and soil or detritus derived mineral nutrients. Both types of resources are variable in space (soil depth, distance from tree) and time (season, host successional series). It is important to understand local diversity because the niche and guild structure among species can reveal the functional significance of EcM diversity.

Seasons impose a set of time gradients in which there is variation in important physical parameters such as temperature, moisture, and nutrient release, and important biological parameters such as activity and abundance of other soil organisms. Fruiting patterns of some (AMF and) EcMF are certainly seasonal, and although it is not known how fruiting and mycelial activity relate, it is obvious that EcMF respond to seasonal cues differently. In most forests production of new roots is likely to occur in more than one

season. Colonization of new roots should be dependent on which fungi (both AMF and EcMF) are active or responsive when susceptible roots are produced.

Ectomycorrhizal fungal species composition changes with ageing of trees, forests and forest soils. Some fungi mainly occur in young, others in older stands (Termorshuizen, 1991; Keizer & Arnolds, 1994; Baar, 1995). Species richness of EcM fungi increases with stand age until canopy closure is reached and litter begins to accumulate, thereafter it decreases (Dighton & Mason, 1985; Jansen, 1991).

Deacon *et al.* (1983) recognized two different groups or guilds of EcMF that have been termed early-stage and late-stage. Early-stage corresponds to a ruderal strategy, while late-stage corresponds to stress-tolerant or combative strategies (Deacon & Fleming, 1992).

There is evidence that at least some EcMF have ability to extract nutrients directly from plant litter and that many relevant enzymatic capabilities vary among EcMF species (Smith & Read, 1997). It follows from these observations that fungi could partition resources by their differential ability to extract them from litter. The differential ability to use various organic nitrogen resources seems particularly important, since nitrogen is most frequently limiting in terrestrial ecosystems and scavenging of organic nitrogen may be the most significant ecosystem role for EcMF (Read, 1991). Abuzinadah & Read (1986) recognized two general classes (with some intermediates) that they termed non-protein fungi and protein fungi based on the ability to use protein as a nitrogen source. While non-protein fungi may be successful in the mineral soil it is likely that with increasing organic matter content selection for fungi with peptidolytic and proteolytic capability occurs.

Another classification of strategies of EcMF, which comes closer to the original concept of r- and K-selection (early- and late stage), has been proposed by Newton (1992). In his epidemiological classification emphasis is put on the relative roles of fungal spores and hyphal networks as dispersal agents. The three classifications of fungal strategies show substantial correlation and, like the classical r-K continuum, predict similar changes to occur during succession after disturbance.

Strategies of AMF have received less attention, but the data would be consistent with strategies where inoculum is mainly determined by spores and colonized root fragments or mainly by intact hyphal networks.

## *Salix repens* AND ITS MYCORRHIZAL ASSOCIATES

*Salix repens* L. is a member of the Salicaceae and is the smallest Dutch *Salix* species (Weeda *et al.*, 1985). While tree height of other *Salix* species is correlated with their age, *Salix repens* is an exception and forms shrubs with heights ranging from 10 cm to 1.5 m. *Salix* species contain male and female plants. The majority of the *Salix* species depend on very wet/moist locations, whereas *S. repens* is also able to grow on very dry locations. Due to their preference for high light conditions, *Salix* species can not compete successfully with other tree species. They are bound to grow on soils, or under climatic conditions, which are too hard for other tree species, viz. arctic and alpine areas, primary dune ecosystems, or regularly flooded sites (Weeda *et al.*, 1985).

*Salix repens* is ecologically different from other Dutch *Salix* species. In wetlands, *S. repens* sometimes co-exists with *S. cinerea* L. and *S. aurita* L., but mainly forms uniform stands. *Salix repens* flowers in spring and its seeds germinate under very wet conditions. After establishment it persists by vegetative growth and spreads to other areas (Weeda *et al.*, 1985).

*Salix repens* has a wide geographical distribution. According to Rechinger (1964) three subspecies can be recognized, of which two occur in western Europe, viz. subsp. *repens* in inland locations and subsp. *argentea (arenaria)* in coastal ecosystems (Oberdorfer, 1994). The limits between both subspecies, however, are very fuzzy (Weeda *et al.*, 1985).

*Salix repens* has a very wide ecological amplitude, occurring from dry to wet, and calcareous to acidic soils. Therefore, a rich diversity of the co-dominant vegetation along the dune successional gradient in *S. repens* shrub is observed (Weeda *et al.*, 1985; Westhoff & Van Oosten, 1991), and similarly over this gradient the EcM and saprotrophic fungal flora in the various vegetation types is quite diverse (Kuyper *et al.*, 1994). Inland *S. repens* communities have a poorer understorey vegetation and less diverse EcM flora. There is, however, no current knowledge on the arbuscular mycorrhizal status (amount of root colonization and AMF species) of *S. repens* in The Netherlands.

For several reasons *Salix repens* has been chosen as model organism in this study. *Salix repens* has great advantages over the mostly used conifers. Favourable qualities of *Salix repens* are: (1) It is able to grow on a variety of soil conditions; it occurs from dry to wet and calcareous to acidic soils; (2) It forms uniform shrubs in which the species dominates and in which it is the only ectomycorrhiza-forming species. In comparison, the amplitude of most trees is narrower and natural homogeneous stands are scarce.

Furthermore, (3) *Salix repens* can form mycorrhizas with a broad range of ectomycorrhizal fungi and, like species of *Populus* and other species of *Salix*, with arbuscular mycorrhizal fungi as well, whereas most tree species are thought to form mycorrhizas of only one kind; and finally (4) it can be multiplied by cuttings. This garantuees genetical homogeneity and relatively strong plants for the experiments compared to seedlings.

However, using cuttings as test material may also complicate the experimental work. Although genetically uniform, the plant material could be physiologically relatively heterogeneous (Ericsson, 1981). Therefore, instead of stem section of the middle part of shoots, shoot tops of one plant were selected, hoping that their physiological state would be similar. Furthermore, the root system is always relatively small initially, i.e. the plants have a (very) high shoot/root ratio. This means that all treatments will show a phase during which the functional equilibrium is readjusted and internal nutrient status is changing because of the developing root systems.

## OUTLINE OF THIS THESIS

With the increasing awareness of the ecological importance of mycorrhizas and their diversity, research must be directed to experiments and surveys that will elucidate quantitative aspects of the distribution of different types and their contribution to the functioning of ecosystems, as opposed to simple records of their occurrence or casual speculation. From an ecological point of view it is important that mycorrhizas increase the tolerance of the host to stress. Effects of mycorrhizal infection are therefore more clear under circumstances of stress, for instance drought or nutrient limitation.

This project concerns a study of the functional significance of different mycorrhizal symbionts of *Salix repens* in relation to survival, growth, biomass allocation and nutrient uptake of the plant under different conditions of pH and availability of nitrogen and phosphorus. The aim of the research is to investigate to what extent the amplitude of *S. repens* is determined by the ecological tolerance of its mycorrhizal symbionts. This relationship is studied in field observations, field experiments, and laboratory experiments. Field observations and experiments each have important advantages. Hypotheses can be generated from results obtained from field observations, and results from laboratory experiments can support understanding of behaviour in the field situation. Therefore, these approaches were combined.

Field observations in 16 *Salix repens* communities in the coastal dunes of the Wadden Isle of Terschelling are presented in CHAPTERS 2 AND 3. The vegetation, soil

characteristics, and the ectomycorrhizal species composition are described in **CHAPTER 2**. A short typology of ectomycorrhizal morphotypes is also provided. Number of EcM species and abundance of sporocarps of EcMF above-ground are related to number of morphotypes and abundance of EcM root tips below-ground. Whereas above-ground studies of fungal species richness and sporocarp abundance last at least three years, below-ground inventories have usually been done only once. In **CHAPTER 3** I provide data on the seasonal dynamics of below-ground diversity and abundance. As *S. repens* is a dual mycorrhizal plant, the arbuscular mycorrhizal community is also described in CHAPTER 3: its species composition, amount of root colonization, and arbuscular mycorrhizal inoculum potential. Correlations between both EcM and AM colonization and (external) soil conditions and (internal) plant nutrient status are calculated.

Observations that *S. repens* is always highly ectomycorrhizal and slightly arbuscular mycorrhizal (CHAPTER 3) lead to questions about the functional importance of both functional types of mycorrhiza and about the ways in which both types interact. Under axenic conditions I determined colonization of two AMF and three EcMF on cuttings of *S. repens* (**CHAPTER 4**). As field data on the amount of colonization give only a static description but do not provide insight in mycorrhizal interactions, I addressed that question explicitly by simultaneous and sequential inoculation of one AMF and one EcMF (different species pairs) under the same conditions. From the plant's perspective, the effects of colonization by one or more mycorrhizal fungi on plant performance are essential. I therefore use data on plant performance (root length, shoot length, shoot biomass, shoot nutrient content) to test initial hypotheses on functional differences between mycorrhizal types (AM versus EcM). In order to be able to relate these observations under axenic conditions to what happens in the field, I repeated this experiment with non-sterile soils from *S. repens* communities with contrasting chemical and physical characteristics both in the lab and in the field (**CHAPTER 8**). An alternative interpretation for dual mycorrhizal colonization (in which previously EcM was supposed to suppress AM) is proposed in **CHAPTER 11**. These experiments did also lead to the question of relative importance of plant and fungal control over mycorrhizal colonization. This question has been investigated both in the lab in a non-sterile field soil and in the field under different moisture conditions (**CHAPTER 10**).

Functional importance of the AM and EcM symbiosis in dual mycorrhizal plants should be derived both from a comparison between functional groups (**CHAPTERS 4 AND 5**) and within functional groups (**CHAPTERS 4 AND 6**). In CHAPTER 6 performance of 11

species of EcMF is determined. As the mycorrhizal symbiosis has been described as multifunctional, a number of parameters have been used to assess its functional importance to the plant: response time, response duration, magnitude of the response both above- and below-ground, changes in foraging behaviour of the plant, and changes in the nutrient pools in the soil. As different mycorrhizal fungi differently affected root length (CHAPTER 6), I question whether it is meaningful to express mycorrhizal benefit as inflow per unit root length and suggest that non-nutritional benefits such as changes in root architecture are more important than hitherto conceived. Expression of multifunctionality is furthermore dependent on soil conditions. For one AMF and two EcMF I therefore have assessed plant performance in a factorial experiment where soil pH and nitrogen/phosphorus ratio have been varied (**CHAPTER 7**).

Genetic variation in both mycorrhizal plant and mycorrhizal fungus could affect their symbiotic effectiveness. This topic is addressed in **CHAPTER 9** where clones of *S. repens* from three provenances were grown on two soils with three different mycorrhizal fungi (one AMF and two EcMF). Similarly, four different isolates from one species of EcMF were grown with one clone of *S. repens*.

The large differences in EcM species composition between different habitats could be due not only to differences in soil characteristics (mainly moisture, pH and N/P ratio), but also to differences in the co-dominant plants that may indirectly affect ectomycorrhizal fungal performance. I determined whether aqueous foliar extracts from a number of co-dominant plants could stimulate or inhibit growth and biomass increment of various EcMF and hence affect EcM community composition (**CHAPTER 12**).

In **CHAPTER 13** the main results are summarized and discussed. Suggestions for further research are given.

2

# Mycorrhizal associations of *Salix repens* L. communities in succession of dune ecosystems. I. Above-ground and below-ground views of ectomycorrhizal fungi in relation to soil chemistry.

**ABSTRACT**

*Diversity of ectomycorrhizal (EcM) communities in 16 stands of* Salix repens *L. growing under a variety of different environmental conditions was studied to assess the possible correspondence between above- and below-ground views of fungal taxa by repeated sampling of EcM sporocarps and ectomycorrhizas. Above- and below-ground views were also related to soil chemistry. On the basis of sporocarps 78 taxa of EcM fungi were found, belonging to 12 genera. The majority of the species found, especially those of the Cortinariaceae, were host-specific for Salicaceae. Canonical Correspondence Analysis (CCA) on the basis of fungal species showed that only pH and the concentration of available phosphorus significantly contributed to the variation explained. CCA on the basis of fungal genera indicated that pH and moisture significantly contributed to the variation explained. Below-ground fifteen different morphotypes were recognized. EcM morphotype composition was different on two sampling dates. CCA on the basis of morphotype composition yielded different results depending on sampling date. Numbers of EcM sporocarps and root tips (at both sampling dates) were not correlated. Diversity above-ground (species and genus diversity) was also not correlated with morphotype diversity below-ground at both sampling dates. Neither diversity nor abundance of EcM fungi above-ground can therefore be used to assess below-ground EcM or abundance. Lack of correlation in below-ground parameters at different sampling dates indicates substantial variation. Causes for temporal variation are discussed. The importance of investigating both above- and below-ground variability in EcM communities is stressed.*

**KEY WORDS:** Sporocarps, ectomycorrhiza, morphotypes, dune ecosystems, *Salix repens*, arbuscular mycorrhiza

## INTRODUCTION

*Salix repens* L. is a common and widespread shrub in western Europe, occurring in a great variety of plant communities, in particular in the coastal dunes of the Wadden Isles. Coastal dune ecosystems, in which *S. repens* occurs, are rich in ectomycorrhizal (EcM) fungi (Courtecuisse, 1984, 1986; Rotheroe, 1993; Watling & Rotheroe, 1989). Moreover, the species is able to form arbuscular mycorrhiza (Harley & Harley, 1987; Read, 1989). On the Dutch Wadden Isle of Terschelling, *S. repens* occurs in a wide variety of habitats, ranging from dry to wet, and from calcareous, humus-poor to acidic, humus-rich soils. In these communities *S. repens* is the only ectomycorrhizal host plant.

Descriptions of the species composition of EcM communities were, until recently, based exclusively on sporocarp surveys. Although above-ground surveys can indicate the presence of an EcM fungus in the soil, its absence cannot indicate the opposite. The question has therefore been raised whether sporocarp production reflects their relative biomass or importance in the soil, and whether diversity above-ground is a reliable estimate of diversity below-ground (Arnolds, 1992; Vogt *et al.*, 1992). Termorshuizen & Schaffers (1989) noted a correlation between the abundance of sporocarps of EcM fungi and the abundance of ectomycorrhizas in Scots pine stands of various ages. Jansen (1991) confirmed that both parameters were correlated in Douglas fir stands of various ages in the Netherlands. She also established a significant positive correlation between the diversity of EcM morphotypes and the diversity of EcM fungi and genera. However, the tacitly assumed relationship between above- and below-ground views has been disproved by Gardes & Bruns (1996), Kårén & Nylund (1997), and Dahlberg *et al.* (1997). In these investigations it was reported that several taxa were common above-ground and rare below-ground and vice versa. In such cases parameters such as EcM diversity or sporocarp abundance or biomass cannot be used to assess the importance of various mycorrhizal fungi in these plant communities.

In this study EcM communities were examined in 16 natural stands of *S. repens* that comprise a natural range of highly different environmental conditions. The aims of this study were: (1) to characterize EcM fungal communities in these sites based on three years of sporocarp surveys; (2) to characterize EcM morphotype composition in these sites at two sampling dates; (3) to relate above-ground species diversity and sporocarp abundance to below-ground morphotype diversity and abundance of ectomycorrhizal roots; (4) to relate above- and below-ground perspectives to soil chemistry. In CHAPTER 3 emphasis is given to arbuscular mycorrhizal (AM) species composition, dual mycorrhizal colonization (EcM and

AM), habitat preferences of arbuscular mycorrhiza and EcM morphotypes, and successional changes, mycorrhizal (seasonal) dynamics and mycorrhizal interference in colonization of both mycorrhizal types.

## METHODS

### *Site description*

Sixteen field sites of 500 $m^2$ were located on the Dutch Wadden Isle Terschelling. The climate is Atlantic with a mean annual temperature of 9.0-9.5 °C (July: 16.0-16.5 °C); January: 2.0-2.5 °C) and precipitation of 700-750 mm. Field sites of *S. repens* comprised a natural range of highly different environmental conditions, both in terms of soil moisture and pH. All sites contained male and female plants. Site descriptions are given in APPENDIX A. The sites 1, 2, 3, 7, 8, 9, 10, and 11 are all on dry soils. The early-successional sites 1 to 3 are situated on dunes with drifting sand at short distance to the sea. The other sites 7 to 11 have a vegetation characteristic for later stages of succession. The sites 4, 5, 6, 12, 13, 14, 15, and 16 are all on moist to wet soils. The sites 4 to 6 are dune slacks with relatively high soil pH, and a vegetation characteristic for early stages of succession. The sites 12 to 16 are dunes with lower pH and a vegetation characteristic for a later stage of succession.

### *Soil analysis*

In October 1993 10 samples (150 $cm^3$; diameter 5 cm, depth 10 cm) were randomly taken with a soil core after removal of the litter layer, and 10 samples of litter (depth 5 cm or less when less litter was present) in each field site. These 10 soil samples were mixed and dried at 40 °C for 4 days. After drying the samples were ground and the mineral soil samples were sieved through a 2 mm sieve in order to remove macro-organic material such as roots. $CaCO_3$ (%) was determined according to the Scheibler method (Houba *et al.*, 1995). The organic matter content was determined by loss-on-ignition, after correction for $CaCO_3$ content. In each sample total N and P were determined after digestion in sulphuric acid, salicylic acid and 30 % hydrogen peroxide in a block digester. The (plant) available nutrients were analysed according to the $CaCl_2$ method described by Houba *et al.* (1990). N dissolved ($N_s$), $N_{org.}$, $NH_4^+$, $NO_3^-$, P dissolved ($P_s$), $K^+$, $Na^+$, and $Mg^{2+}$ were analysed in 0.01M $CaCl_2$ extracts and pH($CaCl_2$) was measured with a standard glass electrode. The acid digests and the $CaCl_2$ extracts were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard.

Soil moisture values were based on indicator values of plant species by taking cover-abundance into account (Ellenberg, 1974). This system is based on grouping plant species, which occur in these 16 sites, into a number of preference groups and assigning numerical scores to these groups. By weighted averaging these scores are converted to scores for individual plots (Jongman *et al.*, 1987).

*Surveys and identification of sporocarps of EcM fungi*

During the autumn in 1990, 1991, and 1992 sporocarps of EcM fungi were counted in these sites two (1991) or three times using mycocoenological methods as suggested by Arnolds (1992). Caps of the sporocarps were removed in order to avoid double counting. Sporocarps were collected for identification when necessary.

*Assessment of EcM colonization and EcM morphotyping*

All sites were sampled in October 1994 and August 1995. Root distribution in a *S. repens* shrub is not random as random sampling using a soil core resulted in 9 out of 10 samples without roots. *Salix repens* (creeping willow) is a clonal plant that spreads through rhizomes and fine roots in the soil are concentrated along the rhizomes. Therefore, soils were searched for rhizomes. In each site, below ten randomly selected rhizomes a sample was cut (sample volume: 1500 $cm^3$; 15 cm length, 10 cm width and 10 cm depth. As rhizomes were found at approximately 10 cm depth, total sampling depth was 20 cm). The samples (rhizome and fine roots attached) were stored at 4 °C in containers with a buffered solution of glutaraldehyde (Alexander & Bigg, 1981) until processed. Samples were immersed in water over a 2 mm sieve to remove most of the soil and rinsed gently to avoid damage of the mycorrhizas. Clean *S. repens* roots were collected for examination of EcM fungi in glutaraldehyde buffer. In each sample, total numbers of EcM root tips and EcM frequency (number of EcM root tips divided by total number of root tips * 100) were determined.

In seven samples per site, EcM morphotypes (between 2,500 and 10,000 maximum EcM root tips were studied per site) were distinguished on the basis of macroscopical and microscopical characteristics. They were assigned to genus level if they matched descriptions of morphotypes (Agerer, 1987-1998; Ingleby *et al.*, 1990). Assigning morphotypes to genera was furthermore supported by matching with EcM root tips collected under sporocarps and with EcM syntheses in the lab, based on sporocarp

cultures. Fifteen EcM morphotypes on *S. repens* roots were described (APPENDIX B). If mantle structures were old or damaged, root tips were designated 'not vital', and 'undeveloped' if their development was not completed. Numbers of EcM root tips were calculated per 20 cm rhizome (sample volume 1500 $cm^3$ soil).

*Data analysis*

*Diversity of EcM sporocarps and morphotypes* - Average number of EcM species, sporocarps, morphotypes and EcM root tips per field site and Margalef's index of diversity were calculated for the three years of above-ground, and for the two seasons of below-ground investigation. Margalef's diversity index ($D_{MG}$) is a function of the number of species recorded (S) and the total number of individuals (N) (Magurran, 1988):

[1] $D_{MG} = (S-1) / \ln N$

*Ordination of EcM sporocarps and morphotypes* - The pattern of EcM diversity (above-ground species diversity; below-ground morphotype diversity) was analysed by ordination using Correspondence Analysis (CA) in the program CANOCO (Jongman *et al.*, 1987). The numbers of sporocarps and ectomycorrhizas of each taxon (species, morphotype) were transformed to the logarithmic scale as proposed by Arnolds (1992).

Correlations between chemical composition of the soil and EcM fungi (above-ground, species and below-ground, morphotypes) were analysed by Canonical Correspondence Analysis (CCA), program CANOCO (Jongman *et al.*, 1987). pH, moisture, $N_s$, $NO_3^-$, $NH_4^+$, $N_{org.}$, $N_{tot}$, $P_s$, $P_{tot}$, $K^+$, $Na^+$, $Mg^{2+}$ and %OM were used as environmental explanatory variables. Standard options of CANOCO were used when data were processed (ter Braak, 1990). Statistical tests of significance were carried out by the Monte Carlo permutation test using 99 permutations of the residuals (Økland & Eilertsen, 1994).

*Statistical tests* - Soil nutrient concentrations, pH, and moisture and numbers of EcM species, EcM sporocarps, EcM morphotypes and their abundance were analysed by multiple analysis of variance (ANOVA). Based on the first and second species environment-correlation axis from the CA of EcM species composition, the 16 field sites were assigned to four habitat types, viz. Calcareous-Dry, Calcareous-Wet, Acidic-Dry and Acidic-Wet. Prior to analysis, soil nutrient concentrations and numbers of EcM species (morphotype) and

sporocarps (ectomycorrhizas) were transformed logarithmically. Differences among means were evaluated with the LSD-test (Sokal & Rohlf, 1995).

Correlations between (i) EcM fungal diversity above- (species, genera) and below-ground (morphotypes) and abundance above- (sporocarps) and below-ground (ectomycorrhizas) and (ii) CA axes based on species and morphotype composition were tested by the Spearman's rank correlation test (Siegel & Castellan, 1988).

## RESULTS

*Species composition and sporocarp abundance of EcM fungi*

Sporocarps of 78 taxa of EcM fungi were found (TABLE 2-1). The Correspondence Analysis-diagram (not shown) based on EcM sporocarp species composition showed that the sites were divided by two axes (eigenvalues resp. 0.55 and 0.53) representing moisture and pH. Although these axes explained only 27.3 % of the variation, they formed the basis for classifying these 16 field sites in four habitats (Calcareous-Dry, Calcareous-Wet, Acidic-Dry, Acidic-Wet).

*Hebeloma leucosarx, H. pusillum, Laccaria laccata*, and *Paxillus involutus* were quite common, whereas *Inocybe agardhii* and *I. dulcamara* were restricted to calcareous field sites, and *Cortinarius cinnamomeoluteus, C. uliginosus*, and *Russula graveolens* to acidic (-wet) field sites. At the genus level *Cortinarius, Hebeloma*, and *Paxillus* were generalists, whereas *Inocybe* was restricted to younger, calcareous sites, and *Lactarius, Russula*, and *Xerocomus* to older, acidic sites. Numbers of species per site varied from 0 to 31. Total numbers of sporocarps ranged from 0 to 10903 per 500 $m^2$. Species number and sporocarp abundance were correlated ($R = 0.64$, $P < 0.01$, Spearman's rank correlation test).

The four different habitats (Calcareous-Dry, Calcareous-Wet, Acidic-Dry and Acidic-Wet) showed no significant differences in mean number of EcM sporocarp species or diversity. The Acidic-Dry habitat always showed the lowest numbers of sporocarps, but this was only significant in 1992 (interaction pH × moisture; $P < 0.015$, ANOVA) (TABLE 2-2).

**TABLE 2-1.** List of species of ectomycorrhizal fungi, and the cumulative abundance of their sporocarps, observed in 16 *Salix repens* field sites (Ca-Dry, Ca-Wet, Acidic-Dry, Acidic-Wet) in autumn 1990, 1991 and 1992.

| Species of ectomycorrhizal fungi | | Calcareous-Dry | | | Calcareous-Wet | | | Acidic-Dry | | | | | Acidic-Wet | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PV* | TD | HD | SB | SO | CR | SBB | DP | Waz | WO | PW | DzP | GS | Myr | TC | JT |
| *Cortinarius* | *ammophilus* A.Pears. | | | 2† | | | | | 4 | 2 | | | | | | | |
| *Cortinarius* | *anomalus* (Fr.: Fr.) Fr. | | | | | | | | | 1 | | | 1 | 2 | | | |
| *Cortinarius* | *aff armeniacus* (Schff.: Fr.) Fr. | | | | | | | | 1 | 2 | | | | | | | |
| *Cortinarius* | *casimiri* (Velen.) Huijsman | | | | | | | | | 4 | | | | | | | |
| *Cortinarius* | *cavipes* J. Favre | 5‡ | | | | | | | | | | | | | | | |
| *Cortinarius* | *cf. cedriolens* (Mos.) Mos | | | | | | | | | | | | | 6 | | | |
| *Cortinarius* | *cinnamomeoluteus* P.D.Orton | | | | | | | | | | | | | 5 | 3 | 7 | 3 |
| *Cortinarius* | *cohabitans* P.Karst. | 2‡ | | | 4 | 4 | | | 3 | 2 | | | | | | | |
| *Cortinarius* | *comptulus* Mos. | | | | | | | | | 1 | | | | | | | |
| *Cortinarius* | *croceoconus* Fr. | | | | | | | | | | | | | | | | 1 |
| *Cortinarius* | *cucumisporus* Mos. | | | | 5 | 2 | | | | | | | | | | | 7 |
| *Cortinarius* | *decoloratus* (Fr.: Fr.) Fr. | | | | | | | | | | | | 2 | 3 | | | 1 |
| *Cortinarius* | *emollitus* Fr. | | | | | | | | | | | | | 1 | | | |
| *Cortinarius* | *fasciatus* (Scop.) Fr. | | | | | | | | | | | | | 1 | | | 5 |
| *Cortinarius* | *flexipes* (Pers.: Fr.) Fr. | | | | 3 | 1 | | | | | | | | | | | |
| *Cortinarius* | *aff. flexipes* (Pers.: Fr.) Fr | | | | | | | | | | | | | 1 | | | 5 |
| *Cortinarius* | *helobius* Romagn. | | | | | | | | | | | | | 1 | | | |
| *Cortinarius* | *aff. helobius* (pelarg.) Romagn | | | | 2 | | | | | | | | | | | | |
| *Cortinarius* | *aff. hemitrichus* (Pers.: Fr.) Fr | | | | | | 2 | | | | | | | | | | |
| *Cortinarius* | *incisus* (Pers.: Fr.) Fr. | | | 2 | 2 | | | | | 4 | | | 1 | 5 | | | 1 |
| *Cortinarius* | *aff. incisus* (Pers.: Fr.) Fr | | | | | | | | | | | | | 4 | | | 4 |
| *Cortinarius* | *junghuhnii* (Fr.: Fr.) Fr. | | | | | 1 | | | | | | | | 1 | | | |
| *Cortinarius* | *obtusus* (Fr.: Fr.) Fr | 6‡ | | | | 1 | | | | | | | | 2 | | | 5 |
| *Cortinarius* | *aff. ochroleucus* (Schff.: Fr.) Fr. | | | | 3 | | | | | | | | | | | | |
| *Cortinarius* | *paleaceus* Fr. | | | | | | | | | 1 | | | | | | | |
| *Cortinarius* | *parvannalatus* Kühn. | 1† | | | | | | | | 2 | | | | | | | |
| *Cortinarius* | *pauperculus* J.Favre | | | | | | | | | 1 | | | 3 | | | | |
| *Cortinarius* | *cf. pauperculus* J.Favre | | | | | 2 | | | | | | | | 3 | | | |
| *Cortinarius* | *privignus* (Fr.) Fr. | | | | | | | | 2 | | | | | | | | |
| *Cortinarius* | *rigidus* sensu Kühn & Rom. | 6‡ | | | 5 | 2 | | | | | | | | | | | |
| *Cortinarius* | *saniosus* (Fr.: Fr.) Fr. | | | | 2 | | | 1 | | | | | | | | | 4 |
| *Cortinarius* | *sertipes* Kühn. | 7‡ | | | | | | | | 1 | | | | 5 | | | |

TABLE 2-1 continued

| Species of ectomycorrhizal fungi | | Calcareous-Dry | | | Calcareous-Wet | | | Acidic-Dry | | | | | Acidic-Wet | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PV* | TD | HD | SB | SO | CR | SBB | DP | Waz | WO | PW | DzP | GS | Myr | TC | JT |
| *Cortinarius* | *trivialis* J.Lange | | | | 5 | | | | | | | | | 1 | | | 6 |
| *Cortinarius* | *uliginosus* Berk. | | | | | | | | | | | | | 3 | 5 | 7 | 4 |
| *Cortinarius* | spec.1. | | | 1 | 5 | 4 | | | 3 | 2 | | | | 1 | | 3 | 3 |
| *Cortinarius* | spec.2. | | | | | | | | | 2 | | | | | | | |
| *Cortinarius* | spec.3. | | | | | | | | | 1 | | | | | | | |
| *Cortinarius* | spec.4. | | | | | | | | | | | | | | | | |
| *Cortinarius* | spec.5. | | | | 2 | | | | | | | | | | | | |
| *Hebeloma* | *circinans* Qúel. | | | | | | | | | | | | | 3 | | | |
| *Hebeloma* | *collariatum* Bruchet | 7‡ | 1 | | 3 | | 1 | | | | | | | | | 1 | |
| *Hebeloma* | *cylindrosporum* | | | | | | | | | | | 3 | | | | | |
| *Hebeloma* | *helodes* J.Favre | | | | | | | | | 2 | | | 2 | 3 | | | |
| *Hebeloma* | *leucosarx* P.D.Orton | 6 | 3 | 2 | 8 | 4 | 2 | | | 1 | | 4 | | 2 | | 7 | |
| *Hebeloma* | *mesophaeum* (Pers.) Qúel. | 5‡ | | | | | | | | 1 | | 4 | | | | | |
| *Hebeloma* | *psammophilum* M.Bon | 7 | 2 | 4 | | | | | 1 | | | | | | | | |
| *Hebeloma* | *pusillum* J.Lange | 6‡ | | | | 4 | | | | | | 1 | 1 | 2 | | 2 | 3 |
| *Hebeloma* | *vaccinum* Romagn. | | | | | 1 | | | | | | | | | | | |
| *Hebeloma* | *velutipes* Bruchet | | | | | | | | | 1 | | | 3 | 4 | | | |
| *Inocybe* | *agardhii* (Lund) P.D.Orton | | | 4 | | 4 | 2 | | | | | | | 1 | | | 5 |
| *Inocybe* | *dulcamara* (Pers.) Kummer | | 4 | 7 | 4 | 4 | 2 | | | | | | | | | | 4 |
| *Inocybe* | *geophylla v. geoph.* (Fr.: Fr.) Kummer | | | | | | | | | | | 1 | | | | | |
| *Inocybe* | *lacera v. helobia* Kuyp. | | | | | | 1 | | | | | | | | | 3 | |
| *Inocybe* | *lacera v. lacera* (Fr.: Fr.) Kummer | | | 3 | | | | | | | | | | | | | |
| *Inocybe* | *rimosa* (Bull.: Fr.) Kummer | | 2 | 5 | 1 | | | | | | | | | | | | |
| *Inocybe* | *salicis* Kühner | | | | 5 | 3 | | | | | | | | 1 | | | 3 |
| *Inocybe* | *serotina* Peck | | 3 | 1 | | | | | | | | | | | | | |
| *Inocybe* | *squarrosa* Rea | | | | | | | | | | | | | | | | 2 |
| *Inocybe* | *vulpinella* Bruylants | 5 | | | | | | | | | | | | | | | 1 |
| *Laccaria* | *laccata* (Scop.: Fr.) Cooke | 1‡ | | | 5 | 3 | 3 | | 4 | 1 | | 2 | 1 | 4 | 3 | 6 | 7 |
| *Laccaria* | *proxima* (Boud.) Pat | | | | | | | | 3 | | | 3 | | | | | |
| *Lactarius* | *controversus* (Pers.: Fr.) Fr. | | | | | | | | 1 | | | | | | | | |
| *Lactarius* | *helvus* (Fr.: Fr.) Fr. | | | | | | | | 3 | | | | 1 | 1 | | | |
| *Lactarius* | *mitissimus* (Fr.: Fr.) Fr. | | | | | | | | | | | 1 | | 1 | | | |
| *Naucoria* | *bohemica* Velen. | | | | | 2 | | | | | | | | | | | |
| *Naucoria* | *salicis* P.D.Orton | | | | 4 | 2 | | | | | | | | | | 3 | |
| *Naucoria* | *tantilla cf.* J.Favre | 2‡ | | | | | | | | | | | | | | | |

TABLE 2-1 continued

| Species of ectomycorrhizal fungi | | Calcareous-Dry | | | Calcareous-Wet | | | Acidic-Dry | | | | | Acidic-Wet | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PV* | TD | HD | SB | SO | CR | SBB | DP | Waz | WO | PW | DzP | GS | Myr | TC | JT |
| *Paxillus* | *involutus* (Batsch: Fr.) Fr. | | 4 | 4 | | | 2 | 1 | 3 | 2 | | 5 | 2 | 1 | | 3 | |
| *Russula* | *atrorubens* Quel. | | | | 4 | | | | | | | | | 2 | | | 7 |
| *Russula* | *graveolens* Romell. | | | | | | | | | | | 2 | 1 | 1 | | | 4 |
| *Russula* | *pectinatoides* Peck | | | | 1 | | | 1 | | | | 3 | | | | | |
| *Russula* | *persicina* Krombh. | | | | | | | | 2 | | | | | | | | |
| *Russula* | spec. | | | | | | | | | | | | | 1 | | | |
| *Russula* | *xerampelina* sl. (Schff.) Fr. | | | | | | | | | | | 2 | | 2 | | | 4 |
| *Thelephora* | *terrestris* Ehrh.: Fr. | | | | | | | | | | | 1 | | | | | |
| *Tomentella* | spec. | | | | | | | | | | | | 1 | 1 | | | |
| *Tricholoma* | *cingulatum* (Amfelt: Fr.) Jacobasch | | | 4 | | | | | 3 | | | | | | | | |
| *Xerocomus* | *rubellus* Kromb.h. | | | | | | | | 1 | | | 1 | | | | | |

* Abbreviation of field sites as follows: PV = Primaire vallei, TD = Tafelduin, HD = Hobbelduin, SB = Schoenus II, SO = Schoenus I, CR = Cranberry, SBB = SBB schuur, DP = Douwesplak, Waz = Noord helling West aan Zee, WO = Vallei Oosterend, PW = Paardenwei, Dz.P = Dazenplak, GS = Groene strand, Myr = Myrica, TC = Telefooncellen, and TD = Jan Thijssensduin.

† Cumulative abundance: 1=1-3 fruitbodies 500 $m^{-2}$; 2=4-10 fruitbodies 500 $m^{-2}$; 3=11-30 fruitbodies 500 $m^{-2}$; 4=31-100 fruitbodies 500 $m^{-2}$; 5=101-300 fruitbodies 500 $m^{-2}$; 6=301-1000 fruitbodies 500 $m^{-2}$; 7=1000-3000 fruitbodies 500 $m^{-2}$; 8=3000-10000 fruitbodies 500 $m^{-2}$.

‡ observed in the autumn of 1990, field site has changed from Calcareous-Wet to Calcareous-Dry by wind and sand drift.

**TABLE 2-2.** Mean numbers of species above ground, mean numbers of sporocarps per 500 $m^2$ and Margalef's diversity index above-ground in *Salix repens* field sites (Ca-Dry, Ca-Wet, Acidic-Dry, Acidic-Wet), in 1990, 1991 and 1992.

| Habitat type | | Mean number of ectomycorrhizal species per field site | | | |
|---|---|---|---|---|---|
| | n | 1990 | 1991 | 1992 | 3-years |
| Calcareous-Dry | 3 | 6.3 a* | 3.3 a | 6.0 a | 11.0 a |
| Calcareous-Wet | 3 | 9.0 a | 7.7 a | 6.3 a | 14.3 a |
| Acidic-Dry | 5 | 5.8 a | 5.0 a | 5.2 a | 11.0 a |
| Acidic-Wet | 5 | 6.8 a | 7.2 a | 10.0 a | 17.0 a |
| | | Mean number of sporocarps per 500 $m^2$ | | | |
| | | 1990 | 1991 | 1992 | 3-years |
| Calcareous-Dry | 3 | 2668 a | 360 a | 1453 a | 4481 a |
| Calcareous-Wet | 3 | 796 a | 1123 a | 229 ab | 2148 a |
| Acidic-Dry | 5 | 41 a | 75 a | 46 b | 162 a |
| Acidic-Wet | 5 | 698 a | 1592 a | 1177 a | 3467 a |
| | | Margalef's diversity index | | | |
| | | 1990 | 1991 | 1992 | 3-years |
| Calcareous-Dry | 3 | 0.81 a | 0.50 a | 0.84 a | 1.34 a |
| Calcareous-Wet | 3 | 1.41 a | 1.07 a | 1.14 a | 2.08 a |
| Acidic-Dry | 5 | 1.23 a | 1.17 a | 1.04 a | 2.04 a |
| Acidic-Wet | 5 | 1.02 a | 1.05 a | 1.45 a | 2.30 a |

* Significant differences between habitat types are indicated by different letters ($P < 0.05$, LSD).

*Morphotype composition and abundance of ectomycorrhizal root tips*

Even though it was difficult to distinguish and identify EcM morphotypes, 15 morphotypes were described. Key morphological characteristics of the morphotypes are given in APPENDIX B. Once key morphological characteristics of EcM morphotypes have been described, morphotyping is a relatively cheap method, and therefore allowed us to examine large numbers (thousands) of EcM root tips. The number of recognizable morphotypes per site ranged from 3 to 8 (TABLE 2-3). *Hebeloma*, *Inocybe*, ITE5 and *Laccaria* were observed in all habitats, while *Cenococcum* was lacking in Calcareous-Dry. The average number of morphotypes between habitats was not significantly different between sampling dates. Morphotype richness (Margalef's Diversity Index), of the Calcareous-Wet habitat was significantly different from the Acidic-Dry habitat in summer (TABLE 2-4).

**TABLE 2-3.** List of types of ectomycorrhizal morphotypes, and the cumulative abundance of their mycorrhizal root tips, observed in 16 *Salix repens* field sites (Ca-Dry, Ca-Wet, Acidic-Dry, Acidic-Wet) in summer and autumn (August 1995 and October 1994, respectively).

| | Calcareous-Dry | | | Calcareous-Wet | | | Acidic-Dry | | | | | Acidic-Wet | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PV* | TD | HD | SB | SO | CR | SBB | DP | Waz | WO | PW | Dzp | GS | Myr | TC | TD |
| August 1995 | | | | | | | | | | | | | | | | |
| Not vital | 5† | 5 | 4 | | 3 | 5 | 5 | 4 | 2 | 6 | 4 | 4 | 5 | 6 | 5 | 3 |
| *Cenococcum* | | | | 1 | 2 | 4 | | 4 | 2 | 3 | | 5 | 6 | | 2 | 5 |
| *Cortinarius* | 2 | | 3 | 3 | 3 | | | | | | | | 6 | | | |
| *Hebeloma* | | 5 | | | 2 | | | | 2 | | | | | | | |
| *Inocybe* | | 4 | 2 | 3 | | | | | | | | | | | | |
| ITE4 | | | | | | | | 3 | | | | | | | | |
| ITE5 | | 5 | 4 | 4 | 4 | 3 | | 4 | 3 | | | 3 | | | | |
| ITE6 | | | | | | | | | | | | | | | | |
| *Laccaria* | 4 | | | 3 | 1 | 5 | 5 | 4 | 3 | 7 | | 4 | 7 | 7 | 5 | 4 |
| *Lactarius* | | | | | | | | | | | | | 2 | | | |
| *Paxillus* | | | | | | | | | | | | | | | | |
| NR4 | | | | | | 3 | | | | | | | | | | |
| *Tuber* | | | 4 | | | | | | | | | | | | | |
| NR1 | | | | | | | | | | | | 4 | 3 | | | |
| NR2 | | 4 | | | 4 | | | | | 3 | | | 5 | | | |
| NR3 | | | | | | | | | | | | | 2 | | | |
| Undeveloped | 5 | 6 | 6 | 5 | 5 | 6 | 5 | 5 | 5 | 7 | 5 | 5 | 6 | 6 | | |
| October 1994 | | | | | | | | | | | | | | | | |
| Not vital | 6 | 6 | 6 | 5 | 6 | 7 | 7 | 1 | 4 | 6 | 6 | 6 | 6 | 8 | 6 | 4 |
| *Cenococcum* | | | | 3 | 4 | | | 5 | 4 | | | 4 | 6 | 5 | 3 | 4 |
| *Cortinarius* | | | | | 1 | | | 3 | | | | 5 | 4 | 8 | | |
| *Hebeloma* | 7 | 6 | 3 | 4 | 6 | 6 | | 1 | 2 | | | 5 | 5 | | 3 | |
| *Inocybe* | 4 | 7 | 2 | 4 | 6 | 8 | | | 3 | | 5 | | 4 | 8 | 6 | |
| ITE4 | | | | | | | | | | | | | | | | |
| ITE5 | 6 | 4 | 4 | 6 | 6 | 6 | | 2 | 2 | 5 | 2 | 6 | 5 | 4 | 4 | |
| ITE6 | | | | 5 | | | | | | | | 6 | | | | |
| *Laccaria* | | | | 3 | 5 | | 7 | 6 | 4 | 6 | 6 | 7 | 3 | | | 5 |
| *Lactarius* | | | | | | | | | 1 | | | 5 | 6 | | | |
| *Paxillus* | | | | | | 3 | | | 3 | | 2 | 2 | | | | |
| NR4 | | | | | | | | | | 5 | | | 5 | | | |
| *Tuber* | | | | | | | | | | | | | | | | |
| NR1 | | | | | | | | | | | | | | | | |
| NR2 | | | | | | | | | | | | | | | | |
| NR3 | | | | | | | | | | | | | | | | |
| undeveloped | 4 | | 6 | 6 | 7 | 7 | | 6 | 5 | 5 | | | 5 | | 6 | 5 |

* Abbreviation of field sites as follows: PV = Primaire vallei, TD = Tafelduin, HD = Hobbelduin, SB = Schoenus II, SO = Schoenus I, CR = Cranberry, SBB = SBB schuur, DP = Douwesplak, Waz = Noord helling West aan Zee, WO = Vallei Oosterend, PW = Paardenwei, Dzp = Dazenplak, GS = Groene strand, Myr = Myrica, TC = Telefooncellen, and TD = Jan Thijssensduin.

† Cumulative abundance: 1=1-3 mycorrhizal root tips; 2=4-10 mycorrhizal root tips; 3=11-30; 4=31-100; 5=101-300; 6=301-1000; 7=1000-3000; 8=3000-10000.

**TABLE 2-4.** Average number of ectomycorrhizal morphotypes (1500 $cm^3$), average number of ectomycorrhizal root tips (1500 $cm^3$), and Margalef's diversity index in summer and autumn (August 1995 and October 1994, respectively) in *Salix repens* sites (Ca-Dry, Ca-Wet, Acidic-Dry, Acidic-Wet).

| | n | Average number of EcM morphotypes | Average number of EcM roottips | Margalef's diversity index |
|---|---|---|---|---|
| Habitat types | | | | |
| pH (Calc.-Acidic) | | Ns | ns | ns |
| Moisture (Dry-Wet) | | Ns | ns | ns |
| Sampling date | | Ns | ** | ns |
| pH × moisture | | * | ns | * |
| August 1995 | | | | |
| Calcareous-Dry | 3 | 3.3 bc[†] | 724 ab | 0.35 bc |
| Calcareous-Wet | 3 | 5.0 bc | 540 a | 0.67 c |
| Acidic-Dry | 5 | 2.4 b | 902 a | 0.23 b |
| Acidic-Wet | 5 | 3.2 bc | 1532 ab | 0.32 bc |
| October 1994 | | | | |
| Calcareous-Dry | 3 | 3.0 bc | 2558 b | 0.26 bc |
| Calcareous-Wet | 3 | 5.6 c | 4617 b | 0.59 bc |
| Acidic-Dry | 5 | 4.0 bc | 1363 ab | 0.46 bc |
| Acidic-Wet | 5 | 5.0 c | 5159 b | 0.50 bc |

* Significance levels in ANOVA table; *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) or not significant (ns). Interactions not presented were ns.

[†] Differences between habitat types and seasons (years) are indicated by different letters ($P < 0.05$, LSD).

**TABLE 2-5.** Correlation of Correspondence Analysis axes between ectomycorrhizal species composition of sporocarps and ectomycorrhizal morphotypes composition in summer ($n$ = 16) and autumn (August 1995, and October 1994, respectively) in16 *Salix repens* field sites.

| | EcM morphotypes | | | | | |
|---|---|---|---|---|---|---|
| | Summer | | | Autumn | | |
| CA axes | First | Second | Third | First | Second | Third |
| Sporocarps | | | | | | |
| First | 0.16 | -0.14 | 0.03 | -0.30 | 0.06 | -0.06 |
| Second | 0.66** | -0.33 | -0.07 | 0.51 | 0.33 | 0.17 |
| Third | -0.01 | 0.63* | 0.14 | 0.11 | -0.23 | 0.24 |
| EcM morphotypes | | | | | | |
| August 1995 | | | | | | |
| First | | 0.13 | -0.07 | 0.49 | 0.01 | 0.20 |
| Second | | | 0.00 | -0.14 | -0.34 | 0.25 |
| Third | | | | -0.08 | -0.26 | 0.05 |
| October 1994 | | | | | | |
| First | | | | | -0.18 | 0.43 |
| Second | | | | | | -0.23 |

* Significant correlations are indicated with * ($P < 0.05$) and **($P < 0.01$) (Spearman's rank correlation test).

**TABLE 2-6.** Nutrient concentrations (mg/kg), pH ($CaCl_2$), $CaCO_3$ (%) and organic matter (%) content of soils of *Salix repens* field sites (Ca-Dry, Ca-Wet, Acidic-Dry, Acidic-Wet) sampled in October 1993.

| Habitat types | n | pH | $CaCO_3$ | OM | $N_{tot}$ | $P_{tot}$ | $NO_3^-$ | $NH_4^+$ | $N_s$ | $N_{org.}$ | $P_s$ | $(N/P)_{tot}$ | $(N/P)_s$ | Moisture |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Habitat type | | | | | | | | | | | | | | |
| pH (Calc.-Acid.) | | *** | *** | ns | *** | * | ns | *** | ns | ns | *** | ** | ns | * |
| Moisture (Dry-Wet) | | ns | *** | ns | ns | ns | ns | ns | ns | ns | * | ns | * | *** |
| pH × moisture | | *** | *** | ns | ns | ns | ns | ns | * | ns | * | ns | ns | ns |
| Calcareous-Dry | 3 | 6.7 a[†] | 0.79 a | 0.8a | 333 a | 114 a | 0.08 a | 2.86 a | 7.71 a | 60.4 a | 0.8 a | 2.86 a | 10.1 a | 6.1 b |
| Calcareous-Wet | 3 | 5.8 b | 0.14 b | 4.1a | 800 ac | 111 a | 0.03 a | 3.83 a | 10.87 a | 65.8 a | 1.0 a | 6.94 ac | 11.2 a | 7.9 c |
| Acidic-Dry | 5 | 4.0 c | 0 c | 11.1a | 2280 b | 199 b | 0.73 a | 12.28 b | 33.32 b | 53.3 a | 12.5 b | 12.67 b | 4.4 b | 4.7 a |
| Acidic-Wet | 5 | 4.6 d | 0 c | 12.3a | 2620 bc | 202 ab | 0.75 a | 11.09 ab | 11.09 a | 53.4 a | 2.3 a | 10.49 bc | 11.6 a | 7.4 bc |

* Significance levels in ANOVA table; *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) or not significant (ns).

[†] Significant differences between habitat types are indicated by different letters ($P < 0.05$, LSD)

*Species and morphotype diversity and abundance*

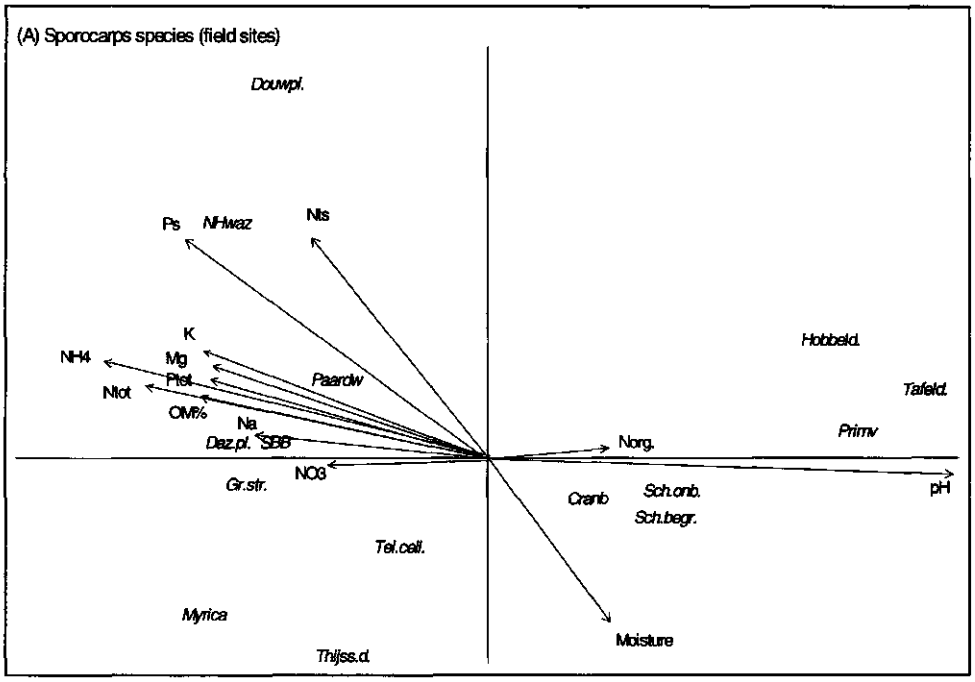
CA of the 16 field sites based on species composition of EcM sporocarps (genus level) and EcM morphotypes was carried out using standard options of CANOCO. Correlations between the three axes obtained per analysis showed that only two significant correlations between EcM sporocarp composition and EcM morphotypes were significant. The first axis of the EcM morphotype composition in summer was significantly correlated with the second axis of the EcM genus composition, and the third axis of the EcM genus composition with the second axis of the EcM morphotype compostion in summer (TABLE 2-5). No significant correlations were found between any of the axes of the EcM morphotype composition at both sampling dates.

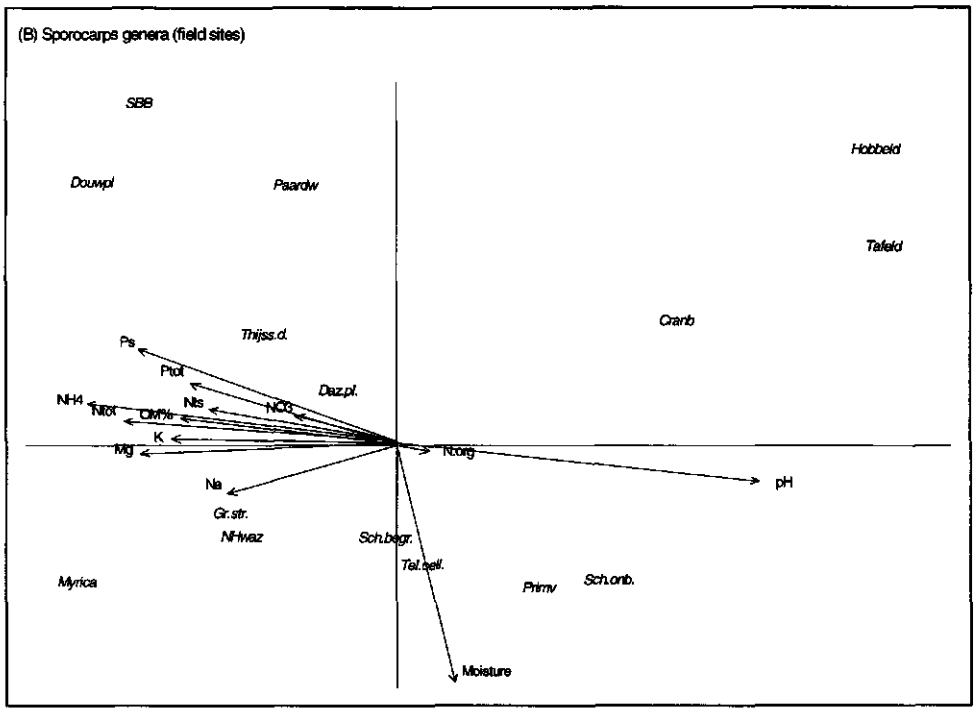
Abundance of EcM sporocarps and EcM root tips were not correlated in autumn ($R$ = -0.06; $P > 0.05$) and even negatively correlated in summer ($R = -0.71$; $P < 0.05$, Spearman's rank correlation test). Species richness was positively correlated with morphotype richness in autumn ($R = 0.67$; $P < 0.05$), but not correlated in summer ($R$ = 0.41; $P > 0.05$). Genus richness was not correlated with morphotype richness at both sampling dates ($R$ = 0.49 and 0.12, for autumn and summer, respectively; $P > 0.05$).

*Soil analysis*

Habitat pH differed significantly in soil $CaCO_3$ , $N_{tot}$, $P_{tot}$, $NH_4^+$, $P_s$, $N/P_t$ , moisture (and of course pH), and habitat moisture only differed in $CaCO_3$, $P_s$, $N/P_s$ (and of course moisture). There was a significant interaction between pH and moisture regarding pH, $CaCO_3$ and to a lesser extent $N_s$ and $P_s$. Organic matter was higher in the Acidic sites than in the Calcareous sites, but due to the large variation between field sites within a habitat the difference was not significant. The Dry habitat classified as Calcareous or Acidic significantly differed for almost all nutrients analysed (TABLE 2-6).

**FIGURE 2-1.** Canonical Correspondence Analysis; diagram with axes 1 and 2, for ectomycorrhizal sporocarp (A) -species and (B) -genera (above ground), in 1990, 1991 and 1992 and nutrient concentrations, pH, organic matter content (%) and moisture. Abbreviation of field sites as follows: Primv = Primaire vallei, Tafeld. = Tafelduin, Hobbeld. = Hobbelduin, Sch.begr. = Schoenus I, Sch.onb. = Schoenus II, Cranb = Cranberry, SBB = SBB schuur, Douwpl. = Douwesplak, NHwaz = Noord helling West aan Zee, Paardw = Paardenwei, Daz.pl. = Dazenplak, Gr.str. = Groene strand, Myrica = Myrica, Tel.cell. = Telefooncellen, and Thijss.d. = Jan Thijssensduin (see opposite page).


(A) Sporocarps species (field sites)
Douwpl.
Ps
NH4waz
Nts
K
NH4
Mg
Ptot
Paardw
Ntot
OM%
Na
Daz.pl.
SBB
NO3
Gr.str.
Hobbeld.
Tafeld.
Primv
Norg.
pH
Cranb
Sch.onb.
Sch.begr.
Tel.cell.
Myrica
Thijss.d.
Moisture


(B) Sporocarps genera (field sites)
SBB
Douwpl
Paardw
Hobbeld
Tafeld
Cranb
Thijss.d.
Ps
Ptot
Daz.pl.
NH4
Ntot
Nts
NO3
OM%
K
Mg
Norg
pH
Na
Gr.str.
NH4waz
Sch.begr.
Tel.cell.
Myrica
Primv
Sch.onb.
Moisture

*Above- and below-ground views of EcM communities in relation to soil chemistry*

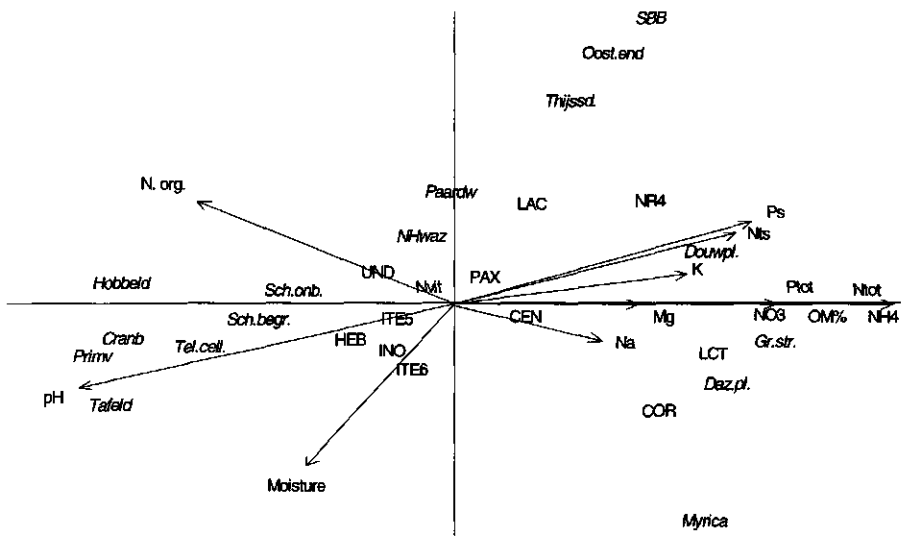
The Canonical Correspondence Analyses-diagrams based on EcM species composition, genus composition or morphotype composition (October and August) and pH, moisture, and soil nutrient concentrations are shown in FIGS. 2-1A, 2-1B (species, genera), 2-2A, and 2-2B (morphotypes), respectively. In all four CCA's forward selection of the environmental variables was performed and in all cases at most 2 of the environmental variables significantly contributed to the explained variation. Therefore, after forward selection, only one or two variables (which significantly contributed to the explained variation) were included in the CCA, axes 3 and 4 were chosen as CA (hybrid option in CANOCO), and the other environmental variables were included as 'passive' variables.

Forward selection of the environmental variables in the CCA of EcM species composition (FIG. 2-1A) showed that only pH and $P_s$ significantly contributed to the explained variation ($P < 0.01$ and $P < 0.05$, respectively). The eigenvalues of axes 1 and 2 were 0.46 and 0.45. These first two axes explained 23.1 % of the variation, and all four axes explained 46.4 %. Monte Carlo permutation tests showed that the first and second axis significantly contributed to the explained variance ($P < 0.05$). The species environment-correlation of the first axis was 0.97 and was defined by pH (r = 0.97), $NH_4^+$ (r = - 0.80) and $N_{tot}$ (r = - 0.72). The species environment-correlation of the second axis is 0.95 and was defined by $P_s$ (r = 0.73) and $N_{tot}$ (r = 0.73). In the field site Vallei Oosterend no sporocarps have been found and therefore it was automatically excluded from the analysis.

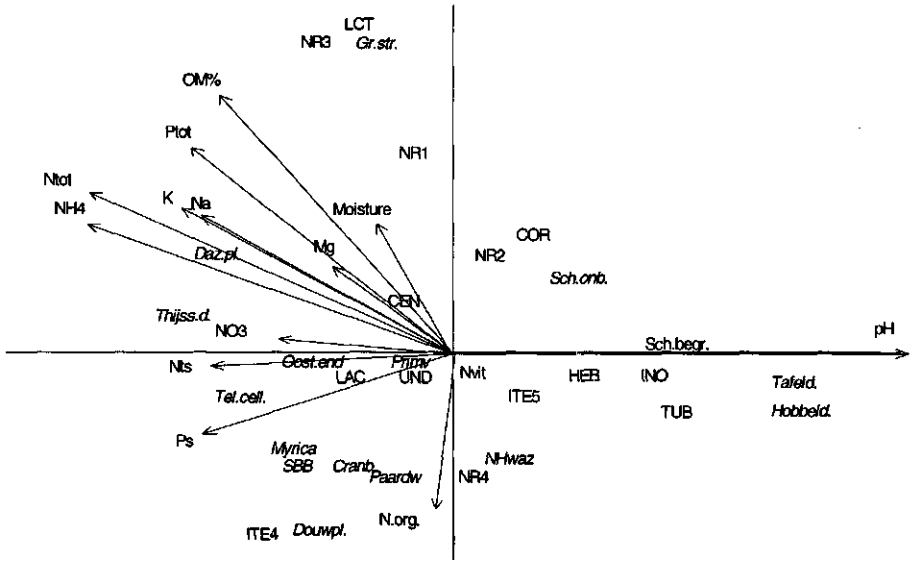
Again the forward selection option was used in the CCA of EcM genus level (FIG. 2-1B). This time pH and moisture were the only variables that significantly contributed to the explained variation ($P < 0.01$ and $P < 0.02$, respectively). The eigenvalues of axes 1 and 2 were 0.17 and 0.12. These first two axes explained 33.9 % of the variation. Monte Carlo permutation tests showed that only the first axis significantly contributed to the explained variance ($P < 0.02$). The genus environment-correlation of the first axis was 0.86 and was defined by pH (r = 0.85), $NH_4^+$ (r = - 0.70), and $N_{tot}$ (r = - 0.63); that of the second axis was 0.80 and was defined by moisture (r = - 0.79).

**FIGURE 2-2.** Canonical Correspondence Analysis; diagram with axes 1 and 2, for ectomycorrhizal morphotypes in (A) October 1994, (B) August 1995 and nutrient concentrations, pH, organic matter content (%) and moisture. Abbreviation of mycorrhizal types: Nvit = 'not vital', CEN = *Cenococcum*, COR = *Cortinarius*, HEB = *Hebeloma*, INO = *Inocybe*, LAC = *Laccaria*, LCT = *Lactarius*, PAX = *Paxillus*, and UND = 'undeveloped'. Abbreviation of field sites as follows: Primv = Primaire vallei, Tafeld. = Tafelduin, Hobbeld. = Hobbelduin, Sch.begr. = Schoenus I, Sch.onb. = Schoenus II, Cranb = Cranberry, SBB = SBB schuur, Douwpl. = Douwesplak, NHwaz = Noord helling West aan Zee, Oost.end = Vallei Oosterend, Paardw = Paardenwei, Daz.pl. = Dazenplak, Gr.str. = Groene strand, Myrica = Myrica, Tel.cell. = Telefooncellen, and Thijss.d. = Jan Thijssensduin (see opposite page).

(A) Mycorrhizal types autumn '94




(B) Mycorrhizal types summer '95



After forward selection in the CCA of EcM morphotype composition in autumn (FIG. 2-2A) only $NH_4^+$ significantly contributed to the explained variation ($P < 0.01$). The eigenvalues of axes 1 and 2 were 0.17 and 0.19. These first two axes explained 38.3 % of the variation. Monte Carlo permutation tests showed that only the first axis significantly contributed to the explained variance ($P < 0.01$). The morphotype environment-correlation of the first axis was 0.88 and was defined by $NH_4^+$ (r = 0.88), $N_{tot}$ (r = 0.78), OM% (r = 0.71), pH (r = - 0.72), $P_{tot}$ (r = 0.67), and $NO_3^-$ (r = - 0.64).

After forward selection in the CCA of EcM morphotype composition in summer (FIG. 2-2B), only pH ($P < 0.01$) was included as explanatory variable. The eigenvalues of axes 1 and 2 were 0.20 and 0.20. They explained 31.8 % of the variation. Monte Carlo permutation tests showed that only the first axis significantly contributed to the explained variance ($P < 0.01$). The morphotype environment-correlation of this first axis was 0.81 and was defined by pH (r = 0.81), $N_{tot}$ (r = - 0.66) and $NH_4^+$ (r = - 0.63).

## DISCUSSION

*Species composition and sporocarps of EcM fungi*

The number of EcM fungal species that can occur with *S. repens* is quite high. Among the 78 taxa of EcM fungi, species belonging to the genus *Cortinarius* were well represented, as at least 30 species have been recorded. Similar observations were made in the British coastal dunes by Watling (1992) and Watling & Rotheroe (1989).

As *S. repens* was the only ectomycorrhizal plant in these plots, host specifity could easily be determined. A large number of species, e.g. *Lactarius controversus, Russula atrorubens, R. persicina,* and *Tricholoma cingulatum* are well-known associates with members of the Salicaceae (*Salix* and *Populus*). Many species of *Cortinarius* (Arnolds & Kuyper, 1995) and *Hebeloma* (Aanen, 1999; Aanen *et al.*, 2000) also preferentially associate with members of the Salicaceae. Only a smaller number of species have larger host ranges and occur with a greater number of tree genera. Because of this host tree specificity species lists of EcM fungi in communities of *S. repens* show a larger resemblance to alpine communities with dwarf willows (Favre, 1955, 1960; Graf, 1994)

than to forest communities with conifers and broad-leaved trees such as oaks, beeches and birches.

Classification of the field sites in four habitat types, viz. Calcareous-Dry, Calcareous-Wet, Acidic-Dry and Acidic-Wet, was supported by CA with the first two axes defined by pH and moisture. However, the explanatory value of these axes accounted only for 27 % of variation, indicating high within-habitat variability. It is therefore not surprizing that EcM species richness, number of sporocarps, or Margalef's diversity index showed no difference between habitats.

It should furthermore be taken into account that several of the sites are dynamic. Site 1 for instance was originally situated in a primary dune valley with low dunes on which *S. repens* occurred. In those shrubs sand accumulated and *S. repens* gradually built up larger and higher dunes in a way that is similar to dune formation by *Ammophila arenaria.*

*Above- and below-ground views in relation to soil chemistry*

All four CCA analyses (species and genus composition above-ground, morphotype composition at two sampling dates) indicated that the first axis significantly contributed to the explained variance. This first axis was defined by pH (positive correlation) and a number of soil nutrients (negative correlation). This axis is best interpreted as a successional gradient where pH drops, due to loss of free $CaCO_3$ and the accumulation of organic matter. As a consequence the total amounts of nitrogen, phosphorus, and major cations increase. These changes occur both in dry and wet habitats. Only in the CCA based on species composition did the second axis significantly contribute to the explained variance as well. The second axis was defined by plant available phosphorus and total plant available nitrogen, indicating that the availability of nutrients might not change in ways that can directly be derived from the knowledge of organic matter accumulation. However, the low amount of variation explained by the first two axes of CCA (between 23 and 38 %) indicates that within-habitat variation is very high.

*Comparing above-ground and below-ground views of EcM fungi*

Fifteen EcM morphotypes could be recognized. This is substantially lower than the number of fungal taxa observed in the same plots (78). A similar difference was noted by Dahlberg *et al.* (1997) who observed in a spruce forest in Sweden 51 taxa of EcM fungi

and 13 morphotypes below-ground. Such discrepancies might in part be due to the fact that many of the ectomycorrhizal fungi might be rare and therefore easily missed in below-ground sampling. More importantly, morphotypes can often not be equated with fungal species. It is possible that morphotypes correspond more closely to fungal genera. In that case morphotypes would comprise 1 (*Cenococcum*-type) to more than 30 (*Cortinarius*-type) species and a more meaningful comparison might therefore be based on genus diversity. However, genus diversity was not correlated with morphotype diversity, whereas species diversity was also not correlated with morphotype diversity in autumn.

Although a few taxa dominated both above- and below-ground (*Hebeloma*, *Laccaria*), other taxa were either common above-ground and rare below-ground and vice versa. Lack of a positive correlation between abundance of sporocarps and EcM root tips indicates that the number of sporocarps cannot be used as an indicator of numbers of EcM root tips, contrary to the data by Termorshuizen & Schaffers (1989) and Jansen (1991) in Dutch forests. On the level of individual EcM genera and morphotypes, abundances were also not correlated. Both high numbers of sporocarps and few EcM root tips and vice versa, were previously reported (Danielson, 1984; Chu-Chou & Grace, 1987; Natajaran *et al.*, 1992; Gardes & Bruns, 1996). It is still unknown for EcM fungi how many EcM root tips are necessary to form a sporocarp. This might depend on the biomass of an individual sporocarp but also on the carbon transfer efficiency of the particular species involved. Therefore, it is likely that parameters such as EcM species composition or abundance of sporocarps cannot easily be used to assess the importance of various mycorrhizal fungi for the functioning of these plant communities. This was most evident in plot vallei Oosterend where no sporocarps had been found despite a high below-ground abundance of EcM root tips (5 morphotypes and ~2,300 and 3,700 EcM root tips per sample in autumn and summer, resp.). Possibly the high grass cover with its dense root mat in this plot suppressed the formation of sporocarps of EcM fungi.

Although a correlation was found between some of the axes of the CA based on diversity of EcM fungi above-ground and below-ground, these correlations do not invalidate the above conclusion that sporocarps cannot be used to assess below-ground importance of various mycorrhizal fungi. Correlation between the CA axes only indicates that both above- and below-ground data support classification of the 16 field sites in four habitats.

This conclusion was supported by the fact that no correlation at all was found between below-ground views of EcM morphotype composition in summer and autumn. This lack of correlation could have a spatial or temporal basis. As only a limited area was covered by the below-ground sampling compared to the size of the plots, it might well be possible that, if data are not spatially autocorrelated, two independent samples are quite different. However, this explanation is considered unlikely, as certain morphotype shifts between both sampling dates were observed in all seven samples from the plots. Seasonal and annual variation between samples could depend on the prevailing weather, but could also be dependent on inherent dynamics of EcM fungi. Potential of different EcM fungi to colonize newly formed roots at the beginning of the growing season may depend on particular environmental conditions in a given year. Thus, every year there is a new opportunity for all EcM (and AM) fungi to compete for root niches in these dune ecosystems. Relative amounts of different mycorrhizal fungi therefore can vary enormously between years in these highly dynamic ecosystems. Surprisingly, while the need for performing sporocarp surveys for at least three years (with a number of visits per year) has often been stressed (Arnolds, 1992), the need to perform below-ground assessments repeatedly (both within and between years) has received less attention. However, dynamics in colonization by different EcM fungi should not be out of mind (CHAPTER 3).

In conclusion, the results of these field observations have demonstrated that the assumption that EcM diversity above-ground is a good indicator of EcM diversity below-ground is not always valid. Neither can abundance of sporocarps be used as an indicator of abundance of ectomycorrhizal root tips. To have a clear understanding of the ecological significance of EcM diversity in these dune ecosystems, above- and below-ground views have to be considered simultaneously. It is conceivable that larger sampling efforts could improve the fit between above- and below-ground descriptions of EcM communities to some extent. However, such efforts would have important trade offs. Increasing sample number per plot would decrease the number of plots that could be assessed and hence make establishing relationships between morphotype diversity and soil chemistry more difficult. Increasing below-ground typing accuracy (with the help of molecular methods) would also substantially reduce the number of root tips that could be checked. This trade-off was clearly shown by Jonsson *et al.* (1999) who could morphotype 7126 ectomycorrhizas (with 20

morphotypes) and successfully apply molecular analyses for 212 ectomycorrhizas (with 42 RFLP-types).

Moreover, *S. repens* is a dual mycorrhizal plant (Harley & Harley, 1987), and therefore interactions with and composition of arbuscular mycorrhizal fungi (CHAPTER 3) should not be out of mind.

3

# Mycorrhizal associations of *Salix repens* L. communities in succession of dune ecosystems II. Mycorrhizal dynamics and interactions of ectomycorrhizal and arbuscular mycorrhizal fungi.

**ABSTRACT**

*Ectomycorrhizal (EcM) and arbuscular mycorrhizal (AM) associations of* Salix repens *were studied in 16 sites in different successional stages of dune ecosystems (calcareous-acidic, dry-wet) in the Netherlands. High EcM, low AM colonization and lack of differences between habitats indicate that ectomycorrhizas do not increase their importance in later successional stages. EcM and AM colonization and plant nutrient status indicate that the relative importance of P and N does not change during the succession, but during season.* Salix repens *showed low levels of AM colonization, but such a colonization nevertheless contributed to cover P-demands of the plant. As a decrease in AM colonization in* S. repens *at the end of the season coincided with a decrease in AM inoculum potential, the seasonal decline of arbuscular mycorrhiza is caused rather by changes in plant demand or soil nutrient availability than by interference with ectomycorrhiza. Regardless of seasonal shifts and possible interaction between ectomycorrhiza and arbuscular mycorrhiza both persist in the plant roots during seasons and throughout succession. Differences in habitat preference of various EcM morphotypes and arbuscular mycorrhiza suggest that mycorrhizal diversity contributes to the broad ecological amplitude of* S. repens.

**KEY WORDS:** arbuscular mycorrhiza, ectomycorrhiza, dual mycorrhiza, dune ecosystems, willow, *Salix repens*

## INTRODUCTION

*Salix repens* L. is a clonal plant that spreads through rhizomes. It is a common shrub in a great variety of plant communities in the coastal dunes of the Wadden Isles of the Netherlands. It occurs in habitats that range from dry to wet, and from calcareous, humus-poor to acidic, humus-rich soils. *S. repens* is furthermore one of the very few plants that form associations with arbuscular (AM) and ectomycorrhizal (EcM) fungi (Harley & Harley, 1987), and therefore might have a selective advantage in this highly dynamic ecosystem.

Read (1989) mentioned the importance of mycorrhizas in the sand dune ecosystem. As the availability of nitrogen and phosphorus changes during successional stages in the dune ecosystem, the occurrence and function of mycorrhizal types - EcM and AM - can change as well. The different successional stages are therefore characterized not only by a typical nutrient status but also by a dominant mycorrhizal type. In the dry foredunes, where phosphorus is the main growth limiting nutrient, arbuscular mycorrhiza dominates. By contrast in the dune slacks, where accumulation of organic matter occurs, nitrogen mineralization is inhibited due to pH reduction, and therefore nitrogen becomes the main growth limiting element and ectomycorrhiza prevails (Read, 1989). Both AM and EcM fungal communities can vary enormously in species richness and composition.

It is widely known for ectomycorrhizas that taking into account only the above-ground views results in incomplete information. Whereas sporocarps of an EcM fungus indicate its presence in the soil, its absence does not indicate the opposite. An assessment of the species richness of AM fungi can only be obtained by below-ground sampling because there are no above-ground structures formed.

Moreover, the interactions between AM and EcM fungi can be studied from a below-ground perspective only. According to Lodge & Wentworth (1990), a negative association between AM and EcM fungi on the same root system can occur. *Salix repens* as a dual mycorrhizal plant can therefore be a suitable model to investigate the interaction of these most common mycorrhizal types.

The aims of this study were: (1) to relate plant nutrient status of *S. repens* to soil chemistry; (2) to characterize species composition and inoculum potential of AM fungi; (3) to study temporal dynamics in colonization of both mycorrhizal types; (4) to assess the interaction between AM and EcM colonization; and (5) to relate AM and EcM colonization to soil chemistry and plant nutrient status.

## MATERIAL AND METHODS

### *Site description*

Sixteen plots of 500 $m^2$ each were established in sites on the Wadden Isle Terschelling. These field sites comprised a natural range of highly different environmental conditions, ranging from dry to wet, and alkaline to acidic soils (drifting sands, slopes, and soils with level surface, water tables that fluctuate with season). All field sites contained both male and female plants of *Salix repens*. Detailed site descriptions are given in CHAPTER 2. The sites 1, 2, 3, 7, 8, 9, 10, and 11 are dry sites. Sites 1-3 are on calcareous, sites 7-11 on acidic soils. The sites 4, 5, 6, 12, 13, 14, 15, and 16 are wet sites. Sites 4-6 are on calcareous soil, 12-16 on acidic soils.

### *Plant and soil analysis*

In April (spring) and in August (summer) 1997 in each field site randomly 5 plants were selected and leaves were collected from top 20 cm of the shoot (5 samples, each 10 g total dry weight). Furthermore, randomly 10 plants were selected and shoots with leaves (10 samples, top 20 cm) were collected. The plant material was dried at 70 °C for 72 hours. After drying and grinding the samples were individually digested in sulphuric acid, salicylic acid and 30 % hydrogen peroxide (Novozamsky *et al.*, 1988). Total N and P were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard. Methods of soil analysis are presented in CHAPTER 2.

### *EcM colonization and morphotyping*

All sites were sampled in October (autumn) 1994 and August (summer) 1995, and only five sites in April (spring) 1996 (the other field sites were inaccessible as protected bird reserves). Methods are presented in CHAPTER 2. In addition to total numbers of EcM root tips and EcM frequency (number of EcM root tips divided by total number of root tips * 100), the percentage of EcM root length (Giovannetti & Mosse, 1980) was determined.

### *AM colonization*

Together with the sampling for EcM colonization, soil-root samples of 1500 $cm^3$ (see methods in CHAPTER 2) were made and stored at 4 °C in containers with 50 % alcohol until processed. Samples were immersed in water over a 2 mm sieve to remove the soil,

and rinsed gently to avoid damage of the mycorrhizas. Clean roots were cleared in 10 % KOH for 3 hours at 90 °C and 1 hour in 10 % $H_2O_2$, acidified in 1 % HCl for 15 minutes, stained with trypan blue in lactophenol for 30 minutes (Phillips & Hayman, 1970; APPENDIX C) and then stored in glycerol. Colonization was evaluated according to McGonigle *et al.* (1990), where a minimum of 100 line intersections per subsample (three subsamples were analysed per sample, in 10 samples per field site) was scored for presence of AM fungal structures (hyphae, hyphal coils, vesicles, arbuscules, and spores). Furthermore, a modification of percentage root length colonization (RLC%) was used, i.e. multiplied with the intensity of colonization (RLCI% = root length colonization (%RLC) multiplied by the (%) of the cross section of the root occupied with AM structures), and total AM root length (cm) was determined.

*Spore trapping and isolation of AM fungi*

In December 1996 soil samples were taken in four sites representative for the different habitat types, viz. site 1 (Calcareous-Dry, two separate samples; one under *Ammophila arenaria* (L.) Link and one under *S. repens*), 4 (Calcareous-Wet), 10 (Acidic-Dry), and 13 (Acidic-Wet). Native plants that emerged from the original soil and sown plants of *Calamagrostis epigejos* (L.) Roth were cultured in the pots for subsequent spore trapping. Spores of AM fungi were extracted by wet sieving and centrifugation in sucrose from soils after one year cultivation in the greenhouse. Multispore subcultures were established on maize grown in vermiculite-sand mixture (1:1 v/v) and AM fungi were identified according to spore morphology. The purity of cultures was checked by isoenzyme analysis of succinate dehydrogenase and esterase according to Dodd *et al.* (1996).

In October 1996 one additional soil sample was taken under *A. arenaria*, the dominant plant species surrounding the young successional field plot 1. AMF species were identified according to spore morphology after three successive cycles of trapping (Stürmer, 1998). First traps were set from the field soil itself, then the second cycle was set up from the first trap by dilution with sterile sand (1:1 v/v). The pots were sown with 50 to 60 seeds of *Sorghum sudanese* (Piper) Staph. The plants were grown for 3 months in a greenhouse. Two soil cores (100 ml each) were taken from both sides of the pot at the harvest and the spores were extracted as described. The plants were cut off and pots were resown and the trapping cycle was repeated twice more.

*Estimation of AM inoculum potential*

Arbuscular mycorrhizal inoculum potential was estimated using an independent trap plant (Brundrett *et al.*, 1996). Ten 100 ml soil samples were randomly collected in April (spring) and in August (summer) 1997 from each site. For each season separately, the samples were pooled to one compound sample from each plot and the sample was divided to ten 100 ml pots. Each pot was sown with 25 seeds of *Trifolium repens* L. A preliminary experiment revealed that rhizobia were present in all field sites. Therefore, no rhizobia were inoculated to the pots. The pots were placed in a growth room (photon flux density 350 $\mu E.m^{-2}.s^{-1}$ (20,000 lux), 16 h day/20 °C/RH 60 %; 8 h night/16 °C/RH nearly 95 %). After 7 and 14 weeks 5 pots from each plot were harvested. The roots were washed, cleared in 10 % KOH at 90 °C for 1 hour, rinsed in tap water, acidified in 1 % HCl for 10 minutes, and stained with trypan blue in lactophenol for 30 minutes. Further procedures were the same as those used to assess AM colonization in *S. repens*.

*Data analysis*

Plant nutrient concentrations, number of EcM morphotypes, number of EcM root tips, total number of root tips, total root length, EcM colonization, AM colonization, and AM fungal inoculum potential were analysed by analysis of variance (ANOVA). Prior to analysis all parameters (but proportional mycorrhizal colonization) were logarithmically transformed. Proportional EcM and AM colonization were arcsine square root transformed. Differences among means were evaluated by the LSD-test (Sokal & Rohlf, 1995). Numbers of the different ectomycorrhizal morphotypes were not normally distributed and were analysed by Kruskall-Wallis and Mann Whitney U tests (Siegel & Castellan, 1988).

Correlations between (i) soil nutrient status and plant nutrient status; (ii) AM colonization and AM inoculum potential; (iii) EcM and AM colonization; and (iv) EcM or AM colonization and soil chemistry and plant nutrient content were tested by Spearman's rank correlation test (Siegel & Castellan, 1988).

## RESULTS

*Plant analysis*

The percent N and the ratio of N/P in shoots showed significant differences among soils with contrasting pH values (TABLE 3-1). In April, the N percent in shoots was lower in the calcareous sites. The percent N in leaves was significantly lower in Calcareous-Dry than in Acidic-Wet. Sampling date strongly significantly affected all parameters and all leaf

percent N had decreased in August, whereas shoot percent N was only lower in the Acidic sites. Shoot and leaf P concentrations were not affected by pH, but differed significantly between moisture regimes (TABLE 3-1). In April, leaf and shoot percent P was higher in Acidic-Dry than in Calcareous-Wet. In August leaf and shoot percent P were usually lower than in April. Leaf and shoot N/P ratios in April were lower than in August.

As total leaf area in August was twice the leaf area in April (data not shown), all habitat types showed higher leaf nutrient concentrations in April than in August. Leaf P, however, decreased to a larger extent than N (67 % versus 50 %). In April leaf nutrient concentrations differed between habitats, whereas in August they did not.

Soil total N/P ratio and the available N/P ratio showed no correlations with leaf and shoot N/P ratio in either April or August (in all cases, $P >> 0.05$, Spearman's rank correlation test).

**TABLE 3-1.** Nutrient concentrations (%), $N_{tot}$, $P_{tot}$ and N/P ratios in leaves and shoots of *Salix repens* from field sites (Calcareous-Dry, Calcareous-Wet, Acidic-Dry, Acidic-Wet) in April and August (1997).

| | | Leaves | | | Shoots | | |
|---|---|---|---|---|---|---|---|
| Habitat types | n | N (%) | P (%) | N/P | N (%) | P (%) | N/P |
| Habitat type | | | | | | | |
| pH (Calc.-Acid.) | | ns | ns | ns | * | ns | ** |
| Moisture (Dry-Wet) | | ns | * | *** | ns | *** | *** |
| Sampling date | | *** | *** | *** | *** | *** | *** |
| PH × moisture | | ns | ns | ns | ns | ns | ns |
| PH × sampling date | | ns | ns | ns | * | ** | ns |
| Moisture × sampling date | | ns | ns | ns | ns | ns | ns |
| PH×moisture×sampling date | | ns | ns | ns | ns | ns | ns |
| April | | | | | | | |
| Calcareous-Dry | 3 | 3.72 b† | 0.58 bc | 6.42 a | 2.13 bc | 0.33 de | 6.44 a |
| Calcareous-Wet | 3 | 3.86 bc | 0.50 b | 7.77 ab | 2.18 bc | 0.28 cd | 7.78 ab |
| Acidic-Dry | 5 | 3.98 bc | 0.60 c | 6.67 a | 2.64 d | 0.38 e | 6.94 a |
| Acidic-Wet | 5 | 4.22 c | 0.56 bc | 7.70 a | 2.64 cd | 0.31 cd | 8.53 b |
| August | | | | | | | |
| Calcareous-Dry | 3 | 1.93 a | 0.20 a | 9.55 bc | 1.83 ab | 0.26 bc | 7.05 a |
| Calcareous-Wet | 3 | 1.96 a | 0.17 a | 11.67 de | 1.94 ab | 0.21 ab | 9.23 b |
| Acidic-Dry | 5 | 2.11 a | 0.20 a | 10.56 cd | 2.02 ab | 0.23 b | 8.78 b |
| Acidic-Wet | 5 | 2.06 a | 0.17 a | 12.68 e | 1.76 a | 0.16 a | 10.99 c |

* Significance levels in ANOVA table; *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) or not significant (ns).
† Significant differences between different habitats and seasons are indicated by different letters ($P < 0.05$, LSD).

**TABLE 3-2.** Average ($n$ = 10) root length (cm), arbuscular mycorrhizal root length (RLCI %, cm mycorrhizal length), ectomycorrhizal root length (% of root length, cm mycorrhizal length), average number of root tips, ectomycorrhizal frequencies (% of number root tips), and total number of ectomycorrhizal root tips in 1500 $cm^3$ soil in *Salix repens* sites (Calcareous-Dry, Calcareous-Wet, Acidic-Dry, Acidic-Wet) in April (1996), August (1995) and October (1994).

| | n | Root length (cm) | AM (%RLCI) | AM (cm) | EcM (%RLC) | EcM (cm) | Number of root tips | EcM frequencies | Number of EcM root tips |
|---|---|---|---|---|---|---|---|---|---|
| Type of habitat | | | | | | | | | |
| pH (Calc.-Acidic) | | ns | ns | ns | * | ns | ns | * | ns |
| Moisture (Wet-Dry) | | ns | ns | ns | ns | ns | ns | ns | ns |
| Sampling date | | ** | *** | *** | ns | ** | ** | ns | ** |
| April (1996) | | | | | | | | | |
| Calcareous-Dry | 2 | 704 abc[†] | 5.0 b | 34.9 abc | 85 abc | 601 abc | 1610 abc | 85 abc | 1361 abc |
| Calcareous-Wet | 1 | 939 abc | 10.8 c | 101.4 bc | 81 abc | 764 abc | 1959 abc | 81 abc | 1577 abc |
| Acidic-Dry | 1 | 314 abc | 5.9 bc | 18.5 abc | 79 abc | 249 abc | 473 abc | 81 abc | 382 abc |
| Acidic-Wet | 1 | 545 abc | 4.9 bc | 26.8 abc | 63 ab | 344 abc | 755 abc | 62b | 465 abc |
| August (1995) | | | | | | | | | |
| Calcareous-Dry | 3 | 504 ab | 4.5 b | 22.8 abc | 84 bc | 423 ab | 874 ab | 84 bc | 735 ab |
| Calcareous-Wet | 3 | 412 a | 6.2 bc | 25.3 a | 80 abc | 329 a | 656 a | 80 abc | 526 a |
| Acidic-Dry | 5 | 352 a | 4.8 b | 17.0 a | 77 abc | 272 a | 1070 a | 76 abc | 813 ab |
| Acidic-Wet | 5 | 1336 abc | 4.0 b | 52.9 c | 69 a | 902 abc | 2213 abc | 68 a | 1507 abc |
| October (1994) | | | | | | | | | |
| Calcareous-Dry | 3 | 1076 abc | 0.6 a | 6.1 a | 88c | 953 abc | 2157 abc | 87 c | 1866 abc |
| Calcareous-Wet | 3 | 3862 bc | 0.7 a | 27.8 a | 86 bc | 3325 bc | 5247 bc | 84 bc | 4412 bc |
| Acidic-Dry | 5 | 1063 abc | 0.2 a | 1.7 a | 88 c | 939 abc | 1904 abc | 85 c | 1622 abc |
| Acidic-Wet | 5 | 4730 c | 0.3 a | 11.7 a | 77 abc | 3647 c | 6139 c | 76 abc | 4659 c |

* Significance levels in ANOVA table; *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) or not significant (ns). All interactions were ns.

[†] Differences between habitat types and season are indicated by different letters ($P < 0.05$, LSD).

*EcM and AM colonization*

Unlike in most other tree species, EcM fungi colonize long stretches of *Salix* and most *Populus* spp. root systems, and not only root tips. Therefore, both percentage EcM root length (%RLC) and EcM frequency (% of root tips) were measured. However, no significant difference occurred between both EcM colonization parameters (%RLC and % of root tips). Root length, number of root tips, number of EcM root tips, and AM colonization (%RLCI and cm) did not differ significantly between the four habitat types, but differences between sampling dates were significant (TABLE 3-2). Root length, number of root tips and number of EcM root tips were highest in October 1994, whereas AM colonization intensity (RLCI%) and AM root length (cm) were lowest in October 1994. Moreover, EcM colonization (%RLC and frequency) was significantly affected by habitat type (pH) and not by sampling date. EcM (%RLC and frequency) in August 1995 was slightly but significantly higher in Calcareous-Dry than in Acidic-Wet, whereas total EcM root length (cm) was not affected. By contrast, total EcM root length (cm) was significantly affected by sampling date, whereas percentage EcM root length (%RLC) was not.

AM colonization was consistently low, less than 11 % in all plots. No differences in relative amount of AM fungal structures (only hyphae and vesicles, relative occurrence 4:1) were noted between sites (data not shown). However, one exception occurred in part of the field site Telefooncellen which was flooded in October 1994. In three root samples collected from the flooded half, roots were occupied by intraradical spores. AM colonization intensity (%RLCI) was signicantly lower in October 1994 than in April 1996 and August 1995. In October 1994 and August 1995 AM (%RLCI) was not significantly different between habitats. Total AM root length (cm) in August 1995 was significantly higher in the Acidic-Wet habitat than in Acidic-Dry and Calcareous-Wet.

*EcM morphotypes*

The number of recognizable morphotypes per plot ranged from 3 to 8. *Hebeloma*, *Inocybe*, ITE5 and *Laccaria* were observed in all habitats, while *Cenococcum* was absent in Calcareous-Dry (TABLE 3-3).

**TABLE 3-3.** The average ($n$ = 7) number of EcM root tips of dominant morphotypes of ectomycorrhizas in *Salix repens* field sites (Calcareous-Dry, Calcareous-Wet, Acidic-Dry, Acidic-Wet), in April (1996), August (1995), and October (1994).

| | n | Total | Nvit* | Cen | Crt | Heb | Ino | ITE5 | ITE6 | Lac | Lct | Pax | Und. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| April (1996) | | | | | | | | | | | | | |
| Calcareous-Dry | 2 | 1380 ab[†] | 239 ab | 0 a | 0 a | 114 abc | 0 a | 0 a | 689 b | 1 a | 0 ab | 142 b | 397 ab |
| Calcareous-Wet | 1 | 1611 ab | 437 ab | 5 ab | 0 ab | 62 c | 29 abc | 0 ac | 0 ab | 58 abc | 17 bc | 0 ab | 683 ab |
| Acidic-Dry | 1 | 386 ab | 76 ab | 66 ab | 0 ab | 0 abc | 0 ab | 0 ac | 0 ab | 118 abc | 0 ab | 0 ab | 121 ab |
| Acidic-Wet | 1 | 454 ab | 138 ab | 0 ab | 0 ab | 0 abc | 0 ab | 0 ac | 0 ab | 0 abc | 0 ab | 0 ab | 316 ab |
| August (1995) | | | | | | | | | | | | | |
| Calcareous-Dry | 3 | 724 a | 146 ab | 0 a | 10 ab | 50 abc | 28 ab | 33 ab | 0 a | 10 a | 0 a | 0 a | 380 ab |
| Calcareous-Wet | 3 | 540 a | 67 a | 26 ab | 12 ab | 2 abc | 4 ab | 54 bc | 0 a | 72 ab | 0 a | 0 a | 297 ab |
| Acidic-Dry | 5 | 903 a | 178 ab | 17 ab | 0 a | 2 abc | 0 a | 18 a | 0 a | 284 b | 0 a | 0 a | 365 ab |
| Acidic-Wet | 5 | 1532 ab | 180 ab | 245 b | 80 ab | 0 ab | 0 a | 2 a | 0 a | 775 c | 1 ab | 0 a | 207 ab |
| October (1994) | | | | | | | | | | | | | |
| Calcareous-Dry | 3 | 2558 b | 478 b | 0 a | 0 a | 639 de | 370 bc | 203 bc | 0 a | 0 a | 0 a | 0 a | 177 ab |
| Calcareous-Wet | 3 | 4617 b | 560 b | 34 ab | 0 a | 451 e | 1816 c | 493 c | 50 b | 46 ab | 0 a | 8 ab | 1174 b |
| Acidic-Dry | 5 | 1363 ab | 574 ab | 31 ab | 4 a | 2 abc | 37 ab | 47 ab | 0 a | 837 c | 0 a | 3 ab | 141 ab |
| Acidic-Wet | 5 | 5159 b | 998 b | 168 b | 1796 b | 95 cd | 1248 c | 145 bc | 62 b | 295 b | 100 c | 1 a | 182 a |

* The EcM morphotypes are abbreviated as follows: Nvit = 'not vital', Cen = *Cenococcum*, Crt = *Cortinarius*, Heb = *Hebeloma*, Ino = *Inocybe*, Lac = *Laccaria*, Lct = *Lactarius*, Pax = *Paxillus*, Und. = 'undeveloped'.

[†] Differences between habitat types and season are indicated by different letters ($P < 0.05$, MWU).

Individual morphotypes had different habitat preferences (TABLE 3-3). *Cenococcum* preferred the Acidic-Wet habitat, having substantially higher numbers of EcM root tips in Acidic-Wet than in Calcareous-Dry in summer and autumn. *Cortinarius* preferred Acidic-Wet habitat, but higher numbers of EcM root tips were found only in autumn. *Laccaria* tended to have higher numbers of EcM root tips in the Acidic habitat. Opposite to the above mentioned morphotypes, *Hebeloma* showed highest numbers of EcM root tips in the Calcareous-Wet habitat in autumn; ITE5 exhibited a similar pattern.

*AM fungal species composition and AM inoculum potential*

Significantly more AM fungal spores were trapped in the two Calcareous sites (537 and 498/kg dry soil, in wet and dry resp.) than in the two Acidic sites (47 and 34/kg dry soil, in wet and dry resp.). Moisture showed no effect on amount of spores (ANOVA, pH: $P < 0.05$; moisture and pH × moisture: $P > 0.05$).

Species composition differed between Calcareous and Acidic habitats, whereas there was no difference between Wet and Dry habitats. In the Calcareous-Wet site, *G. mosseae* and *G. claroideum* (*G. fistulosum* type spores) and in the Calcareous-Dry site, *G. mosseae* and *Acaulospora* sp.were isolated. In both acidic field sites, *G. fasciculatum* and *G. etunicatum* were trapped. In the soil from Calcareous-Dry site collected under *A. arenaria*, *Glomus mosseae*, *Acaulospora* sp., and another *G.* sp. (*G. etunicatum* - *G. geosporum* group) were trapped. Similar species composition was found under *S. repens.*

After three successive cycles of spore trapping with another trap plant, *Sorghum*, eight species of *Glomus* were identified in the soil taken under *A. arenaria* in the Calcareous-Dry site, but only three of them were named. Furthermore, each subsequent trap increased the number of AMF species. This list indicates that AMF species composition in these dunes can be quite high: *G.* sp. isolate 6 (*G. claroideum* 'like'), *G.* sp. isolate 11, *G. intraradices*, *G. pustulatum*, *G. clarum*, *G.* sp. isolate 12 and *G.* sp. isolate 13 were found. Isolate numbers according to Stürmer (1998).

AM inoculum potential was strongly affected by soil pH and sampling date, but not by moisture regime (TABLE 3-4). AM inoculum potential was higher in April than in August and, opposite to spore counts, higher in the acidic sites than in the calcareous sites. AM colonization in *S. repens* was not significantly correlated with the AM inoculum potential (TABLE 3-5), although all assessments of AM inoculum potential showed very high positive correlations between both harvests and sampling dates.

**TABLE 3-4.** Average arbuscular mycorrhizal inoculum potential of the soil from *Salix repens* field sites (Calcareous-Dry, Calcareous-Wet, Acidic-Dry, Acidic-Wet) in April and August (1997). Measured as (% RLCI) after 7 and 14 weeks in bioassay test with *Trifolium repens*.

| | | *Trifolium repens* (AM % RLCI) | |
|---|---|---|---|
| Habitat types | n | 7 weeks | 14 weeks |
| Habitat types | | | |
| pH (Calc.-Acidic) | | *** | *** |
| Moisture (Dry-Wet) | | ns | ns |
| Sampling date | | ** | * |
| April (1997) | | | |
| Calcareous-Dry | 3 | 6.1 ab[†] | 7.1 ab |
| Calcareous-Wet | 3 | 10.5 bcd | 10.8 ac |
| Acidic-Dry | 5 | 20.1 de | 28.5 cd |
| Acidic-Wet | 5 | 29.1 e | 32.6 d |
| August (1997) | | | |
| Calcareous-Wet | 3 | 3.8 a | 4.6 ab |
| Calcareous-Dry | 3 | 4.4 ac | 3.6 a |
| Acidic-Dry | 5 | 13.9 bd | 20.6 cd |
| Acidic-Wet | 5 | 14.7 bd | 25.0 cd |

* Significance levels in ANOVA table; *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) or not significant (ns). All interactions were ns.

[†] Differences between habitat types and season are indicated by different letters ($P < 0.01$, LSD).

**TABLE 3-5.** Correlation matrix of ectomycorrhizal colonization (EcM %RLC), arbuscular mycorrhizal colonization (AM %RLCI) of *Salix repens* in April (1996, $n = 5$), August (1995, $n = 16$) and October (1994, $n = 16$), and arbuscular mycorrhizal inoculum potential (AM %RLCI of *Trifolium repens*) in April and August (1997), after 7 and 14 weeks of 16 *Salix repens* field sites.

| | EcM (%RLC) | | | AM (%RLCI) | | | AM potential (%RLCI) | | |
|---|---|---|---|---|---|---|---|---|---|
| | April 1996 | August 1995 | October 1994 | April 1996 | August 1995 | October 1994 | April 7 wks | April 14 wks | August 7 wks |
| EcM(%RLC) | | | | | | | | | |
| August 1995 | 0.70 | | | | | | | | |
| October 1994 | 0.60 | 0.57 * | | | | | | | |
| AM(%RLCI) | | | | | | | | | |
| April 1996 | -0.80 | -0.80 | -0.90 * | | | | | | |
| August 1995 | -0.60 | -0.22 | -0.17 | 0.90 * | | | | | |
| October 1994 | -0.50 | 0.11 | -0.11 | 0.70 | 0.57 * | | | | |
| AM potential (%RLCI) | | | | | | | | | |
| April '97 7wks | -0.70 | -0.19 | -0.13 | 0.50 | -0.01 | -0.28 | | | |
| April '97 14wks | -0.70 | -0.19 | -0.10 | 0.50 | 0.01 | -0.30 | 0.83 *** | | |
| August'97 7wks | -0.60 | -0.34 | -0.20 | 0.60 | 0.22 | -0.13 | 0.67 ** | 0.82 *** | |
| August'97 14wks | -0.50 | -0.38 | -0.25 | 0.40 | 0.30 | -0.10 | 0.65 ** | 0.81 *** | 0.91 *** |

* Significant correlations are indicated by * ($P < 0.05$). ** ($P < 0.01$) and ***($P < 0.001$) (Spearman's rank correlation test).

**TABLE 3-6.** Correlation between root characteristics in April (1996, $n = 5$), August (1995, $n = 16$) and October (1996, $n = 16$) and soil nutrient concentrations (mg/kg), pH and organic matter (%) in 16 *Salix repens* field sites.

| | Sampl. date | pH | OM (%) | Ntotal | Ptotal | N/Ptotal | $NO_3^-$ | $NH_4^+$ | Nts† | Ps† | N/Ps† |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AM (%RLCI) | April | -0.50 | 0.50 | 0.10 | -0.70 | 0.10 | 0.66 | 0.50 | -0.30 | 0.30 | -0.10 |
| | August | 0.08 | -0.08 | -0.09 | -0.14 | -0.05 | 0.29 | -0.06 | -0.19 | 0.02 | -0.30 |
| | October | 0.51 * | -0.50 * | -0.49 * | -0.51 * | -0.53 * | -0.09 | -0.56 * | -0.56 * | -0.45 | 0.07 |
| EcM (%RLC) | April | 0.70 | -0.70 | -0.50 | 0.50 | -0.50 | -0.56 | -0.70 | 0.30 | -0.30 | -0.10 |
| | August | 0.03 | 0.08 | 0.13 | 0.29 | 0.03 | 0.30 | -0.01 | 0.29 | -0.02 | 0.15 |
| | October | 0.06 | -0.20 | -0.08 | 0.08 | -0.11 | -0.01 | -0.30 | 0.25 | -0.03 | -0.16 |
| No. EcM roottips | April | 0.60 | -0.60 | -0.70 | -0.60 | -0.70 | -0.35 | -0.60 | -0.10 | -0.90 * | 0.80 |
| | August | -0.02 | 0.22 | 0.29 | 0.41 | 0.30 | 0.43 | 0.28 | 0.26 | 0.11 | -0.10 |
| | October | 0.36 | -0.00 | -0.01 | 0.03 | 0.07 | 0.24 | -0.12 | -0.01 | -0.25 | 0.23 |
| No. of root tips | April | 0.60 | -0.60 | -0.70 | -0.60 | -0.70 | -0.35 | -0.60 | -0.10 | -0.90 * | 0.80 |
| | August | -0.02 | 0.11 | 0.18 | 0.27 | 0.18 | 0.33 | 0.21 | 0.20 | 0.08 | -0.15 |
| | October | 0.05 | 0.25 | 0.24 | 0.15 | 0.30 | 0.49 | 0.24 | 0.22 | -0.01 | 0.21 |
| Root length | April | 0.60 | -0.60 | -0.70 | -0.60 | -0.70 | -0.35 | -0.60 | -0.10 | -0.90 * | 0.80 |
| | August | -0.11 | 0.16 | 0.24 | 0.30 | 0.24 | 0.36 | 0.30 | 0.25 | 0.17 | -0.19 |
| | October | 0.04 | 0.29 | 0.29 | 0.21 | 0.34 | 0.51 * | 0.27 | 0.22 | 0.00 | 0.22 |

* Significant correlations are indicated with * ($P < 0.05$), and ** ($P < 0.01$) (Spearman's rank correlation test).

† s in Nts, Ps and N/Ps stands for dissolved.

**TABLE 3-7.** Correlations between root characteristics in April (1996, $n$ = 5), August (1995, $n$ = 16) and October (1994, $n$ = 16) and leaf nutrient concentrations in April and August (1997) of 16 *Salix repens* field sites.

| | | April | | | August | | |
|---|---|---|---|---|---|---|---|
| | Sampling date | N (%) | P (%) | N/P | N (%) | P (%) | N/P |
| AM (%RLCI) | April 1996 | 0.10 | -0.70 | 0.90 * | 0.10 | -0.60 | 0.90 * |
| | August 1995 | -0.22 | -0.08 | -0.02 | 0.37 | 0.10 | 0.22 |
| | October 1994 | 0.06 | 0.00 | 0.02 | 0.36 | 0.14 | 0.23 |
| EcM (%RLC) | April 1996 | -0.50 | 0.50 | -0.90 * | 0.10 | 0.90 * | -0.90 * |
| | August 1995 | 0.28 | 0.63 ** | -0.58 * | 0.17 | 0.43 | -0.39 |
| | October 1994 | 0.22 | 0.51 | -0.47 | 0.15 | 0.61 * | -0.58 * |
| No. EcM root tips | April 1996 | -0.70 | -0.60 | 0.20 | -0.80 | -0.20 | -0.20 |
| | August 1995 | -0.05 | 0.40 | -0.36 | 0.19 | 0.27 | -0.23 |
| | October 1994 | 0.01 | 0.20 | -0.14 | 0.02 | 0.12 | -0.18 |
| No. of root tips | April 1996 | -0.70 | -0.60 | 0.20 | -0.80 | -0.20 | -0.20 |
| | August 1995 | 0.04 | 0.32 | -0.21 | 0.15 | 0.15 | -0.09 |
| | October 1994 | 0.22 | 0.25 | -0.10 | -0.10 | 0.09 | -0.24 |
| Root length | April 1996 | -0.70 | -0.60 | 0.20 | -0.80 | -0.20 | -0.20 |
| | August 1995 | 0.13 | 0.37 | -0.18 | 0.18 | 0.14 | -0.05 |
| | October 1994 | 0.23 | 0.27 | -0.06 | -0.17 | 0.09 | -0.17 |

* Significant correlations are indicated by * ($P < 0.05$) and **($P < 0.01$) ( Spearman's rank correlation test).

*EcM and AM colonization, soil chemistry and plant nutrient content*

AM colonization intensity (%RLCI) in October 1994 was strongly significantly correlated with most of the soil characteristics. AM colonization intensity (RLCI%) was positively correlated with pH, and negatively with OM(%), $N_{tot}$, $P_{tot}$, $N/P_{tot}$, $NH_4^+$, and $N_{ts}$. By contrast, EcM colonization (%RLC and EcM frequency) was not significantly correlated with any of the soil characteristics (TABLE 3-6).

In April 1996 AM colonization showed a positive and EcM colonization (%RLC and frequency) showed a negative correlation with leaf N/P ratio in April 1997. Furthermore, EcM colonization in August 1995 was positively correlated with leaf P and negatively with leaf N/P ratio in April 1997. EcM colonization in October 1994 showed similar positive correlations with leaf P and N/P ratio in August 1997 (TABLE 3-7).

## DISCUSSION

Ideally all parameters presented should be measured in the same year in order to draw conclusions on seasonal dynamics without problems. However, due to the large amount of data that had to be collected, the samplings were performed in different years. Therefore, discussion of the results implying seasonal dynamics should be interpreted cautiously.

*Mycorrhizal colonization: successional stage, habitat and seasonal effects*

These data showing high EcM colonization in all sites, also in the primary dune successional stages (Calcareous sites) contradict Read (1989), who mentioned that ectomycorrhizas play an increasingly important role in later successional stages when nitrogen becomes the main growth limiting factor. Consistently low AM colonization and lack of differences between habitats suggest that the relative importance of phosphorus and nitrogen-limitation during succession does not change. These data are consistent with observations by Kooijman *et al.* (1998), who noted that in dune ecosystems of the Wadden Isles nitrogen is the main limiting factor in all successional stages. However, AM colonization that varied over the different sampling dates (April 1996 ~7 %, August 1995 ~5 % and October 1994 ~0.6 %), could well imply that nutrient limitation (or plant nutrient demand) follows a seasonal pattern, with phosphorus being the major limiting element at the start of the growing season (*S. repens* flowers early in season). For strawberry (*Fragaria* × *ananassa* cv. Hapil) Dunne & Fitter (1989) noted a brief period of high P demand during fruit development, when arbuscular mycorrhizas were essential to explain the inflow, but a lower P demand during vegetative growth, which could even be met without arbuscular mycorrhiza. High leaf P in spring and the larger loss of leaf P than of leaf N are consistent with this hypothesis of the importance of seasonality of nutrient requirements and limitation.

*Salix repens* showed low levels of AM colonization, but the data in this study suggest that these low amounts of colonization significantly contribute to nutrient demands of the plant. Similar low amounts of AM colonization were found to be very effective in terms of plant growth response and fast phosphorus uptake to inoculation in laboratory experiments (CHAPTERS 4 AND 5). Similar positive effects on P uptake by a small amount of *Glomus* hyphae and vesicles in Pinaceae seedling were reported by Smith *et al.* (1998). Jones *et al.* (1991) reported that EcM fungi were very effictive in P uptake in

an early stage of development. In a laboratory study, inoculation of *S. repens* with *Glomus mosseae* resulted in low AM colonization and a fast plant growth response and P uptake (2 weeks), while colonization with three EcM fungi was higher and plant growth responses occurred over a longer period (10 weeks) (CHAPTER 4). There are probably more tree species, previously considered just EcM hosts, that can have similarly low AM colonization, which is hardly detectable and easily overlooked, nevertheless might have significant beneficial effects (maybe depending on season) on plant vigor and growth. In order to measure (temporary) beneficial effects timing is very essential.

*Diversity of both mycorrhizal types*

Even though total EcM colonization did not differ between habitats and sampling dates, habitat preference of the different EcM morphotypes indicated that relative colonization by a certain morphotype of *S. repens* is variable, and can be explained by differences in soil pH (CHAPTER 2) (which coincides with accumulation of organic matter and a change in availability of nutrients) and thus may change over successional stages (Read, 1989). Moreover, occurrence of EcM morphotypes varied between sampling dates. This might be caused by spatial variation within a field site, but in most replicates similar changes in relative abundance were found at the different sampling dates. It is therefore hypothesized that within the functional group of EcM fungi, different functions towards the plant exist; (1) these functions of different fungi may differ in different environments, (2) in different environments these functions are exerted by different EcM fungi. Finally, (3) within one year different EcM morphotypes may contribute during different seasons as their relative abundance may change over seasons.

AM colonization of *S. repens* was not different between Calcareous and Acidic habitat types. This could not be explained by either inoculum amount (spore number) or inoculum potential. Mycorrhizal inoculum potential, determined with *Trifolium* as an independent bait plant, suggests that the Acidic sites had a higher inoculum potential, whereas the two calcareous sites had higher numbers of AM spores than the two acidic sites. A lower mycorrhizal inoculum potential in August coincided with a lower AM colonization in *S. repens* in October. The differences between AM colonization of *S. repens* and of *Trifolium* in the soils from four different habitats suggest that colonization is under plant control. A very low AM inoculum potential of soil near *S. repens* was found also by De Jong *et al.* (1995). The species of AMF present are of interest because of the wide range of pH, and the uniform AM colonization of *S. repens*. However, spore

numbers, identity, and colonization are not necessarily related (Brundrett *et al.*, 1996). Different AM fungal species trapped by different trap plants (*Calamagrostis* and *Sorghum*) would support the hypothesis of plant regulating AM development and AM host specificity. However, two samples taken in the same field site under different host plants, *A. arenaria* (highly colonized by AM fungi), and *S. repens* (slightly colonized by AM fungi), were similar in species composition, which contradicts this hypothesis. Although *S. repens* and *A. arenaria* might share AM fungal species, AM development in roots still can be plant regulated.

*Mycorrhizal interactions*

EcM colonization (October) was only significantly negatively correlated with AM colonization (April). Negative interactions have also been found on the level of individual root tips (Lodge & Wentworth, 1990). These negative correlations have been interpreted as implying that EcM fungi exert a negative influence on AM fungi, e.g. by forming an impenetrable fungal sheath on the root (Chilvers *et al.*, 1987; Lodge, 1989). However, *S. repens* is always slightly AM and highly EcM. Negative interactions between both mycorrhizal types cannot be concluded from negative correlations, but have to be based on comparison of mycorrhizal colonization in experiments where mycorrhizal types are both simultaneously and sequentially inoculated. On the other hand, the lack of negative correlation of EcM and AM inoculum potential in this study suggests that, even if there may be weak interference among both mycorrhizal types, the sustainability of both fungal types are maintained in the plant roots or in the rhizosphere during successive seasons and probably throughout succession. Moreover, as a decrease in colonization at the end of the season (October 1994) coincided with a decrease in inoculum potential as assessed by a different bait plant (*Salix* vs. *Trifolium*) where no competition with EcM fungi for colonization sites occurs, it seems more likely that the (seasonal) decline of AM colonization in this study was caused rather by changes in plant nutrient demand or soil nutrient availability than by interference with EcM fungi.

The interaction of both mycorrhizal types could also be temporarily regulated by small shifts within inner dynamics of edaphic factors. Relative amounts of mycorrhizal associates may fluctuate through seasonal development, or may vary in their inoculum potential depending on environmental characteristics that can differ within years or between years, e.g. high ground water tables or extreme dry and hot summers. Although not measured in this study, hyphal length of mycorrhizal fungi may vary under different

conditions. The importance of hyphal length of various species in the soil might, next to its functional significance for the plant, have significant effects on the interactions between mycorrhizal species. Patchy distributions of fungal inoculum (mycelium, spores, mycorrhizal roots) in the root zone is probably also an important factor determining the distribution of mycorrhizal fungi (between and within types) on the root system. However, in a long term perspective, both mycorrhizal types seem to persist regardless of seasonal shifts and interaction between both.

Fluctuating plant demands, in season or during succession, may contribute to the persistance of both mycorrhizal types. However, situations seem to occur when AM fungi can take over the control and develop relatively high colonization (up to 30 %) under stress conditions of e.g. long term floodings as seen in part of the site Telefooncellen. During such a stress period the AM fungi form large amounts of intraradical spores as an adaptation which is probably not beneficial to the plant but serves only as a survival mechanism. Ectomycorrhiza was suppressed by flooding, particularly hyphal mantles, whereas fractions of the Hartig net survived and ensured post stress development of ectomycorrhiza. Thus changes of both mycorrhizal types previously attributed by Lodge & Wentworth (1990) to negative interaction, or a difference in moisture preference of EcM fungi and interference by some EcM fungi with AM colonization (Lodge, 1989), can in this study in the temporary flooded sites better be explained as a similarity in survival strategy of both mycorrhizal types, viz. internal survival (intraradical spores, Hartig net) in *S. repens* roots. Since, *Salix* roots are known to transport oxygen to their root system (Jackson & Attwood, 1996).

The separate assessment of AM and EcM colonization in the current study did not allow testing for mycorrhizal interactions at the same root fragment, but it was noted that *Inocybe* and *Hebeloma* were often co-occurring with AM fungi on the same root fragment. Furthermore, roots were very often, if not always, colonized by more than one EcM morphotype (even growing through each other). Considering the number of different species of AM and EcM fungi with their individual habitat preferences, interactions between mycorrhizal fungi should not be considered a priori on the level of mycorrhizal types, but on the level of individual species pairs instead.

In conclusion the flexibility and versatility of AM and EcM fungi seem to play a crucial role in the broad ecological amplitude of *S. repens* in dune ecosystems.

# 4

## Mycorrhizal infection of *Salix repens* I: competition or coexistence between AM and EcM fungi.

**ABSTRACT**

*The functional significance of two arbuscular mycorrhizal fungi (AMF) (*Glomus mosseae *and* Acaulospora laevis*) and three ectomycorrhizal fungi (EcMF) (*Hebeloma leucosarx, Inocybe vulpinella *and* Paxillus involutus*) for survival and growth of* Salix repens *cuttings was investigated. Moreover, the interaction between mycorrhizal types was investigated by simultaneous and sequential inoculation. Different mycorrhizal fungi, both within and between mycorrhizal types, had differential effects on plant performance. Generally, root colonization by AMF was very low, but had large effects on plant performance. In contrast, colonization by EcMF was very high, but showed smaller effects.* Glomus mosseae *(AMF) increased shoot length, shoot dry weight and phosphorus uptake very rapidly in the first 6 to 9 weeks of the experiment, whereas the other AMF,* A. laevis, *depressed plant performance. The positive effects of the EcMF became manifested over a much longer period (> 12 weeks). Root length of* S. repens *was increased by* G. mosseae, *but was shorter in the presence of EcMF, compared to the non-mycorrhizal treatment. Contrary to the expectations, considering the proportion of root colonization, colonization by AMF suppressed subsequent EcM colonization, whereas* G. mosseae *was only inhibited by previous colonization by* P. involutus. *When both mycorrhizal types were inoculated at the same time, in general both types colonized similar proportions of the root system as in the single inoculations of the different mycorrhizal species.* Glomus mosseae *and* P. involutus *showed mutual suppression. The persistence of the dual mycorrhizal symbiosis is discussed in relation to differences in colonizing ability of mycorrhizal types and their differential effects on performance of* S. repens.

**KEY WORDS**: arbuscular mycorrhiza, ectomycorrhiza, *Salix repens*

## INTRODUCTION

Mycorrhizas are associations between fungi and plant roots. In general this symbiosis is considered as a mutualistic relationship. The benefit for the fungus is the receipt of carbohydrates from the plant, and the fungus is able to increase resource availability and nutrient uptake of the plant in various ways (Smith & Read, 1997). Besides foraging for nutrients in the soil, several mycorrhizal fungi produce enzymes capable of breaking down organic carbon, nitrogen and phosphorus, and other mycorrhizal fungi are capable of weathering minerals (Smith & Read, 1997). Mycorrhizal fungi can also alleviate abiotic (heavy metals) and biotic (pathogens) stress (Smith & Read, 1997). By this increased resource availability and alleviation of stress mycorrhizas alter plant growth and fitness.

Arbuscular and ectomycorrhiza are the two most common types. Even though most plants form associations with only one (functional) type of mycorrhiza, few plant species regularly form both arbuscular mycorrhiza (AM) and ectomycorrhiza (EcM), and have been considered dual mycorrhizal plants. In these plants field observations have sometimes shown AM to be dominant, sometimes EcM (Lodge, 1989). These observations have been interpreted as a negative association between both types of mycorrhiza in the root system of dual mycorrhizal plants (Lodge & Wentworth, 1990; Chilvers *et al.*, 1987). But it is still not clear which factors favour dominance by AM or EcM.

Lack of resistance is one possible explanation. Arbuscular mycorrhizal fungi (AMF) colonize seedlings due to their supposed initial higher inoculum potential. However, they are rapidly replaced by ectomycorrhizal fungi (EcMF). Chilvers *et al.* (1987) and Lodge (1989) have suggested that such replacements might result from EcMF preventing colonization of newly-formed roots by AMF. The EcM sheath would provide a barrier to subsequent AM infections. Pines and tropical EcM tree species have shown low AM colonization in the seedling stage (Cázares & Smith, 1996; Moyersoen & Fitter, 1999). Moreover, it has become clear that while most temperate woody plants form primarily one type of mycorrhiza, they can form minor amounts of other types, even as mature plants.

A second explanation could be niche differentiation. Lodge (1989) noted that many dual mycorrhizal plant species typically grow in flood plains, and observed dominance by AM under dry and wet conditions, while in moist, but well drained conditions EcM dominated. She suggested that plants with the ability to form both types of mycorrhiza might have a selective advantage in such habitats. Another example of this niche

differentiation, mentioned by Read (1989), is the importance of AM and EcM in dune succession, where the primary effect of AM infection is enhanced phosphorus uptake. The high level of AM infection found in the early stage of dune succession may reflect the requirement for mobilization of phosphate, which is the limiting element in the early successional stages. In the dune slack environment, nitrogen supersedes phosphorus as the main growth limiting element, when litter decomposition and hence nutrient mineralization is retarded by acidic conditions. Thus, these environments preferentially select for EcMF because of its ability to absorb (organic) nitrogen (Read, 1989).

*Salix repens* L. like other species of *Salix, Populus* and *Eucalyptus,* is a dual mycorrhizal plant, and is widespread in a great variety of plant communities, in particular in the coastal areas of Western Europe such as the Wadden Isles in The Netherlands. *Salix repens* occurs in habitats ranging from dry to wet, and from calcareous, humus-poor to acidic, humus-rich soils. Above-ground field observations showed that in some of these communities many species and sporocarps of EcM symbionts were present, while in other communities they were almost lacking (CHAPTER 2). Furthermore, these field sites differed in EcMF species composition and it is known that EcMF vary in their affinity towards inorganic and organic nitrogen and phosphorus compounds. *Salix repens*, having so many different mycorrhizal associates, and being able to grow in such a wide variety of habitats, is the perfect system to investigate the hypothesis that different mycorrhizal fungal species, EcMF as well as AMF, have differential effects on survival and growth of *Salix repens*. The present study focuses on the different EcMF and AMF in primary dune successional stages, as in this dune successional stage plants theoretically depend on AM for P uptake (Read, 1989), whereas *S. repens* was found highly EcM. In another study emphasis is given to the significance of ectomycorrhizal diversity (collected over a range of different successional stages) for *S. repens*. The first objective in the present study is to elucidate: whether mycorrhizal fungi (that occur in the primary dune successional stage), between and within mycorrhizal types, differ in their effects on plant performance (shoot and root growth and nutrient uptake). As this is examined on very poor soil nutrient conditions, comparable to the primary dune successional stages of *S. repens* communities, it is hypothesized that AMF will show higher benefits than EcMF. Moreover, do these two mycorrhizal types show variation in their effects over time; short term (15 weeks) and long term (27 weeks, i.e. a growing season) and how specific are the effects by the different species of AMF and EcMF involved?

Below-ground field observations showed that in all selected habitat types, irrespective of the number of EcMF species or numbers of sporocarps that have been observed, the total EcM colonization was up to 80 % in all seasons, while total AM colonization was very low in all field sites. Although AM colonization varied between habitat types and seasons, it was always less than 11 % (CHAPTER 3). These observations are consistent with the idea that there is a negative association between AM and EcM (Lodge & Wentworth, 1990). However, the way in which these mycorrhizal types interact has not been studied directly. Investigations about how these fungi do interact have not been performed other than accidentally being confronted with the fact that AM-, EcM- or dual mycorrhizal colonization was observed in root samples of *Salix, Populus* or *Eucalyptus* (Lodge, 1989; Chilvers *et al.*, 1987). It is questionable whether observations of associations of mycorrhizal types are sufficient to draw conclusions on interactions.

The second objective in this study therefore was to directly investigate interactions between mycorrhizal types by inoculation of both AMF and EcMF simultaneously and sequentially to the root system of *S. repens*. It was hypothesized, when considering that EcM is the superior competitor in a dual association (AM/EcM) (Chilvers *et al.*, 1987; Lodge & Wentworth, 1990) that, (i) in simultaneous inoculation EcMF would outcompete AMF; colonization by EcMF would be similar to that in the single species inoculation, whereas colonization by AMF would be lower than in the single-species inoculation; (ii) in sequential inoculation where the EcMF was inoculated first, colonization by AMF would even be lower than in the simultaneous inoculation; (iii) in sequential inoculation where the AMF was inoculated first, colonization by EcMF would not be affected; (iv) interaction between mycorrhizal fungi can be generalized across species within functional groups, i.e. the interactions do not depend on the identity of the fungal species within a functional type.

## MATERIAL AND METHODS

### *Field description, plant and fungal material*

The study sites are located on the Wadden Isle of Terschelling and comprise a xerosere and hygrosere of dune successional stages (vegetation and soil chemical description in CHAPTER 2 AND APPENDIX A). This study was focused on the younger, calcareous, humus- and nutrient-poor successional stage with a relatively high soil pH($CaCl_2$) 7.3. Cuttings (shoot tops) of *Salix repens* were collected in December 1994

from the field site 'Schoenus I' (53° 23'50" N, 5° 13'45" E) on the Wadden Isle of Terschelling. All cuttings were collected from the same male plant in order to obtain genetic homogeneity.

Three cultures of EcM fungi collected in the autumn of 1994 from a *S. repens* community on the Isle Terschelling were used in this experiment, viz. *Hebeloma leucosarx* P.D. Orton (L1), *Inocybe vulpinella* Bruylants (L17) and *Paxillus involutus* (Batsch:Fr.)Fr. (L37). Sporocarps were surface sterilized with alcohol (70 %) and sliced in half under sterile conditions. Fungal tissue was cut from the inner side of the cap and transferred to, and maintained on, solid media (alternative Melin Norkrans (AMN)) containing (in g $l^{-1}$): agar (15), malt extract (3), glucose.$H_2O$ (10), $(NH_4)_2HPO_4$ (0.25), $KH_2PO_4$ (0.5), $KNO_3$ (0.5), $MgSO_4.7H_2O$ (0.15), $CaCl_2.H_2O$ (0.67), NaCl (0.025), $FeCl_3$ (0.0012) and thiamine.HCl (100 µg). The choice for these fungal species (isolates) was based on their sporocarp occurrence (over the three years studied) in this field site (CHAPTER 2). *Inocybe vulpinella* is restricted to this calcareous habitat, *H. leucosarx* is also found in older successional stages, but showed much higher numbers of sporocarps in this habitat, and *P. involutus* occurred in all habitat types in equal densities. Two AMF starter cultures were kindly provided by the European Bank of Glomales, *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG 12) and *Acaulospora laevis* Gerd. & Trappe (BEG 13). These cultures were maintained on *Trifolium repens* L. in sandy soil (5 L), mixed with P poor (19.4 mg P/L) Hoagland solution (1 L). *Glomus mosseae* and *Acaulospora* sp. were identified in this field site (CHAPTER 3).

*Inoculation and plant growth conditions*

After storage at 4 °C for a month, the cuttings of *S. repens* were trimmed to 4 cm, surface-sterilized twice in (freshly prepared) 6% $H_2O_2$ for 1 min (in series of 20 in 300 mL), and rinsed three times in (fresh) demineralized water (1 L) for 10 min. Each cutting was placed individually in a culture tube containing 20 ml sterile water agar (1 %). After a 10 weeks rooting period in a climate chamber (photon flux density 120 $\mu E.m^{-2}.s^{-1}$ (11,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 70 %) equally developed cuttings were selected for the experiment.

*Set up for single, simultaneous and sequential inoculations*

Twenty cuttings were harvested at the start of the experiment (= moment of inoculation, t = 0 weeks). For the single inoculations (and for first fungus inoculation of the sequential inoculation, second fungus inoculated at t = 15 weeks) each fungus was individually inoculated to *S. repens* cuttings in 30 replicates, and 30 cuttings were not inoculated (control). At the same day, cuttings were inoculated with two different functional types of mycorrhiza simultaneously. That is 15 replicates were inoculated with *G. mosseae* and *H. leucosarx*, 15 with *G. mosseae* and *I. vulpinella*, and 15 with *G. mosseae* and *P. involutus*.

After 15 weeks eight randomly selected plants of each single fungal treatment and all plants (14 survived) that had been inoculated with *G. mosseae* and an EcMF simultaneously were harvested. From the singly inoculated plants an additional eight were randomly selected for a second harvest at 27 weeks.

The remaining plants (= singly inoculated; 30 at start minus death plants, harvest 1 and selection for harvest 2) were subsequently inoculated with one of the other mycorrhizal types, i.e. the sequential inoculations. *Salix repens* cuttings, previously inoculated with *G. mosseae, A. laevis, H. leucosarx, I. vulpinella* or *P. involutus,* were inoculated with the other mycorrhizal type. Thus, the AMF plants were subsequently inoculated with EcMF; four replicates of *G. mosseae* were inoculated with *H. leucosarx*, four with *I. vulpinella,* and six with *P. involutus*. Three replicates of *A. laevis* were inoculated with *H. leucosarx*, three with *I. vulpinella*, and five with *P. involutus*. The EcMF plants were subsequently inoculated with AMF; eight replicates of *H. leucosarx, I. vulpinella* and *P. involutus* were inoculated with *G. mosseae*. No subsequent inoculation with *A. laevis* was performed in the experimental set up.

*Inoculation procedure and growth conditions*

The isolates of *H. leucosarx, I. vulpinella* and *P. involutus* were precultured twice (successively) for three weeks on solid media in order to obtain sufficient actively growing fungal material. *G. mosseae* and *A. laevis* were precultured on roots of *T. repens* for 15 weeks (inoculum density: roots and soil with AMF to sterilized soil, 10 % v/v). The substrate used was a sand-perlite mixture 1:3 (v/v). For logistical reasons it was not possible to collect soil from the field sites, and therefore this experiment was performed on sand with a similar soil chemical composition. pH ($CaCl_2$) of the sand was 5.8, nutrient

contents were 70 mg $N.kg^{-1}$, 20 mg $P.kg^{-1}$, and 0.17 % organic material. No nutrient solution was supplied during the experiment. Demineralized water was added to the substrate (1:3:1 v/v/v sand:perlite:water) 24 h prior to autoclaving. The substrate was autoclaved twice (1 h, 121 °C, 1 atm.) with a 48 h interval, and left for one week. Root growth chambers (vertically placed Petri dishes 15 cm diameter, with a slit in the edge) were filled with the sterile substrate. Each root growth chamber contained 300 mL substrate, of which 75 mL (*c.* 120 g) was sand. Cuttings were transferred to the root growth chambers (1 cutting per growth chamber), and each cutting was inoculated with 5 mycelial plugs cut from the edge of a precultured EcM fungal colony and the root system was covered with a water agar (1 %) layer (5 cm diameter) to prevent the roots from excessive loss of water. The root growth chambers were sealed with tape and sterilized anhydrous lanolin preventing contamination and loss of water. The control plants were treated similarly, but instead of mycelial plugs they were supplied with 5 plugs of solid medium (5 * 0.15 mL) without fungus. The inoculation of AMF was performed by mixing 20 g freshly washed root material of *T. repens* containing one of the fungi with the sterile substrate (10 L), and 30 root growth chambers were filled with this mixture. Cuttings were transferred to the root growth chambers, the root systems were covered with water agar, root growth chambers were closed and sealed. Because a previous experiment with addition of roots of *T. repens* without AMF, or washings (Koide & Li, 1989) of the AMF inocula to the control and EcM plants showed no effects on plant performance or root colonization when compared with control or EcM plants that were not supplied with roots or washings (APPENDICES E AND F), no attempt was made to provide control and EcM plants with roots of *T. repens* without AMF or washings. All soil compartments were protected from daylight, the growth chambers were placed vertically in transient propagators (in which relative air humity was almost 100 % during the first week) and placed in the climate cell. The plants were arranged in a complete randomized block design, and blocks were randomized every three weeks within the climate cell. The plants were watered every three weeks with 10 mL demineralized water.

*Plant performance, EcMF and AMF colonization*

Every three weeks length of shoots, number of shoots, and number of leaves were determined. At the final harvest shoot weight was determined after 72 hours at 70 °C. The plants were individually digested in sulphuric acid, salicylic acid and 30 % hydrogen

peroxide. Total N and P were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard (Novozamsky *et al.*, 1988).

The roots were immersed in water over a 2 mm sieve to remove most of the soil, and rinsed gently to avoid damage of the mycorrhizas. The cleaned ectomycorrhizal roots were stored in glutaraldehyde buffer (Alexander & Bigg, 1981) and roots of the control plants and the AM plants were stored in 50 % alcohol until they could be processed. The dual mycorrhizal root systems were randomly split in half and stored either in glutaraldehyde or 50 % alcohol. In each sample, root length was determined according to Newman (1966) and the numbers of EcM root tips, and total numbers of root tips were counted. Root biomass was not determined, since different EcM-, AM- or non mycorrhizal root systems contained different (not removable) proportions of sand (CHAPTER 7). EcM frequencies were calculated (100 % * numbers of EcM root tips/total numbers of root tips). EcM root length was not measured in this experiment, since previous studies of mycorrhizal *S. repens* root systems in the field showed that EcM frequency was similar to EcM root length. All root samples that had been inoculated with AMF were cleared with 10 % KOH for three hours in a waterbath at 90 °C, bleached in 10 % $H_2O_2$ for 1 hour, acidified in 1 % HCl for 15 min and stained with trypan blue (Phillips & Hayman, 1970; APPENDIX C) in lactophenol for 30 min. AM colonization was estimated by a modified line intersect method (McGonigle *et al.*, 1990), where a minimum of 100 line intersections per subsample (three subsamples were analysed per sample, in each of the replicates) was scored for the presence of AM structures. Furthermore, a modification of percentage root length colonization (RLC%) was calculated, i.e. multiplied with the intensity of colonization (RLCI% = root length colonization (RLC%) multiplied by the (%) of the cross section of the root occupied with AM structures).

*Statistical analysis*

Data were analysed by Analysis of Variance (ANOVA) using the statistical package STATISTICA (StatSoft). Prior to analysis proportional mycorrhizal colonizations were arcsine square root transformed and plant parameters were logarithmically transformed. Bartlett's test was used to determine whether variances were equal between treatments. The single inoculations were subjected to a one-factor and two-factor ANOVA (for a single harvest or two harvests, respectively). In the two-factor ANOVA mycorrhizal

colonization did not include t = 0 or the control treatment, whereas for all plant parameters t = 0 and control treatment were included. In the one-factor ANOVA the simultaneous and sequential inoculations did not include the control treatment (randomly equal treatment sample sizes were selected before analysis). Differences among means (after the ANOVAS indicated significant effects $P < 0.05$) were evaluated with (an aposteriori) LSD-test, and for the simultaneous and sequential inoculations differences were evaluated with planned comparisons (Sokal & Rohlf, 1995). In addition, orthogonal comparisons were used as a priori tests for pre-planned comparisons of the two functional groups of mycorrhizas separately, or for comparisons between either AMF or EcMF with the simultaneous (&) and sequential (+) dual mycorrhiza treatments (groups: AMF, EcMF, AMF&EcMF, AMF+EcMF and EcMF+AMF) (Sokal & Rohlf, 1995).

## RESULTS

### *Plant performance: single inoculations*

*Survival* - Only 53 % of the non-mycorrhizal *S. repens* cuttings survived (the others died in the first 6 weeks) as compared with 83 % to 93 % of all mycorrhizal treatments (TABLE 4-1). Since only the stronger control plants survived results are likely an underestimate of the effects of mycorrhizal infection.

**TABLE 4-1.** Percentages of survival by *Salix repens* cuttings after inoculation with *Glomus mosseae*, *Acaulospora laevis*, *Paxillus involutus*, *Hebeloma leucosarx*, *Inocybe vulpinella* or not inoculated (control) ($n = 30$).

| | Control | *Glomus mosseae* | *Acaulospora laevis* | *Paxillus involutus* | *Hebeloma leucosarx* | *Inocybe vulpinella* |
|---|---|---|---|---|---|---|
| Survival (%) | 53.3 a* | 93.3 b | 86.7 b | 93.3 b | 86.7 b | 83.3 b |

* Different letters indicate significant differences according to Chi-square test ($P < 0.05$).

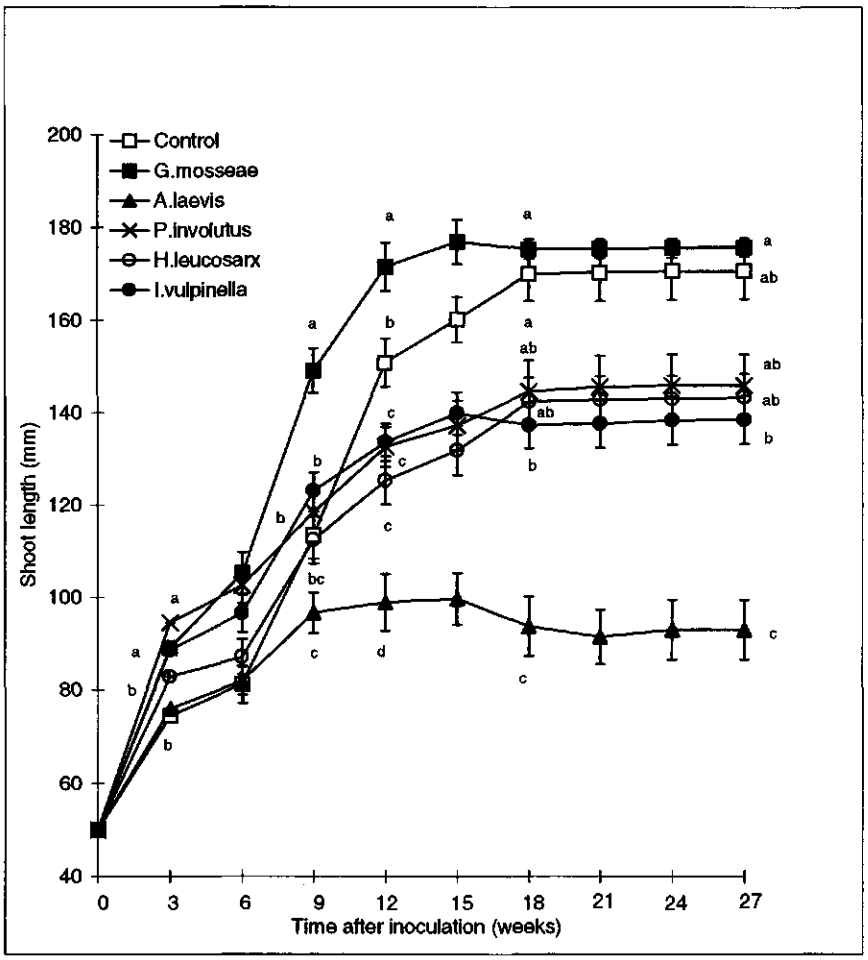



**FIGURE 4-1.** Mean shoot length of *Salix repens* inoculated with *Glomus mosseae, Acaulospora laevis, Paxillus involutus, Hebeloma leucosarx, Inocybe vulpinella* or not inoculated over 27 weeks. Bars represent (±) 1 SE. Until week 15 ($n$ = 30 - dead) and week 27 ($n$ = 8). Different letters next to points at 3, 9, 12, 18 and 27 weeks after inoculation indicate significant different shoot length between mycorrhizal treatments ($P$ < 0.05) according to LSD tests and an ANOVA repeated measures design.

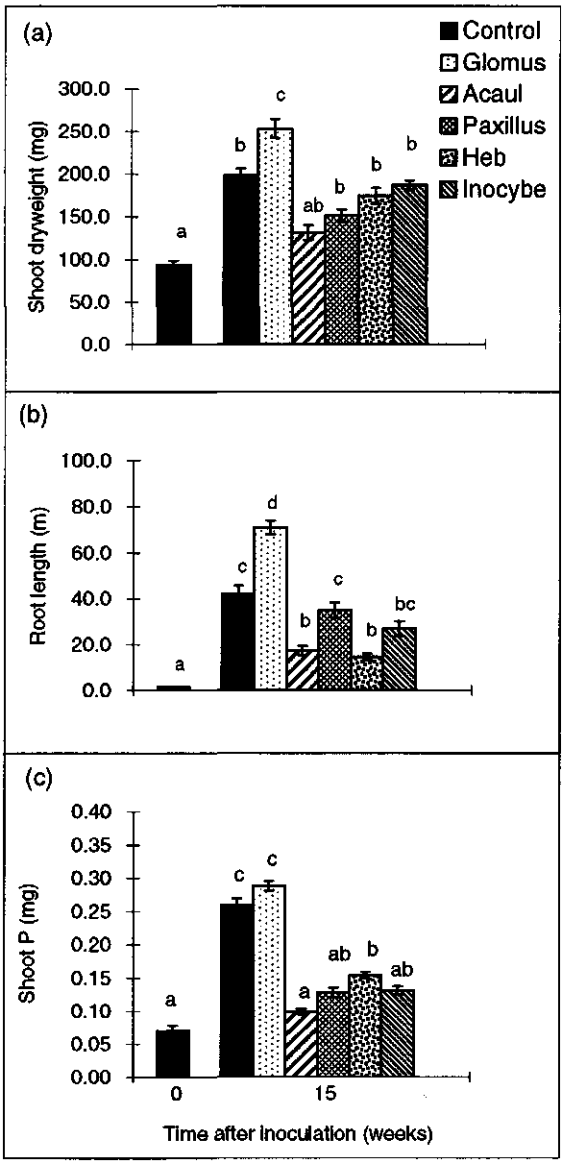



**FIGURE 4-2.** Mean (a) shoot dry weight; (b) root length and (c) shoot P content of *Salix repens* ($n$ = 8) inoculated with *Glomus mosseae, Acaulospora laevis, Paxillus involutus, Hebeloma leucosarx, Inocybe vulpinella* or not inoculated at t = 0 and 15 weeks after inoculation. Bars represent (±) 1 SE. Different letters above bars indicate significant differences according to the LSD test and 1-factor ANOVA ($P < 0.05$).

*Growth response* - *Salix repens* showed the highest growth when inoculated with *G. mosseae*, whereas it was lowest when inoculated with *A. laevis* in the first 12 weeks of the experiment (ANOVA Repeated measures design over 12 weeks, mycorrhizal treatment effect: $F_{5,141} = 10.64$, $P < 0.001$, time effect: $F_{5,705} = 781.8$, $P < 0.001$, interaction mycorrhizal treatment × time: $F_{25,705} = 14.6$, $P < 0.001$) (FIG. 4-1). On the relatively long term, *S. repens* inoculated with *G. mosseae* was not significantly different from the non mycorrhizal plants, or *S. repens* with *P. involutus* and *S. repens* with *H. leucosarx* (FIG. 4-1). *Salix repens* inoculated with *A. laevis* still showed significantly lowest growth rates (ANOVA Repeated measures design over 27 weeks, mycorrhizal treatment effect: $F_{5,46} = 5.25$, $P < 0.001$, time effect: $F_{9,414} = 207.0$, $P < 0.001$, interaction mycorrhizal treatment × time: $F_{45,414} = 5.13$, $P < 0.001$). From week 18 onwards plants had not increased their shoot length, shoot dry weight or nutrient contents, but control plants and to a much larger extent *S. repens* inoculated with *G. mosseae* still significantly increased root length (data not shown).

*Shoot dry weight, root length and shoot P content* - Planned contrasts between AMF and EcMF indicated no significant differences in shoot dry weight, root length or shoot P content ($P > 0.05$). The ANOVA at species level indicated that after 15 weeks dry weight of *S. repens* inoculated with *G. mosseae* was twice the weight of *S. repens* inoculated with *A. laevis* and it was also significantly higher than all of the EcMF treatments ($F_{5,42} = 4.71$, $P < 0.002$. FIG. 4-2a). Furthermore, root length and shoot P-content of *S. repens* as affected by *G. mosseae* were at least twice that of *S. repens* when inoculated either with *A. laevis* or all of the EcMF treatments ($F_{5,42} = 6.29$, $P < 0.001$ and $F_{5,42} = 17.8$, $P < 0.001$, respectively. FIGS. 4-2b,c).

*Plant performance: simultaneous and sequential inoculations*

*Survival* - Cuttings simultaneously inoculated with *G. mosseae* and either one of the EcMF all had a high percentage of survival (93 %). Performances of simultaneously and sequentially inoculated plants were compared with the single mycorrhizal treatments. Due to the experimental design no time effect could be observed. As nutrients were limiting in the soil containers used (see growth in FIG. 4-1), statistical comparisons were appropriate only between the plants simultaneously inoculated and the single inoculations at t = 15 weeks, and between the plants sequentially inoculated and the single inoculations at t = 27 weeks.

**TABLE 4-2.** Shoot dry weight, root length, shoot N and P content and shoot N/P ratio of singly inoculated *Salix repens* as affected by functional types of mycorrhiza, after 15 and 27 weeks; simultaneously inoculated, after 15 weeks; and sequentially inoculated, after 27 weeks.

| Functional type of mycorrhizal fungi | Time after inoculation (weeks) | Shoot dry weight (mg) | Root length (m) | Shoot N content (mg) | Shoot P content (mg) | Shoot N/P ratio |
|---|---|---|---|---|---|---|
| Single inoculations | | | | | | |
| Control | 15 | 198 a* | 42 b | 2.0 b | 0.26 b | 8.0 a |
| AMF | 15 | 192 a | 44 ab | 1.5 a | 0.19 a | 9.3 a |
| EcMF | 15 | 171 a | 25 a | 2.0 b | 0.14 a | 15.4 c |
| Simultaneous inoculations | | | | | | |
| AMF & EcMF | 15 | 174 a | 15 a | 1.8 ab | 0.15 a | 12.7 b |
| Single inoculations | | | | | | |
| Control | 27 | 214 a | 76 b | 1.8 b | 0.30 c | 6.8 a |
| AMF | 27 | 167 a | 63 ab | 1.1 a | 0.13 a | 10.1 b |
| EcMF | 27 | 177 a | 32 a | 1.6 b | 0.14 a | 12.1 c |
| Sequential inoculations | | | | | | |
| AMF + EcMF | 27 | 149 a | 49 ab | 1.0 a | 0.15 ab | 8.0 a |
| EcMF + AMF | 27 | 184 a | 30 a | 1.7 b | 0.17 b | 10.4 b |

* Different letters within a column (with sections) indicate significant differences according to Planned comparisons and one-factor ANOVA ($P < 0.05$).

*Shoot dry weight and root length* – Both the ANOVAS over the grouped treatments AMF, EcMF and AMF&EcMF at t = 15 weeks, and the grouped treatments AMF, EcMF and AMF+EcMF or EcMF+AMF at t = 27 weeks, indicated no significant differences in shoot dry weight and root length ($P > 0.05$) (TABLE 4-2). However, the planned comparisons after ANOVA at species level indicated that after 15 weeks dry weight of *S. repens* inoculated with *G. mosseae* was twice the weight of *S. repens* inoculated with *G. mosseae* and each of the EcMF simultaneously, whereas each of the EcMF treatments did not differ from the treatment in which *G. mosseae* was simultaneously inoculated (TABLE 4-3). Effects for root length were almost similar, but both *G. mosseae* and *P. involutus* individually increased root length to a significantly larger extent than when both were simultaneously inoculated (TABLE 4-3).

**TABLE 4-3.** Shoot dry weight, root length, shoot N content, shoot P content and shoot N/P ratio of *Salix repens* inoculated with *Glomus mosseae, Acaulospora laevis, Paxillus involutus, Hebeloma leucosarx, Inocybe vulpinella*, Simultaneously: *G. mosseae & P. involutus, G. mosseae & H. leucosarx, G. mosseae & I. vulpinella* or not inoculated 15 weeks after inoculation. Data are means (±) 1 SE.

| Mycorrhizal fungi | Shoot dry weight (mg) | | Root length (m) | | Tot N (mg) | | Tot P (mg) | | N/P | |
|---|---|---|---|---|---|---|---|---|---|---|
| Single | | | | | | | | | | |
| Control | 198±6.0 | ab* | 42.0±2.6 | cd | 2.0±0.05 | cd | 0.26±0.008 | e | 8.0±0.2 | b |
| *G.mosseae* | 253±8.1 | c | 71.1±2.1 | d | 1.8±0.03 | c | 0.29±0.005 | e | 6.2±0.1 | a |
| *A.laevis* | 131±6.1 | a | 17.2±1.6 | a | 1.2±0.04 | a | 0.10±0.003 | a | 12.3±0.2 | c |
| *P.involutus* | 151±5.2 | ab | 34.8±2.4 | c | 2.0±0.07 | c | 0.13±0.006 | ab | 16.1±0.3 | d |
| *H.leucosarx* | 175±6.5 | ab | 14.5±1.1 | a | 1.8±0.05 | c | 0.15±0.004 | cd | 11.8±0.3 | c |
| *I.vulpinella* | 187±4.0 | ab | 26.9±2.2 | bc | 2.3±0.06 | d | 0.13±0.004 | ab | 18.4±0.4 | d |
| Simultaneous | | | | | | | | | | |
| *Gm&Pi* | 188±3.9 | b | 18.3±0.6 | ab | 2.2±0.02 | cd | 0.13±0.002 | bc | 17.7±0.3 | d |
| *Gm&Hl* | 159±2.7 | ab | 12.3±0.4 | a | 1.6±0.01 | b | 0.15±0.002 | cd | 10.8±0.1 | b |
| *Gm&Iv* | 174±2.7 | ab | 14.3±0.5 | ab | 1.4±0.02 | ab | 0.16±0.002 | d | 9.4±0.2 | b |
| *P* values† | < 0.001 | | < 0.001 | | < 0.001 | | < 0.001 | | < 0.001 | |

* Different letters within a column indicate significant differences according to the Planned comparisons ($P < 0.05$).

† *P* values presented are obtained in one-factor ANOVA (excl. control and *A. laevis* treatments)

Planned comparisons after ANOVA at species level at t = 27 weeks indicated that shoot dry weight of plants inoculated previously with *G. mosseae* or *A. laevis* had not changed by the subsequent inoculation of each of the EcMF. Root length, however, was depressed after introduction of each of the EcMF for the plants previously inoculated with *G. mosseae* (TABLE 4-4), whereas for plants previously inoculated with *A. laevis* root length had only changed (increased) after subsequent inoculation with *P. involutus*.

**TABLE 4-4.** Shoot dry weight, root length, shoot N content, shoot P content and shoot N/P ratio of *Salix repens* inoculated with *Glomus mosseae, Acaulospora laevis, Paxillus involutus, Hebeloma leucosarx, Inocybe vulpinella*, Sequentially: *G. mosseae* + *P. involutus*, *G. mosseae* + *H. leucosarx*, *G. mosseae* + *I. vulpinella*, *A. laevis* + *P. involutus*, *A. laevis* + *H. leucosarx*, *A. laevis* + *I. vulpinella*, *P. involutus* + *G. mosseae*, *H. leucosarx* + *G. mosseae*, *I. vulpinella* + *G. mosseae* or not inoculated at the final harvest. Data are means (±) 1 SE.

| Mycorrhizal fungi | Shoot dry weight (mg) | | Root length (m) | | Tot N (mg) | | Tot P (mg) | | N/P | |
|---|---|---|---|---|---|---|---|---|---|---|
| Single | | | | | | | | | | |
| Control | 214±6.5 | cd* | 76.5±2.1 | d | 1.8±0.07 | cd | 0.30±0.017 | e | 6.8±0.1 | a |
| *G.mosseae* | 226±9.3 | cd | 99.4±4.5 | e | 1.2±0.03 | ab | 0.19±0.004 | cd | 6.5±0.1 | a |
| *A.laevis* | 108±4.1 | a | 26.5±1.4 | a | 1.0±0.03 | a | 0.07±0.002 | a | 13.7±0.3 | e |
| *P.involutus* | 169±7.7 | bc | 45.7±3.1 | cd | 1.5±0.04 | b | 0.12±0.005 | b | 13.6±0.2 | e |
| *H.leucosarx* | 187±8.4 | bcd | 21.6±1.6 | a | 1.5±0.04 | bc | 0.16±0.004 | cd | 9.3±0.2 | b |
| *I.vulpinella* | 174±5.7 | b | 29.9±2.0 | ab | 1.8±0.05 | cd | 0.14±0.004 | abc | 13.4±0.2 | d |
| Sequential | | | | | | | | | | |
| *Gm+Pi* | 199±8.5 | cd | 68.3±4.9 | d | 1.1±0.04 | a | 0.19±0.008 | cd | 5.7±0.3 | a |
| *Gm+Hl* | 218±5.1 | cd | 55.9±3.5 | d | 1.1±0.02 | a | 0.23±0.008 | d | 4.8±0.1 | a |
| *Gm+Iv* | 193±13.8 | bcd | 77.3±6.0 | de | 1.0±0.05 | a | 0.21±0.014 | cde | 5.1±0.2 | a |
| *Al+Pi* | 92±4.8 | a | 32.9±1.0 | b | 1.0±0.04 | a | 0.09±0.004 | a | 11.4±0.1 | cd |
| *Al+Hl* | 93±8.6 | a | 27.1±4.6 | abc | 1.0±0.07 | a | 0.10±0.010 | a | 9.8±0.4 | b |
| *Al+Iv* | 95±10.8 | a | 30.3±4.5 | ab | 1.0±0.20 | ab | 0.09±0.012 | a | 11.1±0.6 | cd |
| *Pi+Gm* | 181±7.9 | bcd | 34.7±1.5 | b | 1.8±0.06 | cd | 0.15±0.005 | b | 12.1±0.4 | cd |
| *Hl+Gm* | 127±5.4 | ab | 22.6±1.4 | a | 1.3±0.05 | ab | 0.16±0.007 | cd | 8.3±0.2 | b |
| *Iv+Gm* | 243±8.7 | d | 32.2±1.7 | ab | 2.0±0.04 | d | 0.19±0.004 | cde | 10.7±0.2 | c |
| *P* values† | < 0.001 | | < 0.001 | | < 0.001 | | < 0.001 | | < 0.001 | |

* Different letters within a column indicate significant differences according to the Planned comparisons ($P < 0.05$).

† P values presented are obtained in one-factor ANOVA (excl. control treatment)

*Shoot N/P ratio as affected by different functional types of mycorrhiza versus shoot N and P contents in the separate fungal treatments*

Nutrient status of *S. repens* as affected by the functional groups of mycorrhiza, i.e. AM and EcM, showed that shoot N/P ratio of *S. repens* when associated with AMF (*G. mosseae* and *A. laevis*) was 9.3, and *S. repens* when associated with EcMF (*H. leucosarx, I. vulpinella* and *P. involutus*) had a significantly higher shoot N/P ratio of 15.4 (t = 15) (TABLE 4-2). When both functional types (AM and EcM) were inoculated simultaneously shoot N/P ratio was intermediate between the earlier mentioned shoot N/P ratios, namely 12.7. When AMF were inoculated first, the subsequent inoculation with EcMF did not

additionally affect shoot N/P ratio of *S. repens*. But, *S. repens* inoculated first with EcMF and subsequently with AMF, had a N/P ratio significantly lower than *S. repens* inoculated only with EcMF.

*Shoot nutrient status* – ANOVAS on the species level, however, indicated that even though one of the simultaneous inoculations (*G. mosseae* & *P. involutus*) on *S. repens* showed similarity with *S. repens* inoculated only with *G. mosseae*, the addition of EcMF generally changed N or P content significantly from *S. repens* inoculated with *G. mosseae* only (TABLE 4-3). *Salix repens* simultaneously inoculated with *G. mosseae* and *H. leucosarx* showed a significantly lower N content when compared with *H. leucosarx* individually. Presence of *G. mosseae* in combination with *I. vulpinella* significantly depressed N and increased P content when compared to *I. vulpinella* only. Shoot N/P ratio had generally changed in all situations (but for *P. involutus*) when simultaneously inoculated with another fungus.

For plants sequentially inoculated, plant nutrient status was hardly affected by the second fungus inoculated when compared to the plant nutrient status with only the first fungus inoculated (TABLE 4-4). *Salix repens* either inoculated only with *G. mosseae* or *A. laevis*, compared with *G. mosseae* or *A. laevis* and subsequently with each of the EcMF showed no differences at the final harvest in both N or P content. However, even though for *S. repens* inoculated with AMF *G. mosseae* and subsequently with each of the EcMF showed no differences in shoot N/P ratio, *S. repens* inoculated with AMF *A. laevis* previously and subsequently with each of the EcMF showed a significantly lower N/P ratio than plant inoculated with *A. laevis* only. *Salix repens* inoculated with *I. vulpinella* or *P. involutus* and subsequently with *G. mosseae* showed a significant decrease in N/P ratio when compared to *S. repens* inoculated with *I. vulpinella* or *P. involutus* only.

*Mycorrhizal colonization and interactions*

Colonization by AMF was much lower than by the EcMF ($P < 0.001$, planned contrast; data not shown, but compare colonization data in TABLE 4-5a). At both harvests, the ANOVA at the species level indicated that in the single inoculations the five different mycorrhizal fungi had occupied significantly different proportions of the root system of *S. repens* (mycorrhizal treatment effect: $F_{4,73} = 113.46$, $P < 0.001$; time effect and interaction mycorrhizal treatment × time: not significant. TABLE 4-5a). After 15 weeks, both AMF colonized hardly more than 5 % of the root system, and 7.4 to 9.2 % at the second harvest

period (inoculum potential of both AMF after 15 weeks was 60 %RLCI/ 90 %RLC in *Trifolium*). No difference occurred in the percentage of root length occupied by arbuscules and vesicles, neither between AMF nor in time (data not shown). The EcMF *H. leucosarx* and *I. vulpinella* after 15 weeks had almost colonized the complete root system and *P. involutus,* although less, still 5 times more than the AMF. Even though about 40 % of the root system was still uncolonized, colonization by *P. involutus* significantly decreased over time.

**TABLE 4-5a.** Average mycorrhizal colonization (±) SE of *Glomus mosseae, Acaulospora laevis, Paxillus involutus, Hebeloma leucosarx* and *Inocybe vulpinella* on roots of *Salix repens* when individually, simultaneously or sequentially inoculated.

| | After 15 weeks | | After 27 weeks | |
|---|---|---|---|---|
| Mycorrhizal fungi | EcM colonization (%) | AM colonization (%RLCI) | EcM colonization (%) | AM colonization (%RLCI) |
| Single inoculations | | | | |
| Control | 0 | 0 | 0 | 0 |
| *G. mosseae (Gm)* | 0 | 4.6 ± 1.6 | 0 | 7.4 ± 0.3 |
| *A. laevis (Al)* | 0 | 3.7 ± 0.5 | 0 | 9.2 ± 1.6 |
| *P. involutus (Pi)* | 56.5 ± 8.6 | 0 | 37.4 ± 9.8 | 0 |
| *H.leucosarx (Hi)* | 91.6 ± 1.9 | 0 | 85.5 ± 1.9 | 0 |
| *I. vulpinella (Iv)* | 83.1 ± 3.3 | 0 | 86.8 ± 1.4 | 0 |
| Simultaneous inoculations | | | | |
| *Gm & Pi* | 13.6 ± 3.0 | 3.7 ± 0.2 | | |
| *Gm & Hl* | 83.0 ± 1.6 | 4.2 ± 0.2 | | |
| *Gm & Iv* | 80.3 ± 1.8 | 5.4 ± 0.4 | | |
| Sequential inoculations | | | | |
| *Gm + Pi* | 0 | 4.6 ± 1.6 | 16.8 ± 3.2 | 4.7 ± 0.6 |
| *Gm + Hl* | 0 | 4.6 ± 1.6 | 9.7 ± 5.0 | 5.5 ± 0.2 |
| *Gm + Iv* | 0 | 4.6 ± 1.6 | 12.9 ± 1.0 | 3.7 ± 0.6 |
| *Al + Pi* | 0 | 3.7 ± 0.5 | 19.3 ± 4.2 | 4.8 ± 0.6 |
| *Al + Hl* | 0 | 3.7 ± 0.5 | 32.8 ± 8.8 | 3.4 ± 0.4 |
| *Al + Iv* | 0 | 3.7 ± 0.5 | 24.4 ± 9.2 | 3.2 ± 0.4 |
| *Pi + Gm* | 56.5 ± 8.6 | 0 | 45.7 ± 9.5 | 2.2 ± 0.4 |
| *Hl + Gm* | 91.6 ± 1.9 | 0 | 80.2 ± 4.8 | 2.5 ± 0.4 |
| *Iv + Gm* | 83.1 ± 3.3 | 0 | 88.7 ± 1.6 | 2.3 ± 1.2 |

Colonization by the different fungi when simultaneously inoculated can directly be compared with the colonization of the different fungi when singly inoculated. At t = 15 weeks, *P. involutus* was strongly inhibited by the presence of *G. mosseae,* whereas either *H. leucosarx* or *I. vulpinella* and *G. mosseae* colonized the same proportion of the root system as in the single inoculations (TABLE 4-5b).

**TABLE 4-5b.** Matrix with significant interactions of mycorrhizal colonization by *Glomus mosseae, Acaulospora laevis, Paxillus involutus, Hebeloma leucosarx* and *Inocybe vulpinella,* simultaneously inoculated compared to colonization when singly inoculated, after 15 weeks. Data presented are P values of the Planned comparisons ($P < 0.05$).

| Simultaneous inoculations | Single inoculations | | | |
|---|---|---|---|---|
| | *Gm* | *Pi* | *Hl* | *Iv* |
| *Gm&Pi* | NS | 0.0001 | | |
| *Gm&Hl* | NS | | NS | |
| *Gm&Iv* | NS | | | NS |

A number of comparisons can be made in the sequential inoculations. Some possible doubts whether all statistical comparisons are valid can be addressed, as other experiments (CHAPTER 2) showed that colonization by the three different EcM fungi used was about 80-100 % in 10 weeks time. Thus, even though the second fungus in the sequential inoculation was allowed to colonize for only 12 weeks rather than 15 weeks (of the first fungus), both 12 and 15 weeks are considered sufficient time for development of mycorrhizas. Moreover, in previous try out experiments EcM colonization was up to 80-100 % in 10 weeks time on sterile cuttings aging from 10 weeks to 6 months. Effects of root system age on root colonization during this experiment was therefore considered a minor concern when studying interactions between AM and EcM. However, this was not tested previously for AMF. The comparisons made in this sequential inoculation are: (1) colonization of the second fungus to be inoculated (t = 27 = 12 weeks after inoculation) as affected by the fungus already present on the root system compared to t = 15 (single inoculation); (2) colonization of the fungus already present (t = 27) as affected by the second fungus to be inoculated compared to its colonization at t = 27 (single inoculation); and (3) colonization of the fungus already present (t = 27) as affected by the second fungus to be inoculated compared to its colonization at t = 15 (single inoculation).

**TABLE 4-5c.** Matrix with significant interactions of mycorrhizal colonization by *Glomus mosseae*, *Acaulospora laevis*, *Paxillus involutus*, *Hebeloma leucosarx* and *Inocybe vulpinella*, sequentially inoculated. Data presented are P values of the Planned comparisons ($P < 0.05$).

| | Single inoculations | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | After 15 weeks | | | | | After 27 weeks | | | | |
| | *Gm* | *Al* | *Pi* | *Hl* | *Iv* | *Gm* | *Al* | *Pi* | *Hl* | *Iv* |
| Sequential inoculations* | | | | | | | | | | |
| *Gm+Pi* | NS | | 0.0006 | | | 0.0134 | | | | |
| *Gm+Hl* | NS | | | 0.0001 | | NS | | | | |
| *Gm+Iv* | NS | | | | 0.0001 | 0.0355 | | | | |
| *Al+Pi* | | NS | 0.0020 | | | | 0.0123 | | | |
| *Al+Hl* | | NS | | 0.0001 | | | 0.0020 | | | |
| *Al+Iv* | | NS | | | 0.0001 | | 0.0245 | | | |
| *Pi+Gm* | 0.0001 | | NS | | | | | NS | | |
| *Hl+Gm* | NS | | | NS | | | | | NS | |
| *Iv+Gm* | NS | | | | NS | | | | | NS |

* Colonization of the first inoculated fungus is compared with single inoculations after 15 and after 27 weeks, i.e. is colonization significantly affected since t = 15 and is mycorrhizal development different from t = 27 by the appearance of the other fungus. (Colonization of the second inoculated fungus is only compared with the single inoculations at t = 15, i.e. is colonization affected by the presence of the other fungus).

All EcMF showed strongly reduced colonization when inoculated second, even though *G. mosseae* or *A. laevis* had colonized only about 5% of the root system (TABLE 4-5c). In contrast, *G. mosseae* colonized the same proportion of the root system when introduced to *S. repens* that already was colonized by either *H. leucosarx* or *I. vulpinella*. However, colonization of *G. mosseae* was significantly reduced by presence of *P. involutus* on the root system. Colonization by *G. mosseae* was lower at t = 27 weeks, when *P. involutus* or *I. vulpinella* had been introduced to the same root system, when compared to colonization by *G. mosseae* at t = 27 (single inoculation). *Glomus mosseae* did not significantly decrease the proportion of its colonization when compared to its original colonization at t = 15 (single inoculation). *A. laevis* showed a similar behaviour when subsequently inoculated with all EcMF at t = 27. The colonization of the EcMF was not affected after introduction of *G. mosseae* compared to the single inoculations at t = 15 or 27.

## DISCUSSION

*Colonization and significance of mycorrhizal types versus mycorrhizal species*

This study has demonstrated that survival of *S. repens* can strongly be altered by the presence or absence of mycorrhizal fungi. The outcome of the control treatment (fittest plants survived) is likely an overestimate of *S. repens* performance without mycorrhiza. FIGURE 4-1 showed that *G. mosseae* slightly increased shoot dry weight, whereas no significant difference with the control in shoot biomass was observed by the other mycorrhizal fungi. Shoot P content was significantly higher by *G. mosseae* than by the other fungal treatments, but no difference compared to the control was observed. TABLE 4-6 (dead plants in control (and fungal) treatments included) presents the estimated plant P content in order to speculate a more realistic difference in mycorrhizal effects on plant performance. Contrast between *S. repens* inoculated with *G. mosseae* and control plants in shoot length (dry weight) and P content from these calculations are more clear at 15 weeks. Moreover, these effects by *G. mosseae* compared to the control were very clear at three weeks after inoculation already.

**TABLE 4-6.** Correction for 50 % survival bias in control treatment. Estimates of shoot P contents in *Salix repens* at t = 3 and 15 weeks when dead plants are included. Calculated from shoot length of *Salix repens* (*n* = 30 replicates) measured over the first 15 weeks after inoculation.

| | Shoot length (mm) | | Shoot drw/shl* | Shoot P(%)* | Shoot P content (mg) (estimated) | |
|---|---|---|---|---|---|---|
| Fungal treatment | 3 wks n=30 | 15 wks n=30 | 15 wks n=8 | 15 wks n=8 | 3 wks n=30 | 15 wks n=30 |
| Control | 60 | 110 | 1.24 | 0.13 | **0.097** | **0.18** |
| *G. mosseae* | 90 | 175 | 1.42 | 0.12 | **0.153** | **0.30** |
| *A. laevis* | 75 | 90 | 1.28 | 0.08 | 0.077 | 0.09 |
| *P. involutus* | 73 | 135 | 1.12 | 0.08 | 0.085 | 0.12 |
| *H. leucosarx* | 83 | 133 | 1.42 | 0.09 | 0.107 | 0.17 |
| *I. vulpinella* | 90 | 138 | 1.36 | 0.07 | 0.086 | 0.13 |

* Parameters obtained from the eight randomly selected plants harvested at t = 15 weeks (overestimation in the parameters used, shoot dry weight (drw), shoot length (shl) and shoot P(%), of control plants cannot be eliminated).

Mycorrhizal colonization as observed in this experiment and in the field (CHAPTER 3) showed that AMF and EcMF colonize very different proportions of the root system of *S. repens*. It is slowly getting recognized that not only dual mycorrhizal plants, but most ectomycorrhizal plants are, to a lesser extent, simultaneously colonized by AMF. However, such low AM colonization (5 %), as seen in this study, has often been considered negligible and therefore has been neglected. The effects of these AMF on plant performance indicate that the very low colonization observed in the field is not negligible and may actually be of great significance to *S. repens*. In contrast, colonization by all EcMF was high, but showed smaller effects.

Despite similarities on the level of mycorrhizal colonization within functional types, the two AMF showed contrasting effects in plant response, whereas variation within EcMF was much smaller under the circumstances studied. A low colonization of AMF in the field may therefore not easily be explained as favourable or unfavourable for plant growth, as it depends on the species of AMF involved. However, both AMF increased survival.

*Nutrient uptake and plant growth: short and long term effects*

In agreement with Lapeyrie & Chilvers (1985), Chilvers *et al.* (1987), Lodge (1989) and Lodge & Wentworth (1990), but in contrast to Jones *et al.* (1998), in this experiment *G. mosseae* (AMF) showed a fast growth response and high phosphorus uptake shortly after inoculation, whereas the EcMF reacted more slowly and also showed lower net benefits. However, *A. laevis* showed the opposite effect of *G. mosseae*. This indicated that plant nutrient uptake is differentially affected by different mycorrhizal fungi, within and between functional types. Differences in P uptake in cucumber by different species of AMF were also observed by Pearson & Jakobsen (1993). Even though in the present study no extramatrical hyphal lengths were measured, the high effectiveness of low AM colonization could be related to the development of a very large hyphal system exploiting the soil. Alternatively, the mycelium of *G. mosseae,* irrespective of the amount of hyphal length, could be very effective in the uptake of nutrients from the soil, and while roots only showed a very few entry points, nutrient exchange could be very effective. The contrasting effect between both AMF used in this study, can be the result of difference in either external hyphal length or effectiveness of the mycelium.

Contrary to the relatively low P content of *S. repens* cuttings in the EcM treatments in this study, Jones *et al.* (1998) observed a generally better P uptake by two EcMF than three AMF in *Eucalyptus*. However, a study in which a range of different (11) EcM fungi were compared indicated that large variation in their effectiveness for P uptake of *S. repens* exists (CHAPTER 6). Therefore, field data that do not consider species composition and their relative abundance, but only total AM colonization and total EcM colonization should not or cautiously be used for functional explanations.

This was again shown by unpredictable shoot nutrient status of *S. repens* when simultaneously or sequentially inoculated with different combinations of mycorrhizal fungi, as compared to the single inoculations. Effects of these combinations of mycorrhizal fungi on plant performance of *S. repens* are not discussed in this paper, due to differences in nutrient balances of cuttings at the start of the experiment and after 15 weeks at the first harvest.

This nutrient limitation of the soil, on the long term, was clearly shown by plant growth. After 18 weeks all plants stopped growing and this was likely due to nutrient depletion because of the small containers used. This study clearly demonstrated that *G. mosseae* was either able to release a larger amount of nutrients from the soil or *G. mosseae* required less nutrients for its own maintenance, as no additional nutrient uptake in *S. repens* was observed for each of the fungal treatments after 15 weeks (compare TABLES 4-3 and 4-4), while *S. repens* in association with *G. mosseae* had significantly highest shoot biomass, root length and shoot P content at 15 weeks.

The containers (root growth chambers) used in this study were apparently too small and therefore conclusions on long term mycorrhizal performance of both mycorrhizal types are not appropriate. An additional experiment (same fungi, same soil) in larger containers (2 L) will cover the long term effects of both types for *S. repens* under nutrient poor soil conditions.

Root morphology of *S. repens* is affected by mycorrhizal fungi. Root length was significantly increased by *G. mosseae*, whereas all EcMF significantly reduced root length. Hooker & Atkinson (1996) also observed a stimulating effect of AMF on root length. In this study, however, both AMF colonized the same proportion of the root system of *S. repens*, while root length of *S. repens* with *G. mosseae* was three to four times longer than with *A. laevis*.

Nutrient contents and mycorrhizal root length were not related in a straightforward way. This might have been caused by either fungal differences in nutrient uptake. Moreover, root length colonization is not necessarily related to the amount, or the spatial distribution, of the extramatrical mycelium (Jones *et al.*, 1990, 1998; Jakobsen *et al.*, 1992a,b). Hence, the high proportional colonization by EcMF and low proportional colonization by AMF not necessarily implies that EcM plants have access to a larger soil volume.

*Mycorrhizal interactions*

Considering the very low colonization intensity by AMF and the very high colonization by EcMF, it was striking that colonization with both AMF suppressed colonization of all EcMF, whereas colonization of *G. mosseae* was only decreased by *P. involutus* in the sequential inoculations. Both hypotheses that EcMF would not be affected by the presence of AM on the root system, and vice versa, that AMF would even have a lower colonization than in the simultaneous inoculations when EcM was present on the root system had to be rejected. Furthermore, the simultaneous inoculations indicated that both AMF and EcMF in general could independently colonize their proportion of the root system. Therefore, the hypothesis that EcMF would outcompete AMF, resulting in an unaffected EcM colonization and a lower AM colonization than in the single inoculations, again had to be rejected from this experiment. However, some species combinations could result in negative (or positive) interactions, and the hypothesis that interaction between mycorrhizal fungi could be generalized across species within functional groups had to be rejected as well.

In this experiment EcM, despite high colonization, was the weaker competitor. Field observations of *S. repens* roots with low AM and high EcM colonization do not therefore provide evidence for a negative interaction between AM and EcM, in contrast to the interpretations of Lapeyrie & Chilvers (1985); Chilvers *et al.* (1987); Lodge (1989); Lodge & Wentworth (1990). This study, furthermore, does not support the suggested shift of AM colonization from terminal to non terminal roots and the subsequent take-over by EcM, as previously concluded by Dhillion (1994).

In field situations EcMF and AMF may interact by chemicals of fungal or host origin, mechanical barriers, competition for root carbohydrates, or other factors (Last *et*

*al.*, 1983, Duchesne *et al.*, 1988). The type of fungus colonizing a root may also depend on the patchy distribution of the fungal propagules in the root zone of the host plant.

Below-ground field observations in spring versus autumn (CHAPTER 3) have shown that, although the EcM colonization in spring is very high, colonization by AMF is higher (8-11 %) than at the end of the previous year in autumn (1-3 %). Reduction of colonization by AMF has been related to plant age or phenological stage (Wilson & Tommerup, 1992). It is likely that at a certain stage of plant growth AMF colonization of the root may decline allowing EcMF to further increase colonization. Succession from AMF to EcM may thus be a seasonal phenomenon in *S. repens*. I would like to alternatively hypothesize that early in season higher colonization with AMF results in a fast increase of P uptake and growth, and that successively colonization by EcMF increases, which will additionally benefit plant performance. This implies that plant nutrient demand determines the seasonal dynamics of EcM and AM. It is likely that AMF are able to establish, and coexist with EcMF, in the root system of adult ectomycorrhizal plants. Positive effects of *G. mosseae* furthermore indicated that AM colonization in *S. repens* is not a consequence of decreased plant control over AM colonization, but *S. repens* benefits from being dual mycorrhizal.

5

# Differential benefits of AM and EcM infection of *Salix repens*: advantages for being dual mycorrhizal.

**ABSTRACT**

*The functional significance of arbuscular mycorrhiza (AM) and ectomycorrhiza (EcM) for* Salix repens, *a dual mycorrhizal plant, was investigated over three harvest periods (12, 20 and 30 weeks). Cuttings of* S. repens *were collected in December (low shoot P) and March (high shoot P).* Glomus mosseae *(arbuscular mycorrhizal fungus, AMF) resulted in low AM colonization (< 5 %), but showed large short term (< 12 weeks) effects on shoot growth and root length.* Hebeloma leucosarx *(ectomycorrhizal fungus, EcMF) resulted in high EcM colonization (70 %), but benefits occurred over a longer term (> 12 weeks). Furthermore,* G. mosseae *showed larger shoot P uptake, shoot growth, root growth and response duration for* S. repens *collected in December than for those collected in March, whereas* H. leucosarx *and the non-mycorrhizal treatment showed no differences between cuttings collected on different dates. Low AM colonization was functional in the short term for cuttings at both collecting dates. Low AM colonization in* S. repens *occurred irrespective of amount of AM inoculum used. Intensity and relative amounts of AM structures in* S. repens *were compared with those of* Trifolium repens *over three harvest periods (12, 20 and 30 weeks) to assess plant species effects on arbuscular mycorrhizal colonization patterns.*

**KEY WORDS**: Arbuscular mycorrhiza, ectomycorrhiza, *Salix repens*, internal P-status.

## INTRODUCTION

Mycorrhizal fungi form mutualistic associations with plant roots. The two most widespread types are arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EcMF). Even though most plants form only one type of mycorrhiza, some plants, e.g. species of *Alnus* (Molina *et al.*, 1994), *Populus* (Lodge, 1989; Lodge & Wentworth, 1990), *Salix* (Lodge, 1989; Dhillion, 1994) and *Eucalyptus* (Lapeyrie & Chilvers, 1985) form both arbuscular mycorrhiza (AM) and ectomycorrhiza (EcM).

*Salix repens* L. is one of these so-called dual mycorrhizal plant species and is a common and widespread shrub in Western Europe, occurring in a great variety of plant communities, in particular in the coastal dunes of the Wadden Isles. Its habitats range from dry to wet, and from calcareous, humus-poor to acidic, humus-rich soils. In addition, these habitats are strongly influenced by wind and water level fluctuations. As a dual mycorrhizal plant *S. repens* might have a selective advantage in these highly dynamic ecosystems.

The gradient from the drift line to the stabilized back-dunes is characterized by decreasing pH and increasing soil organic matter content. Read (1989) mentioned the importance of mycorrhizas in the sand dune ecosystem. As the relative availability of nitrogen and phosphorus is changing with succession, the occurrence of both mycorrhizal types can change as well. In the dry foredunes, where phosphorus is the main growth limiting nutrient, AM is supposed to predominate. In contrast, in the dune slacks, where accumulation of organic matter occurs, nitrogen mineralization is inhibited due to pH reduction. Therefore nitrogen becomes the main growth limiting element and EcM prevails, with its ability to take up nitrogen (Read, 1989). The different successional stages are therefore characterized not only by a typical nutrient status, but also by a dominant mycorrhizal type.

Contrary to a suggested increasing importance of EcM colonization in this dune successional gradient (Read, 1989), *S. repens* was always found to be slightly AM and highly EcM in 16 field sites comprising successionally different stages of *S. repens* communities on the Dutch Wadden Isles (CHAPTER 3).

A laboratory experiment furthermore showed that high colonization by three different EcMF showed lower shoot P uptake of *S. repens* than did low colonization by *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe (AMF) (CHAPTER 4). The experiment was performed under very nutrient poor conditions similar to the field situation, but under the conditions studied using a small soil volume, total amount of nutrients was limited. As

the experiment showed that small effects of *Hebeloma leucosarx* P.D. Orton (EcMF) opposed to large effects of *G. mosseae* (AMF) seemed to change over time (three versus seven months), the present study focused on the contrast in functional significance of AM versus EcM for *S. repens*. From the previous experiment two fungi, representing one species per functional type were selected, i.e. *G. mosseae* (AMF) and *H. leucosarx* (EcMF). This experiment was again performed under very nutrient poor conditions but in larger soil volumes in order to avoid the total nutrient limitation on the long term (seven months). Besides, since willow species in nature occur in locations with high light intensities, irradiance was also increased (Weeda *et al.*, 1985). Preliminary observations indicated that cuttings collected in spring had higher internal P concentration than cuttings collected autumn and winter. Nutrient analysis of shoots and leaves collected in 16 field sites in April and August (CHAPTER 3) confirmed a higher internal P concentration in April. Therefore the experiment was performed with cuttings from both sampling dates.

Hypotheses that are tested in the present study: (i) *H. leucosarx* (EcMF) and *G. mosseae* (AMF), have different effects on performance of *S. repens* and these effects will show different dynamics, i.e. short term positive effects by *G. mosseae*, long term positive effects by *H. leucosarx*; (ii) short term positive effects are due to P uptake by *G. mosseae*; (iii) positive effects will be higher in cuttings collected in December than in March, because cuttings collected in March have a higher internal P concentration than cuttings collected in December (March 2.1 $g.kg^{-1}$ versus December 0.8 $g.kg^{-1}$).

As difficulties occur when comparing AM and EcM significance, a control experiment was performed. The difficulty is that AM inoculum is supplied as roots containing *G. mosseae* whereas EcM inoculum is supplied as mycelial agar plugs. As *G. mosseae* showed very fast plant responses opposed to *H. leucosarx* (CHAPTER 4), an experiment was performed in order to investigate the possibility that a nutritional effect (caused by supplying mycorrhizal clover roots) might be responsible for the fast short term effect in *S. repens*. Therefore, an experiment was set up using different amounts of AM inoculum. In a further control experiment the ability of the AMF to form mycorrhizas with *S. repens* and with a well-known test plant, *Trifolium repens* L., was compared.

## MATERIAL AND METHODS

*Plant and fungal material*

Cuttings (shoot tops) were collected in March 1996 (experiment 1) and December 1996 (experiment 1 and 2) of *Salix repens* (male) from the field site 'Schoenus I' on the isle of Terschelling (53° 23'50" N, 5° 13'45" E). All cuttings were collected from the same plant in order to obtain genetic homogeneity.

*Hebeloma leucosarx* (L1), collected in the autumn of 1994 from *S. repens* on the Isle Terschelling, was used in this experiment. Sporocarps were surface sterilized with alcohol (70 %) and sliced in half under sterile conditions. Fungal tissue was cut from the innerside of the cap and transferred to, and maintained on, solid media (alternative Melin Norkrans (AMN)) containing (in $g.l^{-1}$): agar (15), malt extract (3), glucose.$H_2O$ (10), $(NH_4)_2HPO_4$ (0.25), $KH_2PO_4$ (0.5), $KNO_3$ (0.5), $MgSO_4.7H_2O$ (0.15), $CaCl_2.H_2O$ (0.67), NaCl (0.025), $FeCl_3$ (0.0012) and thiamine.HCl (100 µg). As *Glomus mosseae* was identified in these dune sites (CHAPTER 3), an AMF starter culture of *G. mosseae* (BEG 12) was kindly provided by the European Bank of Glomales. This culture was maintained on *Trifolium repens* in sandy soil with P-poor Hoagland solution.

*Inoculation and plant growth conditions*

For both experiment 1 and 2, after storage at 4 °C for a month, the cuttings of *S. repens* were trimmed to 4 cm, surface-sterilized twice in freshly prepared 6 % $H_2O_2$ for 1 min (in series of 20 in 300 mL), and rinsed three times in fresh demineralized water (1 L) for 10 min. Each cutting was placed individually in a culture tube containing 20 mL sterile water agar (1 %). After a 10 weeks rooting period in a climate chamber (photon flux density 120 $\mu E.m^{-2}.s^{-1}$ (11,000 lux) day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 70 %) equally developed cuttings were selected for the experiment.

The isolate of *H. leucosarx* was precultured twice (successively) for three weeks on solid media in order to obtain sufficient actively growing fungal material. *Glomus mosseae* was precultured on roots of *T. repens* for 15 weeks (inoculum density: roots and soil with AMF to sterilized soil, 10 % v/v). The substrate used was a sand-perlite mixture 1:3 (v/v). Due to logistical reasons it was not possible to collect soil from the field sites, and therefore these experiments were performed on sand with a similar soil chemical composition to the younger successional stages. pH ($CaCl_2$) of the sand was 5.8, nutrient contents were 70 mg $N.kg^{-1}$, 20 mg $P.kg^{-1}$, and 0.17 % organic matter. No nutrient solution was supplied during the experiment. Demineralized water was added to the substrate

(1:3:1 v/v/v sand:perlite:water) 24 h prior to autoclaving. The substrate was autoclaved twice (1 h, 121 °C, 1 atm.) with 48 h interval, and left for one week. Root growth chambers (vertically placed Petri dishes 15 cm diameter, with a slit in the edge) were filled with the sterile substrate. Each root growth chamber contained 300 mL substrate, of which 75 mL (*c.* 120 g) was sand. Cuttings were transferred to the root growth chambers (1 cutting per growth chamber), each cutting was inoculated with 5 mycelial plugs cut from the edge of a precultured fungal colony, the root system was covered with a water agar (1 %) layer (5 cm diameter) to prevent the roots from excessive loss of water. The root growth chambers were sealed with tape and sterilized anhydrous lanolin preventing contamination and loss of water. The control plants were instead supplied with 5 plugs of solid medium (5 * 0.15 mL) without fungus. The inoculation of the arbuscular mycorrhizal fungus was performed by mixing 33.3 g freshly washed root material of *T. repens* containing *G. mosseae* with the sterile substrate (16.6 L), and 50 growth chambers were filled with this mixture. Cuttings were transferred to the root growth chambers, the root systems were covered with water agar, growth chambers were closed and sealed. As previous experiments with addition of washings (Koide & Li, 1989) or roots of *T. repens* without AMF to the control (and EcM) plants showed no effect on plant performance (APPENDICES E AND F), no attempt was made to provide control plants with roots of *T. repens* without AMF. All soil compartments were protected from daylight, the root growth chambers were placed vertically in transient propagators (relative air humidity almost 100 % in the first week) and placed in the climate chamber. Growth conditions: photon flux density 350 $\mu E.m^{-2}.s^{-1}$ (20,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 60 %.

*Experiment 1: Significance of AMF and EcMF*

The experiment was set up as a complete randomized block design with two (three) factors. One factor, harvest, contained four levels, and the second factor, fungal treatment, contained three levels. The third factor, collection date of cuttings, contained two levels. Planned contrasts were performed with cuttings collected in March 1996 (high P; part 1) and December 1996 (low P; part 2). Each treatment was replicated at least ten times. For both collecting dates, the inoculations with *G. mosseae* and *H. leucosarx* were performed in 50 replicates, and 60 plants were supplied with non-inoculated agar plugs serving as a control for *S. repens*. After 12 weeks (t = 12) ten plants of each combination were harvested randomly. The experiment was continued in 2 L pots. These pots contained 1800

mL substrate of which 450 mL (*c.* 720 g) was sand. Randomly 25 specimens of the remaining *S. repens* inoculated with *G. mosseae* and *H. leucosarx*, and all remaining 20 specimens of non-mycorrhizal *S. repens* (control) were individually transferred to these 2 L pots. Plants were watered sufficiently. At t = 20 and 30 weeks again ten plants were harvested randomly.

*Experiment 2: Different amounts of arbuscular mycorrhizal inoculum*

The experiment was set up in a complete randomized design with two factors. One factor, AMF inoculum amount, contained three levels, and the second factor, harvest, contained four levels, making a total of 12 treatment combinations. *Salix repens* was inoculated with different amounts of clover roots colonized by *G. mosseae* i.e. 0.67, 1.34 and 3.35 g each amount in 45 replicates. After 12 weeks ten plants of each combination were harvested randomly. The experiment was continued in 2 L pots, as in expt 1. Randomly 25 replicates were individually transferred to these pots. Plant were watered sufficiently. At t = 20 and 30 weeks again ten plants were harvested randomly. In addition, AM viability and colonization in *T. repens* was studied when grown on the substrate containing the lowest content of inoculum colonized by *G. mosseae*, and harvested after 12, 20 and 30 weeks.

*Plant performance, EcM and AMF colonization, soil analysis*

Every three weeks length of shoots, number of shoots, and number of leaves were determined. At the final harvest shoot weight was determined after 72 hours at 70 °C. The plants were individually digested in sulphuric acid, salicylic acid and 30 % hydrogen peroxide. Total N and P were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard (Novozamsky *et al.*, 1988).

The roots were immersed in water over a 2 mm sieve to remove most of the soil, and rinsed gently to avoid damage of the mycorrhizas. The cleaned ectomycorrhizal roots were stored in glutaraldehyde buffer (Alexander & Bigg, 1981), and roots of the control plants and the AM plants were stored in 50 % alcohol until they could be processed. In each sample, root length was determined according to Newman (1966) and the numbers of EcM root tips, and total numbers of root tips were counted. Root biomass was not determined, since different EcM-, AM- or non mycorrhizal root systems contain different (not removable) proportions of sand (CHAPTER 7). EcM frequencies were calculated (100

% * numbers of EcM root tips/total numbers of root tips). EcM root length was measured according to Giovanetti & Mosse (1980). All root samples that had been inoculated with AMF were cleared with 10 % KOH for three hours in a waterbath at 90 °C, bleached in 10 % $H_2O_2$ for 1 hour, acidified in 1 % HCl for 15 min and stained with trypan blue (Phillips & Hayman, 1970; APPENDIX C) in lactophenol for 30 min. AM colonization was estimated by a modified line intersect method (McGonigle *et al.*, 1990), where a minimum of 100 line intersections per root sample (replicated three times per sample) was scored for the presence of AM structures. AM root length colonization times intensity (RLCI = RLC * % cover by AM of the cross section of the root colonized) was calculated. Hyphal length of the extramatrical mycelium was not determined because of different amounts of hyphae of non-mycorrhizal fungi, which cannot always unambiguously be separated from hyphae of mycorrhizal fungi (of which length may also be differently affected, due to species-specific interactions between EcMF or AMF mycelia and other soil fungi).

At t = 0, and after 12, 20 and 30 weeks plant-available nutrients in the soil were analysed according to the $CaCl_2$ method described by Houba *et al.* (1990). $N_{ts}$ and $P_s$ were analysed in 0.01M $CaCl_2$ extracts. The $CaCl_2$ extracts were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard.

*Statistical analysis*

For the experiments that were set up in a complete randomized block design, the position of both the blocks and the pots (root growth chambers) within these blocks were randomized every three weeks. Significant block effects did not occur for any of the variables, and therefore no correction for block effect was made.

Data were analysed by Analysis of Variance using the statistical package STATISTICA (StatSoft). Growth measurements were analysed using repeated measures. Prior to analysis proportional mycorrhizal colonizations were arcsine square root transformed, all other data were logarithmically transformed. Bartlett's test was used to determine whether variances were equal between treatments. Differences among means were evaluated with the LSD-test (Sokal & Rohlf, 1995).

Correlations between plant parameters were tested using Spearman's rank tests (Siegel & Castellan, 1988).

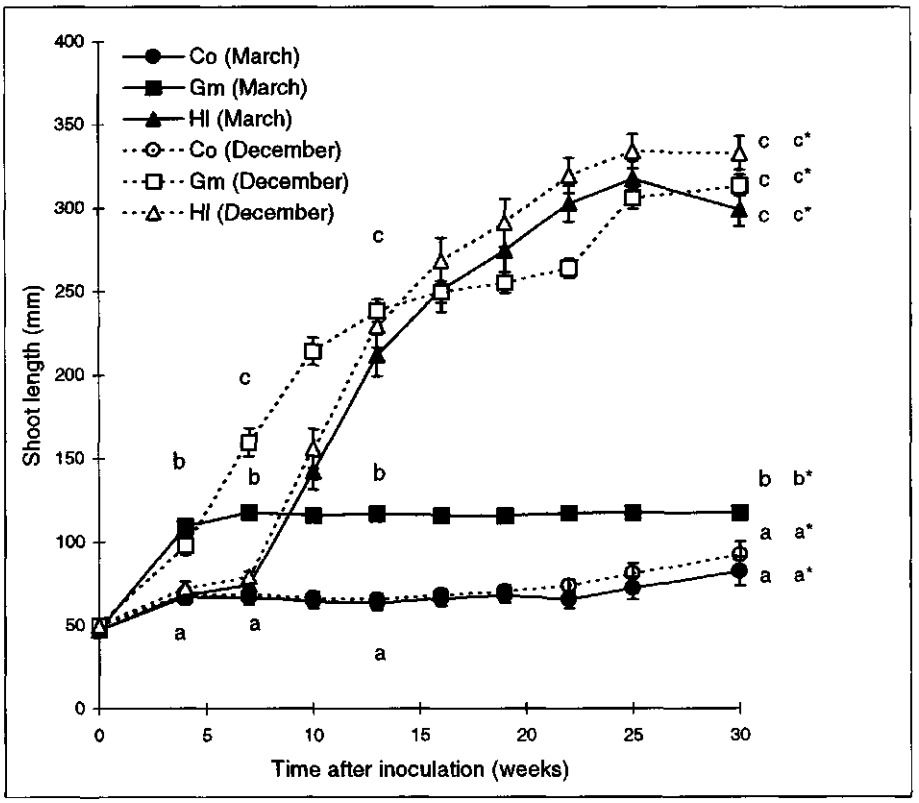



**FIGURE 5-1.** Mean shoot length of *Salix repens* (March 1996; continuous line and December 1996; dotted line and open symbols) inoculated with *Glomus mosseae* and *Hebeloma leucosarx* or not inoculated (control) over 30 weeks. Bars represent (±) 1 SE. Different letters next to points at 4, 7, 10, 13 and 30 weeks after inoculation indicate different shoot length, and different letters and asterisks indicate different growth rates over 30 weeks between mycorrhizal treatments ($P < 0.05$) according to LSD tests.

## RESULTS

*Experiment 1: Significance of AMF and EcMF*

In both experiments only 50 % of the control plants survived, whereas over 90 % of the mycorrhizal plants survived (data not shown), irrespective of the mycorrhizal type involved.

All parameters measured were significantly affected by the mycorrhizal treatment (ANOVA Repeated measures for growth, FIG. 5-1; Factors fungal treatment, harvest and collecting date, TABLES 5-1, 5-2 and FIG. 5-2; ANOVA and Planned contrasts for cuttings collecting date ($P < 0.001$)). Growth responses of *S. repens* inoculated with *G. mosseae* showed large differences with respect to collecting date (FIG. 5-1), whereas *S. repens* inoculated with *H. leucosarx* and the control plants were not affected by collecting date.

*Planned contrasts* between collecting dates March and December for plant parameters as affected by fungal treatments showed that the non-mycorrhizal plants differed only at t = 0, whereas no differences occurred at t = 12, 20 or 30 weeks. Similarly, for *H. leucosarx* no differences occurred between collecting dates March and December for the plant parameters. For *G. mosseae* all plant parameters were significantly different between March and December at t = 12, 20 and 30 weeks (FIG. 5-1 AND 5-2, TABLES 5-1 AND 5-2). Mycorrhizal colonization (%) was different at t = 20 and 30 weeks, while mycorrhizal root length differed at all harvest periods.

Shoot length and dry weight of cuttings collected in March were generally higher when inoculated with *G. mosseae* than the control, while those inoculated with *H. leucosarx* generally performed still better. Cuttings collected in December responded better to *G. mosseae* than to *H. leucosarx* at the short term, whereas the long term showed equal benefit from *G. mosseae* or *H. leucosarx* on shoot length and dry weight (TABLE 5-1, FIGS. 5-2a,b). Root length for both March and December cuttings responded better to *G. mosseae* in the short term, whereas on the long term *H. leucosarx* performed best for cuttings collected in March and *G. mosseae* performed best for cuttings collected from December (TABLE 5-1, FIGS. 5-2c,d). For both March and December cuttings mycorrhizal colonization by *H. leucosarx* decreased after the first harvest period. Colonization by *G. mosseae* did not change significantly over time, but at t = 20 and 30 weeks colonization was lower on cuttings collected in December than on those collected in March. Arbuscular mycorrhizal root length showed the opposite (TABLE 5-1).

**TABLE 5-1.** Shoot length, shoot dry weight, root length, mycorrhizal colonization, mycorrhizal root length (means ± 1 SE, $n \geq 10$) of *Salix repens* (collected in March 1996 and December 1996) inoculated with *Glomus mosseae, Hebeloma leucosarx* or not inoculated (control) grown for 30 weeks.

| Fungal treatment | Shoot length (mm) | Shoot dry weight (mg) | Root length (m) | †Mycorrhizal colon. (%) | †Myc.root length (m) |
|---|---|---|---|---|---|
| **December cuttings** | | | | | |
| Control (t = 0) | 50.0± 1.7 a* | 51.1± 9.2 a | 0.03±0.00 a | 0 a | 0 a |
| Control (t = 12) | 64.9±11.0 b | 59.1± 3.8 b | 1.31±0.29 b | 0 a | 0 a |
| *G. mosseae* | 238.7± 9.3 d | 350.6±11.1 ef | 24.49±2.11 e | 3.1±0.2 b | 0.76±0.11 b |
| *H. leucosarx* | 209.3±15.6 d | 179.9±10.4 d | 6.68±1.32 d | 75.4±2.3 e | 5.04±0.76 e |
| Control (t = 20) | 68.9± 1.9 c | 71.3±1.9 bc | 2.87±0.23 c | 0 a | 0 a |
| *G. mosseae* | 295.4±16.4 e | 410.2±13.2 f | 65.1±5.1 f | 2.3±0.1 b | 1.50±0.09 c |
| *H. leucosarx* | 291.1±15.4 e | 290.1±11.7 e | 54.62±5.64 e | 59.1±3.8 c | 32.28±3.1 f |
| Control (t = 30) | 91.5±5.2 c | 88.5±3.6 c | 8.71±0.81 d | 0 a | 0 a |
| *G. mosseae* | 313.6±12.1 e | 292.3±12.8 e | 130.11±8.51 g | 2.5±0.9 b | 3.25±0.17 d |
| *H. leucosarx* | 323.1±19.3 e | 295.2±7.9 e | 92.32±3.97 f | 66.9±2.1 d | 61.76±2.2 g |
| ANOVA (*P* value) | | | | | |
| Fungus (1) | <<0.001 | <<0.001 | <<0.001 | <<0.001 | <<0.001 |
| Harvest (2) | <<0.001 | <<0.001 | <<0.001 | 0.009 | <<0.001 |
| 1 × 2 | <<0.001 | <<0.001 | <<0.001 | 0.003 | <<0.001 |
| **March cuttings** | | | | | |
| Control (t = 0) | 47.0± 2.5 a | 50.2± 8.7 a | 0.03±0.00 a | 0 a | 0 a |
| Control (t = 12) | 50.7± 9.5 b | 54.1± 7.9 ab | 1.36±0.31 b | 0 a | 0 a |
| *G. mosseae* | 110.9± 8.7 e | 127.6±12.5 d | 17.51±2.38 f | 3.2±0.3 b | 0.57±0.10 b |
| *H. leucosarx* | 166.6±21.7 f | 129.9±18.4 d | 6.18±1.01 d | 70.9±3.3 d | 4.57±0.87 d |
| Control (t = 20) | 62.2±2.5 bc | 61.3±2.0 b | 2.90±0.13 c | 0 a | 0 a |
| *G. mosseae* | 105.2±8.3 de | 136.8±7.8 d | 16.86±0.75 f | 3.4±0.1 b | 0.58±0.04 b |
| *H. leucosarx* | 284.5±19.3 g | 297.3±14.3 e | 53.73±5.75 h | 53.4±2.6 c | 28.67±2.8 e |
| Control (t = 30) | 89.6±8.4 cd | 78.5±6.0 bc | 9.30±0.90 e | 0 a | 0 a |
| *G. mosseae* | 107.1±3.5 e | 94.9±3.2 c | 24.27±0.60 g | 4.3±0.4 b | 1.05±0.08 c |
| *H. leucosarx* | 292.1±10.0 g | 282.9±9.2 e | 95.14±4.00 i | 64.6±1.1 c | 61.45±2.8 f |
| ANOVA (*P* value) | | | | | |
| Fungus (1) | <<0.001 | <<0.001 | <<0.001 | <<0.001 | <<0.001 |
| Harvest (2) | <<0.001 | <<0.001 | <<0.001 | 0.008 | <<0.001 |
| 1 × 2 | <<0.001 | <<0.001 | <<0.001 | 0.004 | <<0.001 |

* Values within a column that are followed by a different letter, differ at $P < 0.05$ according to a LSD test following a two-factor ANOVA (df : fungus = 2, harvest = 3, df Error = 125 or †df: fungus =1, harvest = 2, df Error = 71).

**TABLE 5-2.** Shoot N concentration, shoot P concentration, shoot N content, shoot P content and shoot N/P ratio (means ± 1 SE, $n \geq 10$) of *Salix repens* (collected in March 1996 and December 1996) inoculated with *Glomus mosseae, Hebeloma leucosarx* or not inoculated (control) grown for 30 weeks.

| Fungal treatment | N conc. (g/kg) | P conc. (g/kg) | N total (mg) | P total (mg) | N/P ratio |
|---|---|---|---|---|---|
| | | December cuttings | | | |
| Control (t = 0) | 10.09±0.74 d* | 0.88±0.06 a | 0.52±0.17 a | 0.04±0.01 a | 13.0±0.9 d |
| Control (t = 12) | 7.89±0.12 b | 1.59±0.69 a | 0.47±0.05 a | 0.09±0.02 a | 5.0±0.7 a |
| *G. mosseae* | 10.01±0.43 d | 1.88±0.12 d | 1.73±0.16 d | 0.66±0.02 d | 5.2±0.5 a |
| *H. leucosarx* | 14.98±0.17 e | 1.76±0.13 d | 2.69±0.20 e | 0.32±0.03 b | 8.5±0.6 c |
| Control (t = 20) | 7.43±0.26 bc | 0.78±0.02 a | 0.53±0.02 ab | 0.06±0.01 a | 9.5±0.7 c |
| *G. mosseae* | 8.62±0.19 c | 1.48±0.07 bc | 1.65±0.08 d | 0.61±0.02 d | 5.5±0.6 ab |
| *H. leucosarx* | 8.47±0.21 c | 1.44±0.03 bc | 2.46±0.09 e | 0.42±0.03 c | 5.9±0.4 b |
| Control (t = 30) | 8.18±0.27 c | 1.15±0.04 b | 0.72±0.04 b | 0.10±0.01 a | 7.1±1.3 b |
| *G. mosseae* | 7.23±0.14 b | 1.54±0.06 cd | 1.05±0.05 c | 0.45±0.01 c | 4.6±0.2 a |
| *H. leucosarx* | 6.32±0.10 a | 1.48±0.02 c | 1.87±0.05 d | 0.44±0.02 c | 4.3±0.1 a |
| ANOVA (*P* value) | | | | | |
| Fungus | <<0.001 | <<0.001 | <<0.001 | <<0.001 | <<0.001 |
| Harvest | <<0.001 | <<0.001 | 0.018 | <<0.001 | <<0.001 |
| Fungus × Harvest | <<0.001 | <<0.001 | <<0.001 | <<0.001 | <<0.001 |
| | | March cuttings | | | |
| Control (t = 0) | 10.15±0.74 d | 2.01±0.06 e | 0.88±0.19 c | 0.18±0.02 b | 5.0±0.6 b |
| Control (t = 12) | 7.65±0.21 b | 1.90±0.69 cd | 0.41±0.06 a | 0.10±0.04 a | 7.4±1.3 c |
| *G. mosseae* | 7.67±0.14 b | 0.41±0.02 a | 0.97±0.09 c | 0.05±0.01 a | 19.0±0.7 e |
| *H. leucosarx* | 15.31±0.97 ef | 1.74±0.11 de | 1.86±0.20 d | 0.22±0.03 b | 9.0±0.5 d |
| Control (t = 20) | 7.27±0.16 b | 0.80±0.02 b | 0.44±0.02 ab | 0.04±0.00 a | 9.1±0.2 d |
| *G. mosseae* | 7.11±0.19 b | 0.37±0.01 a | 0.98±0.07 c | 0.05±0.00 a | 18.9±0.5 e |
| *H. leucosarx* | 8.52±0.24 c | 1.42±0.03 cd | 2.43±0.09 e | 0.43±0.02 c | 5.7±0.3 c |
| Control (t = 30) | 8.38±0.19 c | 1.16±0.03 c | 0.65±0.04 b | 0.09±0.01 a | 7.3±1.6 cd |
| *G. mosseae* | 6.35±0.12 a | 0.70±0.02 b | 0.59±0.02 b | 0.06±0.00 a | 9.2±0.2 d |
| *H. leucosarx* | 6.14±0.10 a | 1.45±0.03 cd | 1.72±0.05 d | 0.41±0.01 c | 4.2±0.1 a |
| ANOVA (*P* value) | | | | | |
| Fungus | <<0.001 | <<0.001 | <<0.001 | <<0.001 | <<0.001 |
| Harvest | <<0.001 | <<0.001 | 0.018 | <<0.001 | <<0.001 |
| Fungus × Harvest | <<0.001 | <<0.001 | <<0.001 | <<0.001 | <<0.001 |

* Values within a column that are followed by a different letter, differ at $P < 0.05$ according to a LSD test following a two-factor ANOVA (df : fungus = 2, harvest = 3, df Error = 125).

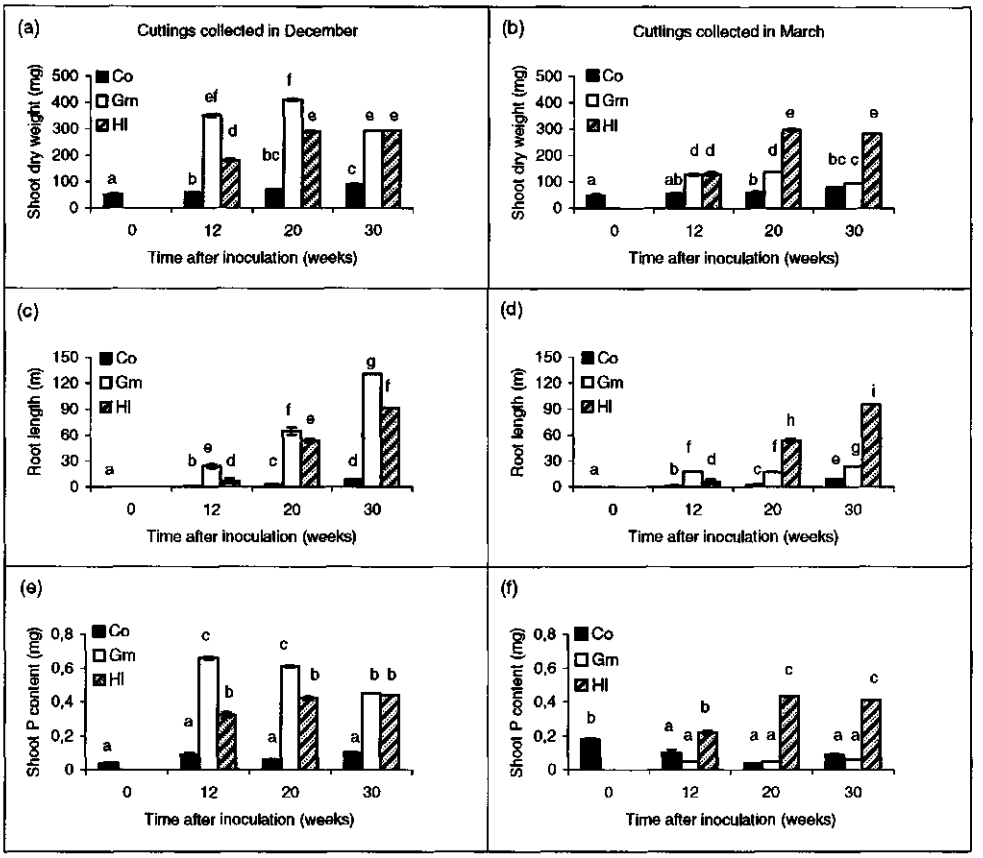



**FIGURE 5-2.** Mean (a,b) shoot dry weight; (c,d) root length and (e,f) shoot P content of *Salix repens* (collected in March 1996 and December 1996) inoculated with *Glomus mosseae, Hebeloma leucosarx* or not inoculated (control), harvested after 0, 12, 20 and 30 weeks. Bars represent (±) 1 SE. Significant differences are indicated by different letters ($P < 0.05$) according to LSD tests.

At t = 0, shoot P concentration differed in cuttings collected in March or December; 2.1 $g.kg^{-1}$ and 0.8 $g.kg^{-1}$, respectively, whereas shoot N concentration was similar. Shoot N and P concentration for cuttings collected in March were generally highest for *H. leucosarx* at t = 12 and 20 weeks, whereas cuttings with *G. mosseae* showed the lowest concentrations. Cuttings collected in December showed positive effects in N concentration at t = 12 and at all three harvest periods in shoot P concentration by both mycorrhizal fungi compared to the control (TABLE 5-2). Shoot N content for cuttings collected in March was positively affected by both mycorrhizal fungi, but to a larger extent by *H. leucosarx*, whereas shoot P content was only positively affected by *H. leucosarx*. However, for cuttings collected in December both N and P contents were positively affected by both fungi, and again to a larger extent by *H. leucosarx* in the long term (TABLE 5-2, FIGS. 5-2e,f).

*Short term effects* (<12 weeks) - Both mycorrhizal fungi had increased shoot length and dry weight as compared to the control plants, irrespective of collecting date. Root length was significantly increased by both mycorrhizal fungi (FIGS. 5-2c,d). Even though *G. mosseae* showed the lowest mycorrhizal colonization (%) (TABLE 5-1), it showed large positive effects on short term shoot growth rate (FIG. 5-1) and root length (FIGS. 5-2c,d). Shoot N concentration was highest in the presence of *H. leucosarx* (TABLE 5-2). *Glomus mosseae* showed the highest shoot P content for cuttings collected in December (FIGS. 5-2e,f), while for cuttings collected in March those with *H. leucosarx* showed equal shoot P content (FIGS. 5-2e,f). Shoot N/P ratio was around 10 for cuttings with *H. leucosarx*, whereas those with *G. mosseae* showed a shoot N/P ratio of 5 for cuttings collected in December and 19 for those collected in March (TABLE 5-2).

*Long term effects* (30 weeks) - Plant growth response was significantly affected by the mycorrhizal treatments (FIG. 5-1, Repeated measures ANOVA: all factors and their interactions, $P < 0.001$). Contrary to similar positive effects on both collecting dates by *G. mosseae* in the first seven weeks of the experiment, over the long term differences occurred in shoot length of cuttings collected in March and December. Cuttings from December with *G. mosseae* continued to increase in shoot length over time similarly to those with *H. leucosarx*, whereas cuttings collected in March no longer increased shoot length after seven weeks when inoculated with *G. mosseae* (FIG. 5-1). Growth measurements over 30 weeks showed that non-mycorrhizal plants had the lowest growth

rate, *H. leucosarx* the highest, while the growth curve of cuttings with *G. mosseae* depended on whether they had been collected in March or December (FIG. 5-1).

Over 30 weeks time, fungal treatments showed significant differences for cuttings collected in December (TABLES 5-1 and 5-2) in shoot length, shoot dry weight, root length, mycorrhizal colonization, mycorrhizal root length, shoot N/P ratio, shoot N concentration, shoot P concentration, shoot N content, and shoot P content (TABLES 5-1 and 5-2, $P < 0.001$). Also interaction with harvest was significant ($P < 0.001$). The significant interaction with harvest for all parameters indicated differential effects of the different treatments over the different harvest periods. Opposite to a very fast growth response and shoot P uptake by *G. mosseae* on the short term, effects were smaller over the longer term (FIG. 5-2). For cuttings collected in March, also all treatments showed significant differences in plant parameters measured over 30 weeks time (TABLES 5-1 and 5-2). For all plant parameters, but mycorrhizal colonization and shoot N total, factors and their interactions showed significant effects at $P < 0.001$.

**TABLE 5-3.** Available $P_s$ and $N_{ts}$ in soils in the pots of *Salix repens* (March and December 1996) inoculated with *Glomus mosseae*, *Hebeloma leucosarx* or not inoculated (control) after harvesting at 0, 12, 20 and 30 weeks (means ± SE, $n \geq 10$).

| | Available nutrients (mg/kg) | | | |
|---|---|---|---|---|
| | December cuttings | | March cuttings | |
| Fungal treatment | $P_s$ | $N_{ts}$ | $P_s$ | $N_{ts}$ |
| t = 0 | 0.29 ±0.02 d* | 11.86 ±0.02 e | 0.31 ± 0.01 e | 11.94 ±0.04 e |
| t = 12 | | | | |
| Control | 0.30 ± 0.09 d | 9.73 ± 1.02 d | 0.27 ± 0.10 d | 9.87 ± 1.20 d |
| *Glomus mosseae* | 0.06 ± 0.01 b | 2.60 ± 0.07 ab | 0.06 ± 0.02 ab | 12.80 ± 0.59 e |
| *Hebeloma leucosarx* | 0.15 ± 0.07 bc | 7.47 ± 1.46 cd | 0.26 ± 0.17 d | 9.48 ± 0.47 d |
| t = 20 | | | | |
| Control | 0.17 ± 0.02 c | 4.04 ± 0.17 bc | 0.07 ± 0.02 b | 4.94 ± 0.24 ab |
| *Glomus mosseae* | 0.00 a | 2.33 ± 0.15 a | 0.00 a | 4.04 ± 0.16 a |
| *Hebeloma leucosarx* | 0.07 ± 0.01 b | 3.23 ± 0.23 b | 0.05 ± 0.02 b | 4.24 ± 0.29 a |
| t = 30 | | | | |
| Control | 0.10 ± 0.01 b | 4.99 ± 0.21 c | 0.09 ± 0.01 b | 5.19 ± 0.25 bc |
| *Glomus mosseae* | 0.00 a | 2.28 ± 0.07 a | 0.00 a | 4.04 ± 0.35 a |
| *Hebeloma leucosarx* | 0.17 ± 0.03 c | 5.12 ± 0.19 c | 0.14 ± 0.04 c | 5.72 ± 0.27 c |
| Kruskal-Wallis test | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 |

* Significant differences between fungal treatments or time are indicated by different letters (Kruskal Wallis and Mann-Whitney-U; $P < 0.05$).

*Shoot nutrient uptake versus nutrients in the soil available pool by two mycorrhizal types*

Shoot N and P uptake was highest in the presence of *H. leucosarx* for both March and December cuttings. However, shoot P uptake over the period 0 - 12 weeks was highest in the presence of *G. mosseae*, but only for cuttings collected in December (TABLE 5-2).

Soil analyses showed that available N and P in the soil, after harvesting, differed between fungal treatments (TABLE 5-3). $P_s$ significantly decreased over time. In the treatment with *G. mosseae* all available P was removed (March and December cuttings), whereas at the final harvest period in the treatment with *H. leucosarx*, $P_s$ was higher than in the second harvest period.

*Experiment 2:Different amounts of arbuscular mycorrhizal inoculum*

This fast growth response by *G. mosseae* occurred irrespective of the amount of inoculum and the growth response of *S. repens* did not last longer when supplied with a higher amount of inoculum. No significant differences were found in plant parameters and mycorrhizal colonization, when *S. repens* was supplied with three different amounts of inoculum, interaction between amount of inoculum × harvest being not significant either (TABLE 5-4).

**TABLE 5-4.** Results of ANOVA (*P* values) executed for shoot length, shoot biomass, root length, mycorrhizal colonization, mycorrhizal root length, shoot N and P concentration and content and shoot N/P ratio of *Salix repens* ($n \geq 10$) inoculated with *Glomus mosseae*.

| Response variable | Source of variation | | |
|---|---|---|---|
| | Inoculum* (df = 2) | Harvest* (df = 2) | Inoculum × harvest (df = 4) |
| Shoot length | 0.875 | 0.019 | 0.754 |
| Shoot biomass | 0.896 | 0.004 | 0.898 |
| Root length | 0.256 | <0.001 | 0.170 |
| Mycorrhizal colonization | 0.527 | 0.041 | 0.053 |
| Mycorrhizal root length | 0.894 | <0.001 | 0.431 |
| Shoot N concentration | 0.612 | <0.001 | 0.871 |
| Shoot N total | 0.848 | <0.001 | 0.548 |
| Shoot P concentration | 0.617 | <0.001 | 0.408 |
| Shoot P total | 0.560 | <0.001 | 0.354 |
| Shoot N/P ratio | 0.269 | <0.001 | 0.891 |

* The factor 'inoculum' represents 3 different amounts of inoculum (0.67, 1.34 or 3.35 g fw. clover roots with *Glomus mosseae*), and the factor 'harvest' represents three harvest periods (12, 20 and 30 weeks).

## DISCUSSION

*Significance of AMF and EcMF*

Benefits by two functional types of mycorrhiza (AM and EcM) were demonstrated in this study. *Glomus mosseae* was very beneficial on the short term (12 weeks), whereas *H. leucosarx* was very beneficial over the long term (seven months). Newman & Eason (1989) suggested that mycorrhizal links between dying and living roots can contribute to nutrient cycling. However, in the present study the very fast growth response and shoot P uptake shortly after inoculation by *G. mosseae* was not caused by a nutritional bias from clover roots, since no additional benefits occurred when the amount of inoculum (roots) was raised.

The present experiment was performed under higher light intensity, 350μE.$m^{-2}$.$s^{-1}$, opposed to 120 μE.$m^{-2}$.$s^{-1}$ in the experiment previously carried out. The former experiment indicated that *G. mosseae* also showed large positive short term effects unlike *H. leucosarx* (CHAPTER 4). Long term effects, however, showed that both *G. mosseae* and *H. leucosarx* did not increase plant weight or shoot nutrient uptake compared to the non-mycorrhizal treatment. This was partly explained by selection of the fittest 50 % surviving non-mycorrhizal plants that biased those results. However, in the present experiment also only 50 % of the non-mycorrhizal plants survived and still large positive effects (opposed to the non-mycorrhizal treatment) generally occurred in both mycorrhizal treatments. Therefore, a better explanation may be that *Salix repens,* like almost all willow species, is adapted to very high light intensities. Mycorrhizal net benefits have been found to be higher under higher irradiance (Johnson *et al.*, 1997). Besides, the present experiment was performed (continued) in 2 L pots, whereas the previous experiment was performed in small root growth chambers (75 mL soil). Therefore, another explanation may be that even though soil conditions were similarly poor in this experiment, the amounts of nutrients were not too low (by use of a larger soil volume of nutrient poor soil) and hyphal networks of both mycorrhizal fungi actually could increase resource availability. Both these circumstances may explain the larger benefits of both mycorrhizal fungi in this study.

*Differential effect of AM and EcM*

Over a relatively short period (12 weeks) very different effects on plant performance of *S. repens* by different mycorrhizal types have occurred. Multiple

harvesting up to 30 weeks showed that these effects furthermore vary in time, duration, magnitude and nature.

Responses to the two mycorrhizal types varied considerably in time and duration. *G. mosseae* (this study and CHAPTER 4) showed large effects on a short term (the first 7 weeks), whereas benefits of *H. leucosarx*, had to be considered over a longer period. Lapeyrie & Chilvers (1985) also concluded that most of the growth promotion of *Eucalyptus* in calcareous soils was due to AM, which were later succeeded by EcM.

Mycorrhizal types differed in their magnitude of response as related to the amount of root colonization. There was no reason to just assume that low infection implied low plant response, and high colonization implied high plant response. This was strikingly demonstrated by *G. mosseae*. Only 5 % of the root was colonized and very strong effects on short term shoot dry weight, shoot P uptake and root length were shown. The contrasting effect by *G. mosseae* on *S. repens* collected in either March or December was striking. At the start of the experiments, these cuttings only differed in shoot P concentration (and physiological state). These results indicated that nutrient uptake of *S. repens* benefits from *G. mosseae* when P is the limiting element. Apparently, shoot P-demand (and hence P-internal concentration) of *S. repens* is very high at the start of the growing (and flowering) season. This involves a higher dependency on AM during that time. In agreement with this a higher AM colonization was observed in spring than in autumn in the field (CHAPTER 3). Shoot P-inflow by *H. leucosarx* was about 50 % of that of *G. mosseae* in the short term. As a flush of soil nutrients in natural systems is released in spring, the relatively high (yet low in absolute terms) colonization by AMF in *S. repens* observed early in spring and its very fast response in P uptake, as observed in this study, explained the functional significance of AM in P limited *S. repens*.

For *S. repens* collected in March (already high in shoot P-concentration), this study generally confirmed the conclusions of Jones *et al.* (1998) who studied AM and EcM colonization in *Eucalyptus*. As in that study, growth rates and shoot nutrient uptake of *S. repens* were also stimulated more by each of the EcM fungi than by *G. mosseae*. However, their study lasted for 13 weeks and in the present study conclusions were supported when examined over 30 weeks. The positive effects, in the present study, of *G. mosseae* (cuttings from both collecting dates) within the first 7-10 weeks were most definitely beneficial (shoot growth and root length). In the first 12 weeks, *G. mosseae* still showed positive effects similar to those of the EcM fungus. Moreover, when *S. repens* was

collected in December (low shoot P), *G. mosseae* in this study showed similar benefits to *H. leucosarx* on the long term as well.

Unlike *Eucalyptus* (Jones *et al.*, 1998), colonization by different mycorrhizal fungi and mycorrhizal root length of *S. repens* varied enormously between the different fungal types. Therefore, not only one parameter (e.g. mycorrhizal percentage, mycorrhizal root length or hyphal length) can be used as an explanatory variable.

Furthermore, in this study also short term (12 weeks) growth responses and nutrient contents were correlated, whereas over 30 weeks they were not. This indicates that when studying mycorrhizal significance of different fungi experiments should last longer than 10-12 weeks. In this study, benefits were also examined over a period of 30 weeks, and thus the initial response to mycorrhizal infection could be a function of lifestage of the mycorrhizal association (McGonigle & Fitter, 1988). AM can go through cycles of activity, with many arbuscules being produced during periods of growth in young roots, and vesicles predominating at other times (Douds & Chaney, 1982). It is likely that similar cycles of activity occur in EcM (Downes *et al.*, 1992). In this study, *G. mosseae* showed no differences in relative amounts of structures (i.e. hyphae, vesicles) over time (or between *S. repens* with different internal shoot P-status). The fast shoot P uptake could therefore not be related to certain structures (arbuscules). The temporarily high shoot P uptake on the short term, however, still can be related to a certain AM developmental state, which is not expressed in arbuscule formation in *S. repens*.

*Differential effects on soils and shoot nutrient uptake*

In the first 12 weeks, in accordance with the relative importance of AM for P uptake and EcM for N uptake in the different dune successional stages mentioned by Read (1989), shoot P uptake was very high by *G. mosseae*, and shoot N uptake was very high by *H. leucosarx*. This indicates a very effective (N or P specific) nutrient uptake of the mycorrhizal fungi. These high nutrient uptakes by shoots of *S. repens* might be a result of the extremely low root/shoot ratio of cuttings at the start of the experiment. Maybe due to the unnaturally low root/shoot ratio, these extreme uptakes of N and P in the shoot could be measured in the first 12 weeks.

Even though this study was performed in a very poor sandy soil in which nutrient availabilities and total nutrient contents were low (and 0.17 % organic matter), different mycorrhizal types had access to different amounts of nutrients from the mineral soil.

Mycorrhizas can alter soil chemistry of the soil in their vicinity so that more exchangeable N or P comes in solution, or by increasing the volume of the soil to which *S. repens* has access via their external hyphae (Tinker, 1975). However, *G. mosseae* absorbed almost all P from the available pool in the first 12 weeks, whereas *H. leucosarx* was able to translocate more P to the shoots of *S. repens* over 30 weeks compared to *G. mosseae.* Still P remained detectable in the available pool of EcM treatments. Thus, nutrient uptake was not only different by different types, but also at different time scales. Furthermore, different mycorrhizal types had access to different amounts of nutrients, and at different rates, from the mineral soil.

No attempt was made in this experiment to measure root dry weight and nutrient contents. Dry weight measurement, followed by loss on ignition indicated that mycorrhizal root systems with *H. leucosarx* contained 7 times the amount of sand (in the mycelial mantle) compared to non mycorrhizal root systems (CHAPTER 7). This sand could not be removed by thorough washing. Furthermore, above mentioned differences in their weathering capacity of surfaces of mineral soil made calibration with a certain amount of (unweathered) sand inappropriate.

*Root foraging plasticity as affected by different mycorrhizal types*

For many years it was believed that colonization by an AMF did not alter root system architecture (Harley & Smith, 1983). In this study, however, *G. mosseae* strongly increased root length of *S. repens* in the first 12 weeks (and even for 30 weeks in the *S. repens* collected in December) compared to *H. leucosarx* and the control. This is in agreement with Hooker *et al.* (1992), who also reported increased lateral root branching of *Populus* after infection with three AMF species. These alterations to root system architecture will clearly have major impact on root system function and long-term development.

*Arbuscular mycorrhizal colonization in Salix repens*

Arbuscular mycorrhizal colonization intensity (RLCI%) versus AM root length colonization (RLC%) used by others needs clarification. In this study AM colonization intensity (%) was used. In the field differences in AM colonization intensities in *S. repens* were found between certain habitats (e.g. very wet) and seasons, and due to older coarser root systems in the field (CHAPTERS 3 AND 10). For that reason AM root colonization was

considered an inappropriate measure, and therefore the term RLCI was used. The term intensity is used for comparison with field observations and field experiments. However, in the present experiments root length was stimulated by *G. mosseae*, resulting in a very fine long root system, and very low amounts of vesicles and no arbuscules were observed. Unlike coarse field roots, in these fine long roots AM colonization intensity of a cross-section of the root was almost always 50 %. Therefore, in this study 5 % AM colonization intensity (RLCI) was similar to 10 % AM root length (RLC).

Differences in AM colonization in *S. repens* versus the well known AM colonization (structures and intensity) in *Trifolium repens* deserve clarification as well. Mycorrhizas formed by AM fungi can be quite variable, the same fungus forming extensive intracellular coils and rather few arbuscules in some species of plant, and intercellular hyphae and many arbuscules in others. When the relative amount of AM colonization structures in *S. repens* was compared with *T. repens* (data not shown), in *S. repens* the structures mainly consisted of internal hyphae, few vesicles, and very incidental or no occurrence of hyphal coils, whereas AM colonization in roots of *T. repens* showed a complete spectrum of AM structures; internal hyphae, vesicles, and arbuscules (no hyphal coils were observed). Besides, over time, even though RLC in *Trifolium* remained around 90 %, RLCI changed over time (40, 90, 60 %, respectively). This coincided with a change in structures from mainly internal hyphae and arbuscules (few vesicles) to lots of vesicles and internal hyphae (few arbuscules), and finally mainly internal hyphae and high amounts of external spores attached to the external mycelium. Furthermore, AM colonization by *G. mosseae* in *T. repens* showed dynamics of development over 30 weeks time, whereas in *S. repens* RLC, RLCI and relative amount of (few) structures remained the same througout the different harvest periods. It is therefore likely that low AM colonization (root length, intensity, structure diversity) in *S. repens* was not due to a failure of mycorrhiza synthesis, but should be interpreted as the structural (and functional) development of the symbiosis between *S. repens* and *G. mosseae*.

In conclusion, AM and EcM showed differences in their significance towards *S. repens* depending on the internal (shoot) P-status. Low AM colonization seems to be the norm in *S. repens*, but this has to be considered a functional symbiosis. The supposed temporary beneficial effects of AM early in the season in (shoot) P uptake was supported in this study by the fact that *G. mosseae* was more beneficial for *S. repens* that was P

limited, than for *S. repens* that already had obtained a higher shoot P concentration. This may indicate that the supposedly non-functional low AM colonization, previously reported in ectomycorrhizal tree species (Cázares & Trappe, 1993), can in contrast provide a functional symbiosis depending on internal nutrient status or season (plant/tree nutrient demand).

In the present study only one AMF and one EcMF were used as model symbionts for both functional types. Functional significance of AMF and EcMF diversity for *S. repens,* therefore, deserves further research.

# 6

## Foraging strategies of 11 ectomycorrhizal fungi of *Salix repens*: ecological consequences of root manipulation versus root replacement.

**ABSTRACT**

*The ecological significance of a diversity of ectomycorrhizal fungal species, associated with* Salix repens, *was investigated under controlled conditions. Ectomycorrhizal diversity increased plant benefits in various ways. Effects of 11 ectomycorrhizal fungi on short-term (12 weeks) and long-term (20 and 30 weeks) plant performance were compared. Different fungi increased plant benefits in different ways and none exerted the full range of mycorrhizal benefits. Two strategies of EcM fungi were recognized, root manipulation and root replacement. Species with a root manipulation strategy strongly increased root length and had a more effective nitrogen economy than species with the root replacement strategy. As a consequence of these different strategies two parameters of efficiency of nutrient acquisition, viz. shoot nutrient inflow per unit root length and total shoot nutrient uptake, showed no correlation. Plant nutritional status indicated that mycorrhiza not only increased uptake, but also the size of the plant-available pool. Differences in magnitude of mycorrhizal response were not related to the amount of root colonization. Low colonization could not be equated with low plant response, and high plant response not with high colonization. Only in the short term was plant nutrient content positively correlated with root length colonized. Over a whole growing season plant nutrient content could not be predicted from root length colonized. Effects of mycorrhizal fungi also occurred with aqueous extracts of the fungus and, hence, were partly independent of the formation of ectomycorrhizas.*

**KEY WORDS**: Ectomycorrhiza, diversity, multifunctionality, fungal strategies, non-nutritional beneftis, nutrient uptake efficiencies, *Salix repens*.

## INTRODUCTION

Mycorrhizas are mutualistic associations between fungi and plant roots. The benefit for the fungus is the receipt of carbohydrates from the plant and the fungus generally increases resource availability and nutrient uptake of the plant in various ways. Besides foraging for nutrients in the soil, several mycorrhizal fungi produce enzymes capable of breaking down organic carbon, nitrogen, and phosphorus, and other mycorrhizal fungi can weather mineral soil substances. Furthermore, mycorrhizal fungi can alleviate abiotic (heavy metals) and biotic (pathogens) stress. By increasing resource availability and alleviating stress mycorrhizas generally improve plant growth and fitness (Smith & Read, 1997). Preliminary investigations indicated differential effects by different fungi on root length which might be a non-nutritional effect of the mycorrhizal symbiosis (CHAPTERS 4 AND 5).

The two most widespread types of mycorrhizal associations are ectomycorrhiza and arbuscular mycorrhiza. Most plant species form one type of association, but species from genera such as *Salix* (Fontana, 1962; Lodge, 1989), *Populus* (Vozzo & Hackskaylo, 1974; Lodge, 1989), *Eucalyptus* (Lapeyrie & Chilvers, 1985; Jones *et al.*, 1998), and *Alnus* (Molina, 1994) form both arbuscular mycorrhiza and ectomycorrhiza. In these dual mycorrhizal plants field observations have sometimes shown arbuscular mycorrhiza to be dominant and sometimes ectomycorrhiza (Lodge, 1989), but it is still unclear which factors favour dominance by each functional group. On the Dutch Wadden isle of Terschelling, *Salix repens* L. (Salicaceae), a dual mycorrhizal plant, was, however, consistently highly ectomycorrhizal (usually above 80-85 %) and slighty arbuscular mycorrhizal (less than 11 %) in all successional stages (CHAPTERS 2 AND 3).

The ectomycorrhizal fungal flora in these *S. repens* communities was (highly) diverse. In a three year sporocarp survey 74 taxa of ectomycorrhizal fungi were found in 16 field sites. These sites showed large differences in species number and abundance of sporocarps. Below-ground observations in these plots yielded 15 ectomycorrhizal morphotypes, plots again showing large differences in morphotype number and abundance of ectomycorrhizas. However, no significant correlation was found between fungal species and morphotype diversity, and between abundance of carpophores and ectomycorrhizas (CHAPTER 2).

High ectomycorrhizal species and morphotype diversity within stands suggests that the ectomycorrhizal fungi show substantial niche differentiation. Niche differentiation could occur through resource partitioning (Bruns, 1995). Resources could be partitioned in time or in space. Obvious temporal gradients are related to season, age of the host, and age of the

stand. Spatial niche dimensions of ectomycorrhizal fungi do not only include the soil where the external mycelium forages, but also the plant-fungus interface. If different mycorrhizal fungi have differential effects on plants and if plants can to some extent control mycorrhizal colonization, coexistence of different mycorrhizal fungi could also be explained by different plant benefits.

Expression of benefits depends on fungal species, plant species, soil characteristics, and duration of the experiment. Newsham *et al.* (1995b) introduced the concept of multifunctionality of the mycorrhizal symbiosis: individual mycorrhizal fungal species do not exert the full suite of mycorrhizal benefits (increased nutrient uptake and carbon gain, stress alleviation, non-nutritional benefits). For that reason a diverse fungal community might be essential from the plant's perspective. Multifunctionality of the ectomycorrhizal symbiosis, related to ecological and physiological differences between fungal species, has received less attention. This study was therefore designed to explicitly address the importance of ectomycorrhizal fungal diversity for the multifunctionality of the mycorrhizal symbiosis. Specifically it addresses questions about (1) response time, response duration, and magnitude of plant response in relation to extent of colonization; (2) non-nutritional benefits of mycorrhizal fungi on plant performance; (3) effects of mycorrhizal fungi on soils. Although fungal behaviours are individualistic (no two fungal species have identical effects on the plant), classification of fungal behaviours in strategies, eventhough it is an oversimplification to some extent might be helpful. This study therefore also addresses the question whether different ectomycorrhizal strategies could be recognized and how they relate to life history strategies of the ectomycorrhizal fungi.

## MATERIAL AND METHODS

### *Plant and fungal species*

*Salix repens* L. (Salicaceae) is a dual mycorrhizal shrub, common and widespread in the coastal dunes in western Europe. It occurs in a wide variety of habitats, ranging from dry to wet, and from calcareous, humus-poor to acidic, humus-rich soils. With its ability to grow in a very wide variety of habitats and with its highly diverse ectomycorrhizal fungal communities, the species is ideally suited to investigate the multifunctionality of the ectomycorrhizal symbiosis.

Experiments were conducted with cuttings (shoot tops) collected from male plants of *S. repens* from the field site Schoenus I on the Isle of Terschelling (53° 23'50" N, 5° 13'45" E). All cuttings were collected from the same individual to obtain genetic homogeneity.

Eleven cultures of ectomycorrhizal fungi collected from sporocarps in the autumn of 1994 and 1995 from *S. repens* from the Isle of Terschelling were used, viz. *Cortinarius pauperculus* J. Favre (L110), *C. trivialis* J. Lange (L89), *Hebeloma leucosarx* P.D. Orton (L1), *H. psammophilum* M. Bon (L129), *Inocybe lacera* (Fr.: Fr.) Kummer (L15), *Laccaria laccata* (Scop.: Fr.) Cooke (L153), *Lactarius controversus* (Pers.: Fr.) Fr. (L14), *L. helvus* (Fr.: Fr.) Fr. (L12), *Paxillus involutus* (Batsch: Fr.) Fr. (L82), *Scleroderma verrucosum* (Bull.: Pers.) Pers. (L69) and *Xerocomus rubellus* Krombh. (L114). *Cortinarius pauperculus* is restricted to members of the genus *Salix*, occurring both in alpine ecosystems and in the dunes (Arnolds & Kuyper, 1995). On Terschelling the species grew on dry to wet, acidic, humus-rich soils. *Cortinarius trivialis* associates with members of the Salicaceae (*Populus, Salix*) and with *Quercus*. On Terschelling it grew in calcareous to acidic, wet habitats and was very abundant in a regularly mown grassland with *S. repens*. *Hebeloma leucosarx* sensu auct. neerl. is exclusively associated with the genus *Salix*. The species was found to consist of 2 biological species (Aanen & Kuyper, 1999) that could not be separated morphologically. It is a generalist species, growing from dry to wet, and from calcareous to acidic conditions. *Hebeloma psammophilum* is exclusively associated with *S. repens*, growing in dry, humus-poor, calcareous sand. However, the taxonomy of this complex is not well known and vicariant taxa (or phenotypic variants) occur on wet and acidic sites (Vesterholt, 1989). *Inocybe lacera* is a generalist, associating with conifers and deciduous trees. On Terschelling it was found in calcareous, humus-poor, dry sand. *Laccaria laccata* is also a generalist species, which was found in all habitats. It was, however, more common on wet sites than on dry sites. *Lactarius controversus* is a common associate with *Populus* and also occurs in *S. repens* shrub. It was found on dry, acidic sand where rabbit activity had locally disturbed the soil. *Lactarius helvus* associates both with conifers and deciduous trees. On Terschelling it was found in dry to wet, acidic sites. *Paxillus involutus* has a very broad host range. However, the morphospecies consists of 3 biological species (Fries, 1985) and specimens under *S. repens* have generally shorter, more thick-set and often excentric stipes compared to the typical variant. It was found in all habitats, ranging from dry to wet, and from calcareous to acidic. *Scleroderma verrucosum*, known to associate with various deciduous trees, has only once been found in association with *S. repens* in a dry, acidic grassland with *S. repens*. *Xerocomus rubellus* is a collective species. Both collections, which may be referable to the recently described *X. riparíellus*, were made on dry, acidic sand. Voucher specimens are preserved at WAG.

Sporocarps were surface sterilized with alcohol (70 %) and sterilely sliced in half. Fungal tissue was cut from the innerside of the cap and transferred to, and maintained on, solid media (alternative Melin Norkrans (AMN)) containing (in $g.l^{-1}$): agar (15), malt extract (3), glucose.$H_2O$ (10), $(NH_4)_2HPO_4$ (0.25), $KH_2PO_4$ (0.5), $KNO_3$ (0.5), $MgSO_4.7H_2O$ (0.15), $CaCl_2.H_2O$ (0.67), NaCl (0.025), $FeCl_3$ (0.0012) and thiamine.HCl (100 µg).

*Inoculation and plant growth conditions*

After storage at 4 °C for a month, the cuttings of *S. repens* were trimmed to 4 cm, surface-sterilized twice in (freshly prepared) 6 % $H_2O_2$ for 1 min (in series of 20 in 300 mL), and rinsed three times in (fresh) demineralized water (1 L) for 10 min. Each cutting was placed individually in a culture tube containing 20 mL sterile water agar (1 %). After a 10 weeks rooting period in a climate chamber (photon flux density 120 µE. $m^{-2}.s^{-1}$ (11,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 70 %) equally developed cuttings were selected for the experiment.

The isolates of the EcM fungi were precultured twice (successively) for three weeks on solid media in order to obtain sufficient actively growing fungal material. Each fungus was individually inoculated to *S. repens*, and control cuttings were not inoculated. For each experiment 20 cuttings were harvested at the moment of inoculation (t = 0). The substrate used was a sand-perlite mixture 1:3 (v/v). Due to logistical reasons it was not possible to collect soil from the field sites, and therefore the experiments were performed on sand with a comparable soil chemical composition to the younger successional stages. pH ($CaCl_2$) of the sand was 5.8, nutrient contents were 70 mg $N.kg^{-1}$, 20 mg $P.kg^{-1}$, and 0.17 % organic material. No nutrient solution was supplied during the experiment. Demineralized water was added to the substrate (1:3:1 v/v/v sand:perlite:water) 24 h prior to autoclaving. The substrate was autoclaved twice (1 h, 121 °C, 1 atm.) with a 48 h interval, and left for one week. Root growth chambers (vertically placed Petri dishes of 15 cm diameter, with a slit in the edge) were filled with the sterile substrate. Each root growth chamber contained 300 mL substrate, of which 75 mL (*c.* 120 g) was sand. Cuttings were transferred to the root growth chambers (1 cutting per root growth chamber), each cutting was inoculated with 5 mycelial plugs cut from the edge of a precultured fungal colony, the root system was covered with a water agar (1 %) layer (5 cm diameter) to prevent the roots from excessive loss of water. The root growth chambers were sealed with tape and

sterilized anhydrous lanolin preventing contamination and loss of water. The control plants were instead supplied with 5 plugs of solid medium (5 * 0.15 mL) without fungus. All soil compartments were protected from daylight. Root growth chambers were placed vertically in transient propagators (relative air humidity almost 100 % in the first week) and placed in the climate chamber. Growth conditions: photon flux density 350 $\mu E.m^{-2}.s^{-1}$ (20,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 60 %.

*Experiment 1: Functional significance of ectomycorrhizal diversity for Salix repens*

The experiment was set up as a complete randomized block design with two factors. One factor, EcM treatments, contained 11 (part 1) or eight (part 2) levels, and the second factor, harvest (time), contained two (part 1) or four (part 2) levels. Each treatment was replicated at least ten times. Cuttings were collected in December 1995. Each of the 11 EcM fungi was individually inoculated to *S. repens* in 50 replicates, and 60 cuttings served as a control. After 12 weeks ten plants of each combination (except *L. helvus*) were harvested randomly (Part 1). The experiment was continued with a selection of the EcM fungi (Part 2). Randomly 25 replicates of the remaining *S. repens* inoculated with *C. pauperculus, C. trivialis, H. leucosarx, H. psammophilum, L. laccata, L. helvus* and *P. involutus* and all remaining 20 replicates of non mycorrhizal *S. repens* (control) were transferred to 2 L pots (individually). These pots contained 1800 mL substrate of which 450 mL (*c.* 720 mg) was sand. Plants were watered sufficiently. After 20 and 30 weeks again ten plants were harvested randomly. *Cortinarius trivialis* was examined over 30 weeks in three harvest periods in eight replicates instead of 10 due to low amount of mycelium that could be obtained (slow mycelial growth). *Lactarius helvus* was harvested after 30 weeks only, due to slow formation of mycorrhizas.

*Experiment 2: Plant performance as affected by different amounts of inoculum or water soluble extract of Cortinarius trivialis*

The experiment was set up as a complete randomized design with one factor. This factor comprised three different amounts of inoculum or a culture extract of *C. trivialis* plus two controls. Cuttings were collected in December 1996. *Cortinarius trivialis* was individually inoculated to *S. repens* in 10 replicates, and 15 cuttings served as a control. The substrate used was a sand-perlite mixture 1:3 (v/v). pH ($CaCl_2$) of the sand was 5.5, nutrient contents were 6 mg $N.kg^{-1}$, 6 mg $P.kg^{-1}$, and 0 % organic matter. Ten replicates of

cuttings were inoculated with 4, 8 or 16 mycelial plugs cut from the edge of a precultured fungal colony. In addition, 12, 8 or 0 non-inoculated agar plugs (respectively) of solid medium were supplied so that all cuttings were supplied with 16 plugs either with or without mycelium. The control plants were treated similarly, but instead of mycelial plugs they were supplied with 16 non-inoculated agar plugs of solid medium (16 * 0.15 mL).

Another 15 *S. repens* cuttings were supplied with a water soluble extract of a *C. trivialis* culture. Fifteen *S. repens* cuttings were supplied with a water soluble extract of non-inoculated AMN solid medium (i.e. without fungal culture, serving as a control). Water soluble extracts were made by shaking 46 g of AMN agar (with or without fungal tissue) in 150 mL sterile water for 2 hours. Immediately after extraction these extracts were filter sterilized and supplied to *S. repens* (10 mL per plant every three weeks, the other treatments were simultaneously supplied with 10 mL demineralized water). After 18 weeks randomly ten plants of each combination were harvested.

*Plant performance, EcM and AM colonization, soil analysis*

Every three weeks shoot length was measured. At the final harvest shoot weight was determined after 72 hours at 70 °C. The plants were individually digested in sulphuric acid, salicylic acid, and 30 % hydrogen peroxide. Total N and P were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard (Novozamsky *et al.*, 1988).

The roots were immersed in water over a 2 mm sieve to remove most of the soil, and rinsed gently to avoid damage of the mycorrhizas. The cleaned roots were stored in glutaraldehyde buffer (Alexander & Bigg, 1981) until they could be processed. In each sample, root length was determined according to Newman (1966) and the numbers of EcM root tips, and total numbers of root tips were counted. Root biomass was not determined, since different EcM- or non-mycorrhizal root systems contain different (not removable) proportions of sand (CHAPTER 7). EcM frequencies were calculated (100 % * numbers of EcM root tips/total numbers of root tips). EcM root length was measured according to Giovanetti & Mosse (1980). Hyphal length of the extramatrical mycelium was not determined because of different amounts of hyphae of non-mycorrhizal fungi, which cannot always unambiguously be separated from hyphae of mycorrhizal fungi (of which length may also be differently affected, due to species specific interactions between EcMF mycelia and other soil fungi).

At t = 0, and after 12, 20 and 30 weeks (plant) available nutrients in soils were analysed according to the $CaCl_2$ method described by Houba *et al.* (1990). $N_{ts}$ and $P_s$ were analysed in 0.01M $CaCl_2$ extracts. The $CaCl_2$ extracts were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard.

*Statistical analysis*

For the experiments that were set up in a complete randomized block design, the position of both the blocks and the pots (root growth chambers) within these blocks were randomized every three weeks. Significant block effects did not occur for any of the variables, and therefore no correction for block effect was made.

Data were analysed by Analysis of Variance using the statistical package STATISTICA (StatSoft). Growth measurements were analysed using repeated measures. Prior to analysis proportional mycorrhizal colonizations were arcsine square root transformed, all other data were logarithmically transformed. Bartlett's test was used to determine whether variances were equal between treatments. Differences among means were evaluated with the LSD-test (Sokal & Rohlf, 1995).

Correlations between mycorrhizal colonization (% of root length), mycorrhizal root length (m), or root length (m) and the other plant parameters measured, were tested using Spearman's rank test (Siegel & Castellan, 1988).

Shoot nutrient uptake ($\delta N$ and $\delta P$) was calculated as the difference in N and P content of shoots between two harvest periods. Shoot nutrient uptake is considered an estimate of mycorrhizal effectiveness. Nutrient inflow rate (N and P) was calculated according to Brewster & Tinker (1972) with the following formula:

$$[2] \quad (C_2 - C_1) \cdot \log_n(RTL_2 - RTL_1) \;/\; (RTL_2 - RTL_1) \cdot (t_2 - t_1),$$

where in this study C is shoot nutrient content (mg), RTL is root length (m), t is time (days), 1 represents the earlier and 2 the latter harvest period of the time intervals studied. Shoot nutrient inflow rate is considered an estimate of (mycorrhizal) root system efficiency. Calculations of shoot uptake and shoot inflow were based on averages per harvest period, hence statistics were not applied (McGonigle & Fitter, 1988).

## RESULTS

*Experiment 1: functional significance of ectomycorrhizal diversity for Salix repens; Response time, response duration, and magnitude of plant response in relation to extent of colonization*

Survival of ectomycorrhizal plants was over 90 % for most species (data not shown). All plants inoculated with *X. rubellus* died after ectomycorrhiza was established. Only 50 % of the control plants survived.

**TABLE 6-1a.** Shoot length, shoot dry weight, root length, mycorrhizal colonization and mycorrhizal root length (means ± SE, $n$ = 10) of *Salix repens* inoculated with *Cortinarius pauperculus, C. trivialis, Hebeloma leucosarx, H. psammophilum, Inocybe lacera, Laccaria laccata, Lactarius controversus, Paxillus involutus, Scleroderma verrucosum, Xerocomus rubellus* or not inoculated (control) grown for 12 weeks.

| Fungal treatment | Shoot length (mm) | Shoot dry weight (mg) | Root length (m) | Myc. colonization (%) | Myc.root length (m) |
|---|---|---|---|---|---|
| Control (t = 0) | 40.0± 2.5 a* | 50.3± 8.3 a | 0.03±0.00 a | 0 a | 0 a |
| Control (t = 12) | 50.7± 9.5 a | 54.3± 8.0 a | 1.33±0.32 b | 0 a | 0 a |
| *C. pauperculus* | 109.5±24.3 bc | 88.3±16.9 bc | 4.77±1.74 cd | 68.0±6.4 ef | 3.02±1.07 cd |
| *C. trivialis* | 135.6±15.4 cd | 103.3± 9.6 bc | 7.87±0.94 de | 29.2±4.8 b | 2.17±0.34 cd |
| *H. leucosarx* | 163.6±17.2 d | 127.9±16.4 c | 6.26±1.11 d | 70.6±3.6 f | 4.42±0.85 e |
| *H. psammophilum* | 160.8±27.3 d | 111.2±16.2 bc | 9.45±3.71 e | 68.5±5.2 f | 6.54±2.62 e |
| *I. lacera* | 65.0±17.3 ab | 56.1±10.1 a | 5.13±0.50 d | 45.3±4.3 cd | 2.35±0.41 cde |
| *L. laccata* | 54.5± 6.1 a | 72.5± 5.6 b | 3.55±0.57 c | 73.1±6.5 f | 2.60±0.50 cde |
| *L. controversus* | 90.4± 7.3 b | 84.3± 6.1 bc | 6.32±1.00 d | 57.3±8.5 de | 3.41±40.0 e |
| *P. involutus* | 88.3±10.6 b | 72.5± 4.9 b | 3.87±0.40 c | 45.9±7.6 c | 1.85±0.40 c |
| *S. verrucosum* | 88.1±10.0 b | 64.1± 8.3 ab | 6.07±0.53 d | 50.8±2.7 cd | 3.12±0.45 de |
| *X. rubellus* | 62.2±22.0 ab | 49.2±17.3 a | 3.07±1.65 b | 27.6±2.5 b | 0.85±0.49 b |
| (*P* values) | | | | | |
| 2 factor ANOVA | | | | | |
| Fungus | <0.001 | 0.024 | <0.001 | <0.001 | <0.001 |
| Harvest | <0.001 | 0.012 | <0.001 | | |
| Fungus×harvest | <0.001 | 0.024 | <0.001 | | |
| 1 factor ANOVA† | <0.001 | 0.005 | 0.001 | <0.001 | <0.001 |

* Values within a column that are followed by a different letter differ at $P < 0.05$ according to a LSD test, and a two-factor ANOVA (including control and t = 0, df: fungus = 10, harvest = 1, df Error = 108, or † a one factor ANOVA without control and t = 0, df: fungus = 9, df Error = 98).

*Short term effects* (part 1, 0-12 weeks) - Over the first 12 weeks all parameters measured were strongly significantly affected by the treatment both when the non-mycorrhizal treatment was included and when it was excluded from the analysis (TABLES 6-1a,b). Shoot length, shoot dry weight, and root length were correlated ($P < 0.001$). Root length was also correlated with shoot N and P content of shoots ($P < 0.001$). Shoot length

and dry weight of plants inoculated with *I. lacera* and *X. rubellus* was not different from that of control plants. Plants with *L. laccata* had higher shoot dry weight and plants with *S. verrucosum* had higher shoot length only than the control. Plants with *C. trivialis, H. leucosarx* and *H. psammophilum* showed the highest shoot lengths and those with *H.leucosarx* the highest shoot dry weights. Root length was significantly increased by all mycorrhizal fungi except *X. rub*ellus. Ectomycorrhizal colonization by *L. laccata* was highest and by *C. trivialis* and *X. rubellus* lowest. Shoot N concentration was highest by *C. trivialis, H. leucosarx*, and *H. psammophilum*, while *I. lacera, L. controversus*, and *S. verrucosum* showed very low shoot N concentrations. *Cortinarius pauperculus* showed relatively low shoot P concentration. Shoot P content in the presence of *C. trivialis* and both *Hebeloma* species was high. Shoot N/P ratio was around 10 in the presence of *Cortinarius* and *Hebeloma*, and around 5 in the presence of the other EcM species.

**TABLE 6-1b.** Shoot N concentration, shoot P concentration, shoot N content, shoot P content and shoot N/P ratio (means ± SE, $n$ = 10) of *Salix repens* inoculated with *Cortinarius pauperculus, C. trivialis, Hebeloma leucosarx, H. psammophilum, Inocybe lacera, Laccaria laccata, Lactarius controversus, Paxillus involutus, Scleroderma verrucosum* or not inoculated (control) grown for 12 weeks.

| Fungal treatment | N conc. (g/kg) | | P conc. (g/kg) | | N total (mg) | | P total (mg) | | N/P ratio | |
|---|---|---|---|---|---|---|---|---|---|---|
| Control (t = 0) | 10.14±0.63 | d* | 0.87±0.05 | ab | 0.87±0.16 | b | 0.08±0.02 | a | 11.5±0.6 | d |
| Control (t = 12) | 7.65±0.21 | c | 1.09±0.69 | b | 0.41±0.06 | a | 0.06±0.04 | a | 6.9±1.3 | b |
| *C. pauperculus* | 8.11±1.07 | cd | 0.79±0.16 | a | 0.83±0.26 | ab | 0.09±0.03 | ab | 10.2±0.8 | d |
| *C. trivialis* | 16.80±0.52 | f | 1.88±0.11 | d | 1.72±0.14 | c | 0.19±0.01 | c | 9.1±0.5 | cd |
| *H. leucosarx* | 15.29±0.87 | ef | 1.64±0.11 | cd | 1.99±0.19 | c | 0.21±0.02 | c | 9.3±0.5 | c |
| *H. psammophilum* | 13.14±1.05 | e | 1.66±0.17 | cd | 1.49±0.24 | c | 0.20±0.04 | c | 8.2±0.5 | c |
| *I. lacera* | 5.61±0.50 | a | 1.16±0.48 | bc | 0.31±0.04 | a | 0.07±0.02 | a | 4.8±0.5 | ab |
| *L. laccata* | 9.70±0.44 | d | 1.65±0.18 | cd | 0.70±0.05 | b | 0.12±0.02 | b | 6.4±0.6 | b |
| *L. controversus* | 6.05±0.42 | ab | 1.27±0.10 | c | 0.50±0.01 | a | 0.10±0.01 | ab | 4.8±0.3 | ab |
| *P. involutus* | 8.49±0.32 | cd | 1.56±0.18 | cd | 0.61±0.04 | b | 0.11±0.01 | b | 6.0±0.6 | b |
| *S. verrucosum* | 5.92±0.64 | a | 1.39±0.11 | c | 0.38±0.05 | a | 0.09±0.01 | ab | 4.2±0.2 | a |
| (*P* values) | | | | | | | | | | |
| 2 factor ANOVA | | | | | | | | | | |
| Fungus | <0.001 | | 0.001 | | <0.001 | | 0.005 | | <0.001 | |
| Harvest | 0.002 | | <0.001 | | 0.180 | | <0.001 | | <0.001 | |
| Fungus×harvest | <0.001 | | 0.001 | | <0.001 | | 0.005 | | <0.001 | |
| 1 factor ANOVA† | <0.001 | | <0.001 | | <0.001 | | 0.002 | | <0.001 | |

* Values within a column that are followed by a different letter differ at $P < 0.05$ according to a LSD test, and a two-factor ANOVA (including control and t = 0, df: fungus = 9, harvest = 1, df Error = 98, or

†a one factor ANOVA without control and t = 0, df: fungus = 8 and df Error = 88).

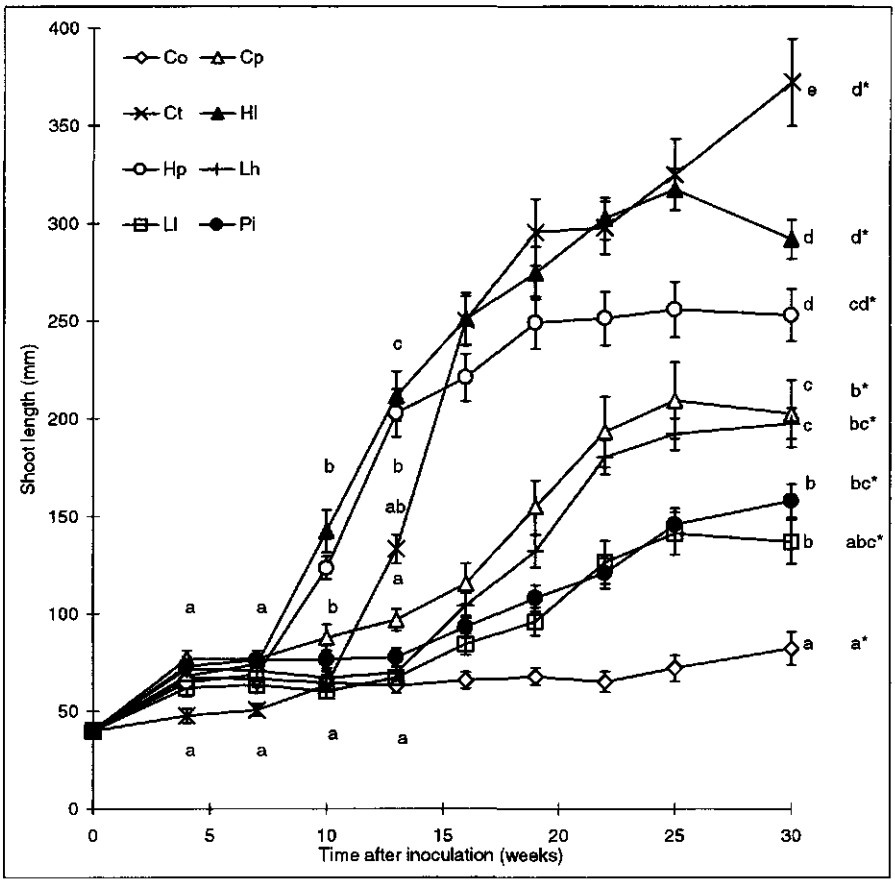



**FIGURE 6-1.** Mean shoot length of *Salix repens* inoculated with *Cortinarius pauperculus, C. trivialis, Hebeloma leucosarx, H. psammophilum, Laccaria laccata, Lactarius helvus, Paxillus involutus* or not inoculated (control) over 30 weeks. Bars represent (±) 1 SE. Different letters next to points at 4, 7, 10, 13 and 30 weeks after inoculation indicate different shoot length, and different letters and asterisks indicate different growth rates over 30 weeks between mycorrhizal treatments ($P < 0.05$) according to LSD tests.

**TABLE 6-2.** Results of ANOVA (*P* values) executed for shoot length, shoot biomass, root length, mycorrhizal colonization, mycorrhizal root length, shoot N and P concentration and content and shoot N/P ratio of *Salix repens* ($n \geq 10$).

| | Fungus | | Harvest | | Fungus × harvest | |
|---|---|---|---|---|---|---|
| Response variable | (df = 6) | without Co (df = 5) | (df =3) | without Co (df = 3) | (df = 18) | without Co (df = 15) |
| Shoot length | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Shoot dry weight | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Root length | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Mycorrhizal colonization | | <0.001 | | 0.026 | | <0.001 |
| Mycorrhizal root length | | <0.001 | | <0.001 | | 0.083 |
| Shoot N concentration | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Shoot N content | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Shoot P concentration | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Shoot P content | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Shoot N/P ratio | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

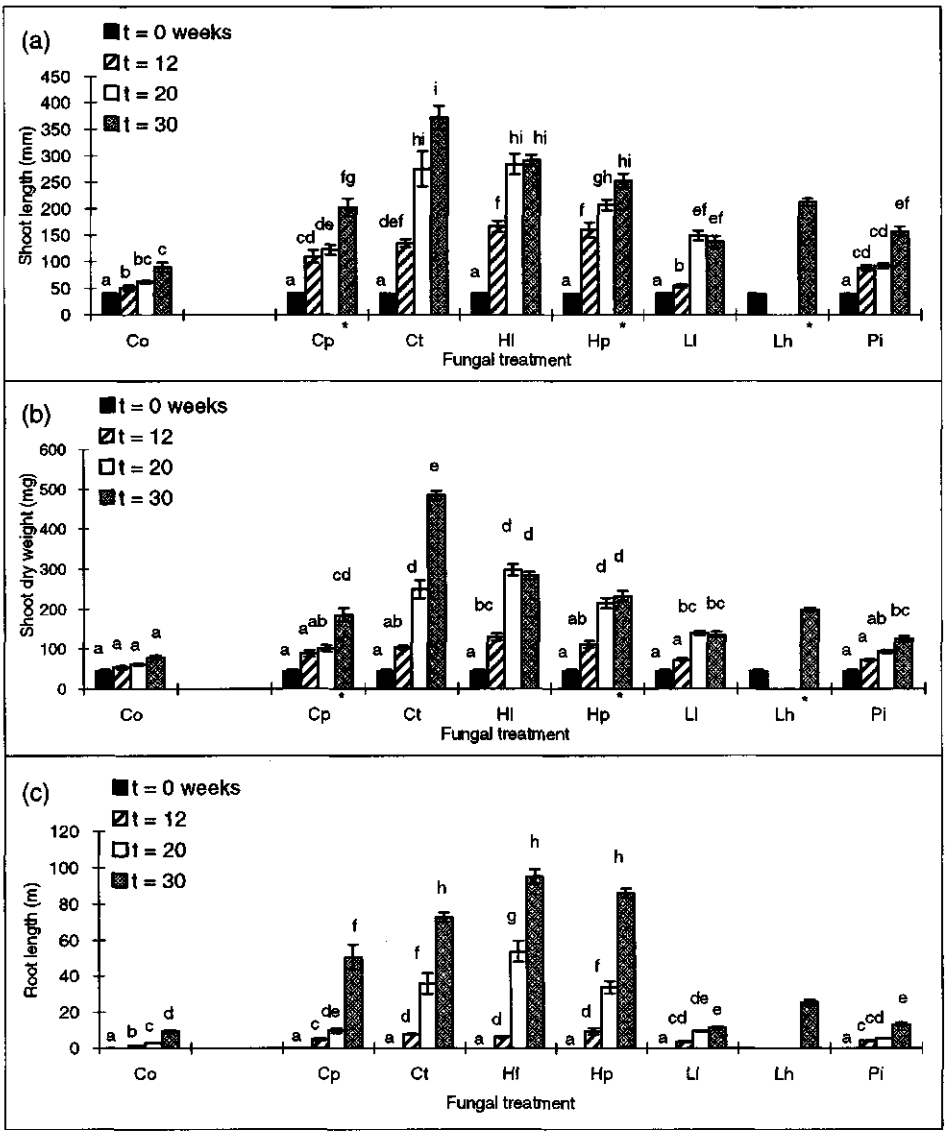
* df Error = 248, and without control df Error = 218.

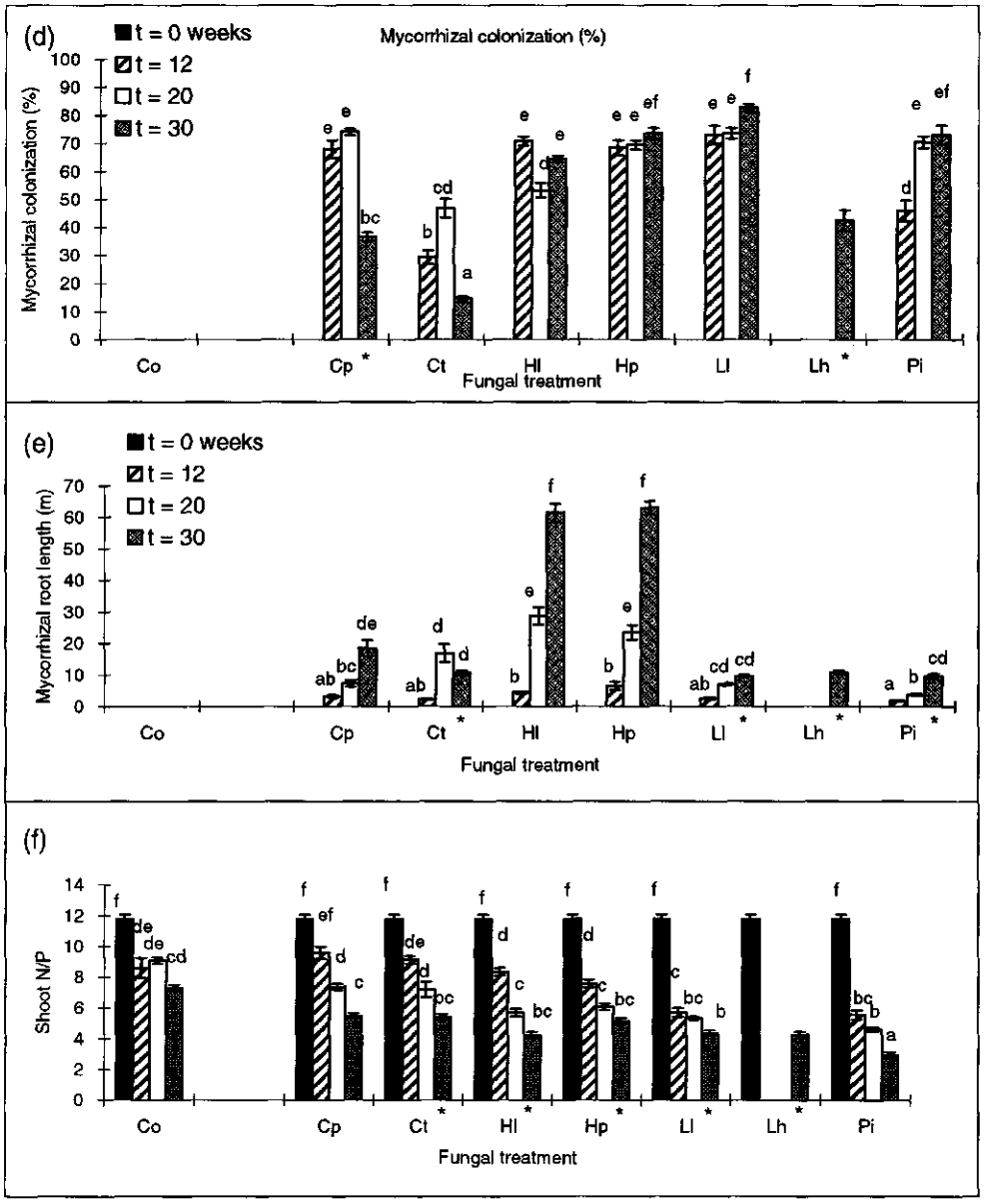
*Long term effects* (part 2, 12-30 weeks) - Plant growth response was significantly affected by the mycorrhizal treatments (FIG. 6-1, Repeated measures ANOVA: fungus, harvest, interaction between fungus × harvest, $P < 0.001$). Plant growth curves measured over 30 weeks showed that non-mycorrhizal plants had the lowest growth rate, whereas both *Hebeloma* species and *C. trivialis* showed the highest growth rates of *S. repens*. *Cortinarius trivialis* still increased shoot length of *S. repens* at the final harvest, while the other fungi did no longer increase shoot length.

In the long term (30 weeks) all plant parameters were significantly affected by the mycorrhizal treatment, harvest time, and the interaction between treatment and harvest time (TABLE 6-2; $P < 0.001$ in all cases). Mycorrhizal colonization was less strongly affected by time ($P = 0.026$), while mycorrhizal root length was not affected by the interaction between fungal treatment and time. The significant interaction with time for all parameters indicated differential effects of the various mycorrhizal fungi over the different periods. *Cortinarius pauperculus, C. trivialis, H. leucosarx*, and *H. psammophilum* strongly increased shoot length, shoot dry weight, and root length of *S. repens*, whereas *P. involutus, L. laccata*, and *L. helvus* had smaller effects on shoot dry weight and root length (FIGS. 6-2a,b,c). Non-mycorrhizal plants showed the lowest performance. After 30 weeks shoot length, shoot dry weight and root length did not show significant correlations. Root length and shoot N and P content (FIGS. 6-2i,j) after 30 weeks also did not show a significant correlation.

Correlations between mycorrhizal root length (FIG. 6-2e) and shoot nutrient concentrations were different in the various harvest periods. In the short term these correlations were positive, over 20 weeks no significant correlations were noted, whereas over 30 weeks nitrogen (but not phosphorus) was negatively correlated with mycorrhizal root length (TABLE 6-3). On the other hand, ectomycorrhizal colonization (%) (FIG. 6-2d) did not show significant correlations with shoot nutrient content over the first 12 weeks and after that period even showed significantly negative correlations.

**FIGURE 6-2.** Mean (a) shoot length; (b) shoot dry weight; (c) root length; (d) mycorrhizal colonization; (e) mycorrhizal root length; (f) shoot N/P ratio; (g) shoot N concentration; (h) shoot P concentration; (i) shoot N content and (j) shoot P content of *Salix repens* inoculated with *Cortinarius pauperculus, C. trivialis, Hebeloma leucosarx, H. psammophilum, Laccaria laccata, Lactarius helvus, Paxillus involutus* or not inoculated (control), harvested after 0, 12, 20 and 30 weeks. Bars represent (±) 1 SE. Significant differences are indicated by different letters, and asterisks indicate a not significant difference between fungal treatment at 30 weeks with *Lactarius helvus* ($P < 0.05$) according to LSD tests (see pages 124, 125 and 126).


(a)
t = 0 weeks
t = 12
t = 20
t = 30
Shoot length (mm)
Co
Cp
Ct
Hl
Hp
Ll
Lh
Pi
Fungal treatment
(b)
Shoot dry weight (mg)
(c)
Root length (m)


(d)
t = 0 weeks
t = 12
t = 20
t = 30
Mycorrhizal colonization (%)
Mycorrhizal colonization (%)
Co
Cp
Ct
Hl
Hp
Ll
Lh
Pi
Fungal treatment
(e)
Mycorrhizal root length (m)
(f)
Shoot N/P

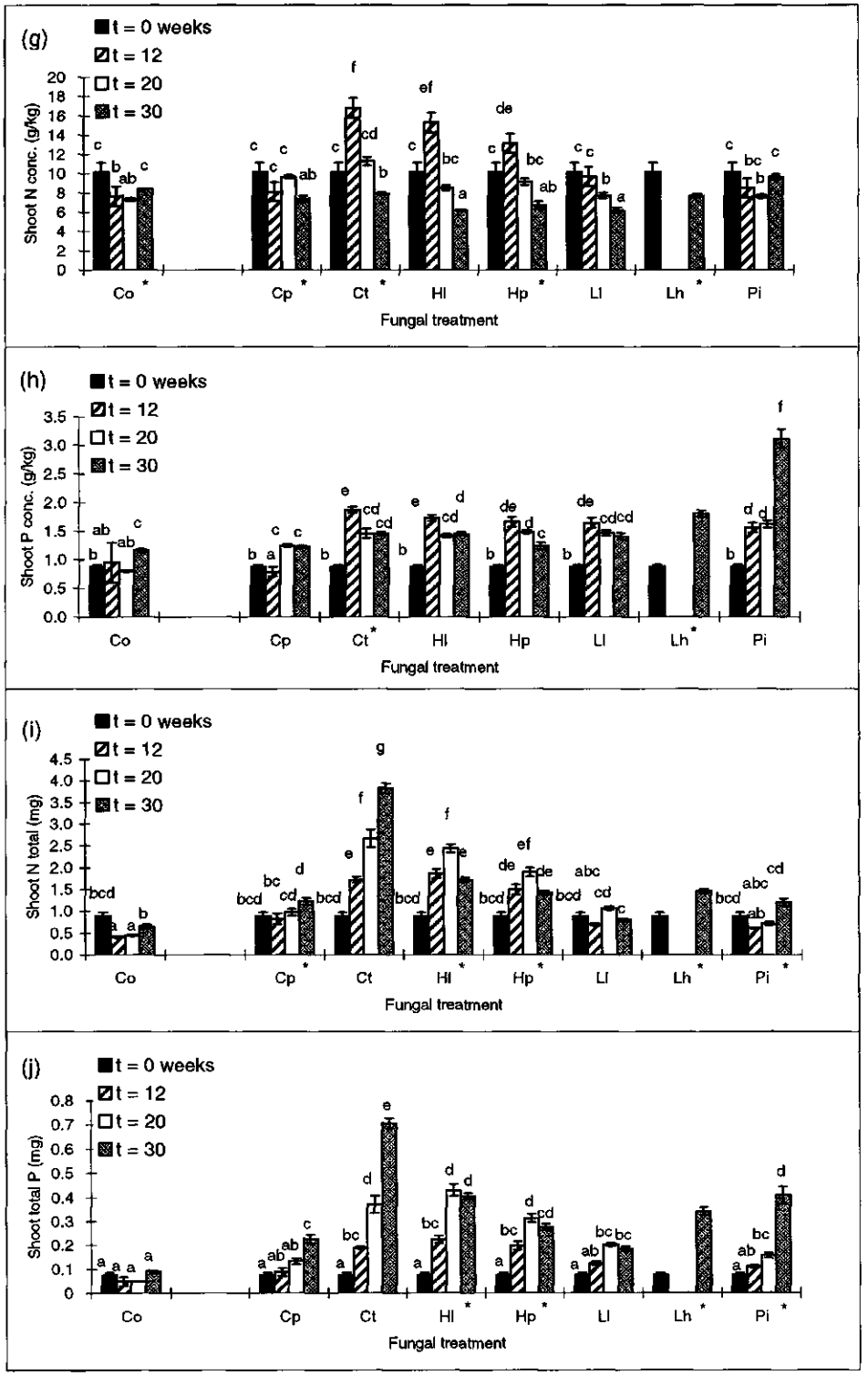

(g)
t = 0 weeks
t = 12
t = 20
t = 30
Shoot N conc. (g/kg)
Co
Cp
Ct
Hl
Hp
Ll
Lh
Pi
Fungal treatment
(h)
Shoot P conc. (g/kg)
(i)
Shoot N total (mg)
(j)
Shoot total P (mg)

**TABLE 6-3.** Correlations* between shoot length, shoot weight, root length, shoot N concentration, shoot P concentration, shoot N content or shoot P content and mycorrhizal colonization (%), mycorrizal root length or root length of *Salix repens* over three harvest periods (12, 20 and 30 weeks).

| | Mycorrhizal colonization (%) | | Mycorrhizal root length | | Root length | |
|---|---|---|---|---|---|---|
| | *R* | *P* | *R* | *P* | *R* | *P* |
| 0-12 weeks $n = 58$ | | | | | | |
| Shoot length | 0.00 | 0.990 | 0.65 | <0.001 | 0.74 | <0.001 |
| Shoot biomass | 0.09 | 0.497 | 0.70 | <0.001 | 0.77 | <0.001 |
| Root length | -0.15 | 0.274 | 0.79 | <0.001 | | |
| Myc. Root length | 0.39 | 0.002 | | | | |
| Shoot N conc. | -0.21 | 0.112 | 0.30 | 0.023 | 0.44 | <0.001 |
| Shoot P conc. | -0.05 | 0.721 | 0.38 | 0.003 | 0.42 | 0.001 |
| Shoot N content | -0.01 | 0.952 | 0.66 | <0.001 | 0.75 | <0.001 |
| Shoot P content | 0.03 | 0.798 | 0.75 | <0.001 | 0.83 | <0.001 |
| 0-20 weeks $n = 58$ | | | | | | |
| Shoot length | -0.25 | 0.058 | 0.89 | <0.001 | 0.91 | <0.001 |
| Shoot biomass | -0.30 | 0.019 | 0.87 | <0.001 | 0.91 | <0.001 |
| Root length | -0.28 | 0.029 | 0.97 | <0.001 | | |
| Myc. Root length | -0.11 | 0.401 | | | | |
| Shoot N conc. | -0.03 | 0.808 | -0.09 | 0.474 | -0.09 | 0.478 |
| Shoot P conc. | -0.11 | 0.405 | 0.14 | 0.296 | 0.13 | 0.327 |
| Shoot N content | -0.34 | 0.009 | 0.80 | <0.001 | 0.84 | <0.001 |
| Shoot P content | -0.36 | 0.005 | 0.85 | <0.001 | 0.89 | <0.001 |
| 0-30 weeks $n = 62$ | | | | | | |
| Shoot length | -0.35 | 0.006 | 0.55 | <0.001 | 0.71 | <0.001 |
| Shoot biomass | -0.40 | 0.001 | 0.47 | <0.001 | 0.72 | <0.001 |
| Root length | -0.33 | 0.009 | 0.84 | <0.001 | | |
| Myc. Root length | 0.13 | 0.297 | | | | |
| Shoot N conc. | -0.17 | 0.176 | -0.41 | 0.001 | -0.31 | 0.014 |
| Shoot P conc. | 0.09 | 0.496 | -0.16 | 0.202 | -0.21 | 0.091 |
| Shoot N content | -0.418 | <0.001 | 0.40 | 0.001 | 0.64 | <0.001 |
| Shoot P content | -0.27 | 0.034 | 0.31 | 0.013 | 0.50 | <0.001 |

* EcM fungi; *Cortinarius pauperculus, C. trivialis, Hebeloma leucosarx, Hebeloma psammophilum, Laccaria laccata* and *Paxillus involutus.* Significant correlations according to Spearman's rank tests.

*Shoot nutrient uptake, shoot nutrient inflow (effectiveness, efficiency) versus nutrients in the soil available pool in the presence of different EcM species*

Shoot nutrient uptake and shoot nutrient inflow clearly differed between the different EcM treatments (TABLE 6-4). Shoot uptake rates (mycorrhizal effectiveness) were generally higher for plants inoculated with ectomycorrhizal fungi that had the strongest positive effect on root length, whereas shoot inflow rates (mycorrhizal root efficiency) were usually higher for plants with short roots. Shoot uptake and shoot inflow of nitrogen and phosphorus were uncorrelated. Over the short term plants showed either positive or negative shoot N uptake, depending on fungal species, whereas shoot P uptake was positive for all species. Over the longer term the behaviour of nitrogen and phosphorus became more similar.

**TABLE 6-4.** Shoot phosporus and nitrogen uptake and inflow per unit root length of *Salix repens*, over 30 weeks and three different time intervals (0-12, 12-20 and 20-30 weeks), inoculated with *Cortinarius pauperculus, C. trivialis, Hebeloma leucosarx, H. psammophilum, Laccaria laccata, Lactarius helvus* (0-30 weeks), *Paxillus involutus* or not inoculated (control) (means, $n = 10$).

| | Shoot nutrient uptake (µg/day) | | Shoot nutrient inflow (µg.day$^{-1}$.m$^{-1}$ root length) | |
|---|---|---|---|---|
| Fungal treatment | δ N | δ P | N | P |
| 0 - 30 weeks | | | | |
| Control | -1.11 | 0.07 | -0.68 | 0.041 |
| *Cortinarius pauperculus* | 1.64 | 0.71 | 0.25 | 0.105 |
| *Cortinarius trivialis* | 13.99 | 3.01 | 1.50 | 0.322 |
| *Hebeloma leucosarx* | 3.99 | 1.57 | 0.34 | 0.133 |
| *Hebeloma psammophilum* | 2.57 | 0.96 | 0.24 | 0.089 |
| *Laccaria laccata* | -0.35 | 0.52 | -0.17 | 0.270 |
| *Lactarius helvus* | 2.75 | 1.27 | 0.74 | 0.339 |
| *Paxillus involutus* | 1.54 | 1.59 | 0.71 | 0.737 |
| 0 – 12 weeks | | | | |
| Control | -5.56 | 0.13 | -16.05 | 0.751 |
| *Cortinarius pauperculus* | -0.62 | 0.14 | -0.64 | 0.153 |
| *Cortinarius trivialis* | 9.98 | 1.35 | 7.10 | 0.956 |
| *Hebeloma leucosarx* | 11.64 | 1.76 | 10.11 | 1.526 |
| *Hebeloma psammophilum* | 7.23 | 1.45 | 4.44 | 0.887 |
| *Laccaria laccata* | -2.18 | 0.57 | -2.91 | 0.775 |
| *Paxillus involutus* | -3.22 | 0.41 | -4.06 | 0.526 |
| 12 – 20 weeks | | | | |
| Control | 0.53 | -0.86 | 0.26 | -0.421 |
| *Cortinarius pauperculus* | 2.49 | 0.79 | 0.36 | 0.112 |
| *Cortinarius trivialis* | 16.88 | 3.26 | 0.91 | 0.177 |
| *Hebeloma leucosarx* | 10.28 | 3.68 | 0.46 | 0.167 |
| *Hebeloma psammophilum* | 7.22 | 2.05 | 0.37 | 0.107 |
| *Laccaria laccata* | 6.60 | 1.37 | 1.09 | 0.227 |
| *Paxillus involutus* | 1.87 | 0.80 | 0.43 | 0.175 |
| 20 – 30 weeks | | | | |
| Control | 2.91 | 0.57 | 0.55 | 0.103 |
| *Cortinarius pauperculus* | 3.69 | 1.34 | 0.15 | 0.054 |
| *Cortinarius trivialis* | 16.50 | 4.79 | 0.32 | 0.092 |
| *Hebeloma leucosarx* | -10.22 | -0.34 | -0.14 | -0.005 |
| *Hebeloma psammophilum* | -6.74 | -0.50 | -0.12 | -0.009 |
| *Laccaria laccata* | -3.75 | -0.23 | -0.36 | -0.022 |
| *Paxillus involutus* | 6.98 | 3.63 | 0.79 | 0.417 |

Measured over 30 weeks control plants and cuttings with *L. laccata* had a negative shoot N-uptake or inflow (N-balance). Cuttings with *C. trivialis* had the largest positive shoot N- and P-balance, but *H. leucosarx* and *P. involutus* also had a large positive shoot P-balance. All plants had a positive shoot P-balance. The different fungi showed different shoot nutrient balances over shorter time periods. *Salix repens* inoculated with *C. pauperculus, L. laccata,* and *P. involutus* showed a negative shoot N-balance in the first 12 weeks, whereas *C.*

*trivialis, H. leucosarx*, and *H. psammophilum* showed a positive shoot N-balance over the first 20 weeks. In the final harvest period (between week 20 and 30) cuttings with *C. trivialis* still showed a positive shoot N- and P-balance, whereas cuttings with both *Hebeloma* species showed negative shoot uptake of N and P. *Laccaria laccata* only had a positive shoot N-balance in the second period (between weeks 12 and 20) and then even had the highest efficiency.

**TABLE 6-5.** Available $P_s$ and $N_{ts}$ in soils in the pots of *Salix repens* inoculated with *Cortinarius pauperculus, C. trivialis, Hebeloma leucosarx, H. psammophilum, Laccaria laccata, Paxillus involutus* or not inoculated (control) after harvesting at 0, 12, 20 and 30 weeks (means ± SE, $n \geq 10$).

| | Available nutrients (mg/kg) | |
|---|---|---|
| Fungal treatment | $P_s$ | $N_{ts}$ |
| t = 0 | 0.31 ± 0.01 e* | 8.20 ± 0.03 d |
| t = 12 | | |
| Control | 0.27 ± 0.10 d | 9.14 ± 1.12 e |
| *Cortinarius pauperculus* | 0.27 ± 0.25 d | 9.80 ± 0.77 e |
| *Cortinarius trivialis* | 0.26 ± 0.07 d | 9.41 ± 1.36 e |
| *Hebeloma leucosarx* | 0.26 ± 0.17 d | 9.94 ± 2.46 e |
| *Hebeloma psammophilum* | 0.25 ± 0.12 d | 9.52 ± 1.02 e |
| *Laccaria laccata* | 0.27 ± 0.12 d | 9.19 ± 0.66 e |
| *Paxillus involutus* | 0.26 ± 0.15 d | 9.02 ± 0.59 e |
| t = 20 | | |
| Control | 0.07 ± 0.02 b | 4.51 ± 0.22 a |
| *Cortinarius pauperculus* | 0.07 ± 0.02 b | 5.41 ± 0.87 b |
| *Cortinarius trivialis* | 0.07 ± 0.02 b | 8.55 ± 1.37 bcd |
| *Hebeloma leucosarx* | 0.05 ± 0.02 b | 3.88 ± 0.27 a |
| *Hebeloma psammophilum* | 0.06 ± 0.02 b | 4.52 ± 0.17 a |
| *Laccaria laccata* | 0.08 ± 0.01 b | 5.28 ± 0.32 b |
| *Paxillus involutus* | 0.06 ± 0.02 b | 4.98 ± 0.23 b |
| t = 30 | | |
| Control | 0.09 ± 0.01 b | 4.73 ± 0.24 ab |
| *Cortinarius pauperculus* | 0.09 ± 0.01 b | 6.08 ± 0.27 c |
| *Cortinarius trivialis* | 0.09 ± 0.01 b | 8.94 ± 0.56 d |
| *Hebeloma leucosarx* | 0.14 ± 0.04 c | 5.31 ± 0.28 b |
| *Hebeloma psammophilum* | 0.10 ± 0.03 bc | 4.08 ± 0.53 a |
| *Laccaria laccata* | 0.07 ± 0.02 b | 4.16 ± 0.83 ab |
| *Paxillus involutus* | 0.10 ± 0.02 bc | 5.06 ± 0.46 b |
| Kruskal-Wallis test | $P < 0.0001$ | $P < 0.0001$ |

* Significant differences between fungal treatments or time are indicated by different letters (Kruskal Wallis and Mann-Whitney-U; $P < 0.05$).

Soil analyses showed that at the end of the experiment available N and P in the soil differed between fungal treatments (TABLE 6-5). Differences in available phosphorus, even though small, significantly decreased over time. After 12 weeks $N_{ts}$ was higher in all treatments than at the start of the experiment. At the final harvest period in pots with cuttings with *H. leucosarx* available phosphorus increased in soil compared to the second harvest time. Available nitrogen ($N_{ts}$) also decreased over time, but in the treatment with *C. trivialis* (and to a lesser extent with *C. pauperculus*) $N_{ts}$ only slightly decreased over the different harvest periods, while shoot N uptake was largest.

*Experiment 2: Indirect ectomycorrhizal benefits for Salix repens*

Despite its low mycorrhizal colonization, *C. trivialis* strongly increased root length of *S. repens* (FIG. 6-2c). Different amounts of inoculum of *C. trivialis* showed no differences in plant performance over 18 weeks (TABLE 6-6), although higher amount of inoculum tended to show positive growth response earlier (Average leaf surface at t = 9 weeks was significantly higher when inoculated with 16 *C. trivialis* mycelial plugs as opposed to 0, 4 or 8 mycelial plugs; data not shown). The water soluble extract of *C. trivialis*, however, showed a significantly longer root system than both control treatments (TABLE 6-6), but not different from the root systems of *S. repens* inoculated with *C. trivialis* mycelia.

## DISCUSSION

*Response time, response duration, and magnitude of plant response in relation to extent of colonization*

*Xerocomus rubellus* did not show positive effects as all cuttings died after mycorrhizas were formed. Few sporocarps of this species have been observed in late successional sites on dry, acid soils but mycorrhizas of the species have not yet been identified. Species composition of ectomycorrhizal fungi changes with ageing of trees or forest soils (Termorshuizen, 1991; Deacon & Fleming, 1992; Baar, 1995). Deacon *et al.* (1983) recognized two different strategies within ectomycorrhizal fungi that were termed early stage and late stage. Late stage fungi are generally K-selected and their carbon demands are much higher than for early stage fungi (Gibson & Deacon, 1990). As *X. rubellus* occurred in later successional stages only, it is possible that its carbon costs were too high for the cuttings used in the experiment.

**TABLE 6-6.** Shoot length, shoot dry weight, root length, mycorrhizal colonization, shoot N concentration, shoot P concentration, shoot N content, shoot P content and shoot N/P ratio (means) of *Salix repens* either inoculated with *Cortinarius trivialis* (4, 8 or 16 mycelial plugs), or supplied with water soluble extracts of *Cortinarius trivialis* cultures or AMN agar plates without fungal tissue, or not inoculated (control) grown for 18 weeks.

| Treatment | n | Shoot length (mm) | Shoot biomass (mg) | Root length (m) | †Myc. (%) | N conc. (g/kg) | P conc. (g/kg) | N total (mg) | P total (mg) | N/P ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| *C. trivialis* | | | | | | | | | | |
| 4 plugs | 10 | 169.4 b* | 160.9 b | 15.47 b | 47.7 b | 6.88 a | 1.15 a | 1.09 c | 0.18 bc | 6.00 b |
| 8 plugs | 10 | 184.5 b | 144.0 b | 14.92 b | 42.8 b | 7.25 a | 1.23 ab | 1.02 bc | 0.17 b | 5.93 b |
| 16 plugs | 10 | 186.3 b | 163.9 b | 16.17 b | 15.7 a | 7.62 a | 1.31 ab | 1.19 c | 0.21 bcd | 5.77 b |
| Extracts | | | | | | | | | | |
| AMN medium | 8 | 169.0 b | 132.8 b | 10.94 a | 0 | 6.35 a | 1.65 bc | 0.77 b | 0.20 c | 3.85 a |
| *C. trivialis* | 9 | 151.4 b | 138.3 b | 16.62 b | 0 | 6.75 a | 1.80 c | 0.92 bc | 0.24 d | 3.88 a |
| Control | 6 | 78.1 a | 71.6 a | 11.00 a | 0 | 6.95 a | 1.45 b | 0.45 a | 0.10 a | 4.52 a |
| ANOVA (*P* value) | | <0.001 | <0.001 | 0.002 | 0.001 | ns | <0.001 | 0.002 | 0.002 | <0.001 |

* Values within a column that are followed by a different letter, differ at $P < 0.05$ according to a LSD test, and a one factor ANOVA (df: treatment = 5, df Error = 47 or † df: treatment = 2, df Error = 27) detected differences amongst treatments.

Experiments with ectomycorrhizal systems often involved only one harvest (Termorshuizen & Ket, 1991; Baar & Oude Elferink, 1996). The duration of the experiment might then be crucial. Harvesting plants over various periods of time (12, 20, 30 weeks) indicated that the ectomycorrhizal fungal species × harvest time interaction was significant for all parameters studied. These significant interactions were due to the fact that both response time and response duration were variable. Both parameters acted independently. *Cortinarius trivialis* responded rapidly and for a long time and *P. involutus* responded more slowly but positive effects extended for a long time. *Hebeloma leucosarx* and *H. psammophilum* showed shoot N and P uptake in the first 20 weeks but in the last 10 weeks there was a loss of both nutrients. *Laccaria laccata* finally was only positive between 12 and 20 weeks showing negative shoot uptake values both in the first and last time period.

Negative shoot uptake could be due to the fact that cuttings have a very high shoot to root ratio and after outplanting will initially readjust their functional equilibrium. Habte & Byappanahalli (1994), using cuttings of cassava (*Manihot esculenta*) also noted a negative shoot P-balance of leaves after outplanting. Decline of P leaf status continued for about 40 days, irrespective of the P-status of the soil. Differences between *C. trivialis* and both *Hebeloma* species on the one hand (no initial negative shoot N-balance) and *L. laccata* and *P. involutus* on the other (initial negative shoot N balance) might reflect differences in strategies of ectomycorrhizal fungi [see below].

Differential responses in time suggest that species-specific mycorrhizal benefits might be temporary and only occur in certain stages of the life cycle (establishment as a seedling, flowering or fruiting). Temporary benefits of arbuscular mycorrhizas have also been shown for *S. repens* (CHAPTERS 3, 4 AND 5) and these were related to the high shoot P-demand at the start of the growing season as the plant flowers before its leaves are fully expanded. Similar temporary benefits of arbuscular mycorrhizal fungi have been observed for *Hyacinthoides non-scripta* (Merryweather & Fitter, 1995), *Ranunculus adoneus* (Mullen & Schmidt, 1994) and *Fragaria* x *ananassa* (Dunne & Fitter, 1989). In ecosystems with prominent nutrient pulses temporary benefits of the ectomycorrhizal symbiosis are likely, related to the storage function of the fungal sheath (Newbery *et al.*, 1997, 1998; Lussenhop & Fogel, 1999). Plant response, either assessed as shoot dry weight (carbon gain) or nutrient content (nitrogen or phosphorus gain) was only weakly correlated to extent of colonization. Whereas both *Hebeloma* species showed a high colonization and a high plant response (at least during the first 20 weeks), *C. trivialis* had the lowest colonization but the highest plant

response in terms of carbon and nutrient gain. There is therefore no reason to simplistically assume that low colonization implies low response or that high response implies high colonization. This is in accordance with a similar conclusion on the arbuscular mycorrhizal fungus *Glomus mosseae,* which showed a very low colonization (around 5 %) but a very rapid plant response (CHAPTERS 4, 5 AND 10). This lack of correlation could have several causes: (1) ectomycorrhizas might already be physiologically active before a well-developed sheath is visible. In the present study plant responses occurred about one week before clearly developed ectomycorrhizas could be observed. Similar observations were made by Frankland & Harisson (1985) who equally noted plant response before birch ectomycorrhizas had well-developed and visible mantle structures. The effectiveness of ectomycorrhizal fungi for nutrient supply at early stages of colonization could indicate that the significance of the mantle concerns pathogen resistance or nutrient storage; (2) mycorrhizal colonization depends on relative rates of growth of root and fungus. If roots outgrow the fungus, colonization might decrease at least temporarily as shown for *C. pauperculus, C. trivialis,* and *H. leucosarx.* However, this lower colonization will not necessarily translate into lower performance; and (3) there is unclarity whether % colonization, number of root tips, mycorrhizal root length, total root length or hyphal length should theoretically correlate best with nutrient uptake. For willow cuttings and eucalypt seedlings Jones *et al.* (1990, 1998) claimed that the length of the extramatrical mycelium was the best predictor of plant response. They also noted that proportional colonization was not correlated with plant response. External hyphal length could for certain ectomycorrhizal species be a good predictor of plant response. In the present study, however, the length of the extramatrical mycelium was not determined because differences in mycelial structures (Agerer, 1987-1998: hyphae, rhizomorphs) might make such a comparison between different species of limited applicability. Furthermore, variation in inflow rates per unit of mycorrhizal hyphal length of different species is unknown (Jakobsen *et al.*, 1992a). On the basis of data in Jones *et al.* (1998) it can be calculated that correlation of root length with plant performance will also yield the same very high correlations as that between hyphal length and plant performance. Apparently, root length and hyphal length could themselves be significantly correlated. As the various ectomycorrhizal fungi affected root length to various degrees [see below] the importance of root length, mycorrhizal root length, and hyphal length might vary with species. In the present study it is hypothesized that ectomycorrhizal fungi that restrict root elongation will have larger additional effects of hyphal length than ectomycorrhizal fungi that increase root length.

When three different arbuscular mycorrhizal fungi were compared, length of the extramatrical mycelium turned out to be an unreliable indicator of plant performance (Jakobsen *et al.* 1992b). One further reason why hyphal length might not be the most suitable parameter is that hyphal length might be optimal from the perspective of fungal fitness which not necessarily coincides with the optimum for plant fitness.

Differences between the behaviour of nutrient content and nutrient concentration (FIGS. 6-2g,h,i,j) suggest that shoot nutrient uptake and carbon gain go through different phases. Such phases have both been described for arbuscular mycorrhizal fungi, where arbuscules are produced during periods of growth in young roots and the number of vesicles increase in later stages (Douds & Chaney, 1982) and ectomycorrhizal fungi (Downes *et al.*, 1992; Lussenhop & Fogel, 1999). Jones *et al.* (1991) noted for willow plants a maximum P benefit at an early stage of infection whereas the carbon drain came at a later time. Such pulses of nutrient uptake, growth and below-ground carbon expenditure can also explain the observations that plants cannot regulate their internal P status over the short term (Clarkson & Hanson, 1980).

*Non-nutritional effects of mycorrhizal fungi*

Non-nutritional benefits of the mycorrhizal symbiosis have always provided a contested issue. Under the standard model of mycorrhizal functioning (Tinker *et al.*, 1992) many supposedly non-nutritional benefits are better interpreted as being consequent upon the improved phosphorus status of the mycorrhizal plant.

The second experiment with *C. trivialis* indicated that non-nutritional benefits could occur in the absence of formation of ectomycorrhizas, as the aqueous extract of a mycelial culture stimulated root length of *S. repens* similar to the effect of the fungal inoculum. Considering the similarities in root elongation in the treatments with both *Cortinarius* and *Hebeloma* species (members of the Cortinariaceae) this non-nutritional effect might occur more commonly. Such effects might be achieved by the production of auxines such as IAA which are produced by species of *Cortinarius* (Moser, 1959) and *Hebeloma* (Gay *et al.*, 1994). Stimulation of plant growth by isolates of *Suillus* species in the absence of formation of ectomycorrhizas has also been reported and attributed to the production of auxines by Sen (1990). Changes in root architecture might also be considered non-nutritional effects of the mycorrhizal symbiosis.

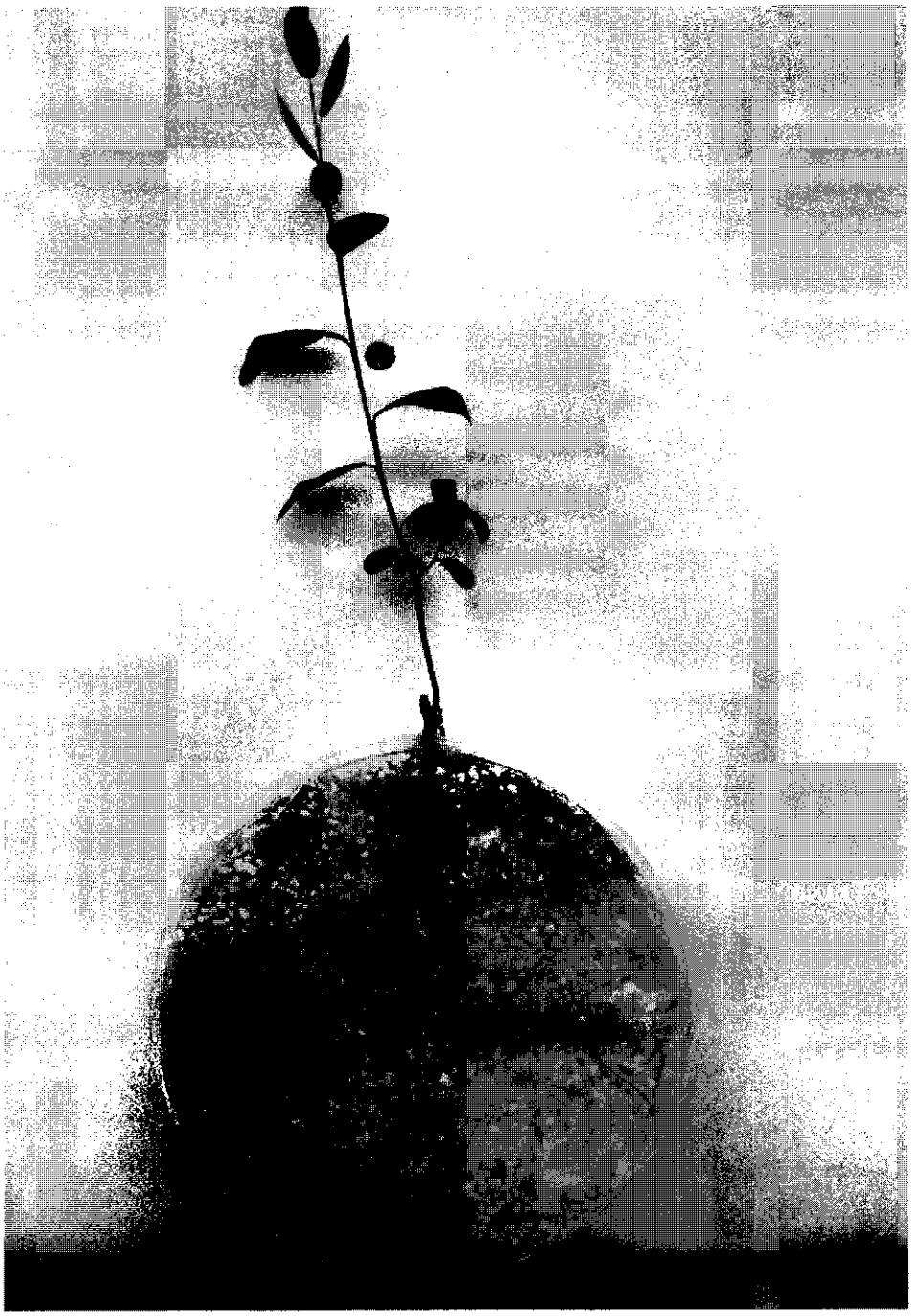


**FIGURE 6-3.** *Salix repens* cutting in root growth chamber, 10 weeks after inoculation: Mycorrhizas of *Paxillus involutus* (left) and *Hebeloma leucosarx* (right) on the root system.

Two different mycorrhizal strategies with regard to root architecture can be recognized in the present study. First, the external mycelium of the ectomycorrhizal fungus exploits the soil beyond the root depletion zone around roots (Tinker, 1975). Second, the ectomycorrhizal fungus can alter root length of *S. repens* and thereby both mycorrhizal root system and external mycelium of the fungus exploit the soil. These changes in root system architecture could have major impacts on long-term root functioning and dynamics.

Mycorrhiza-induced changes in root length (and hence in root foraging ability) are apparently localized. This can be concluded from observations on root systems of cuttings that were inoculated with 5 plugs of *H. leucosarx* and 5 plugs of *P. involutus* at different sides of the root system. Harvest after 10 weeks showed long slender roots where *H. leucosarx* was inoculated and shorter and much branched roots where *P. involutus* was inoculated (FIG. 6-3) (coinciding with larger total root length of *H. leucosarx*)

*Effect of ectomycorrhizal fungi on soil*

Even though this study (expt 1) was performed in a sandy soil that was very low in organic matter (0.17 %) and nutrients, different ectomycorrhizal fungi had access to different amounts of nutrients from the soil. This conclusion is based on the large differences in shoot P content between treatments with different ectomycorrhizal species and similar amounts of soil solution P at various harvest dates. Shoot N content also showed large differences between fungal species but total amount of soluble N (ammonium, nitrate, dissolved organic nitrogen) in the soil showed smaller differences. However, amount of N taken up and amount of soluble N remaining were not correlated as *C. trivialis* both had highest shoot N content and N remaining in the soil solution. *Paxillus involutus*, known for its ability to break down organic material (Laiho, 1970) had access to a large pool of N and P from this mineral soil but this effect was only shown in the longer term.

Mycorrhizal fungi can alter soil chemistry in the mycorrhizosphere and hyphosphere so that more exchangeable N and P come into solution. Over a period of 30 weeks ectomycorrhizal fungi were able to translocate more P to the shoot of *S. repens* compared to the arbuscular mycorrhizal fungus *G. mosseae* (CHAPTER 5) but in the ectomycorrhizal treatment soil solution P remained detectable whereas it decreased to zero within 12 weeks with *G. mosseae*.

Unfortunately, soils at the end of the experiment were not analysed for total amount of N and P. Therefore no complete nutrient balance for this soil-plant system can be provided and further research is needed to obtain a complete balance. Part of the nutrients will be

found in the root material. However, root dry weight and nutrient content were not measured. Preliminary dry weight measurements, followed by loss on ignition, indicated that mycorrhizal root systems with *P. involutus* contained four times the amount of sand (in the mycorrhizal sheath) and *H. leucosarx* seven times the amount of sand as the non-mycorrhizal control. This sand could not be removed by thorough washing. Root dry weight and nutrient analyses will therefore require an enormous amount of controls before reliable data can be obtained.

*Strategies of ectomycorrhizal fungi*

In the present study, two kinds of ectomycorrhiza formation could be observed, viz. loose, discontinuous mantles by species of *Cortinarius* and *Hebeloma*, and tight, thick mantles by *L. laccata, L.helvus* and *P. involutus*. These differences coincided with differences in plant root length. In the former group long and slender root systems were formed, whereas in the latter group roots were much shorter and thicker. This effect was already weakly shown after 12 weeks when *H. psammophilum* and *C. trivialis* had already highest root length. In the short term (12 weeks) plants inoculated with these fungi also had highest shoot length, shoot dry weight and higher shoot nitrogen and phosphorus content and concentration. The initial higher shoot nutrient uptake could, however, not always be sustained over the duration of the experiment and in the final part of the experiment (between 20 and 30 weeks) both *H. leucosarx* and *H. psammophilum* showed negative shoot uptake (shoot inflow rates). Species that showed initial negative shoot uptake (shoot inflow rates) (*L. laccata, P. involutus*) had positive shoot uptake (inflows) during the progression of the experiment.

These data suggest the existence of two fungal strategies which might be called root manipulation strategy and the root replacement strategy. The first strategy results in rapid above- and below-ground biomass increment and root growth might in these cases be more rapid than fungal growth. As a consequence proportional colonization might decrease or remain low as was observed for *C. trivialis*. If the root outgrows the fungus, competition for nutrients between plant and fungus could shift in the direction of the plant. Much higher initial shoot nutrient uptake would be consistent with such a mechanism. The root replacement strategy conforms more to the standard view of mycorrhizal funtioning, where the nutrient uptake depends on the fungus extending depletion zones of relatively immobile elements such as phosphorus. In such cases of decreased root growth rate, proportional colonization could consequently be higher. As the fungus now is better competitor for

nutrients than the plants, initial N-uptake would be lower. Nutrient uptake and inflow patterns indicate that mycorrhizal strategies affect the behaviour of nitrogen and phosphorus differentially. The ratio of Nitrogen / Phosphorus translocated to the shoot was much higher in ectomycorrhizas that exhibited the root manipulation strategy (TABLE 6-4, over 30 weeks N/P uptake > 2.2 for root manipulators, and < 1.0 for root replacers).

How do these strategies compare to other classifications of strategies of ectomycorrhizal fungi? Baar *et al.* (1997) compared nitrophobic and nitrotolerant strategies. Their nitrotolerant species were *P. involutus* and *Laccaria bicolor*, a close relative of *L. laccata*. These fungi had an intrinsically high nitrogen demand, which is consistent with observations in the present study of an initial negative shoot N-balance for cuttings inoculated with *P. involutus* and *L. laccata*. As mineral nitrogen generally represses phytohormone production by ectomycorrhizal fungi (Nylund, 1988), experiments under relatively nutrient-rich conditions might well have selected for fungi with the root replacement strategy. It is unclear how organic nitrogen affects phytohormone production by ectomycorrhizal fungi, and it is therefore not yet possible to relate strategies obtained from the present study to the classes recognized by Abuzinadah & Read (1986) as protein fungi and non-protein fungi, based on the differential ability to use simple organic compounds as nitrogen sources.

One interesting implication of these different mycorrhizal strategies should finally be mentioned. Increased nutrient inflow rates of mycorrhizal plants compared to non-mycorrhizal plants has been attributed to the root replacement strategy. Inflow rates, which are considered as an indicator for the efficiency of the mycorrhizal system, are based on changes in root length during a given period as in the formula of Brewster & Tinker (1972). However, when mycorrhizal fungi have a large impact on root length, inflow rates (efficiency) will not necessarily correlate with uptake (effectiveness). This is evident from TABLE 6-4 where shoot nitrogen uptake of plants inoculated with *P. involutus* was only 40-60 % of that of plants inoculated with both *Hebeloma* species, but shoot inflow rates were 2-3 times higher.

7

# Conditionality of mycorrhizal benefits for *Salix repens*: role of pH and relative availabilities of nitrogen and phosphorus.

**ABSTRACT**

*Responses of one arbuscular mycorrhizal fungus* (Glomus mosseae) *and two ectomycorrhizal fungi* (Hebeloma leucosarx, Paxillus involutus) *to a range of soil conditions were investigated experimentally. Non-mycorrhizal controls were also included. Soil conditions included three levels of relative availabilities of nitrogen and phosphorus, ranging from N limitation to P limitation (N/P ratio 5.4, 16.2, 48.6), and three pH's, ranging from acidic to alkaline (pH 4, 5.5, 7), in a full factorial experiment. Plant parameters (carbon gain, N and P-content, root length) were significantly affected by fungus, soil pH and soil N/P, and their interactions. Mycorrhizal benefits were larger after 26 weeks than after 12 weeks. Mycorrhizal benefits by EcMF were generally larger than by the AMF.* Hebeloma leucosarx *and* P. involutus *were equally effective, despite differences in proportional colonization. Proportional colonization of* H. leucosarx *was always close to 100 % and of* P. involutus *ranged from 45 to 90 %. Absolute root length colonized by* P. involutus *indicated optimal conditions at pH 5.5 and N/P 5.4 or 16.2, whereas* H. leucosarx *was negatively affected at N/P 48.6 only.* Hebeloma leucosarx *was able to expand niche width of* S. repens *towards alkaline conditions. Results are discussed in the framework of a dune successional gradient from young, calcareous, humus-poor towards old, acidic, humus-rich soils.*

**KEY WORDS**: Ectomycorrhiza, arbuscular mycorrhiza, conditionality, nitrogen, phosphorus, pH, *Salix repens*.

## INTRODUCTION

Mutualisms are ubiquitous and extremely diverse in nature (Boucher *et al.*, 1982). It is increasingly evident that the costs and benefits that determine net effects of mutualistic associations vary greatly both in space and time. Consequently, the type of a particular interrelationship is likely to change when the interactors change (due to evolutionary responses), or when the environmental factors constraining the mutualists change. This leads to an ecological or evolutionary continuum within which mutualism is just one possible interrelationship (Bronstein, 1994). When the outcome of interactions between species changes along environmental gradients, such mutualistic relationships show conditionality. Both abiotic and biotic settings in which the interaction takes place affect conditionality (Bronstein, 1994).

One of the most important mutualistic relationships is the one between plant roots and fungi - mycorrhiza - which has repeatedly been reported to improve growth and survival of the plant, e.g., by enhancing nutrient uptake and providing protection against pathogens (Smith & Read, 1997). The costs caused by the fungi may be high, with estimates ranging between 7 and 60 % of the photosynthate production supporting the mycorrhizal fungus (Stribley *et al.*, 1980; Finlay & Söderström, 1992; Rygiewicz & Andersen, 1994).

Mycorrhiza is especially important in nutrient-poor habitats, where plant survival may not otherwise be possible (Chapin, 1980; Read, 1990). It has been suggested that mycorrhizal fungi become less or not beneficial to their hosts in fertile soils (Fitter, 1977; Bowen, 1980), shifting the relationship from mutualism to parasitism (Johnson *et al.*, 1997). The outcome of a plant-fungus interaction may then not be pre-determined, but could vary along an environmental gradient, therefore showing conditionality in relation to soil fertility.

The two most widespread types of mycorrhizal associations are ectomycorrhiza (EcM) and arbuscular mycorrhiza (AM). The distribution of mycorrhizal types over the various terrestrial biomes is not random. Read (1991) noted a pattern with the natural climax of arctic (and alpine) biomes being dominated by ericoid mycorrhizal plants, boreal and temperate biomes by ectomycorrhizal plants, and (sub)tropical biomes by arbuscular mycorrhizal plants. Within biomes the occurrence of ectomycorrhizal and arbuscular mycorrhizal plants is also not random. In temperate regions, ectomycorrhizal trees occur on acidic soils where litter accumulates and a mor humus profile develops, whereas arbuscular mycorrhizal trees develop on the most fertile, neutral soils where litter is easily

degraded and a mull humus profile develops. This pattern might be related to differential availability of phosphorus and nitrogen on nutrient-poor and rich sites, where phosphorus is usually the limiting element under richer conditions and nitrogen under nutrient-poor conditions. Finally, within successional series a replacement of vegetation types with different mycorrhizal types can occur. Read (1989) described the importance of mycorrhizas in the sand dune ecosystem. As the relative availability of nitrogen and phosphorus changes during succession, the occurrence of both mycorrhizal types changes as well. In the dry foredunes, where phosphorus is the main growth limiting nutrient, AM predominates. In contrast in the dune slacks, where accumulation of organic matter occurs, nitrogen mineralization is inhibited due to pH reduction, and nitrogen becomes the main growth limiting element, as a consequence of which EcM prevails, with its ability to take up nitrogen (Read, 1989). The different successional stages are, therefore, characterized not only by a typical nutrient status, but also by a dominant mycorrhizal type.

*Salix repens* L. is a common shrub in a great variety of plant communities in the coastal dunes. It occurs in habitats that range from dry to wet, and from calcareous, humus-poor to acidic, humus-rich soils. *Salix repens* is one of the very few plants that form associations with AM and EcM fungi (Harley & Harley, 1987; CHAPTER 3), and therefore might have a selective advantage in this highly dynamic ecosystem.

With the increasing awareness of the ecological importance of mycorrhizas and their diversity, research must be directed to experiments that compare the functional differences between AM and EcM symbioses, and the significance of diversity within functional types. Such research has led to the conclusion that the mycorrhizal symbiosis is multi-functional (Newsham *et al.* 1995b) in three respects: differential effects of different fungi on the same plant; differential effects of the same fungus on different plants; differential effects of the same fungus on the same plant under different environmental conditions (conditionality). Earlier research with *Salix repens* indicated that a number of parameters were necessary to assess the functional importance of the mycorrhizal symbiosis. Different mycorrhizal fungi varied in response time, response duration, magnitude of response as related to amount of root colonization, changes in root foraging behaviour of the plant, and changes in the nutrient pools in the soil (CHAPTERS 5 AND 6).

In this study, conditionality was assessed in a factorial experiment where soil pH and Nitrogen / Phosphorus ratio were varied. Three mycorrhizal fungi were used, viz. *Glomus mosseae* (AMF), *Hebeloma leucosarx* (EcMF) and *Paxillus involutus* (EcMF). In an earlier study (CHAPTERS 5 AND 6) those species differed in response time and response

duration; *H. leucosarx* and *P. involutus* furthermore differed in plant biomass response under nutrient-poor conditions.

Questions that will be addressed in this study are: (1) Will EcM colonization (potential) decrease when nitrogen availability increases? (2) Will AM colonization (potential) increase when nitrogen availability increases (and hence P limitation becomes stronger)? (3) Will EcM colonization be higher under more acidic conditions? (4) Will AM colonization be higher under more alkaline conditions? (5) Does mycorrhizal benefit decrease when nutrient availability increases? (6) Does relative benefit of *H. leucosarx* and *P. involutus* change when nutrient availability increases?

## MATERIAL AND METHODS

### *Field description, plant and fungal material*

Cuttings (shoot tops) of *Salix repens* were collected in December 1996 from the field sites Schoenus on the isle of Terschelling. All cuttings were collected from the same male plant in order to obtain genetic homogeneity.

Two cultures of EcMF were collected in the autumn of 1994 and 1995 from *S. repens* from the Isle Terschelling, i.e. *Hebeloma leucosarx* (L1: Thijssensduin: acidic, humus-rich soil) and *Paxillus involutus* (L82: Paardenwei: acidic, humus-rich soil). Sporocarps were surface sterilized with alcohol (70 %) and sliced in half under sterile conditions. Fungal tissue was cut from the innerside of the cap and transferred to and maintained on solid media (alternative Melin Norkrans (AMN)) containing (in $g.l^{-1}$): agar (15), malt extract (3), glucose.$H_2O$ (10), $(NH_4)_2HPO_4$ (0.25), $KH_2PO_4$ (0.5), $KNO_3$ (0.5), $MgSO_4.7H_2O$ (0.15), $CaCl_2.H_2O$ (0.67), NaCl (0.025), $FeCl_3$ (0.0012) and thiamine.HCl (100 µg). An AMF starter culture was kindly provided by the European Bank of Glomales (France), *Glomus mosseae* (BEG 12). This culture was maintained on *Trifolium repens* L. in sandy soil, supplied with P-poor Hoagland solution.

The selection of the fungal species was based on their contrasting effects on a number of parameters of *S. repens* under poor soil conditions. *Hebeloma leucosarx* and *P. involutus* occurred in all habitat types; *G. mosseae* was identified only in the calcareous plots on Terschelling (CHAPTER 3).

*Inoculation and plant growth conditions*

After storage at 4 °C for one month, the cuttings of *S. repens* were trimmed to 4 cm, surface-sterilized twice in (freshly prepared) 6 % $H_2O_2$ for 1 min (in series of 20 in 300 mL), and rinsed three times in (fresh) demineralized water (1 L) for 10 min. Each cutting was placed individually in a culture tube containing 20 mL sterile water agar (1 %). After a 10 weeks rooting period in a climate chamber (photon flux density 120 $\mu E.m^{-2}.s^{-1}$ (11,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 70 %) equally developed cuttings were selected for the experiment.

Soil used in this experiment was a sandy soil, classified as nutrient- and humus-poor: pH ($CaCl_2$) 5.5, nutrient contents 6 mg $N_{tot}.kg^{-1}$, 6 mg $P_{tot}.kg^{-1}$, and 0 % organic matter.

A four-factorial experimental design was used. One factor, fungal treatment, contained four levels (including non-mycorrhizal control), the second factor, soil pH($CaCl_2$), contained three levels (4, 5.5, 7), the third factor, soil N/P ratio, contained three levels (5.4, 16.2, 48.6), and finally the fourth factor, time, contained two levels (12 and 26 weeks). Each treatment was replicated 6 times. The experiment was set up in a complete randomized design.

Twenty cuttings were harvested at the moment of inoculation (t = 0). Each fungus was individually inoculated to *S. repens* in 15 replicates (in order to end with two harvests of 6 replicates), and 15 cuttings were supplied with non-inoculated agar plugs (control) per treatment combination (soil pH and N/P ratio). The isolates of *H. leucosarx* and *P. involutus* were precultured twice (successively) for three weeks on solid media (AMN) in order to obtain sufficient actively growing fungal material. *G. mosseae* was precultured on roots of *T. repens* for 15 weeks (inoculum density: roots and soil with AMF to sterilized soil, 10 % v/v).

Different soil N/P ratios were obtained by regular supply of a Hoagland nutrient solution with N/P ratio of 5.4. The amount of nitrogen in the solution was increased 3 times and 9 times in order to obtain N/P ratios 16.2 and 48.6. Soil pH 4 was adjusted with $H_2SO_4$, whereas soil pH 7 was obtained by mixing $CaCO_3$ with the sandy soil, two weeks before nutrient treatment, and pH of the nutrient solution was adjusted with KOH. Measurement of soil pH ($CaCl_2$) and nutrient analyses ($CaCl_2$ extraction according to Houba *et al.*, 1990) were performed at the start of the experiment, at the first harvest, at

the start of the second period and at the final harvest in all soils, including a complete set of soil treatments without plants.

The substrate used was a sand-perlite mixture 1:3 (v/v). The different nutrient solutions were added to the substrate (1:3:1 v/v/v sand:perlite:nutrient solution) 24 h prior to autoclaving. The substrate was autoclaved twice (1 h, 121 °C, 1 atm.) with a 48 h interval, and left for one week. Root growth chambers (vertically placed Petri dishes 15 cm diameter, with a slit in the edge) were filled with the sterile substrate. Each root growth chamber contained 300 mL substrate, of which 75 mL (*c.* 120 g) was sand. Cuttings were transferred to the root growth chambers (1 cutting per growth chamber), each cutting was inoculated with 5 mycelial plugs cut from the edge of a precultured EcM fungal colony, the root system was covered with a water agar (1 %) layer (5 cm diameter) to prevent the roots from excessive loss of water. The root growth chambers were sealed with tape and sterilized anhydrous lanolin preventing contamination and loss of water. The control plants were supplied with 5 plugs of solid medium (5 * 0.15 mL) without fungus instead. The inoculation of AMF was performed by mixing 10 g freshly washed root material of *T. repens* containing *G. mosseae* with the (each of the pH, N/P ratios) sterile substrates (5 L), and 15 growth chambers were filled with this mixture. Cuttings were transferred to the root growth chambers, the root systems were covered with water agar, root growth chambers were closed and sealed. As in previous experiments with addition of AMF inoculum washings (Koide & Li, 1989) or roots of *T. repens* without AMF to the control and EcM plants showed no effect on plant performance in 12 weeks (APPENDICES E AND F), control and EcM plants were not provided with washings or roots of *T. repens* without AMF. Mycorrhizal inoculum viability of *G. mosseae* was tested using *T. repens* as a control host plant grown on the same dosis of inoculum in the same nine soil treatments. All soil compartments were protected from daylight, the growth chambers were placed vertically in transient propagators (relative air humidity almost 100 % in the first week) and placed in the climate chamber. Growth conditions: photon flux density 350 μE $\mu E.m^{-2}.s^{-1}$ (20,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 60 %. The plants were arranged in a complete randomized block design, and blocks were randomized every three weeks within the climate chamber. The plants were watered every three weeks with 10 mL of their nutrient solution. After 12 weeks 6 plants of each combination (fungus, pH and N/P ratio) were harvested randomly.

From the remaining plants randomly 6 of each combination were transported to 2 L pots (individually) filled with pH and N/P ratio adjusted substrates. Plants were sufficiently watered, and every three weeks pots were percolated with 450 mL of the adjusted nutrient solutions in order to standardize the soil conditions between fungal treatments (pot; soil water content 400 mL). After 26 weeks all plants were harvested.

*Plant performance, mycorrhizal effectiveness, EcMF and AMF colonization*

Every three weeks length of shoots was determined. After the harvest shoot weight was determined after 72 hours at 70 °C. The plants were individually digested in sulphuric acid, salicylic acid and 30 % hydrogen peroxide. Total N and P were analyzed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard (Novozamsky *et al.*, 1988).

In this study mycorrhizal effectiveness (responsiveness) is used as a modification of the term mycorrhizal dependency as defined by Plenchette *et al.* (1983). Mycorrhizal effectiveness was calculated for (1) biomass, (2) root length, (3) N- and (4) P content as:

[*3*] Mycorrhizal effectiveness = $1 - (b / a)$,

where $a$ is the mean biomass (or root length or N- or P-content) of one of the mycorrhizal treatments and, $b$ is the mean biomass (or root length or N- or P-content) of the non-mycorrhizal control.

The roots were immersed in water over a 2 mm sieve to remove most of the soil, and rinsed gently to avoid damage of the mycorrhizas. The cleaned ectomycorrhizal roots were stored in glutaraldehyde buffer (Alexander & Bigg, 1981) and roots of the control plants and the AM plants were stored in 50 % alcohol until they could be processed. In each sample, root length was determined according to Newman (1966) and the numbers of EcM root tips, and total numbers of root tips were counted. Root biomass was determined after 72 hours at 70 °C, and since different EcM-, AM- or non mycorrhizal root systems contained different (not removable) proportions of sand, root weight was corrected for this proportion of sand by its weight after loss on ignition (550 °C). EcM frequencies were calculated (100 % * numbers of EcM root tips/total numbers of root tips). EcM root length (%) was measured according to Giovannetti & Mosse (1980). All root samples that had been inoculated with AMF were cleared with 10 % KOH for three hours in a waterbath at

90 °C, bleached in 10 % $H_2O_2$ for 1 hour, acidified in 1 % HCl for 15 min and stained with trypan blue (Phillips & Hayman, 1970; APPENDIX C) in lactophenol for 30 min. AM colonization was estimated by a modified line intersect method (McGonigle *et al.*, 1990), where a minimum of 100 line intersections per root sample (replicated three times) was scored for the presence of AM structures. AM root length times intensity (RLCI = RLC * % cover by AM of the cross section of the root colonized) was calculated.

*Statistical analysis*

Data were analysed by Analysis of Variance using the statistical package STATISTICA (StatSoft). A four-factorial statistical design was used. Preliminary analyses (TABLE 7-1) showed large effects of time and fungus. For that reason, experiments were separately analyzed for each fungus and each harvest period.

The parameters mycorrhizal colonization (%) and mycorrhizal root length were analyzed without the non-mycorrhizal treatment. Prior to analysis proportional mycorrhizal colonizations were arcsine square root transformed and plant parameters were logarithmically transformed. Bartlett's test was used to determine whether variances were equal between treatments. Differences among means were evaluated with the LSD-test (Sokal & Rohlf, 1995).

## RESULTS

*Soil analysis*

In control pots (without plants) soil pH ($CaCl_2$) at the start of the experiment and at the start after transplantation to larger pots were 4.0, 5.5 and 7.2, after the first harvest at t = 12 weeks 4.5, 5.5 and 7.1, and at the final harvest 4.8, 5.2 and 7.6. Nutrient analysis at the start of the experiment resulted in N/P ratios of approximately 6.7, 18.3 and 45. In treatment pH 7 total available nutrient amounts were lower and N/P ratios were slightly different, 8.1, 19.7 and 49.1. However, as the pots (with plants) were percolated every three weeks with an adjusted nutrient solution and with an adjusted pH, the factorial treatment with pH and relative nutrient availability as factors was successful.

Soil pH and remaining amount of N and P per treatment were generally not significantly different between fungal treatments, However, in the fungal treatment with *G. mosseae* no P was detected in soils, irrespective of the pH or N/P ratio.

**TABLE 7-1** ANOVA results (*P* values) executed for each combination of fungal and plant parameters. The factor time consists of two periods, the factor fungal treatment consist of three different fungi and a non-mycorrhizal treatment, the factor soil pH consists of three different pH values, and the factor soil N/P ratio consists of three different N/P ratios.

| Parameter | Time(1) (df=1) | Fungi(2) (df=3) | pH (3) (df=2) | N/P (4) (df=2) | 1×2 (df=3) | 1×3 (df=2) | 2×3 (df=6) | 1×4 (df=2) | 2×4 (df=6) | 3×4 (df=4) | 1×2×3 (df=6) | 1×2×4 (df=6) | 1×3×4 (df=4) | 2×3×4 (df=12) | 1×2×3×4 (df=12) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shoot: | | | | | | | | | | | | | | | |
| Length | <0.001 | <0.001 | <0.001 | <0.001 | 0.015 | <0.001 | 0.124 | <0.001 | 0.002 | <0.001 | 0.320 | 0.065 | 0.016 | <0.001 | <0.001 |
| Dry weight | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.385 | 0.021 | 0.032 | 0.785 | 0.334 | 0.086 | <0.001 |
| N (%) | <0.001 | <0.001 | <0.001 | <0.001 | 0.002 | 0.048 | 0.793 | <0.001 | <0.001 | <0.001 | 0.324 | 0.101 | 0.765 | 0.712 | 0.051 |
| N content | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.023 | 0.011 | 0.042 | 0.996 | 0.292 | 0.069 | <0.001 |
| P (%) | <0.001 | 0.005 | <0.001 | 0.002 | <0.001 | <0.001 | 0.049 | 0.101 | 0.118 | <0.001 | 0.052 | 0.027 | 0.021 | 0.126 | <0.001 |
| P content | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.217 | 0.058 | <0.001 | 0.405 | 0.419 | <0.001 | <0.001 |
| N/P ratio | <0.001 | 0.679 | <0.001 | <0.001 | <0.001 | <0.001 | 0.240 | 0.001 | 0.036 | <0.001 | 0.240 | 0.089 | 0.167 | 0.040 | 0.017 |
| Root: | | | | | | | | | | | | | | | |
| Length | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.003 | <0.001 | <0.001 | 0.017 | 0.375 | 0.954 | 0.747 | 0.586 | 0.064 | 0.299 |
| Myc. (%)† | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.673 | <0.001 | 0.029 | 0.748 | <0.001 | 0.009 | 0.020 | 0.040 | <0.001 | 0.001 |
| Myc. rtl† | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.021 | <0.001 | <0.001 | 0.087 | 0.023 | 0.054 | 0.050 | 0.027 | 0.002 |

* df Error = 394

† Fungus (df = 2), 1×2 (df = 4), 2×3 (df = 4), 1×2×3 (df = 4), 1×2×4 (df = 4), 2×3×4 (df = 8), 1×2×3×4 (df = 8), df Error = 247.

*Overall treatment effects*

Analysis of variance in which all factors (harvest time, fungal species, pH, and N/P ratio) were included, indicated that for almost all parameters all main factors were highly significantly different (TABLE 7-1). Most two-way interactions were also highly significant, and several higher-order interactions were also significant. As the factor time was highly significant for all parameters ($P << 0.001$), subsequent analyses were done per harvest period (mycorrhizal colonization: TABLE 7-2; plant parameters: TABLE 7-4a).

*Mycorrhizal colonization*

On the short term (12 weeks), colonization (%) of *G. mosseae, H. leucosarx* and *P. involutus* was significantly affected by pH and N/P ratio (TABLE 7-2), whereas their absolute length of colonization (m) was significantly affected by pH for *G. mosseae* and by N/P ratio for *H. leucosarx* and *P. involutus*. On the longer term (26 weeks), colonization (%) of *G. mosseae* was affected by both pH and N/P ratio, that of *P. involutus* was affected by pH and that of all three fungi by the interaction between pH and N/P ratio (TABLE 7-2). Absolute length of colonization (m) was affected by N/P ratio for *G. mosseae* and for *H. leucosarx*, whereas that of *P. involutus* was affected by pH and N/P ratio.

Even though mycorrhizal inoculum potential of *G. mosseae* in *T. repens* showed an optimum at N/P ratio 16.2 (irrespective of pH), in *S. repens* a significant increase in AM (%) was observed with higher N/P ratio, irrespective of pH, after 12 and 26 weeks (TABLE 7-3). Arbuscular mycorrhizal colonization was higher at pH 7 than at the other pH's. Absolute length of colonization by *G. mosseae* was generally lower at pH 7 at both harvests.

*Hebeloma leucosarx* showed very little or no differences in amount of root colonization (TABLE 7-3). At t = 12 weeks, colonization (%) was slightly lower at pH 7 in combination with N/P ratios 5.4 and 16.2 and at pH 4, N/P ratio 5.4. However, at t = 26 weeks colonizations increased to maximal colonizations at whatever treatment. Absolute length of colonization was generally lower at N/P ratio 48.6, at both harvests (TABLE 7-3).

Colonization by *P. involutus* showed less clear patterns in relation to pH and N/P ratio (TABLE 7-3). After 12 weeks, colonization tended to be higher with increasing amount of nitrogen. After 26 weeks mycorrhizal colonization generally decreased, but at pH 4, N/P ratio 48.6 it was not different and at pH 5.5, N/P ratio 16.2 colonization increased and mantles were much better developed than at all other treatments.

**TABLE 7-2.** ANOVA results (*P* values) executed for mycorrhizal colonization (% and m ) of *Glomus mosseae, Hebeloma leucosarx* and *Paxillus involutus* per harvest period as affected by soil pH, soil N/P ratio, and their interaction.

| Parameter | Fungus | Soil pH (df = 2) | Soil N/P (df = 2) | pH × N/P (df = 4) |
|---|---|---|---|---|
| After 12 weeks | | | | |
| Mycorrhizal colonization (%) | *Glomus mosseae* | 0.019 | <0.001 | 0.037 |
| | *Hebeloma leucosarx* | <0.001 | <0.001 | 0.128 |
| | *Paxillus involutus* | <0.001 | 0.010 | 0.624 |
| Mycorrhizal root length colonized (m) | *Glomus mosseae* | <0.001 | 0.130 | 0.027 |
| | *Hebeloma leucosarx* | 0.188 | <0.001 | 0.347 |
| | *Paxillus involutus* | 0.125 | 0.016 | 0.868 |
| After 26 weeks | | | | |
| Mycorrhizal colonization (%) | *Glomus mosseae* | <0.001 | <0.001 | <0.001 |
| | *Hebeloma leucosarx* | 0.174 | 0.334 | 0.002 |
| | *Paxillus involutus* | 0.016 | 0.711 | <0.001 |
| Mycorrhizal root length colonized (m) | *Glomus mosseae* | 0.648 | <0.001 | <0.001 |
| | *Hebeloma leucosarx* | 0.202 | <0.001 | 0.074 |
| | *Paxillus involutus* | <0.001 | <0.001 | 0.107 |

**TABLE 7-3.** Mycorrhizal colonization (root length (%), root length (m); $n = 6$) by *Glomus mosseae* (on *Trifolium repens* and *Salix repens*), *Hebeloma leucosarx* and *Paxillus involutus* on *Salix repens* as affected by soil pH (7, 5.5, 4) and N/P ratio (5.4, 16.2, 48.6) after 12 and 26 weeks (SE within parentheses).

| | | Mycorrhizal colonization | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *T. repens* | *Salix repens* | | | | | | | | | | | |
| | | *G. mosseae* | *Glomus mosseae* | | | | *Hebeloma leucosarx* | | | | *Paxillus involutus* | | | |
| | | %RLCI | %RLCI | | RLC (m) | | %RLC | | RLC (m) | | %RLC | | RLC (m) | |
| pH | N/P | Wks:12 | 12 | 26 | 12 | 26 | 12 | 26 | 12 | 26 | 12 | 26 | 12 | 26 |
| 7 | 5.4 | 13.0 c* | 1.9 a | 2.3 b | 0.30 b | 4.68 c | **79.6 a** | **96.5 a** | 15.0 b | 253.9 d | 55.1 a | 66.9 a | 15.9 b | 91.4 b |
| | | (0.78) | (0.1) | (0.1) | (0.01) | (0.05) | **(3.5)** | **(0.1)** | (0.06) | (0.00) | (2.4) | (7.5) | (0.17) | (3.02) |
| 7 | 16.2 | 44.8 e | 2.5 ab | 3.2 c | 0.25 b | 2.41 a | **79.6 a** | **97.8 b** | 8.7 a | 169.0 c | 60.7 ab | 45.7 a | 17.0 b | 82.6 b |
| | | (2.11) | (0.1) | (0.2) | (0.01) | (0.06) | **(2.2)** | **(0.5)** | (0.05) | (0.09) | (6.1) | (3.3) | (0.30) | (2.24) |
| 7 | 48.6 | 27.3 d | **3.6 bc**† | **11.4 e** | 0.11 a | 1.69 a | **94.5 cd** | **100 b** | 9.2 a | 32.5 a | 64.1 ab | 46.9 a | 8.7 a | 25.9 a |
| | | (3.71) | **(0.4)** | **(0.8)** | (0.01) | (0.12) | **(1.1)** | **(0)** | (0.12) | (0.00) | (2.6) | (2.7) | (0.10) | (0.18) |
| 5.5 | 5.4 | 7.6 b | **2.4 a** | **0.7 a** | 0.68 c | 2.39 a | **94.1 cd** | **100 b** | 14.4 b | 243.8 d | 55.4 a | 61.8 a | 18.8 b | 212.9 c |
| | | (0.39) | **(0.3)** | **(0.1)** | (0.04) | (0.03) | **(1.3)** | **(0)** | (0.05) | (0.00) | (5.0) | (6.9) | (0.23) | (2.94) |
| 5.5 | 16.2 | 40.0 e | 2.6 ab | 2.6 b | 0.51 bc | 8.37 d | **93.7 cd** | **99.2 b** | 14.0 b | 160.5 c | **47.8 a** | **87.7 b** | 13.9 ab | 207.7 c |
| | | (2.07) | (0.1) | (0.1) | (0.00) | (0.05) | **(1.0)** | **(0.5)** | (0.03) | (0.19) | **(6.2)** | **(4.8)** | (0.36) | (0.52) |
| 5.5 | 48.6 | 26.4 d | 4.0 c | 4.3 cd | 0.51 bc | 3.32 b | 96.5 d | 96.7 b | 7.7 a | 53.5 b | **73.0 b** | **51.9 a** | 12.2 a | 35.3 a |
| | | (2.71) | (0.3) | (0.4) | (0.01) | (0.09) | (1.3) | (0.7) | (0.02) | (0.07) | **(5.8)** | **(3.1)** | (0.22) | (0.25) |
| 4 | 5.4 | 3.6 a | 2.3 a | 1.9 b | 0.37 bc | 4.89 c | **80.3 ab** | **100 b** | 20.7 b | 198.2 c | 73.1 b | 61.2 a | 19.5 b | 177.1 c |
| | | (0.21) | (0.1) | (0.1) | (0.01) | (0.07) | **(5.6)** | **(0)** | (0.18) | (0.37) | (5.0) | (8.4) | (0.41) | (2.26) |
| 4 | 16.2 | 41.8 e | 2.6 ab | 3.0 bc | 0.75 c | 5.69 c | **89.5 bc** | **98.7 b** | 15.8 b | 179.1 c | 69.1 b | 51.0 a | 16.2 b | 94.6 b |
| | | (2.91) | (0.1) | (0.4) | (0.00) | (0.10) | **(1.9)** | **(2.0)** | (0.06) | (0.57) | (4.3) | (3.3) | (0.18) | (1.50) |
| 4 | 48.6 | 27.0 d | 6.6 d | 5.8 d | 0.59 c | 2.86 a | **94.0 cd** | **99.9 b** | 6.7 a | 21.1 a | 88.3 c | 88.2 b | 10.3 a | 23.6 a |
| | | (2.89) | (0.2) | (2.5) | (0.00) | (0.29) | **(2.0)** | **(0.1)** | (0.01) | (0.61) | (4.2) | (3.7) | (0.08) | (0.24) |

* Different letters within a column (per harvest period) indicate significant differences according to a three factor ANOVA and LSD tests ($P < 0.05$).

† Significantly different colonization over the two harvest periods are printed in bold. (Mycorrhizal root length was always significantly different between two harvests).

**TABLE 7-4a.** ANOVA results (*P* values) executed for each plant parameter per harvest period as affected by fungal treatment, soil pH, soil N/P ratio, and their interactions.

| Parameters | Fungus (1) (df = 3) | Soil pH (2) (df = 2) | Soil N/P (3) (df = 2) | 1×2 (df = 6) | 1×3 (df = 6) | 2×3 (df = 4) | 1×2×3 (df = 12) |
|---|---|---|---|---|---|---|---|
| After 12 weeks (df Error = 176) | | | | | | | |
| Shoot:Length | <0.001 | <0.001 | <0.001 | 0.548 | 0.780 | 0.099 | 0.914 |
| Dry weight | <0.001 | <0.001 | <0.001 | 0.178 | 0.310 | 0.368 | 0.617 |
| N (%) | <0.001 | 0.622 | <0.001 | 0.345 | 0.001 | 0.015 | 0.103 |
| N content | <0.001 | <0.001 | <0.001 | 0.006 | 0.043 | 0.048 | 0.192 |
| P (%) | <0.001 | 0.006 | 0.108 | 0.227 | 0.941 | 0.208 | 0.216 |
| P content | <0.001 | <0.001 | <0.001 | 0.019 | 0.076 | 0.026 | 0.686 |
| N/P | <0.001 | 0.004 | <0.001 | 0.106 | 0.541 | 0.024 | 0.081 |
| Root: Length | <0.001 | <0.001 | <0.001 | 0.153 | 0.283 | 0.921 | 0.167 |
| After 26 weeks (df Error = 172) | | | | | | | |
| Total plant dry weight | <0.001 | <0.001 | <0.001 | 0.001 | 0.637 | 0.030 | 0.021 |
| Shoot:Length | <0.001 | <0.001 | <0.001 | 0.159 | 0.002 | <0.001 | <0.001 |
| Dry weight | <0.001 | <0.001 | <0.001 | 0.002 | 0.756 | 0.088 | 0.004 |
| N (%) | 0.065 | <0.001 | <0.001 | 0.704 | 0.001 | 0.023 | 0.467 |
| N content | <0.001 | <0.001 | <0.001 | 0.009 | 0.520 | 0.121 | 0.008 |
| P (%) | 0.004 | <0.001 | 0.013 | 0.051 | 0.030 | <0.001 | 0.012 |
| P content | <0.001 | <0.001 | <0.001 | <0.001 | 0.478 | 0.300 | <0.001 |
| N/P | 0.005 | <0.001 | <0.001 | 0.406 | 0.016 | <0.001 | 0.022 |
| Root: Length | <0.001 | <0.001 | <0.001 | 0.008 | 0.081 | 0.209 | 0.119 |
| Dry weight | 0.658 | <0.001 | <0.001 | 0.045 | 0.077 | 0.023 | 0.294 |
| Specific root length | <0.001 | 0.190 | <0.001 | 0.007 | 0.003 | 0.004 | <0.001 |

**TABLE 7-4b.** Average total plant biomass, shoot length, shoot biomass, shoot N and P concentration and content, shoot N/P ratio, root length, root biomass and specific root length over all experimental soil treatments (soil pH and N/P ratios) between fungal treatments (nd = not determined).

| | Plant dry mass (g) | Shoot length (mm) | Shoot dry mass (mg) | Shoot N conc.(%) | Shoot N cont. (mg) | Shoot P conc. (%) | Shoot P cont. (mg) | Shoot N/P | Root length (m) | Root dry mass (mg) | Spec..rtl. ($cm.g^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 12 weeks | | | | | | | | | | | |
| Control | nd | 219.2 a* | 377.6 b | 1.82 b | 6.81 b | 0.091 a | 0.332 b | 21.6 c | 29.2 b | nd | nd |
| *G. mosseae* | | 203.9 a | 307.2 a | 1.65 a | 4.99 a | 0.095 a | 0.273 a | 18.7 b | 27.4 b | | |
| *H. leucosarx* | | 292.6 c | 488.7 c | 1.95 c | 9.34 c | 0.119 b | 0.547 c | 17.4 ab | 16.3 a | | |
| *P. involutus* | | 269.1 b | 499.4 c | 1.93 c | 9.53 c | 0.130 b | 0.596 d | 16.4 a | 26.7 b | | |
| 26 weeks | | | | | | | | | | | |
| Control | 2.78 a | 625.0 a | 1821.3 a | 2.10 ab | 36.80 a | 0.184 b | 3.10 a | 13.6 a | 168.2 a | 932.1 a | 166.4 a |
| *G. mosseae* | 3.20 a | 756.0 a | 2293.6 a | 2.07 a | 46.31 a | 0.154 a | 3.24 a | 14.3 ab | 256.0 b | 907.0 a | 309.0 c |
| *H. leucosarx* | 3.97 b | 876.7 b | 3086.6 b | 2.10 a | 59.03 b | 0.141 a | 4.30 b | 15.5 b | 147.7 a | 885.2 a | 192.4 a |
| *P. involutus* | 2.88 a | 657.4 a | 2099.4 a | 2.21 b | 44.29 a | 0.164 ab | 2.97 a | 16.8 b | 165.2 a | 773.2 a | 224.5 b |

* Different letters within a column (per harvest period) indicate significant differences according to a three factor ANOVA and LSD tests ($P < 0.05$).

Absolute mycorrhizal root length (m) showed no differences between N/P ratios 5.4 and 16.2, irrespective of pH and harvest period, but at N/P ratio 48.6 it was significantly lowest (TABLE 7-3). Mycorrhizal colonization (%) at pH 5.5, N/P 16.2 and pH 4, N/P ratio 48.6 was not different, but absolute length of the colonization was strongly significantly different; this was also the case for *H. leucosarx*.

*Plant performance*

Over 12 weeks, all plant parameters were significantly affected by the fungal treatment, and the majority of the plant parameters were significantly affected by pH (but not shoot N %) and N/P ratio (but not shoot P %) (TABLE 7-4a). Over 26 weeks these parameters were significantly affected by fungal treatment (but not shoot N %), pH and N/P ratio. Root dry weight was not affected by fungal treatment and specific root length ($m.g^{-1}$ root dry weight) was not affected by pH. Significant interactions between the main factors can be found in TABLE 7-4a.

Both *H. leucosarx* and *P. involutus* were beneficial compared to the non-mycorrhizal treatment, whereas *G. mosseae* was generally disadvantageous at t = 12 weeks (TABLE 7-4b). After 26 weeks generally the rank order from the most beneficial to least beneficial was *H. leucosarx* > *P. involutus* > *G. mosseae* = non-mycorrhizal. However, for root length and specific root length the rank order was different: *G. mosseae* > *H. leucosarx* ≈ *P. involutus* ≈ non-mycorrhizal (TABLE 7-4b).

*Above-ground plant performance*

Short term growth responses showed that for non-mycorrhizal plants shoot length and shoot biomass optimum were generally largest at pH 4 and 5.5 with N/P ratios 5.4 and 16.2 (FIGS. 7-1a,b). When plants were inoculated with *G. mosseae* shoot length significantly increased from pH 7 towards pH 4, and with N/P ratio from 48.6 towards N/P ratio 5.4; this pattern was less clear for shoot biomass. Shoot biomass was lower at pH 7 with N/P ratio 48.6. *Salix repens* inoculated with *H. leucosarx* showed no significant differences among soil treatments. This was also found for shoot length when plants were inoculated with *P. involutus*, but for shoot biomass a similar significant trend as for non-mycorrhizal plants was found (FIGS. 7-1 a,b). Over 26 weeks patterns for non-mycorrhizal plants were similar to the harvest after 12 weeks, but differences between treatments were larger (FIGS. 7-2a,b).

**FIGURE 7-1.** Shoot length (a), shoot dry weight (b) and shoot N/P ratio (c) of *Salix repens* ($n$ = 6) when inoculated with *Glomus mosseae, Hebeloma leucosarx* and *Paxillus involutus* or not inoculated as affected by soil pH (7, 5.5, 4) and N/P ratio (5.4, 16.2, 48.6) after 12 weeks. Different letters per fungal treatment indicate significant differences among soil treatments according to 2-factor ANOVA and LSD-tests ($P < 0.05$) (see opposite page).

For plants inoculated with *G. mosseae* the trend found after 12 weeks that shoot length and biomass were larger at pH 4 and 5.5 in combination with N/P ratios 5.4 and 16.2, was much stronger and significant after 26 weeks. Even though after 26 weeks, shoot length and biomass were not correlated for plants inoculated with *H. leucosarx* at N/P ratios 5.4 or 16.2 with pH's 4, 5.5 and 7, these parameters were clearly larger than at N/P ratio 48.6. Shoot length and biomass for plants inoculated with *P. involutus* was correlated and showed an optimum at pH 5.5 with N/P 5.4 or 16.2 and pH 4 with N/P ratio 5.4 after 26 weeks (FIGS. 7-2 a,b).

Mycorrhizal effectiveness of *G. mosseae* was generally low after 12 weeks (TABLE 7-5; at pH 7 with N/P ratio 5.4 or 48.6 and pH 4 with N/P ratio 5.4 shoot biomass was even significantly lower than the non-mycorrhizal treatment), whereas that of *H. leucosarx* or *P. involutus* was generally positive and shoot biomass was significantly higher than the non-mycorrhizal plants at pH 4 with N/P ratios 16.2 and 48.6 (TABLE 7-5). However, after 26 weeks all fungi showed positive mycorrhizal effectiveness at pH's 4 and 5.5 considering shoot biomass and at pH 7 *H. leucosarx* generally performed best (TABLE 7-5).

After 12 weeks, shoot N/P ratio was generally lowest at N/P 5.4 irrespective of pH (FIG. 7-1c). Shoot N/P ratios of plants at N/P ratios 16.2 and 48.6 were not linearly related but showed interactions with soil pH. After 26 weeks, plant N/P ratios showed less significant differences at different soil N/P ratios (FIG. 7-2c). Clearly patterns between shoot N/P ratio at the different soil N/P ratios were different at the different fungal treatments (FIGS. 7-1c AND 7-2c).

Shoot P concentrations at the short term were not different between soil treatments for non-mycorrhizal plants or inoculated with *G. mosseae* or *P. involutus*. However, *H. leucosarx* showed higher P (%) at pH 5.5 and 7 with N/P ratio 5.4 or 16.2 (TABLE 7-6), whereas after 26 weeks no differences were found. Non-mycorrhizal plants after 26 weeks showed decreasing P (%) at pH 7 and 5.5 with increasing N/P ratio (5.4 and 16.2 to 48.6),

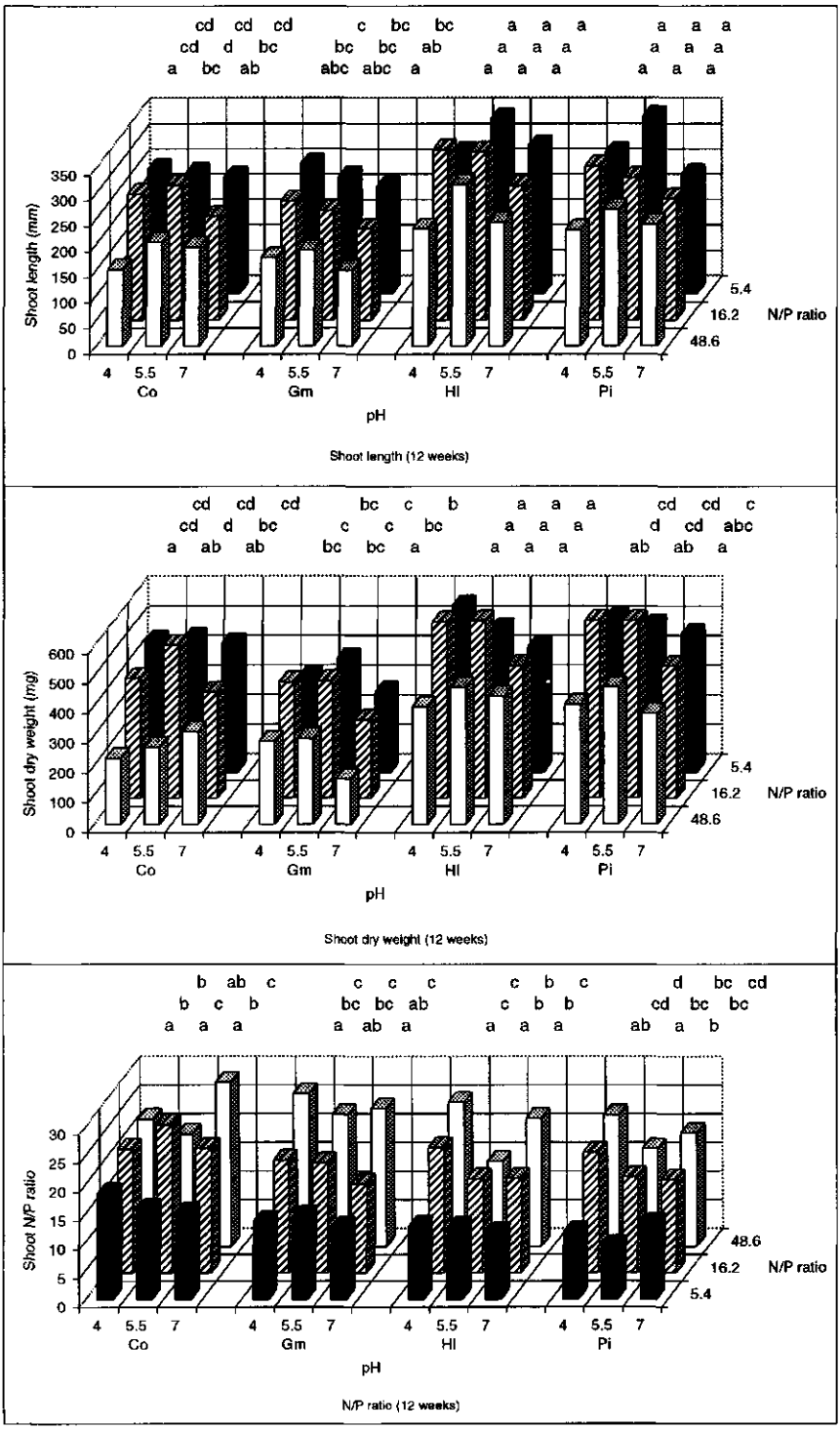

cd cd cd c bc bc a a a a a a
cd d bc bc bc ab a a a a a a
a bc ab abc abc a a a a a a a
Shoot length (mm)
350
300
250
200
150
100
50
0
5.4
16.2
48.6
N/P ratio
4 5.5 7 4 5.5 7 4 5.5 7 4 5.5 7
Co Gm Hl Pi
pH
Shoot length (12 weeks)
cd cd cd bc c b a a a cd cd c
cd d bc c c bc a a a d cd abc
a ab ab bc bc a a a a ab ab a
Shoot dry weight (mg)
600
500
400
300
200
100
0
5.4
16.2
48.6
N/P ratio
4 5.5 7 4 5.5 7 4 5.5 7 4 5.5 7
Co Gm Hl Pi
pH
Shoot dry weight (12 weeks)
b ab c c c c c b c d bc cd
b c b bc bc ab c b b cd bc bc
a a a a ab a a a a ab a b
Shoot N/P ratio
30
25
20
15
10
5
0
48.6
16.2
5.4
N/P ratio
4 5.5 7 4 5.5 7 4 5.5 7 4 5.5 7
Co Gm Hl Pi
pH
N/P ratio (12 weeks)

**FIGURE 7-2.** Shoot length (a), shoot dry weight (b) and shoot N/P ratio (c) of *Salix repens* ($n = 6$) when inoculated with *Glomus mosseae*, *Hebeloma leucosarx* and *Paxillus involutus* or not inoculated as affected by soil pH (7, 5.5, 4) and N/P ratio (5.4, 16.2, 48.6) after 26 weeks. Different letters per fungal treatment indicate significant differences among soil treatments according to 2-factor ANOVA and LSD-tests ($P < 0.05$) (see opposite page).

but the opposite trend occurred at pH 4 (TABLE 7-6). No pattern related to soil N/P ratio was found for *G. mosseae* and *P. involutus* showed a very high P (%) at pH 7 with N/P ratio 5.4. Shoot N concentrations at the short term were generally increasing with increasing soil N/P ratio, but plants inoculated with *H. leucosarx* or *P. involutus* showed no difference between N/P ratios 16. 2 and 48.6 (TABLE 7-6). On the long term all fungal treatments generally showed increasing N (%) with increasing soil N/P ratio.

Data on shoot N and P contents were not related to shoot N and P concentrations. Both the non-mycorrhizal plants and plants inoculated with *G. mosseae* indicated that P content was lowest at N/P 48.6 (TABLE 7-7). After 12 weeks, this was also found at pH 4 for plants inoculated with *H. leucosarx* and *P. involutus*, but not at the other pH's. However, after 26 weeks, P contents were generally lowest for all fungal treatments at N/P ratio 48.6, irrespective of soil pH. Shoot N contents were generally highest at N/P ratio 16.2, irrespective of fungal treatment. After 26 weeks, all fungi significantly increased shoot N content compared to the non-mycorrhizal treatment at pH 5.5 with N/P ratio 16.2 (and for *G. mosseae* and *H. leucosarx* also at pH 4) (TABLE 7-8). Furthermore, after 12 weeks the effectiveness of *G. mosseae* concerning shoot N content was significantly lower in six out of nine soil treatment combinations, but was generally positive after 26 weeks. Both *H. leucosarx* and *P. involutus* showed generally positive effectiveness (TABLE 7-8). When considering shoot P content, both *H. leucosarx* and *P. involutus* showed positive effectiveness over both harvests. In contrast, *G. mosseae* was ineffective over the first 12 weeks, while it was generally effective over 26 weeks, but only significantly better than the control at pH 4 with N/P ratio 16.2 (TABLE 7-8).

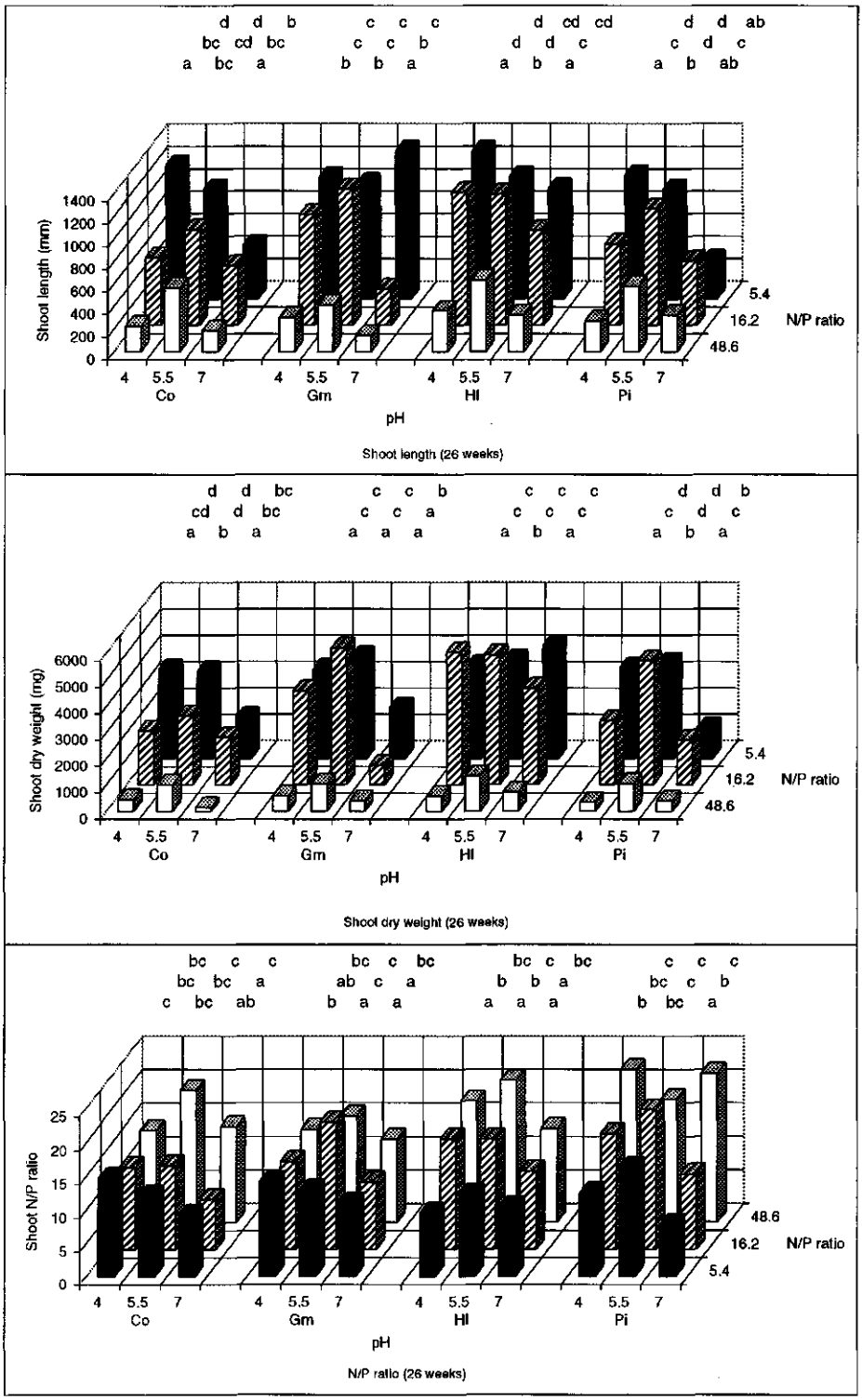

d d b
bc cd bc
a bc a
c c c
c c b
b b a
d cd cd
d d c
a b a
d d ab
c d c
a b ab
Shoot length (mm)
1400
1200
1000
800
600
400
200
0
5.4
16.2
48.6
N/P ratio
4 5.5 7
Co
Gm
Hl
Pi
pH
Shoot length (26 weeks)
d d bc
cd d bc
a b a
c c b
c c a
a a a
c c c
c c c
a b a
d d b
c d c
a b a
Shoot dry weight (mg)
6000
5000
4000
3000
2000
1000
0
5.4
16.2
48.6
N/P ratio
4 5.5 7
Co
Gm
Hl
Pi
pH
Shoot dry weight (26 weeks)
bc c c
bc bc a
c bc ab
bc c bc
ab c a
b a a
bc c bc
b b a
a a a
c c c
bc c b
b bc a
Shoot N/P ratio
25
20
15
10
5
0
48.6
16.2
5.4
N/P ratio
4 5.5 7
Co
Gm
Hl
Pi
pH
N/P ratio (26 weeks)

**TABLE 7-5.** Mycorrhizal effectiveness (shoot biomass and root length) of *Salix repens* ($n = 6$) when inoculated with *Glomus mosseae*, *Hebeloma leucosarx* and *Paxillus involutus* as affected by soil pH (7, 5.5, 4) and N/P ratio (5.4, 16.2, 48.6) after 12 and 26 weeks.

| | | Shoot biomass | | | Root length | | |
|---|---|---|---|---|---|---|---|
| pH | N/P | *G.mosseae* | *H. leucosarx* | *P. involutus* | *G.mosseae* | *H. leucosarx* | *P. involutus* |
| 12 weeks | | | | | | | |
| 7 | 5.4 | **-0.60** a* | -0.02 b | +0.07 b | -0.18 a | -0.45 a | +0.05 a |
| 7 | 16.2 | -0.35 a | +0.20 b | +0.20 ab | -0.20 ab | **-0.50** a | +0.41 b |
| 7 | 48.6 | **-1.05** a | +0.28 b | +0.17 b | **-1.62** a | -0.21 ab | +0.12 b |
| 5.5 | 5.4 | -0.18 a | +0.07 a | +0.09 a | +0.06 b | -1.63 a | -0.19 b |
| 5.5 | 16.2 | -0.30 a | +0.14 b | +0.14 b | -0.16 b | **-1.25** a | -0.53 b |
| 5.5 | 48.6 | +0.10 a | +0.44 a | +0.44 a | +0.35 b | -0.59 a | +0.25 b |
| 4 | 5.4 | **-0.37** a | +0.21 b | +0.15 b | -0.28 a | -0.19 a | -0.15 a |
| 4 | 16.2 | -0.03 a | **+0.32** b | **+0.44** b | +0.40 b | **-0.44** a | -0.08 ab |
| 4 | 48.6 | +0.20 a | **+0.44** a | **+0.45** a | +0.22 b | -0.47 a | +0.10 ab |
| 26 weeks | | | | | | | |
| 7 | 5.4 | +0.20 ab | **+0.64** b | -0.33 a | +0.36 a | +0.26 a | -0.43 a |
| 7 | 16.2 | **-1.46** a | +0.51 b | -0.05 b | -0.37 a | -0.06 a | -0.01 a |
| 7 | 48.6 | +0.62 a | **+0.79** a | +0.62 a | +0.19 a | +0.45 a | **+0.68** a |
| 5.5 | 5.4 | +0.14 a | +0.12 a | +0.09 a | **+0.32** b | -0.38 a | 0.00 a |
| 5.5 | 16.2 | **+0.50** a | **+0.47** a | **+0.44** a | **+0.43** b | -0.68 a | -0.14 a |
| 5.5 | 48.6 | +0.02 a | +0.25 a | +0.03 a | **+0.65** a | +0.26 a | +0.41 a |
| 4 | 5.4 | 0.00 a | +0.03 a | +0.01 a | +0.26 b | -0.43 a | -0.10 a |
| 4 | 16.2 | **+0.58** ab | **+0.59** b | +0.15 a | **+0.46** b | +0.15 a | +0.17 a |
| 4 | 48.6 | +0.25 a | +0.23 a | +0.33 a | **+0.66** b | -0.20 a | +0.05 a |

* Values printed in bold indicate that average shoot biomass (or root length) is significantly different from the non-mycorrhizal treatment, and different letters within a row indicate that average shoot biomass (or root length) is significantly different between fungal treatments according to LSD and three-factor ANOVA ($P < 0.05$).

**TABLE 7-6.** Shoot N and P concentrations of *Salix repens* ($n$ = 6) when inoculated with *Glomus mosseae*, *Hebeloma leucosarx* and *Paxillus involutus* or not inoculated as affected by soil pH (7, 5.5, 4) and N/P ratio (5.4, 16.2, 48.6) after 12 and 26 weeks.

| | | Control | | *G. mosseae* | | *H. leucosarx* | | *P. involutus* | |
|---|---|---|---|---|---|---|---|---|---|
| pH | N/P | N (%) | P (%) | N (%) | P (%) | N (%) | P (%) | N (%) | P (%) |
| 12 weeks | | | | | | | | | |
| 7 | 5.4 | 1.57 a* | 0.107 a | 1.46 a | 0.126 a | 1.48 a | 0.140 b | 1.51 a | 0.117 a |
| 7 | 16.2 | 1.84 b | 0.095 a | 1.38 a | 0.090 a | 2.11 b | 0.133 b | 2.08 b | 0.135 a |
| 7 | 48.6 | 2.17 c | 0.066 a | 1.77 bc | 0.077 a | 2.27 b | 0.102 a | 2.04 b | 0.143 a |
| 5.5 | 5.4 | 1.51 a | 0.103 a | 1.23 a | 0.089 a | 1.56 a | 0.131 b | 1.32 a | 0.148 a |
| 5.5 | 16.2 | 1.97 bc | 0.080 a | 1.75 bc | 0.098 a | 2.08 b | 0.131 b | 2.23 b | 0.144 a |
| 5.5 | 48.6 | 1.97 bc | 0.107 a | 2.03 cd | 0.095 a | 1.91 b | 0.117 ab | 2.30 b | 0.144 a |
| 4 | 5.4 | 1.45 a | 0.084 a | 1.37 a | 0.105 a | 1.28 a | 0.103 a | 1.58 a | 0.138 a |
| 4 | 16.2 | 1.84 b | 0.090 a | 1.54 ab | 0.083 a | 2.15 b | 0.100 a | 1.98 b | 0.096 a |
| 4 | 48.6 | 2.02 bc | 0.095 a | 2.32 d | 0.098 a | 2.37 b | 0.099 a | 2.38 b | 0.108 a |
| 26 weeks | | | | | | | | | |
| 7 | 5.4 | 1.87 a | 0.263 c | 1.73 a | 0.170 b | 1.50 a | 0.148 a | 1.95 a | 0.357 b |
| 7 | 16.2 | 1.85 a | 0.277 c | 1.79 a | 0.182 b | 1.84 bc | 0.158 a | 1.98 a | 0.185 a |
| 7 | 48.6 | 2.18 ab | 0.118 a | 2.33 c | 0.125 a | 2.48 d | 0.143 a | 2.48 bc | 0.133 a |
| 5.5 | 5.4 | 1.87 a | 0.155 b | 1.91 ab | 0.152 ab | 1.57 a | 0.130 a | 1.87 a | 0.117 a |
| 5.5 | 16.2 | 2.13 ab | 0.185 b | 2.10 bc | 0.113 a | 2.11 c | 0.138 a | 2.47 bc | 0.127 a |
| 5.5 | 48.6 | 2.36 b | 0.135 ab | 2.39 c | 0.152 ab | 2.57 d | 0.124 a | 2.30 b | 0.135 a |
| 4 | 5.4 | 1.97 a | 0.137 ab | 1.89 a | 0.142 ab | 1.75 b | 0.156 a | 1.82 a | 0.158 a |
| 4 | 16.2 | 2.15 ab | 0.182 b | 2.13 bc | 0.178 b | 2.11 c | 0.130 a | 2.20 b | 0.140 a |
| 4 | 48.6 | 2.49 b | 0.208 bc | 2.36 c | 0.173 b | 3.00 e | 0.140 a | 2.85 c | 0.138 a |

* Different letters within a column (per harvest period) indicate significant differences according to a three factor ANOVA and LSD tests ($P < 0.05$).

**TABLE 7-7.** Shoot N and P contents of *Salix repens* ($n = 6$) when inoculated with *Glomus mosseae*, *Hebeloma leucosarx* and *Paxillus involutus* or not inoculated as affected by soil pH (7, 5.5, 4) and N/P ratio (5.4, 16.2, 48.6) after 12 and 26 weeks.

| | | Control | | *G. mosseae* | | *H. leucosarx* | | *P. involutus* | |
|---|---|---|---|---|---|---|---|---|---|
| pH | N/P | N (mg) | P (mg) | N (mg) | P (mg) | N (mg) | P (mg) | N (mg) | P (mg) |
| 12 weeks | | | | | | | | | |
| 7 | 5.4 | 6.53 bc* | 0.438 b | 3.80 ab | 0.295 cd | 6.16 a | 0.556 b | 6.69 a | 0.519 ab |
| 7 | 16.2 | 6.54 bc | 0.307 ab | 3.62 ab | 0.236 b | 9.31 ab | 0.563 b | 9.23 b | 0.556 ab |
| 7 | 48.6 | 6.54 bc | 0.188 a | 2.64 a | 0.118 a | 9.77 ab | 0.441 ab | 7.60 ab | 0.413 a |
| 5.5 | 5.4 | 6.43 bc | 0.412 b | 4.65 c | 0.323 d | 7.08 a | 0.567 b | 6.25 a | 0.678 cd |
| 5.5 | 16.2 | 10.21 d | 0.420 b | 6.82 d | 0.359 d | 12.61 b | 0.769 c | 13.44 c | 0.841 d |
| 5.5 | 48.6 | 5.26 ab | 0.268 a | 6.31 cd | 0.252 bc | 8.07 ab | 0.439 b | 10.85 b | 0.599 b |
| 4 | 5.4 | 6.39 bc | 0.362 b | 4.34 bc | 0.317 cd | 7.02 a | 0.568 b | 7.70 ab | 0.650 bc |
| 4 | 16.2 | 7.39 c | 0.350 b | 6.00 cd | 0.304 cd | 12.73 b | 0.584 b | 14.18 c | 0.688 d |
| 4 | 48.6 | 4.64 a | 0.204 a | 6.56 cd | 0.247 b | 9.94 ab | 0.366 a | 9.86 b | 0.425 a |
| 26 weeks | | | | | | | | | |
| 7 | 5.4 | 28.00 bc | 2.498 c | 34.79 ab | 2.961 bc | 64.01 c | 6.281 b | 21.80 b | 2.742 c |
| 7 | 16.2 | 33.07 bc | 5.034 d | 14.28 a | 1.300 a | 67.97 c | 5.720 b | 33.96 c | 3.088 cd |
| 7 | 48.6 | 3.26 a | 0.163 a | 6.26 a | 0.334 a | 12.78 a | 0.750 a | 9.94 a | 0.579 a |
| 5.5 | 5.4 | 64.06 d | 5.123 d | 73.42 c | 5.811 d | 58.66 c | 4.855 b | 67.65 c | 4.188 de |
| 5.5 | 16.2 | 56.90 d | 4.627 d | 108.17 c | 5.872 d | 107.34 d | 6.518 b | 115.63 d | 6.014 e |
| 5.5 | 48.6 | 23.02 b | 1.364 bc | 23.56 ab | 1.506 ab | 33.66 b | 1.615 a | 23.19 b | 1.383 b |
| 4 | 5.4 | 66.95 d | 4.643 d | 64.35 b | 4.269 cd | 50.93 c | 4.538 b | 62.50 c | 5.248 e |
| 4 | 16.2 | 44.63 cd | 3.516 cd | 75.61 c | 5.991 d | 104.74 d | 6.565 b | 54.11 c | 3.092 cd |
| 4 | 48.6 | 10.19 a | 0.895 ab | 13.20 a | 0.977 a | 14.25 a | 0.620 a | 9.52 a | 0.439 a |

* Different letters within a column (per harvest period) indicate significant differences according to a three factor ANOVA and LSD tests ($P < 0.05$).

**TABLE 7-8.** Mycorrhizal effectiveness (shoot N and P contents) of *Salix repens* ($n = 6$) when inoculated with *Glomus mosseae*, *Hebeloma leucosarx* and *Paxillus involutus* as affected by soil pH (7, 5.5, 4) and N/P ratio (5.4, 16.2, 48.6) after 12 and 26 weeks.

| | | Shoot N content | | | Shoot P content | | |
|---|---|---|---|---|---|---|---|
| pH | N/P | *G.mosseae* | *H. leucosarx* | *P. involutus* | *G.mosseae* | *H. leucosarx* | *P. involutus* |
| 12 weeks | | | | | | | |
| 7 | 5.4 | **-0.72** a* | -0.06 b | +0.02 b | **-0.48** a | +0.21 b | +0.16 b |
| 7 | 16.2 | **-0.81** a | +0.30 b | +0.29 b | -0.30 a | **+0.45** b | **+0.45** b |
| 7 | 48.6 | **-1.48** a | +0.33 b | +0.14 b | **-0.59** a | **+0.57** b | **+0.54** b |
| 5.5 | 5.4 | **-0.38** a | +0.09 b | -0.03 b | **-0.28** a | **+0.27** b | **+0.39** c |
| 5.5 | 16.2 | **-0.50** a | +0.19 b | +0.24 b | -0.17 a | **+0.45** b | **+0.50** b |
| 5.5 | 48.6 | +0.17 a | +0.35 a | +0.52 a | -0.06 a | **+0.39** b | **+0.55** b |
| 4 | 5.4 | **-0.47** a | +0.09 b | +0.17 b | -0.14 a | **+0.36** b | **+0.44** b |
| 4 | 16.2 | -0.23 a | **+0.42** b | **+0.48** b | -0.15 a | **+0.40** b | **+0.49** b |
| 4 | 48.6 | +0.29 a | **+0.53** a | **+0.53** a | +0.17 a | **+0.44** b | **+0.52** b |
| 26 weeks | | | | | | | |
| 7 | 5.4 | +0.20 ab | **+0.56** b | -0.28 a | +0.16 a | **+0.60** b | +0.09 a |
| 7 | 16.2 | **-1.32** a | +0.51 b | +0.03 b | **-2.87** a | +0.12 b | -0.63 b |
| 7 | 48.6 | +0.48 a | **+0.74** a | **+0.67** a | +0.51 a | **+0.78** a | +0.72 a |
| 5.5 | 5.4 | +0.13 a | -0.09 a | +0.05 a | +0.12 b | -0.06 ab | -0.22 a |
| 5.5 | 16.2 | **+0.47** a | **+0.47** a | **+0.50** a | +0.21 a | +0.29 a | +0.23 a |
| 5.5 | 48.6 | +0.02 a | +0.32 a | +0.01 a | +0.09 a | +0.16 a | +0.01 a |
| 4 | 5.4 | -0.04 a | -0.31 a | -0.07 a | -0.09 a | -0.02 a | +0.12 a |
| 4 | 16.2 | **+0.41** ab | **+0.57** b | +0.18 a | **+0.41** b | **+0.46** b | -0.14 a |
| 4 | 48.6 | +0.23 b | **+0.28** b | -0.07 a | +0.08 b | -0.44 ab | **-1.04** a |

* Values printed in bold indicate that average shoot N content (or P content) is significantly different from the non-mycorrhizal treatment, and different letters within a row indicate that average shoot N content (or P content) is significantly different between fungal treatments according to LSD and three-factor ANOVA ($P < 0.05$).

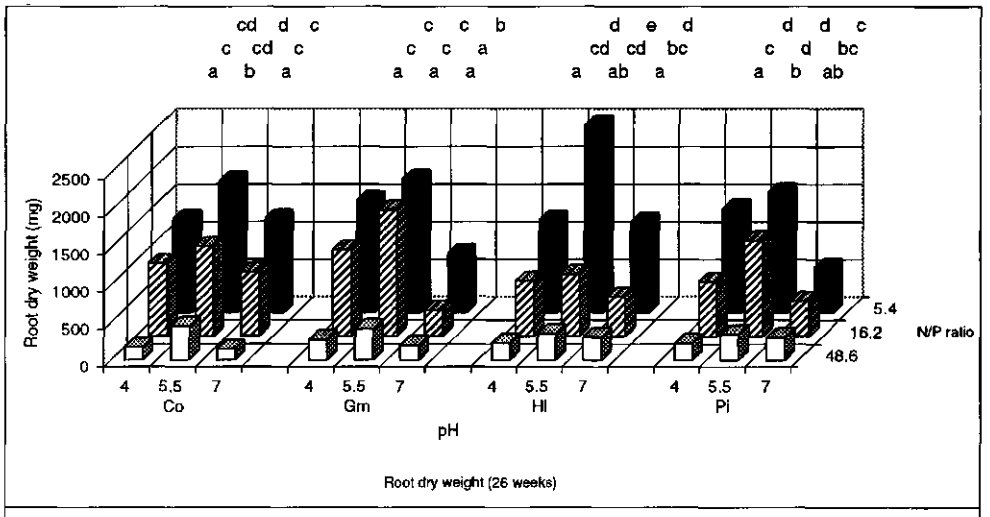
*Below-ground plant performance*

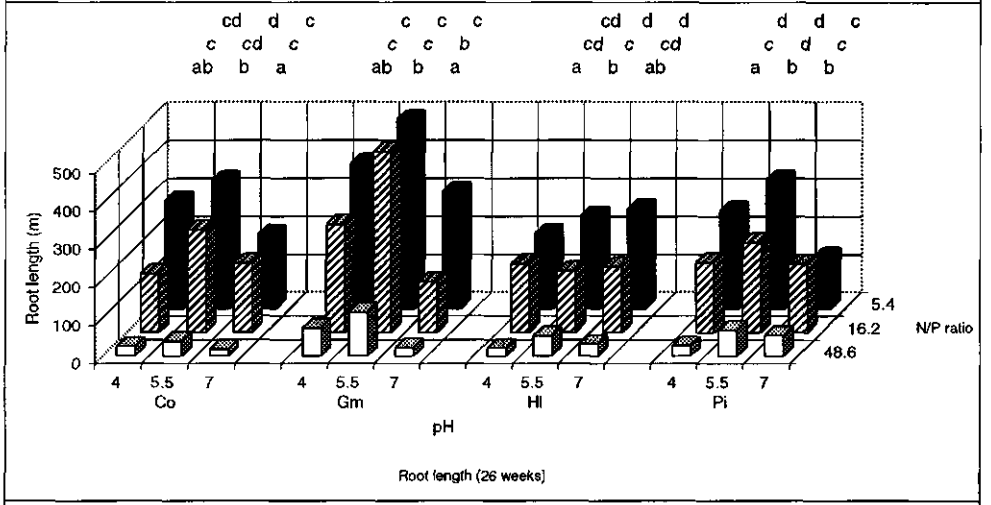
The effectiveness of the different fungi for root length after 12 weeks was generally lowest for *H. leucosarx* (TABLE 7-5). After 26 weeks, effectiveness of *G. mosseae* was positive in eight out of nine soil treatments, whereas for *H. leucosarx* and *P. involutus* effectiveness was both positive and negative. Moreover, for *G. mosseae* at pH 5.5, irrespective of N/P ratio and at pH 4 with N/P ratios 16.2 and 48.6 root length was larger than the non-mycorrhizal treatment (TABLE 7-5 AND FIG. 7-3b). The pattern of root length and root biomass for the non-mycorrhizal treatment over the soil treatment combinations was comparable (FIGS. 7-3a,b). Root length as affected by *G. mosseae* was very high, but root biomass was not very different from the non-mycorrhizal plants and plants inoculated with *P. involutus*. For *H. leucosarx* root biomass in the soil treatment pH 5.5 and N/P ratio 5.4 was very high compared to its root length. This resulted in a generally high specific root length for plants inoculated with *G. mosseae*, whereas specific root length of plant inoculated with *H. leucosarx* at pH 5.5 with N/P ratio 5.4 was very low (FIG. 7-3c).

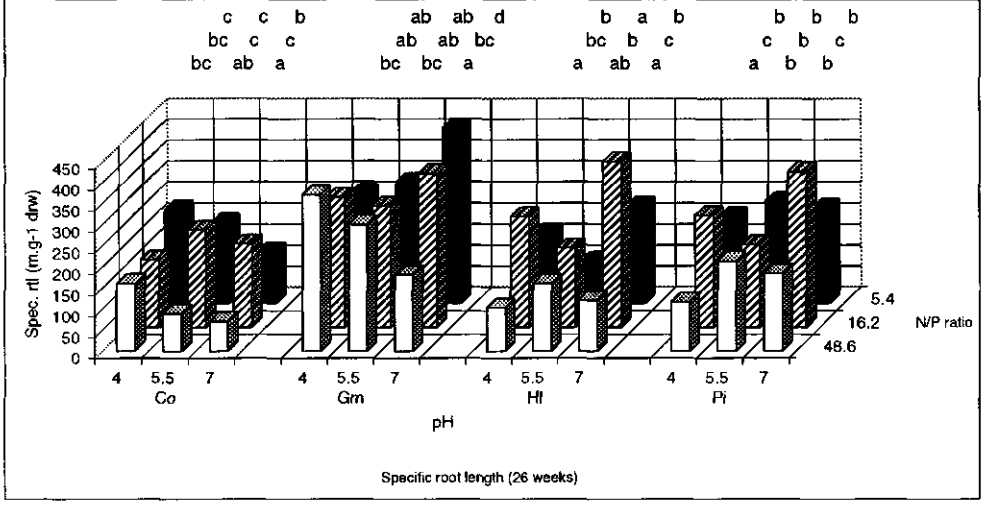
At the second harvest, at soil N/P ratio 48.6 all plants were very small, irrespective of fungal symbiont (FIG. 7-4). Furthermore, *H. leucosarx* increased biomass at N/P ratios 5.4 and 16.2 irrespective of pH compared to the non-mycorrhizal plants, whereas *G. mosseae* and *P. involutus* increased biomass at N/P ratio 5.4 with pH 4 or 5.5 and at N/P ratio 16.2 with pH 5.5 (FIG. 7-4).

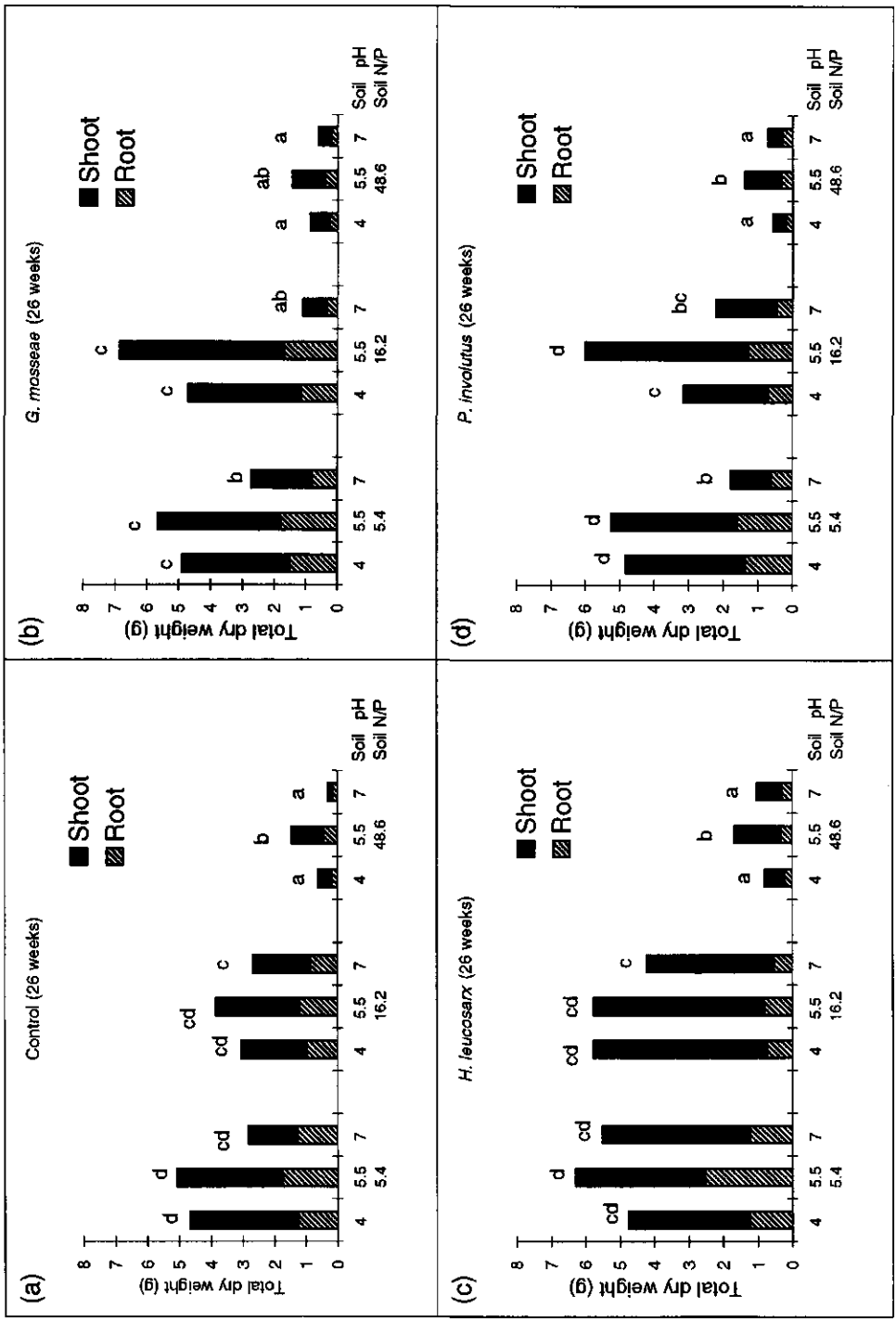
**FIGURE 7-3.** Root dry weight (a), root length (b) and specific root length (c) of *Salix repens* ($n = 6$) when inoculated with *Glomus mosseae*, *Hebeloma leucosarx* and *Paxillus involutus* or not inoculated as affected by soil pH (7, 5.5, 4) and N/P ratio (5.4, 16.2, 48.6) after 26 weeks. Different letters per fungal treatment indicate significant differences among soil treatments according to 2-factor ANOVA and LSD-tests ($P < 0.05$) (see page 163).

**FIGURE 7-4.** Total plant dry weight of *Salix repens* ($n = 6$) not inoculated (a) of when inoculated with *Glomus mosseae* (b), *Hebeloma leucosarx* (c) and *Paxillus involutus* (d) as affected by soil pH (7, 5.5, 4) and N/P ratio (5.4, 16.2, 48.6) after 26 weeks. Different letters per fungal treatment indicate significant differences among soil treatments according to 2-factor ANOVA and LSD-tests ($P < 0.05$) (see page 164).


cd d c
c cd c
a b a
c c b
c c a
a a a
d e d
cd cd bc
a ab a
d d c
c d bc
a b ab
Root dry weight (mg)
2500
2000
1500
1000
500
0
5.4
16.2
48.6
N/P ratio
4 5.5 7
Co
Gm
Hl
Pi
pH
Root dry weight (26 weeks)


cd d c
c cd c
ab b a
c c c
c c b
ab b a
cd d d
cd c cd
a b ab
d d c
c d c
a b b
Root length (m)
500
400
300
200
100
0
5.4
16.2
48.6
N/P ratio
4 5.5 7
Co
Gm
Hl
Pi
pH
Root length (26 weeks)


c c b
bc c c
bc ab a
ab ab d
ab ab bc
bc bc a
b a b
bc b c
a ab a
b b b
c b c
a b b
Spec. rtl (m.g-1 drw)
450
400
350
300
250
200
150
100
50
0
5.4
16.2
48.6
N/P ratio
4 5.5 7
Co
Gm
Hl
Pi
pH
Specific root length (26 weeks)


(a)
Control (26 weeks)
(b)
G. mosseae (26 weeks)
(c)
H. leucosarx (26 weeks)
(d)
P. involutus (26 weeks)
Shoot
Root
Total dry weight (g)
Soil pH
Soil N/P

## DISCUSSION

### *Soil properties*

The plant nutrient that normally limits production in temperate forest ecosystems is nitrogen. The trees dominating these ecosystems are usually ectomycorrhizal and nutrient uptake is thus dependent on the activity of the ectomycorrhizal fungal partners (Read, 1991). Read (1989) also mentioned that over a dune successional gradient a shift of vegetation associated with AMF towards a vegetation associated with EcMF occurs. Fertilization experiments in England (Willis, 1963) confirmed that initial vegetation stages responded more to P-fertilization and later stages to N-fertilization. However, dune ecosystems on the Wadden Isles in the Netherlands were N-limited over the whole successional range (Kooijman *et al.*, 1998). Possibly, changes in nutrient limitation during succession are dependent on initial pH and rate of decalcification. For that reason, both relative availabilities of nitrogen and phosphorus, and pH were varied independently. It should be borne in mind, however, that in the field both variables are not independent, as the actual behaviour of the various ions is pH dependent (Marschner, 1995). At pH 7, presence of $CaCO_3$ will reduce availability of P. In order to minimize this effect we decided to percolate the pots at regular times with the nutrient solution. Soil analysis indicated that this treatment was largely successful.

### *Mycorrhizal establishment and colonization as affected by pH and N/P ratio*

The experiment included two harvests, viz. after 12 weeks (short term) and 26 weeks (long term, i.e. a growing season). Short-term effects relate mainly to the effects of pH and nutrient availability on mycorrhizal establishment, long-term effects relate to effects on mycorrhizal fungal and plant development.

Mycorrhizal colonization by *P. involutus* increased with higher nitrogen availabilities in the first 12 weeks. *Hebeloma leucosarx* also increased proportional colonization with increased N/P ratios, in the first 12 weeks, although to a smaller extent. For both EcMF species, however, root length (and also absolute root length colonized) decreased with higher nitrogen availabilities. A lower investment in roots has been often reported in N-fertilization trials (Marschner, 1995). Lower root length with increasing nitrogen availabilities was also observed for plants colonized by *G. mosseae* and non-mycorrhizal plants. Mycorrhizal colonization intensity by *G. mosseae* also increased with increasing N-availabilities. Fertilization experiments have often indicated a negative effect of nitrogen on the extramatrical mycelium (Arnebrant, 1994) or the amount of root

colonization (Termorshuizen & Ket, 1991; Wallander & Nylund, 1991). The larger sensitivity of the external mycelium compared to the proportional colonization of the EcMF, over approximately 10 weeks, has also been confirmed by Wallander & Nylund (1992) and Ekblad *et al.* (1995). Sensitivity of both parameters is also species-specific. Observations of mycelial growth in the root growth chamber and of the thickness of the ectomycorrhizal sheath indicated that the extramatrical mycelium of *H. leucosarx* was not affected by N-availability in the first 12 weeks; however, its sporocarp formation was exclusively observed at pH 5.5 in combination with N/P ratio 5.4, coinciding with a much higher root/mycorrhiza biomass after 26 weeks. Both the extramatrical mycelium and mantle thickness of *P. involutus* were higher with the higher N/P ratio in the first 12 weeks. However, after 26 weeks thick mantles of *P. involutus* were only observed at N/P 16.2 in combination with pH 5.5.

pH had a smaller effect on extent of EcM colonization, although the proportional colonization tended to be higher at lower pH. This effect was largely independent of effects of pH on root length (and hence absolute root length colonized). Ek *et al.* (1994) and Erland & Söderström (1991) noted an optimum growth of *P. involutus* at pH values below 5 (in water). The high colonization found in the present study at pH 4 (in $CaCl_2$) in combination with N/P 48.6 is better explained by the very short root length of *S. repens* under these conditions (occurring irrespective of the fungal treatment). In the present study on the long term mycorrhizal colonization and development of *P. involutus* was best at pH 5.5 (in $CaCl_2$), but only in combination with N/P 16.2. However, *P. involutus* is a highly versatile species which is adapted to a wide range of pH's (Laiho, 1970; Cairney, 1999). Soil acidity has long been known to affect mycorrhiza formation and hyphal development, to a degree dependent on the plant species and the mycorrhizal fungus involved (Sharpe & Marx, 1986). The ability of *Pisolithus tinctorius* inoculum to infect plant roots declined when the pH (in water) of a nursery soil was raised 4.8 to 6.8 (Marx, 1990). In the present study colonization by *P. involutus* was also lower at pH 7 than pH 4 (in $CaCl_2$), but for *H. leucosarx* it was not. Erland & Söderström (1990) found that infection of five mycorrhizal types on *Pinus sylvestris* rose from 70 % to nearly 100 % with an increase of pH in pine forest soil from 4 to 5 (in water). They also reported that the number of mycorrhizal root tips declined beyond pH 5, whereas in the present study they were not affected by pH. However, pH 4 (in water) is even slightly more acidic than the pH 4 treated in the present study and sensitivity of the EcMF used in this study might be higher for such low pH as well. *Hebeloma leucosarx* seemed to be very tolerant and showed very high infection

levels over all nine soil treatment combinations, and was only slightly suppressed at pH 7 in combination with N/P ratios 5.4 and 16.2. Comparisons between these studies, however, are difficult as different methods, substrates and fungal isolates were used. Moreover, soil nutrient availabilities were not discussed, although changes in pH would have changed nutrient availability as well.

pH had almost no effect on intensity of root colonization by *G. mosseae*, while root length and absolute root length colonized somewhat increased at lower pH. Establishment and growth of a mycorrhizal fungus at an optimum pH and N/P was not just a fungal species character, but depended on the interaction with the plant host. Mycorrhizal colonization of *G. mosseae* on *T. repens* was highest at N/P 16.2, but on *S. repens* at N/P 48.6 (irrespective of pH). This difference may indicate plant control over the amount of AM colonization (CHAPTERS 3 AND 10).

In the present study mycorrhizal colonization changed over time. Thus not only establishment of the mycorrhizal association was affected by the different soil treatments, but their development was different between the different fungal species as well. Whereas *H. leucosarx* showed no difference between the different soil treatments and increased over 26 weeks towards 100 % colonization, the differences in colonization by *P. involutus* over 26 weeks became more clearly affected by the different soil treatments. *Paxillus involutus* showed an optimum colonization at pH 5.5 in combination with N/P ratio 16.2, but with N/P ratio 48.6 its colonization significantly declined. Colonization by *G. mosseae* over 26 weeks showed a similar trend as over 12 weeks, higher colonization was observed with increasing nitrogen and thus might be related to P limitation in *S. repens*. However, the significantly increased colonization at pH 7 in combination with N/P 48.6, was due to higher amounts of intraradical spore formation. Intraradical spore formation was earlier observed in *S. repens* after waterlogging and in flooded parts of field sites (CHAPTER 10). This phenomenon was interpreted as a reaction of AMF to unfavourable environmental conditions. It is possible that the same fungal strategy was observed in this experiment as plant performance under that condition was very low (indicating non-optimal soil conditions).

*Mycorrhizal effect on plant performance as affected by pH and N/P ratio*

Increases in nitrogen and phosphorus contents after colonization by arbuscular and ectomycorrhizal fungi, often connected with plant growth increase, are well documented (Smith & Read, 1997). Using methods that separate hyphae from the mycorrhizal roots

long distance N and P transport by external hyphae of arbuscular mycorrhizal fungi (George *et al.*, 1995) and ectomycorrhizal fungi (Brandes *et al.*, 1998) has been quantified. In the field both the plant roots and mycorrhizal mycelium are simultaneously exposed to the same substrate (nutrients and soil pH). In the present study hyphae and mycorrhizal roots were therefore not separated. It should be noted, however, that nutrients simultaneously supplied to plant and fungus can possibly lead to a down-regulation of C allocation from the plant to the fungus, whereas nutrients supplied exclusively to the extramatrical mycelium may have completely different effects on reaction of the mycelium (Brandes *et al.*, 1998).

*Glomus mosseae* seemed, under these fertilized conditions, rather ineffective in the short term with respect to shoot biomass and nutrient uptake. However, it was clearly effective over the longer term. In comparison with the other fungal treatments its effect on root length and specific root length was tremendous. Arbuscular mycorrhizal fungal effects were manifested over a longer term, whereas EcMF effects became manifested over 12 weeks. The opposite temporal pattern was found in previous experiments performed in nutrient poor soil, although effects of *G. mosseae* on root structure were the same (CHAPTERS 4 AND 5). *Glomus mosseae* used in this experiment was obtained from a culture collection. Different isolates of an AMF species can be physiologically different or adapted to the environmental conditions of their origin, and this might explain why this isolate of *G. mosseae* did not extend the niche of *S. repens* towards growth under alkaline conditions as hypothesized. Colonization of *T. repens* by *G. mosseae* was much higher than that of *S. repens* by the same isolate. In fact all studies in this THESIS indicated very low colonization intensity (except when intraradical spores were formed). The possibility that this consistently low colonization was due to P suffiency in the soils used was tested in this experiment where a very high N/P ratio must have resulted in strong P limitation. Similarly low AM colonization at N/P 48.6 demonstrates that *S. repens* has an intrinsic low AM colonization. Low AM colonization might be more common in dual mycorrhizal plants related to the negative effects of AMF on EcMF (CHAPTERS 4, 8 AND 11).

In an earlier experiment in the absence of fertilizer addition (CHAPTER 6) *P. involutus* turned out to be more nitrophilic than *H. leucosarx* (also in vitro shown in CHAPTER 12). In the present study, after 12 weeks *P. involutus* had a larger benefit than in the previous study in agreement with hypothesis 5. However, observations after 26 weeks suggested that even at high N availabilities *H. leucosarx* was equally beneficial as *P. involutus*. *Hebeloma leucosarx* extended the niche of *S. repens* towards growth under

alkaline conditions. It performed best in eight out of nine combinations and had mycorrhizal colonization towards 100 % in all nine soil treatment combinations. *Hebeloma leucosarx* and *P. involutus* were obtained from these dune *S. repens* communities. However, plant performance in this experiment was not related to the fact that these particular isolates were collected from acidic field sites. Sporocarps of both species were found in all habitat types (calcareous-wet, calcareous-dry, acidic-wet, acidic-dry), but mycorrhizal root tips of both species were generally higher in the calcareous plots. However, the number of mycorrhizas (and ectomycorrhizal root length) formed by *H. leucosarx* and its numbers of sporocarps was higher than by *P. involutus* (CHAPTERS 2 AND 3).

When the two EcMF and the AMF species are compared under these conditions, plant parameters showed less differences than in a previous experiment in nutrient poor soil with pH 5.8 (in $CaCl_2$). These earlier studies in nutrient-poor soil contradicted the results of Jones *et al.* (1998) who suggested that all EcMF treatments were more effective than all AMF treatments in their study. However, in the present study, effects of AMF versus EcMF over 12 weeks are in agreement with Jones *et al.* (1998).

Multifunctionality of mycorrhizal associations involves three elements, of which conditionality (differential effects due to environmental variation) is one. This study provides ample evidence that conditionality of mycorrhizal responses to a variety of soil conditions is substantial, both in terms of magnitude of response and timing of response. The fact that, even in the face of conditionality, *S. repens* under field conditions is always highly EcM and only slightly AM suggests that conditionality can be constrained by plant control over mycorrhizal colonization.

# 8

## Mycorrhizal infection of *Salix repens* II: Interactions between AM and EcM in *Salix repens* communities with contrasting soil chemical and physical characteristics.

**ABSTRACT**

*Interactions between arbuscular mycorrhizas and ectomycorrhizas on roots of* Salix repens *were investigated in the field and in field soil under laboratory conditions. The interaction was studied in two soils that represent a young dune successional stage (calcareous, humus-poor soil) and an old dune successional stage (acidic, humus-rich soils). Initial inoculation with the arbuscular mycorrhizal fungus (AMF)* Glomus mosseae *negatively affected subsequent colonization by native EcM fungi, but initial inoculation with the ectomycorrhizal fungus (EcMF)* Hebeloma leucosarx *did not affect subsequent colonization by native arbuscular mycorrhizal fungi. The negative interaction between mycorrhizal types was transient and hardly noticeable after 24 weeks. Uninoculated plants that were simultaneously exposed to AMF and EcMF did not show a negative interaction between both mycorrhizal types. Inoculum potential of both AMF and EcMF was higher in the younger site. Young and old sites differed in EcM morphotype composition, but this difference did not affect the outcome of the interaction with AMF. Plant response to inoculation with* H. leucosarx *was initially higher than to inoculation with* G. mosseae. *These differences persisted after outplanting, and uninoculated cuttings relatively performed best in terms of carbon gain and nitrogen and phosphorus uptake. Arbuscular mycorrhizal fungi transiently mobilize plant-defence reactions. The ecological implications of this mobilization are discussed for the evolution of dual mycorrhizas.*

**KEY WORDS**: Ectomycorrhiza, arbuscular mycorrhiza, interaction, dual mycorrhiza, *Salix repens*, plant-defence systems.

## INTRODUCTION

One of the most important mutualistic relationships is the one between plant roots and fungi - mycorrhiza - which is repeatedly reported to improve growth and survival of the plant, e.g., by enhancing nutrient uptake and providing protection against pathogens (Smith & Read, 1997). The costs caused by the fungi may be high, with estimates ranging between 7 and 60 % of the photosynthate production supporting the mycorrhizal fungus (Stribley *et al.*, 1980; Finlay & Söderström, 1992; Rygiewicz & Andersen, 1994). This relationship is considered to be especially important in nutrient-poor habitats, where plant survival may not otherwise be possible (Chapin, 1980; Read, 1990). It has been suggested that mycorrhizal fungi become less or not beneficial to their hosts in fertile soils (Fitter, 1977; Bowen, 1980), shifting the relationship from mutualism to parasitism (Johnson *et al.*, 1997).

The two most widespread types of mycorrhizal associations are ectomycorrhiza (EcM) and arbuscular mycorrhiza (AM). Even though most plants form only one type of mycorrhizal association, some plants, e.g. *Salix*, *Populus*, *Alnus* and *Eucalyptus*, can form both AM and EcM associations (Harley & Harley, 1987). *Salix repens* L. is one of these dual mycorrhizal plant species. *Salix repens* occurs in a wide variety of habitats, ranging from dry to wet, and from calcareous, humus-poor to acidic, humus-rich soils. Below-ground observations showed that *S. repens* had consistently low AM (less than 11 %) and high EcM (usually above 85 %) colonization (CHAPTER 3). Considering the differences between both mycorrhizal types, the question may be raised whether dual mycorrhizal plants can increase their niche breadth depending on the kind of mycorrhizal association.

Previously, in a laboratory study under controlled conditions, the interaction between mycorrhizal types was investigated by simultaneous and sequential inoculation (CHAPTER 4). Colonization by *Glomus mosseae* suppressed subsequent colonization by three ectomycorrhizal fungi (*Hebeloma leucosarx*, *Inocybe vulpinella* and *Paxillus involutus*), whereas *G. mosseae* was not inhibited by previous colonization by *H. leucosarx* and *I. vulpinella* and only slightly by *P. involutus*. When both functional types were inoculated at the same time, both types colonized similar proportions of the root system as in their single inoculations (CHAPTERS 4 AND 5). As these results contradicted an earlier hypothesis about negative interactions between AM and EcM in which EcM was considered as the stronger competitor (Lodge & Wentworth, 1990; Chilvers *et al.*, 1987), the necessity for examination under natural conditions was evident. However, in the field a number of influences occur (climate, vegetation type, pathogens, shade, soil structure,

variable ground water table and spatial occurrence of large or small mycorrhizal mycelia) that might interfere with the outcome of the interaction. Therefore, an experiment performed in field soil under controlled conditions was considered an important intermediate step. This combination of a field experiment and laboratory experiment investigated the interaction between, and the functional significance of, both mycorrhizal types in two different dune successional stages, viz. a calcareous, humus-poor *Salix repens* community (young) and an acidic, humus-rich *Salix repens* community (old). During succession in the dune ecosystem a shift from P-limitation towards N-limitation is suggested (Read, 1989), and consequently a shift in mycorrhizal type (AM towards EcM).

Hypotheses tested in this study were that (1) *G. mosseae* will suppress EcM colonization; (2) this suppression will be less in the older successional stage, as plant N demands may interfere with the outcome of the interaction; (3) suppression of EcM by AM is stronger in the lab than in the field, as in the lab (in field soil) inoculum potential of mycelia will be less; (4) control plants will show no interference between EcM and AM colonization and behave similar to the simultaneous inoculation in the previous experiment; (5) *G. mosseae* will show a higher significance for *S. repens* in the younger successional site, whereas *H. leucosarx* will show the higher significance in the older successional site, both in the lab (in field soil) and in the field.

**TABLE 8-1.** Soil chemical characteristics of the two *Salix repens* communities: Schoenus (relatively young dune successional stage) and Groene strand (relatively old dune successional stage).

| | Schoenus (young successional stage) | Groene strand (old successional stage) |
|---|---|---|
| pH ($CaCl_2$) | 6.2 | 4.3 |
| $CaCO_3$ content (%) | 0.4 | 0 |
| Organic matter (%) | 5.0 | 35.1 |
| N total ($mg.kg^{-1}$) | 796 | 6412 |
| P total ($mg.kg^{-1}$) | 112 | 458 |
| N dissolved ($mg.kg^{-1}$) | 19.7 | 48.9 |
| P dissolved ($mg.kg^{-1}$) | 1.6 | 4.5 |
| N/P total | 7.1 | 14.0 |
| N/P dissolved | 12.3 | 10.9 |
| Moisture (Ellenberg) | 7.3 | 7.0 |

## MATERIAL AND METHODS

*Field description, plant and fungal material*

Two field sites on the isle of Terschelling were selected for this experiment: Schoenus (calcareous, humus-poor) and Groene strand (acidic, humus-rich). These field sites cover the extremes of a dune successional gradient for *Salix repens* communities. Soil characteristics of these field sites are presented in TABLE 8-1. Soil moisture values were based on indicator values of plant species by taking cover-abundance into account (Ellenberg, 1974). This system is based on grouping plant species into a number of preference groups and assigning numerical scores to these groups. By weighted averaging these scores are converted to scores for individual plots (Jongman *et al.*, 1987). Vegetation, EcM sporocarp number and species composition, and below ground colonization (AM and EcM) are presented in APPENDIX A AND CHAPTERS 2 AND 3.

Cuttings (shoot tops) of *Salix repens* were collected in December 1996 from the field site Schoenus. All cuttings were collected from the same male plant in order to obtain genetic homogeneity.

*Hebeloma leucosarx* (L1) was collected in the autumn of 1994 from *S. repens* from an acidic, humus-rich soil. Sporocarps were surface sterilized with alcohol (70 %) and sliced in half under sterile conditions. Fungal tissue was cut from the innerside of the cap and transferred to and maintained on solid media (alternative Melin Norkrans (AMN)) containing (in $g.l^{-1}$): agar (15), malt extract (3), glucose.$H_2O$ (10), $(NH_4)_2HPO_4$ (0.25), $KH_2PO_4$ (0.5), $KNO_3$ (0.5), $MgSO_4.7H_2O$ (0.15), $CaCl_2.H_2O$ (0.67), NaCl (0.025), $FeCl_3$ (0.0012) and thiamine.HCl (100 μg). An AMF starter culture was kindly provided by the European Bank of Glomales (France), *Glomus mosseae* (BEG 12). This culture was maintained on *Trifolium repens* L. in sandy soil, supplied with P-poor Hoagland solution.

The selection of the fungal species *H. leucosarx* and *G. mosseae* was based on their contrasting effects on a number of parameters of *S. repens* under poor soil conditions and the occurrence of *H. leucosarx* in all habitat types (but dominant in the calcareous plots). *Glomus mosseae* was identified in the calcareous plots only.

*Inoculation and plant growth conditions*

After storage at 4 °C for one month, the cuttings of *S. repens* were trimmed to 4 cm, surface-sterilized twice in (freshly prepared) 6 % $H_2O_2$ for 1 min (in series of 20 in 300 mL), and rinsed three times in (fresh) demineralized water (1 L) for 10 min. Each

cutting was placed individually in a culture tube containing 20 mL sterile water agar (1 %). After a 10 weeks rooting period in a climate chamber (photon flux density 120 $\mu E.m^{-2}.s^{-1}$ (11,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 70 %) equally developed cuttings were selected for the experiment.

In the inoculation procedure a sandy soil, classified as nutrient- and humus-poor: pH ($CaCl_2$) 5.5, nutrient contents 6 mg $N_{tot}.kg^{-1}$, 6 mg $P_{tot}.kg^{-1}$, and 0 % organic matter was used. Twenty cuttings were harvested at the moment of inoculation (t = 0). Each fungus was individually inoculated to *S. repens* in 300 replicates and 500 cuttings were supplied with non-inoculated agar plugs (control). The isolate of *H. leucosarx* was precultured twice (successively) for three weeks on solid media (AMN) in order to obtain sufficient actively growing fungal material. *Glomus mosseae* was precultured on roots of *T. repens* for 15 weeks (inoculum density: roots and soil with AMF to sterilized soil and clover seeds, 10 % v/v).

The substrate used was a sand-perlite mixture 1:3 (v/v). Hoagland nutrient solution was added to the substrate (1:3:1 v/v/v sand:perlite:nutrient solution) 24 h prior to autoclaving. The substrate was autoclaved twice (1 h, 121 °C, 1 atm.) with a 48 h interval, and left for one week. Transparant root growth chambers (vertically placed Petri dishes 15 cm diameter, with a slit in the edge) were filled with the sterile substrate. Each root growth chamber contained 300 mL substrate. Cuttings were transferred to the root growth chambers (1 cutting per growth chamber), each cutting was inoculated with 5 mycelial plugs cut from the edge of a precultured *H. leucosarx* colony, the root system was covered with a water agar (1 %) layer (5 cm diameter) to prevent the roots from excessive loss of water. The root growth chambers were sealed with tape and sterilized anhydrous lanolin preventing contamination and loss of water. The control plants were supplied with 5 plugs of solid medium (5 * 0.15 mL) without fungus instead. The inoculation of AMF was performed by mixing 10 g freshly washed root material of *T. repens* containing *G. mosseae* with the sterile substrates (5 L), and 15 growth chambers were filled with this mixture (repeated 20 times in order to obtain 300 inoculated cuttings). Cuttings were transferred to the root growth chambers, the root systems were covered with water agar, root growth chambers were closed and sealed. As a previous experiment with addition of AMF inoculum washings (Koide & Li, 1989) or roots of *T. repens* without AMF to the control or EcM plants showed no effect on plant performance in 12 weeks (APPENICES E AND F), no control plants with roots of *T. repens* without AMF were provided.

All soil compartments were protected from daylight, the root growth chambers were placed vertically in transient propagators (relative air humidity almost 100 % in the first week) and placed in the climate chamber. Growth conditions: photon flux density 350 $\mu E.m^{-2}.s^{-1}$ (20,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 60 %. The plants were arranged in a complete randomized block design, and blocks were randomized every three weeks within the climate chamber. The plants were watered every three weeks with 10 mL of Hoagland nutrient solution.

*Experiment 1: Laboratory experiment in field soil*

Soil was collected from the two selected field sites. Per field site soil was very well ground and mixed in order to reduce inoculum potential of the existing mycelia and propagules. For each field site, soils were divided over 48 0.2 L pots, and 16 control cuttings, 16 cuttings with *G. mosseae* and 16 cuttings with *H. leucosarx* were individually planted in these pots. The plants had been precultured with their mycorrhizal treatment for ten weeks before the experiment started (t = 0). All plants were arranged in a randomized block design in a climate chamber, and blocks were randomized every three weeks. Growth conditions: photon flux density 350 $\mu E.m^{-2}.s^{-1}$ (20,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 60 %. The plants were watered sufficiently with demineralized water.

*Experiment 2: Field experiment*

After 8 weeks, the precultured plants were placed outside in order to adjust to weather influences. Two weeks afterwards (t = 0, moment of outplanting), 100 replicates of control plants, cuttings with *G. mosseae,* and cuttings with *H. leucosarx* were planted randomly in the two selected field sites of 500 $m^2$ (in four plots of 1 m * 1 m). Plants were sufficiently watered after outplanting and also two weeks later.

*Plant performance, EcMF and AMF colonization*

Eight weeks after outplanting (t = 8 weeks), 8 plants of each combination in expt 1 and 12 (three in each plot) plants of each combination in expt 2 were harvested randomly. At t = 24 weeks, these harvests were repeated. After the harvest shoot length was measured and shoot weight was determined after 72 hours at 70 °C. The plants were individually digested in sulphuric acid, salicylic acid and 30 % hydrogen peroxide. Total

N and P were analyzed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard (Novozamsky *et al.*, 1988).

The roots were immersed in water over a 2 mm sieve to remove most of the soil, and rinsed gently to avoid damage of the mycorrhizas. All root samples were split in two samples. For ectomycorrhiza assessment, roots were stored in glutaraldehyde buffer (Alexander & Bigg, 1981) and for AM assessment roots were stored in 50 % alcohol until they could be processed. In each sample, root length was determined according to Newman (1966). For EcM assessment, numbers of EcM root tips per morphotype and total numbers of root tips were counted. EcM frequencies were calculated (100 % * numbers of EcM root tips/total numbers of root tips). EcM root length (%) was measured according to Giovannetti & Mosse (1980). For AM assessment root samples were cleared with 10 % KOH for three hours in a waterbath at 90 °C, bleached in 10 % $H_2O_2$ for 1 hour, acidified in 1 % HCl for 15 min and stained with trypan blue (Phillips & Hayman, 1970; APPENDIX C) in lactophenol for 30 min. AM colonization was estimated by a modified line intersect method (McGonigle *et al.*, 1990), where a minimum of 100 line intersections per root sample (replicated three times) was scored for the presence of AM structures. AM root length times intensity (RLCI = RLC * % cover by AM of the cross section of the root colonized) was calculated.

The experiments involved three comparisons with the previous laboratory study (CHAPTER 4): Uninoculated plants in the field and field soils are compared with simultaneous inoculations; plants inoculated with *G. mosseae* in the field and in field soil are compared with plants inoculated with *G. mosseae* in the sequential inoculation; plants inoculated with *H. leucosarx* in the field and in field soil are compared with plants inoculated with *H. leucosarx* in the sequential inoculation. Interactions between AM and EcM colonization were tested by comparing EcM on AM plants, resp. AM on EcM plants, with the control. As *G. mosseae* and *H. leucosarx* were present before outplanting the differences in colonization between the uninoculated control and AM on AM plants, resp. EcM on EcM plants, are not of interest.

*Statistical analysis*

Data were analysed by Analysis of Variance using the statistical package STATISTICA (StatSoft). As plot effect was not significant in the analysis a three-factorial (fungus, site and time) statistical design was used (ANOVA).

Prior to analysis proportional mycorrhizal colonizations were arcsine square root transformed and plant nutrient concentrations were logarithmically transformed. Due to differential effects of the different fungal treatments that occurred in the 10 weeks inoculation period, all plant parameters (except shoot N/P ratio) were significantly different at the start (moment of outplanting, t = 0 weeks) of experiment 1 and 2 (TABLE 8-2). Therefore, shoot dry weight, root length, shoot N and P content were expressed in percentage of t = 0 weeks, prior to analysis square root transformed and analysed (ANOVA) excluding t = 0 weeks. Mycorrhizal colonization (%) and shoot N and P concentration (%) were analysed (ANOVA) including t = 0 weeks. Bartlett's test was used to determine whether variances were equal between treatments. Differences among means were evaluated with the LSD-test (Sokal & Rohlf, 1995).

**TABLE 8-2.** Shoot length, shoot dry weight, root length, AM and EcM colonization, N and P concentration, N and P content and shoot N/P ratio (means ± SE) of *Salix repens* after 10 weeks inoculation period (t = 10' equals t = 0 start) with *Glomus mosseae, Hebeloma leucosarx* or not inoculated before outplanting in the field and field soil.

| | Control | *Glomus mosseae* | *Hebeloma leucosarx* |
|---|---|---|---|
| Shoot length (cm) | 26 (2.0) a* | 25 (2.2) a | 35 (3.8) b |
| Shoot dry weight (mg) | 269 (36) a | 191(30) a | 446 (90) b |
| Root length (m) | 9.4 (1.5) a | 10.5 (1.3) a | 9.4 (1.9) a |
| AM (%) | 0 a | 3.5 (1.4) b | 0 a |
| EcM (%) | 0 a | 0 a | 87.1 (1.3) b |
| N concentration (%) | 1.58 (0.06) a | 2.09 (0.12) b | 2.04 (0.09) b |
| P concentration (%) | 0.142 (0.01) a | 0.182 (0.01) b | 0.166 (0.01) ab |
| N content (mg) | 4.21 (0.55) a | 4.18 (0.84) a | 9.56 (2.19) b |
| P content (mg) | 0.372 (0.05) a | 0.343 (0.06) a | 0.751 (0.175) b |
| N/P ratio | 11.3 a | 12.2 a | 12.7 a |

* Different letters indicate significant differences after ANOVA and LSD tests ($P < 0.05$).

## RESULTS

### *AM and EcM colonization and their interaction in field soils and field sites*

Analysis of variance showed for both experiments that AM colonization was affected by site, fungal treatment and time, whereas EcM colonization was affected by site in the field experiment, but not in field soil (TABLE 8-3).

**TABLE 8-3.** Results of ANOVA (*P*-values) executed for AM and EcM colonization, N and P concentration, shoot dry weight, root length, and N and P content of *Salix repens* inoculated with *Glomus mosseae, Hebeloma leucosarx* or not inoculated when grown in field soil collected from or planted in the field sites Schoenus (young) and Groene strand (old) after 8 and 24 weeks.

| | Field site (1) (df = 1) | Fungus (2) (df = 2) | Time (3) (df = 1 or 2‡) | 1 × 2 (df = 2) | 1 × 3 (df = 1 or 2‡) | 2 × 3 (df = 2 or 4‡) | 1 × 2 × 3 (df = 2 or 4‡) |
|---|---|---|---|---|---|---|---|
| Field soil: | | | | | | | |
| AM (%)† | *** <0.001 | *** <0.001 | *** <0.001 | * 0.045 | *** <0.001 | *** <0.001 | ** 0.006 |
| EcM (%)† | ns 0.744 | *** <0.001 | *** <0.001 | ** 0.002 | ** 0.003 | *** <0.001 | ** 0.004 |
| N conc. (%)† | * 0.016 | ns 0.088 | *** <0.001 | ns 0.714 | ns 0.141 | *** <0.001 | ns 0.614 |
| P conc. (%)† | ns 0.431 | *** <0.001 | *** <0.001 | ns 0.820 | ns 0.832 | * 0.018 | ns 0.976 |
| Dry weight (%t0) | ns 0.588 | * 0.049 | ns 0.209 | ns 0.790 | ns 0.121 | ns 0.913 | ns 0.392 |
| Root length (%t0) | ** 0.002 | *** <0.001 | *** <0.001 | ns 0.212 | ns 0.080 | ns 0.835 | ns 0.217 |
| N content (%t0) | ns 0.746 | *** <0.001 | * 0.034 | ns 0.904 | ns 0.065 | ns 0.939 | ns 0.215 |
| P content (%t0) | ns 0.205 | * 0.018 | ns 0.374 | ns 0.862 | ns 0.052 | ns 0.886 | ns 0.453 |
| Field: | | | | | | | |
| AM (%)‡ | *** <0.001 | *** <0.001 | *** <0.001 | ns 0.715 | *** <0.001 | *** <0.001 | ns 0.591 |
| EcM (%)‡ | *** <0.001 | *** <0.001 | *** <0.001 | ns 0.210 | *** <0.001 | *** <0.001 | * 0.025 |
| N conc. (%)‡ | ns 0.609 | *** <0.001 | *** <0.001 | ns 0.328 | ns 0.846 | *** <0.001 | ns 0.407 |
| P conc. (%)‡ | ** 0.002 | *** <0.001 | *** <0.001 | ns 0.234 | * 0.010 | ns 0.087 | ns 0.355 |
| Dry weight (%t0) | ns 0.971 | *** <0.001 | ns 0.807 | ns 0.982 | ** 0.009 | ns 0.194 | ns 0.768 |
| Root length (%t0) | ns 0.094 | *** <0.001 | ns 0.216 | ns 0.594 | ns 0.129 | ns 0.137 | ns 0.232 |
| N content (%t0) | ns 0.686 | *** <0.001 | ns 0.898 | ns 0.777 | * 0.038 | * 0.035 | ns 0.818 |
| P content (%t0) | ns 0.158 | *** <0.001 | * 0.015 | ns 0.779 | ** 0.003 | * 0.032 | ns 0.697 |

* Level of significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).
† df Error = 84, 120 for field soil and field experiment resp., ‡including t = 10', df Error = 138, 170 resp.

**TABLE 8-4.** Mycorrhizal colonization (± SE) of *Salix repens*, inoculated with *Glomus mosseae, Hebeloma leucosarx* or not inoculated, planted in soil collected from Schoenus (relatively young, calcareous) or from Groene strand (relatively old, acidic) after 0, 8 and 24 weeks. See text for relevant statistical comparisons.

| | Control | | *G. mosseae* | | *H. leucosarx* | |
|---|---|---|---|---|---|---|
| Field soils/ Harvest(weeks) | AM (%) | EcM (%) | AM (%) | EcM (%) | AM (%) | EcM (%) |
| t = 0 (start) | 0 | 0 | 3.5 ± 0.1 c* | 0 | 0 | 87.1±1.3 H* |
| t = 8 | | | | | | |
| 'young' | 4.7 ± 0.4 de | 62.2±1.1 BCD | 5.2 ± 0.4 e | **37.7±1.9 A** | 4.4 ± 0.4 de | 82.3±2.5 G |
| 'old' | 3.5 ± 0.3 c | 54.4±1.5 B | 3.4 ± 0.2 c | **43.6±1.3 A** | 3.1 ± 0.1 b | 71.4±2.8 E |
| t = 24 | | | | | | |
| 'young' | 3.1 ± 0.2 b | 56.6±6.5 BC | 4.1 ± 0.3 d | 62.3±5.4 CD | 3.8 ± 0.4 c | 81.8±1.4 G |
| 'old' | 2.9 ± 0.2 b | 73.6±2.2 EF | 4.0 ± 0.2 d | 69.2±3.5 DE | 2.2 ± 0.2 a | 77.8±1.9 FG |

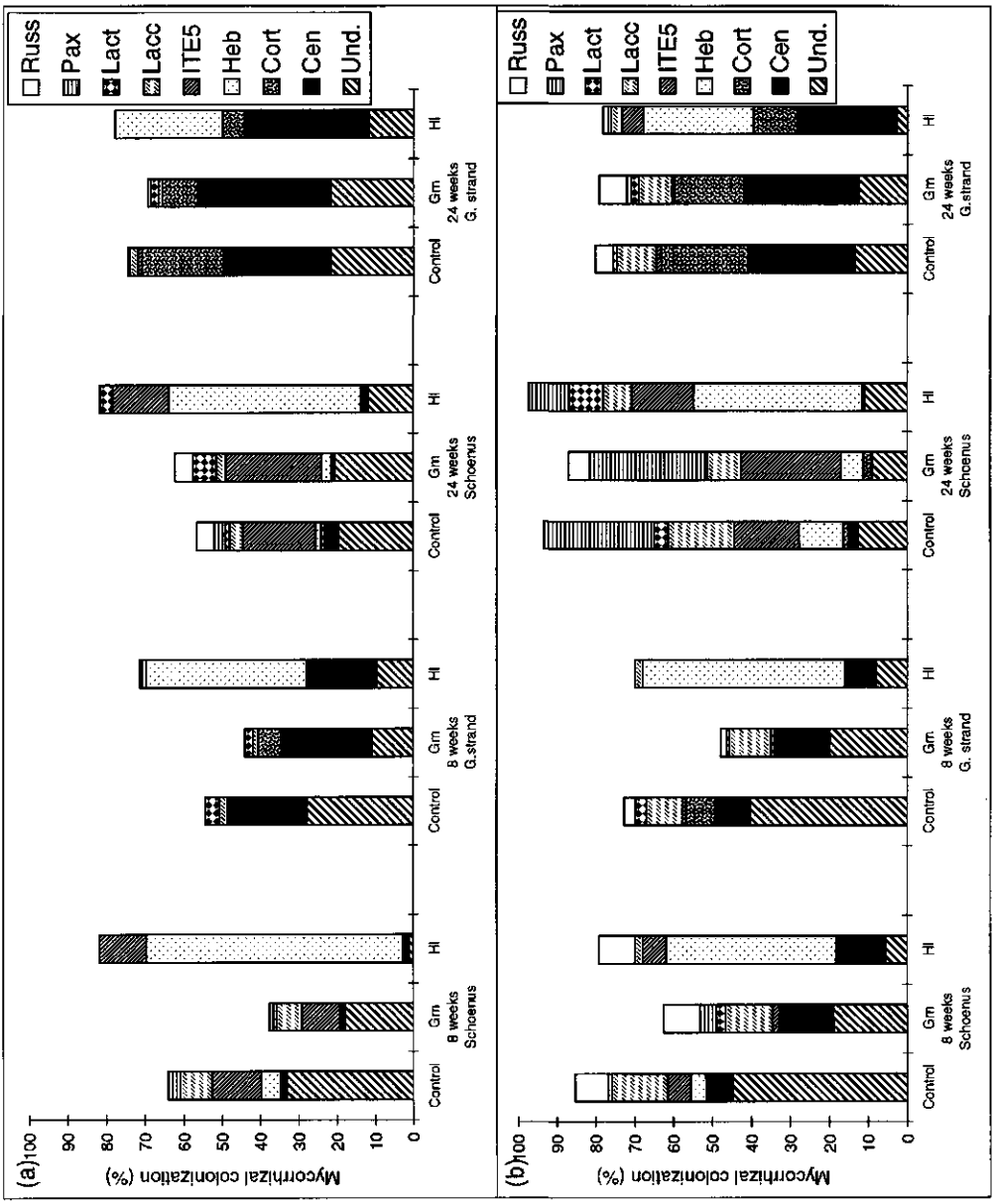
* Significant differences (tested by a three-way factorial ANOVA and LSD values; $P < 0.05$) within functional types of mycorrhiza are indicated by different letters in small letters for AM and in capitals for EcM.

**TABLE 8-5.** Mycorrhizal colonization (± SE) of *Salix repens*, inoculated with *Glomus mosseae, Hebeloma leucosarx* or not inoculated, planted in Schoenus (relatively young, calcareous) or in Groene strand (relatively old, acidic) after 0, 8 and 24 weeks. See text for relevant statistical comparisons.

| | Control | | *G. mosseae* | | *H. leucosarx* | |
|---|---|---|---|---|---|---|
| Field sites/ Harvest(weeks) | AM (%) | EcM (%) | AM (%) | EcM (%) | AM (%) | EcM (%) |
| t = 0 (start) | 0 | 0 | 3.5 ± 0.1 abc* | 0 | 0 | 87.1±1.3 F* |
| t = 8 | | | | | | |
| 'young' | 5.0 ± 0.5 d | 85.4±2.1 EF | 6.1 ± 0.9 e | **62.1±2.5 B** | 4.1 ± 0.5 c | 79.4±2.1 D |
| 'old' | 3.4 ± 0.3 abc | 71.0±1.3 C | 3.9 ± 0.2 bc | **47.8±3.4 A** | 3.0 ± 0.2 a | 69.1±2.3 C |
| t = 24 | | | | | | |
| 'young' | 3.5 ± 0.2 abc | 93.2±1.5 G | 3.7 ± 0.3 bc | 87.7±2.7 F | 3.7 ± 0.2 b | 96.9±0.8 H |
| 'old' | 3.3 ± 0.2 ab | 80.1±2.8 DE | 3.0 ± 0.2 a | 77.6±2.5 D | 2.9 ± 0.1 a | 77.3±2.4 D |

* Significant differences (tested by a three-way factorial ANOVA and LSD values; $P < 0.05$) within functional types of mycorrhiza are indicated by different letters in small letters for AM and in capitals for EcM.

**FIGURE 8-1.** Different ectomycorrhizal morphotypes: undeveloped, *Cenococcum*, *Cortinarius*, *Hebeloma*, ITE5, *Laccaria*, *Lactarius*, *Paxillus*, *Russula*, of *Salix repens*, inoculated with *Glomus mosseae, Hebeloma leucosarx* or not inoculated, (a) planted in soil collected from Schoenus (relatively young, calcareous) or from Groene strand (relatively old, acidic), (b) planted in field site Schoenus or Groene strand, after 8 and 24 weeks (see opposite page).


(a)
(b)
Mycorrhizal colonization (%)
Russ
Pax
Lact
Lacc
ITE5
Heb
Cort
Cen
Und.
Control
Gm 8 weeks Schoenus
HI
Gm 8 weeks G.strand
Gm 24 weeks Schoenus
Gm 24 weeks G.strand

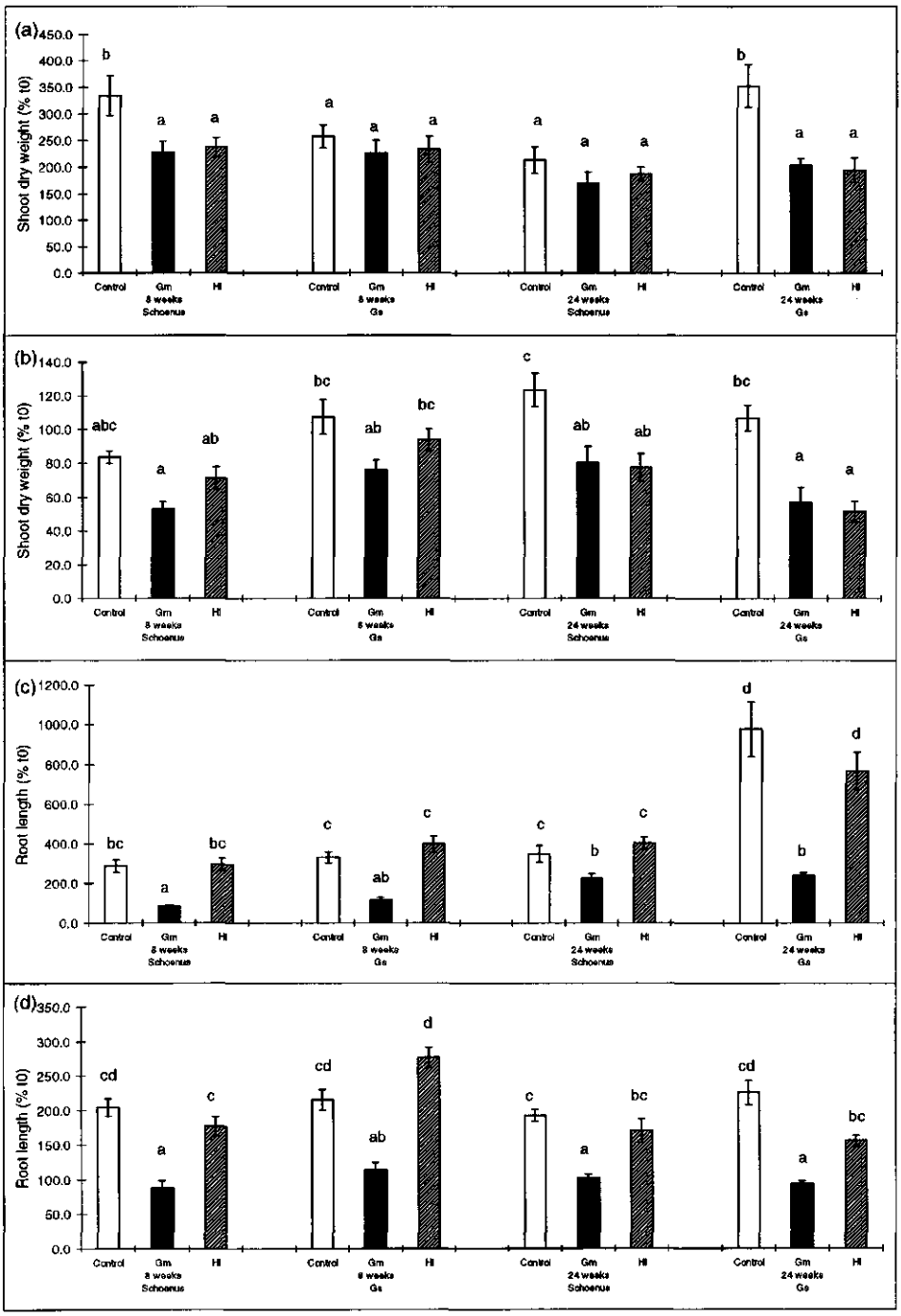
After eight weeks in the field and in field soil EcM colonization on *S. repens* previously inoculated with *G. mosseae* was significantly lower than on the control cuttings, whereas in the field soil AM colonization on *S. repens* that was previously inoculated with *H. leucosarx* was only different from the control in the young site. In the field AM colonization in the young site was higher on the control cuttings than on cuttings previously inoculated with *H. leucosarx*. Effects after 24 weeks in the field and in the field soil were much weaker and usually not significant (TABLES 8-4 AND 8-5).

Ectomycorrhizal inoculum potential was higher in the field than in field soil (control cuttings in TABLES 8-4 AND 8-5), and was significantly higher in the young than in the old site. This was similar on the long term (at t = 24 weeks) in the field, whereas in field soil the opposite was found. Arbuscular mycorrhizal inoculum potential was not different between field soil and the field, and was higher in the young than in the old site at eight weeks, but not at 24 weeks (control cuttings in TABLES 8-4 AND 8-5).

After eight weeks, both AM and EcM colonization in the field and field soil were higher in the young than in the old site. After 24 weeks, AM colonization in the field and field soil were not different between sites, and EcM colonization in the field was higher in the younger site, but in the field soil lower in the younger site (TABLES 8-4 AND 8-5).

The different EcM morphotypes that could be identified (FIG. 8-1) indicated that *H. leucosarx* initially slightly depressed EcM morphotype diversity, both in field soil and in the field in both sites. However, at t = 24 weeks in the field this was no longer found. No obvious differences occurred between EcM morphotype richness on control cuttings and cuttings previously inoculated with *G. mosseae*. In the field EcM diversity on all cuttings was generally higher than in field soil, whereas total number of EcM morphotypes identified were the same. *Cenococcum* was dominant in the old site, both in field soil and in the field. ITE5 was dominant at t = 24 weeks in the young site, both in field soil and in the field, whereas in the field *Paxillus* was a codominant EcM morphotype. The relative proportion of *Hebeloma* was higher in the young than in the old site.

**FIGURE 8-2.** Shoot dry weight (a, b) and Root length (c, d) of *Salix repens*, inoculated with *Glomus mosseae* ■, *Hebeloma leucosarx* ▨ or not inoculated □, (a, c) planted in soil collected from Schoenus (relatively young, calcareous) or from Groene strand (relatively old, acidic), (b, d) planted in field site Schoenus or Groene strand, after 8 and 24 weeks (see opposite page).


(a)
Shoot dry weight (% t0)
(b)
Shoot dry weight (% t0)
(c)
Root length (% t0)
(d)
Root length (% t0)
Control
Gm 8 weeks Schoenus
Hl
Control
Gm 8 weeks Gs
Hl
Control
Gm 24 weeks Schoenus
Hl
Control
Gm 24 weeks Gs
Hl

*Plant performance as affected by AM and EcM in field soil and in the field*

Shoot dry weight was not affected by the factor field site (both in field soil and in the field) but was affected by the factor fungal treatment (TABLE 8-3). Furthermore, shoot dry weight in field soil, but not in the field, was affected by time (TABLE 8-3). In field soil, shoot dry weight (%t0) of control cuttings in the young site decreased over time (t = 8 to 24 weeks), whereas dry weight of control cuttings in the old site increased over time (FIG. 8-2a). In the field in both field sites at t = 8 weeks no differences in shoot dry weight (%t0) occurred, but at t = 24 weeks in both field sites the control cuttings were significantly larger (FIG. 8-2b).

Even though root length showed similar patterns between field soil and the field, and for the fungal treatments (FIGS. 8-2c,d), root length was significantly affected by the factors site, fungal treatment and time in field soil, whereas root length in the field was only affected by the factor fungal treatment (TABLE 8-3). Root length (%t0) in field soil and the field in both field sites was lower on cuttings that were previously inoculated with *G. mosseae* than the other treatments. Both in field soil and in the field in the young site root length did not change over time. In the old site, however, in the field root length (%t0) of cuttings previously inoculated with *H. leucosarx* decreased over time, whereas in field soil in the lab, root length of control cuttings and cuttings previously inoculated with *H. leucosarx* increased over time.

**TABLE 8-6.** Shoot N and P concentration (± SE) of *Salix repens*, inoculated with *Glomus mosseae*, *Hebeloma leucosarx* or not inoculated, planted in field soil collected from Schoenus (relatively young, calcareous) or in Groene strand (relatively old, acidic) after 0, 8 and 24 weeks.

| | Control | | *G. mosseae* | | *H. leucosarx* | |
|---|---|---|---|---|---|---|
| Field soils/ Harvest(weeks) | N (%) | P (%) | N (%) | P (%) | N (%) | P (%) |
| t = 0 (start) | 1.58 e* | 0.142 B* | 2.09 f | 0.182 C | 2.04 f | 0.166 BC |
| t=8 | | | | | | |
| 'young' | 1.03 d | 0.060 A | 0.81 bc | 0.068 A | 0.87 c | 0.064 A |
| 'old' | 0.79 bc | 0.060 A | 0.76 ab | 0.073 A | 0.80 bc | 0.067 A |
| t = 24 | | | | | | |
| 'young' | 0.77 abc | 0.061 A | 0.71 ab | 0.064 A | 0.76 abc | 0.060 A |
| 'old' | 0.73 ab | 0.063 A | 0.66 a | 0.069 A | 0.68 ab | 0.073 A |

* Significant differences (tested by a three-way factorial ANOVA and LSD values; $P < 0.05$) within functional types of mycorrhiza are indicated by different letters in small letters for N(%) and in capitals for P(%).

**TABLE 8-7.** Shoot N and P concentration (± SE) of *Salix repens*, inoculated with *Glomus mosseae*, *Hebeloma leucosarx* or not inoculated, planted in Schoenus (relatively young, calcareous) or in Groene strand (relatively old, acidic) after 0, 8 and 24 weeks.

| | Control | | *G. mosseae* | | *H. leucosarx* | |
|---|---|---|---|---|---|---|
| Field sites/ Harvest(weeks) | N (%) | P (%) | N (%) | P (%) | N (%) | P (%) |
| t = 0 (start) | 1.58 B* | 0.142 ef* | 2.09 C | 0.182 g | 2.04 C | 0.166 fg |
| t = 8 | | | | | | |
| 'young' | 1.02 A | 0.091 a | 1.17 A | 0.113 b | 1.12 A | 0.096 a |
| 'old' | 1.07 A | 0.087 a | 1.04 A | 0.093 a | 1.13 A | 0.098 a |
| t = 24 | | | | | | |
| 'young' | 1.15 A | 0.135 de | 1.11 A | 0.153 ef | 1.19 A | 0.152 ef |
| 'old' | 1.24 A | 0.125 cd | 1.12 A | 0.124 c | 1.07 A | 0.118 bc |

* Significant differences (tested by a three-way factorial ANOVA and LSD values; $P < 0.05$) within functional types of mycorrhiza are indicated by different letters in small letters for P(%) and in capitals for N(%).

Shoot N and P concentration were differently affected by the factor site, in the field or field soil. In field soil, shoot N concentration was significantly affected by the factor site, whereas shoot P concentration was not. In the field, shoot P concentration was significantly affected by the factor site, whereas shoot N concentration was not (TABLE 8-3). In field soil, at t = 8 and 24 weeks, all plants showed lower shoot P concentrations than at t = 0 weeks, and did not differ between sites, fungal treatments or harvests (TABLE 8-6). In the field the same was found, except for shoot N concentration (TABLE 8-7). Shoot N and P concentrations were initially (at t = 8 weeks) lower in field soil than in the field. In field soil shoot N was decreasing over time and in the field shoot P concentration was increasing over time.

Both in field soil and in the field, shoot N and P content were unaffected by site, but were affected by the fungal treatment. Time significantly affected shoot N content in field soil and shoot P content in the field (TABLE 8-3). In general, shoot N and P content (%t0) was higher in field soil than in the field (FIG. 8-3). Overall pattern in shoot N content between field soil and field, sites and fungal treatment were similar (FIGS. 8-3a,b). However, in field soil shoot N content of control cuttings in the young site decreased over time, whereas in the field it increased. All other treatments in field soil did not change over time. In the field, shoot N content in cuttings previously inoculated with *H. leucosarx* decreased over time. In field soil, shoot P content of control cuttings in the young site

decreased over time, whereas shoot P content of control cuttings in the old site increased over time (FIG. 8-3c). In the field, shoot P content of control cuttings in both sites increased over time (FIG. 8-3d).

In summary, shoot weight, shoot N and P concentration, shoot N and P content of cuttings that were previously inoculated with *G. mosseae* generally showed no differences between sites or over time. Cuttings previously inoculated with *H. leucosarx* only showed decreased shoot weight, shoot N content and increased P concentration in the field in the old site over time.

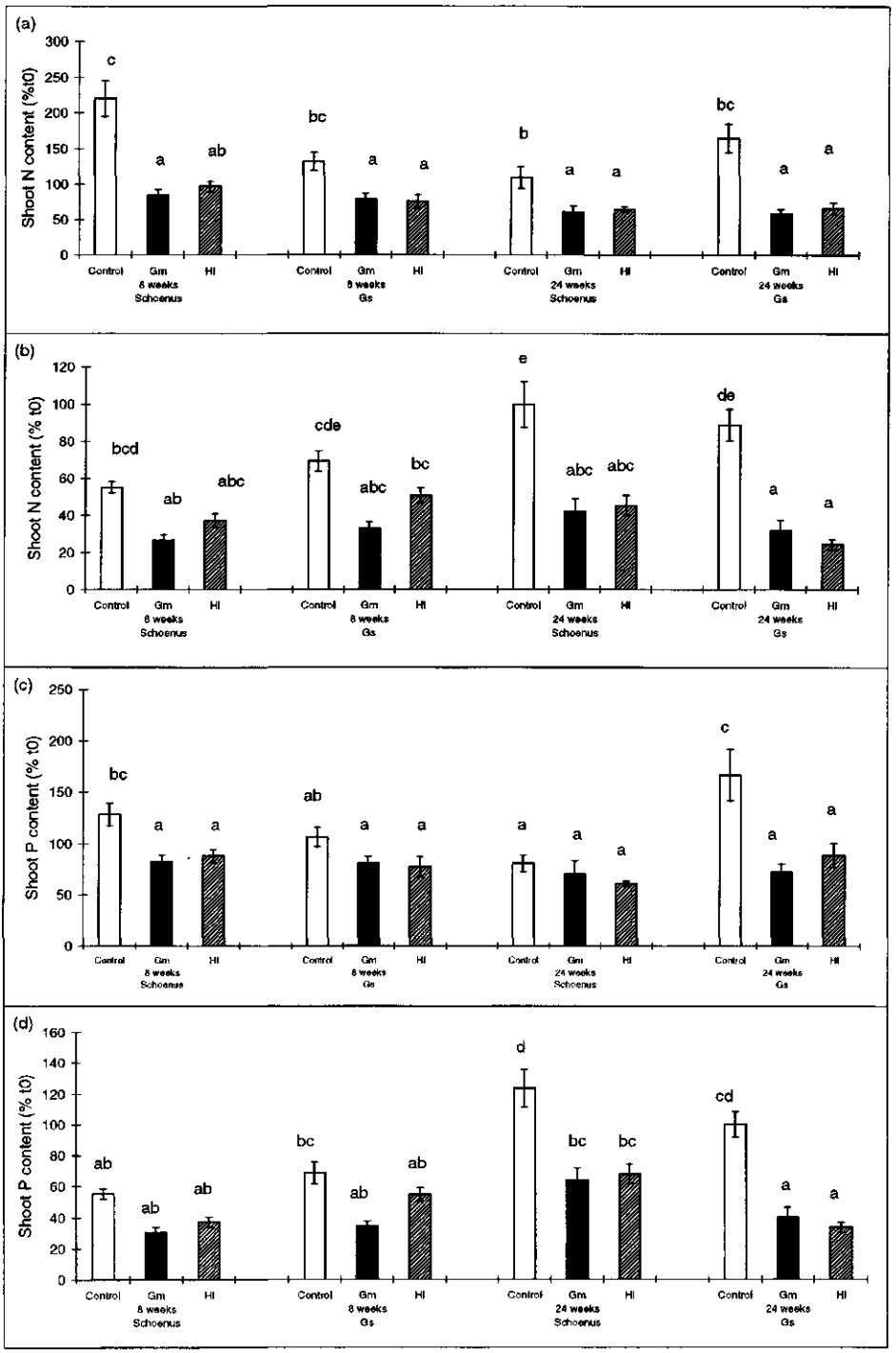
## DISCUSSION

### *Mycorrhizal colonization and interaction between AM and EcM*

The non-mycorrhizal *S. repens* cuttings planted in both habitats, after 8 weeks in field soil and in the field showed both AM and EcM colonization and EcM morphotype diversity similar to that on native *S. repens* plants in both habitats (CHAPTER 3), indicating that field soil with mycorrhizal inoculum can be used as a model for different habitats.

Leaf nutrient analysis of the native *S. repens* community in August (young site: N/P = 13.0 and old site: N/P = 14.5) did not indicate differences in relative nutrient availability. However, root colonization of the native *S. repens* community in August (young: 77 % EcM, 6 % AM and old: 88 % EcM, 2 % AM) did suggest a shift in relative importance of both mycorrhizal types between those two successional stages of *S. repens* communities. Furthermore, bait plants (non-mycorrhizal *S. repens* cuttings with initial N/P = 11.3) after 8 and 24 weeks showed higher EcM colonization in the young site (85 and 93 %, resp.) than in the old site (71 and 80 %, resp.). Simultaneously, after 8 weeks AM colonization was higher in the young site (5 % opposed to 3.4 % in the old site). Apparently, mycorrhizal inoculum potential of both types is higher in young sites than in old sites.

**FIGURE 8-3.** Shoot N (a, b) and P content (c, d) of *Salix repens*, inoculated with *Glomus mosseae* ■, *Hebeloma leucosarx* ▨ or not inoculated □, (a, c) planted in soil collected from Schoenus (relatively young, calcareous) or from Groene strand (relatively old, acidic), (b, d) planted in field site Schoenus or Groene strand, after 8 and 24 weeks (see opposite page).


(a)
Shoot N content (%t0)
300
250
200
150
100
50
0
c
a
ab
bc
a
a
b
a
a
bc
a
a
Control
Gm 8 weeks Schoenus
HI
Control
Gm 8 weeks Gs
HI
Control
Gm 24 weeks Schoenus
HI
Control
Gm 24 weeks Gs
HI
(b)
Shoot N content (% t0)
120
100
80
60
40
20
0
bcd
ab
abc
cde
abc
bc
e
abc
abc
de
a
a
Control
Gm 8 weeks Schoenus
HI
Control
Gm 8 weeks Gs
HI
Control
Gm 24 weeks Schoenus
HI
Control
Gm 24 weeks Gs
HI
(c)
Shoot P content (% t0)
250
200
150
100
50
0
bc
a
a
ab
a
a
a
a
a
c
a
a
Control
Gm 8 weeks Schoenus
HI
Control
Gm 8 weeks Gs
HI
Control
Gm 24 weeks Schoenus
HI
Control
Gm 24 weeks Gs
HI
(d)
Shoot P content (% t0)
160
140
120
100
80
60
40
20
0
ab
ab
ab
bc
ab
ab
d
bc
bc
cd
a
a
Control
Gm 8 weeks Schoenus
HI
Control
Gm 8 weeks Gs
HI
Control
Gm 24 weeks Schoenus
HI
Control
Gm 24 weeks Gs
HI

The hypothesis by Read (1989) about a shift in vegetation dominated by AM plants towards a vegetation dominated by EcM plants in a dune succession driven by changes in relative availabilities of nitrogen and phosphorus, could not be confirmed for the dual mycorrhizal plant *S. repens*.

Initial colonization by *H. leucosarx* on the long term in the field hardly affected EcM morphotype diversity, but it ensured its relatively high colonization when compared to the other treatments. In field soil presence of *H. leucosarx* did suppress EcM morphotype diversity in soil from both sites, while the *Hebeloma* morphotype did not or hardly occur in the other treatments. The larger proportion of EcM root tips formed by *H. leucosarx* in the field soil is explained by its greater competitive ability compared to the indigenous ectomycorrhizal flora due to small soil volume, thus smaller mycelia, and disturbance of these mycelia that were originally present. In the lab in field soil and in the field, in both sites *S. repens* previously inoculated with *G. mosseae* did not clearly affect EcM diversity.

In a previous study interaction between AM and EcM was studied under controlled conditions. Two AMF species (*Acaulospora laevis* and *G. mosseae*) suppressed subsequent colonization of the three EcM species studied (*Inocybe vulpinella, H. leucosarx* and *Paxillus involutus*), whereas only *P. involutus* suppressed subsequent colonization by *G. mosseae*. When both mycorrhizal types were simultaneously inoculated, only *P. involutus* was suppressed, while the others colonized similar proportions of the roots as in their individual inoculations (CHAPTER 4). The suppressive effect of AM on subsequent EcM colonization has now been confirmed in a lab study in field soil, and moreover in the field in two different successional stages. Apparently, this interaction outcome is a general phenomenon. Earlier publications reported negative interaction between AM and EcM, in which EcM was supposed to be the stronger competitor (Lodge & Wentworth, 1990; Chilvers *et al.*, 1987). However, these conclusions were based on static data. Since *S. repens* is always slightly AM and highly EcM, interactions between AM and EcM in *S. repens* should therefore be studied over time (repeated sampling) and by simultaneous and sequential inoculations of both AM and EcM.

*Plant performance as affected by dual colonization in two extreme habitats*

During the inoculation procedure plants were supplied with Hoagland nutrient solution in order to provide all treatments with sufficient N and P, and to arive at similar

plant size. Despite this nutrient supply, *H. leucosarx* significantly increased biomass and nutrient uptake compared to the control. *Glomus mosseae* even decreased biomass and nutrient uptake compared to the control. These differences suggest absence of nutritional benefits of AMF in a P sufficient nutrition, while costs persist. Possibly nutritional benefits of EcM involve a larger range of nutrients than phosphorus. Negative effects of *G. mosseae* and positive effects of *H. leucosarx* have been confirmed in some later studies (CHAPTERS 5, 7 AND 9), both under nutrient-poor and nutrient richer conditions. However, *G. mosseae* was rather consistently beneficial on the short term (< 12 weeks). Although in all these experiments AM colonization was low (< 5 %) and EcM colonization was high (> 85 %), it is possible that these differences in colonization do not reflect carbon costs accurately.

Plant performance (shoot biomass and nutrient uptake) expressed in percentage of t = 0 (start of outplanting) by *G. mosseae* and *H. leucosarx* in both field sites (or field soils) generally was depressed compared to the non-mycorrhizal plants. Various explanations for this effect can be put forward: (1) *Glomus mosseae* suppresses subsequent native EcM colonization, whereas non-mycorrhizal plants associate with the native mycorrhizal population immediately (TABLES 8-4 AND 8-5). (2) Both *G. mosseae* and *H. leucosarx* might negatively interact with the native mycorrhizal fungi which are probably best adapted to the circumstances in the two field sites and which have relatively large mycelia, which exploited the substrate already. The non-mycorrhizal cuttings could immediately have taken up nutrients, whereas the mycorrhizal fungi of the mycorrhizal cuttings would have had to start exploitation of the substrate. Competition (in this case for root space) might involve rather high carbon costs. (3) The fittest non-mycorrhizal plants were selected for these experiments, as under the fertilized pre-treatment survival of non-mycorrhizal plants was c. 75 % (compared to > 90 % of the mycorrhizal plants).

Root length of cuttings inoculated with *H. leucosarx* was not different from the control, whereas root length on cuttings with *G. mosseae* was suppressed. This observation contrasts with previous experiments, in sterile soils, that indicated strong increased root length of *S. repens* when colonized by *G. mosseae*. Decrease of root length was not caused by the fertilizer treatment as longer root were also observed in another experiment in which fertilizers were used, however, only after 26 weeks (CHAPTER 7).

*Plant control over AM colonization?*

The consistent suppressive effect of AM on subsequent EcM indicates that AM colonization in *S. repens* triggers the plant defence system. This activated plant defence system prevents roots from EcM colonization. However, this effect was only transient and was absent or weak after 24 weeks. The same mechanism may occur more generally and could explain why the protection of an annual grass from root pathogenic fungi in the field as suggested by Newsham *et al.* (1995a,b) was found after 62 days but no longer occurred after 90 days. Population dynamics of *Fusarium* over a 90 day period also indicated that the interference by the AMF was transient. The opposite, suppression of AM by EcM fungi was absent and occurred only incidentally under laboratory conditions (CHAPTER 4).

One could speculate whether the consistently low AM colonization, which has recently been found in other EcM tree species as well (Cázares & Smith, 1996; Moyersoen & Fitter, 1999) is still functional. However, even in a functional symbiosis the benefits of AM have to be traded off against suppression of EcM. Ectomycorrhizal trees may have solved this problem, while primitively retaining the ability to maintain AM, by increasing plant control over AM colonization. This might constrain the evolution of *S. repens* (and maybe all EcM tree species) towards non-AM-host. This supposed ubiquity of dual mycorrhiza in EcM tree genera implies that AM fungi offer benefits to these plant species that sometimes cannot easily be established in a simple experimental design, but depend on plant physiological state or/and season.

9

# Does origin of mycorrhizal fungus or mycorrhizal plant influence effectiveness of the mycorrhizal symbiosis?


## ABSTRACT

*Mycorrhizal effectiveness depends on the compatibility between fungus and plant. Therefore genetic variation in plant and fungal species affect the effectiveness of the symbiosis. The importance of mycorrhizal plant and mycorrhizal fungus origin was investigated in two experiments. In the first experiment clones (cuttings) of* Salix repens *L. from three different origins (two coastal dune and one inland ecosystem) were inoculated with three mycorrhizal fungi (the ectomycorrhizal fungi* Hebeloma leucosarx *and* Paxillus involutus*; the arbuscular mycorrhizal fungus* Glomus mosseae*) on two soils with different nutrient availabilities. In the second experiment homogeneous plant material (one clone of* S. repens*) was inoculated with four isolates of* H. leucosarx*, all found in association with* S. repens*. Plant origin had a large effect on symbiotic effectiveness, assessed by three different criteria (shoot biomass, shoot N-content, shoot P-content). This effect was probably mediated through genetical differences in root parameters. There was also a large plant origin × soil type interaction, with the plants from the nutrient-poor habitat performing better on the poorest soil. The plants from the more nutrient-rich habitat performed better on the relatively rich soil. Effects of plant origin on symbiotic effectiveness were also fungal-species specific. Fungal origin had only a minor effect on symbiotic effectiveness. Studies concerning the ecological significance of mycorrhizal symbiosis should be more explicit about both mycorrhizal plant and fungus origin and compatibility.*


**KEY WORDS**: Arbuscular mycorrhiza, ectomycorrhiza, plant- or fungal origin, *Salix repens*, symbiotic effectiveness.

## INTRODUCTION

Mycorrhizas can be defined in structural terms as associations between symbiotic soil fungi and plant roots. Mycorrhizas are often considered to be classical mutualisms: many experimental investigations have shown that both plant and fungal symbionts benefit from the reciprocal exchange of mineral and organic resources (Smith & Read, 1997).

Differences in nutrient absorption by tree roots are partially due to genetic variation in their growth, morphology and mycorrhizal associations (Bowen, 1984). The extent to which the host plant benefits from improved nutrient absorption is strongly influenced by root morphology. Root morphology again depends on genetic constitution, plant age, site conditions, and other organisms i.e. pathogens or mycorrhizal fungi (Fitter, 1985). As intraspecific genetic variation in root characteristics exists (Dixon *et al.*, 1987), the benefits derived from a mycorrhizal association may vary depending on host genotype and thus on host origin. Also, intraspecific variation in the ability of host plants to form ectomycorrhizas has been well documented (Dixon *et al.*, 1987; Tonkin *et al.*, 1989; Thomson *et al.*, 1990). Fungal isolates of one species vary in mycorrhizal effectiveness. When tested on a single host plant species mycorrhizal fungal isolates can increase, decrease, or have little effect on plant growth (e.g. Molina, 1979; Miller *et al.*, 1985; Dosskey *et al.*, 1990; Burgess *et al.*, 1994). Because of this intraspecific variation of both plant and fungus, mycorrhiza formation and effectiveness may differ when either origin of the fungal isolates (of a certain species) or of the host plants are varied.

In order to investigate the significance of genetic variation in the host species with genetically uniform fungal material, or to examine the significance of genetic variation in the fungal species with genetically uniform plant material, *Salix repens* L., a clonal plant, is a perfect host model organism. *Salix repens* can be multiplied by cuttings, thereby genetically uniform host plants can be obtained. Besides, *S. repens* is a dual mycorrhizal plant and is widespread in a great variety of plant communities. *Salix repens* furthermore has a very broad ecological amplitude and occurs in habitats ranging from dry to wet, and from calcareous, humus-poor to acidic, humus-rich soils. Finally, *S. repens* has a wide geographical distribution.

The effects of origin of mycorrhizal plant and mycorrhizal fungus on symbiotic effectiveness were separately investigated. Even though a clear concept of mycorrhizal (symbiotic) effectiveness is lacking (Janos, 1993), in the present study mycorrhizal effectiveness is used as a modification of the term mycorrhizal dependency as defined by

Plenchette *et al.* (1983), based on the relationship between dry mass of plants inoculated with a mycorrhizal fungus and the dry mass of uninoculated plants. Here the term is used for three parameters (biomass, N-content and P-content) to describe mycorrhizal (symbiotic) effectiveness. When mycorrhizal effectiveness > 0, the mycorrhizal association is considered beneficial for the plant.

In the present study it was hypothesized that (i) symbiotic effectiveness would be higher for clones from poorer sites; (ii) symbiotic effectiveness would be higher on the poorer soil; (iii) symbiotic effectiveness would depend on the interaction between clone origin and soil condition, with the clone from the poorest site showing a higher effectiveness on the poorer soil and the clone from the richest site showing a higher effectiveness on the richer (less poor) soil; (iv) variation in plant origin is more important, i.e. showing stronger differential responses, than variation in fungal origin. The latter hypothesis would follow from the fact that spores of EcMF spread more efficiently than willow seeds.

## MATERIAL AND METHODS

### *Field description, plant and fungal material, and soils*

This study included three field sites (TABLE 9-1). Two study sites on the Wadden Isle of Terschelling comprised two extremes of dune successional stages: 1) Schoenus: a young, calcareous, humus- and nutrient-poor site with a relatively high soil pH($CaCl_2$) 7.3; 2) Oosterend: an older, acidic, humus- and nutrient-rich site with a relatively low soil pH($CaCl_2$) 3.6; and finally 3) Vledder: an inland *Salix repens* field site located in the province of Drenthe, soil characteristics similar to Oosterend, but 1.5 times richer in total N. All cuttings within a field site were collected from the same male plant in order to obtain genetic homogeneity.

Five cultures of EcMF (TABLE 9-1) were collected in the autumn of 1994 and 1995 from *S. repens*, four fungal cultures from the Isle Terschelling. Sporocarps were surface sterilized with alcohol (70 %) and sliced in half under sterile conditions. Fungal tissue was cut from the innerside of the cap and transferred to and maintained on solid media (alternative Melin Norkrans (AMN)) containing (in $g.l^{-1}$): agar (15), malt extract (3), glucose.$H_2O$ (10), $(NH_4)_2HPO_4$ (0.25), $KH_2PO_4$ (0.5), $KNO_3$ (0.5), $MgSO_4.7H_2O$ (0.15), $CaCl_2.H_2O$ (0.67), NaCl (0.025), $FeCl_3$ (0.0012) and thiamine.HCl (100 µg). An AMF starter culture was kindly provided by the European Bank of Glomales (France), *Glomus*

*mosseae* (BEG 12). This culture was maintained on *Trifolium repens* L. in sandy soil, supplied with P-poor Hoagland solution.

The selection of the fungal species *H. leucosarx, P. involutus* and *Glomus mosseae* was based on occurrence of *H. leucosarx* and *P. involutus* in all three selected field sites. However, *Glomus mosseae* was identified in the calcareous plots only on Terschelling.

Soils used in these experiments were two sandy soils, both classified as nutrient- and humus-poor soils, but soil I was extremely poor. Soil I: pH ($CaCl_2$) 5.5, nutrient contents 6 mg $N_{tot}.kg^{-1}$, 6 mg $P_{tot}.kg^{-1}$, and 0 % organic matter. Soil II: pH ($CaCl_2$) 5.8, nutrient contents 70 mg $N_{tot}.kg^{-1}$, 20 mg $P_{tot}.kg^{-1}$, and 0.17 % organic matter.

**TABLE 9-1.** Fungal species, code number, host tree species, geographical location, site, habitat of the selected mycorrhizal fungi and cuttings.

| | Code | Expts | Host | Geographical | Site | Habitat |
|---|---|---|---|---|---|---|
| Fungal isolates | | | | | | |
| *H. leucosarx* | L1 | 1, 2 | *S. repens* | Terschelling | Thijssensduin | Acidic-Wet |
| *H. leucosarx* | L73 | 2 | *S. repens* | Drenthe | Vledder | Acidic-Wet |
| *H. leucosarx* | L143 | 2 | *S. repens* | Terschelling | Schoenus | Calcareous-Wet |
| *H. leucosarx* | L144 | 2 | *S. repens* | Terschelling | Schoenus | Calcareous-Wet |
| *P. involutus* | L82 | 1 | *S. repens* | Terschelling | Paardenwei | Acidic-Dry |
| *G. mosseae* | BEG 12 | 1 | unknown | England | | |
| Cuttings | | 1, 2 | *S. repens* | Terschelling | Schoenus | Calcareous-Wet |
| | | 1 | *S. repens* | Terschelling | Oosterend | Acidic-Dry |
| | | 1 | *S. repens* | Drenthe | Vledder | Acidic-Wet |

*Inoculation and plant growth conditions*

After storage at 4 °C for one month, the cuttings of *S. repens* were trimmed to 4 cm, surface-sterilized twice in (freshly prepared) 6% $H_2O_2$ for 1 min (in series of 20 in 300 mL), and rinsed three times in (fresh) demineralized water (1 L) for 10 min. Each cutting was placed individually in a culture tube containing 20 mL sterile water agar (1 %). After a 10 weeks rooting period in a climate chamber (photon flux density 120 $\mu E.m^{-2}.s^{-1}$ (11,000 lux) day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 70 %) equally developed cuttings were selected for the experiment.

The substrate used was a sand-perlite mixture 1:3 (v/v). No nutrient solution was supplied during the experiment. Demineralized water was added to the substrate (1:3:1

v/v/v sand:perlite:water) 24 h prior to autoclaving. The substrate was autoclaved twice (1 h, 121 °C, 1 atm.) with a 48 h interval, and left for one week. Root growth chambers (vertically placed Petri dishes 15 cm diameter, with a slit in the edge) were filled with the sterile substrate. Each root growth chamber contained 300 mL substrate, of which 75 mL (*c.* 120 g) was sand. Cuttings were transferred to the root growth chambers (1 cutting per root growth chamber), each cutting was inoculated with 5 mycelial plugs cut from the edge of a precultured EcM fungal colony, the root system was covered with a water agar (1 %) layer (5 cm diameter) to prevent the roots from excessive loss of water. The root growth chambers were sealed with tape and sterilized anhydrous lanolin preventing contamination and loss of water. The control plants were supplied with 5 plugs of solid medium (5 * 0.15 mL) without fungus. The inoculation of AMF was performed by mixing 20 g freshly washed root material of *T. repens* containing *G. mosseae* with the sterile substrate (10 L), and 30 root growth chambers were filled with this mixture. Cuttings were transferred to the root growth chambers, the root systems were covered with water agar, root growth chambers were closed and sealed. As a previous experiment with addition of roots of *T. repens* without AMF or AMF washings to the control (and EcM) plants showed no effect on plant performance (APPENDICES E AND F), no attempt was made to provide control (and EcM) plants with roots of *T. repens* without AMF or AMF washings. All soil compartments were protected from daylight, the root growth chambers were placed vertically in transient propagators (relative air humidity almost 100 % in the first week) and placed in a climate chamber. Growth conditions: photon flux density 350 $\mu E.m^{-2}.s^{-1}$ (20,000 lux), day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 60 %. The plants were arranged in a complete randomized block design, and blocks were randomized every three weeks within the climate chamber. The plants were watered every three weeks with 10 mL demineralized water. After 12 weeks the plants were harvested. In experiment 1 ten plants of the 15 of each fungus-cutting combination were harvested randomly, and seven control cuttings of each origin due to poor survival. In experiment 2 all surviving plants were harvested.

*Experiment 1: One fungus origin and three different host plant origins*

Cuttings of *S. repens* were collected in March. A factorial experimental design was used: two different soil types (soils I and II), and three different origins (clones) of

cuttings, each analyzed for the effects of three different mycorrhizal fungi and a non-mycorrhizal treatment. Twenty cuttings of each field site were harvested at the moment of inoculation (t = 0). Each fungus was individually inoculated to the three different origins of *S. repens* in 15 replicates, and 15 cuttings were supplied with non-inoculated agar plugs (control). The isolates of *H. leucosarx* and *P. involutus* were precultured twice (successively) for three weeks on solid media (AMN) in order to obtain sufficient actively growing fungal material. *G. mosseae* was precultured (inoculum density: AMF propagules to sterilized soil, 10 % v/v) on roots of *Trifolium repens* for 15 weeks.

*Experiment 2: One host origin and four different isolates of H. leucosarx*

Cuttings were collected in December 1995 in field site Schoenus. Isolates of *H. leucosarx* again comprized different habitat origins and different geographical locations. This experiment was performed on soil type I. Twenty cuttings were harvested at the moment of inoculation (t = 0). Four isolates of *H. leucosarx* were individually inoculated to *S. repens* in 12 replicates, and 12 cuttings were supplied with non-inoculated agar plugs (control). The isolates of *H. leucosarx* were precultured twice (successively) for three weeks on solid media in order to obtain sufficient actively growing fungal material.

*Plant performance. EcMF and AMF colonization*

Every three weeks length of shoots was determined. After the harvest shoot weight was determined after 72 hours at 70 °C. The plants were individually digested in sulphuric acid, salicylic acid and 30 % hydrogen peroxide. Total N and P were analyzed spectrophoto-metrically with a continuous flow analyser using samples with known chemical composition as standard (Novozamsky *et al.*, 1988).

The roots were immersed in water over a 2 mm sieve to remove most of the soil, and rinsed gently to avoid damage of the mycorrhizas. The cleaned ectomycorrhizal roots were stored in glutaraldehyde buffer (Alexander & Bigg, 1981) and roots of the control plants and the AM plants were stored in 50 % alcohol until they could be processed. In each sample, root length was determined according to Newman (1966) and the numbers of EcM root tips, and total numbers of root tips were counted. EcM frequencies were calculated (100 % * numbers of EcM root tips/total numbers of root tips). EcM root length (%) was not measured in this experiment, since previous studies of mycorrhizal *S. repens* root systems showed that EcM frequency (%) was similar to percentage of the root length

colonized by EcM (CHAPTERS 3, 5, 6, 7, AND 8). All root samples that had been inoculated with AMF were cleared with 10 % KOH for three hours in a waterbath at 90 °C, bleached in 10 % $H_2O_2$ for 1 hour, acidified in 1 % HCl for 15 min and stained with trypan blue (Phillips & Hayman, 1970; APPENDIX C) in lactophenol for 30 min. AM colonization was estimated by a modified line intersect method (McGonigle *et al.*, 1990), where a minimum of 100 line intersections per root sample (replicated three times) was scored for the presence of AM structures. AM root length times intensity (RLCI = RLC * % cover by AM of the cross section of the root colonized) was calculated.

*Data analysis*

After 10 weeks rooting period, at t = 0 in experiment 1, cuttings from different field sites (origins) showed differences in all plant parameters measured. Therefore, data of t = 0 per host origin were set to 100 % and all plant parameters after 12 weeks were expressed in a percentage of t = 0.

*Calculation of symbiotic effectiveness* - Symbiotic (mycorrhizal) effectiveness was calculated for (1) shoot biomass, (2) N-content and (3) P-content as:

[*3*] Mycorrhizal effectiveness = 1 – ( b / a ),

where a is the mean plant biomass (or N-content or P-content) of one of the mycorrhizal treatments, and b is the mean plant biomass (or N-content or P-content) of the non-mycorrhizal control.

*Statistical analysis* - Data were analysed by Analysis of Variance using the statistical package STATISTICA (StatSoft). Prior to analysis proportional mycorrhizal colonizations were arcsine square root transformed and plant parameters in experiment 2 were logarithmically transformed. Data (percentages) in experiment 1 all were arcsine transformed prior to analysis. Plant growth curves were analysed with repeated measures ANOVA. Bartlett's test was used to determine whether variances were equal between treatments. Differences among means were evaluated with the LSD-test (Sokal & Rohlf, 1995).

**TABLE 9-2.** Results of ANOVA (*P* values) executed for shoot length, shoot biomass, root length, mycorrhizal colonization, shoot N and P concentration and content of *Salix repens* cuttings (Schoenus, Oosterend, Vledder) ($n$ = 10) inoculated with *Glomus mosseae, Hebeloma leucosarx, Paxillus involutus* or not inoculated (control) harvested after 12 weeks.

| | Source of variation | | |
|---|---|---|---|
| Response variable | Soil type (df = 1) | Host provenance (df = 2) | Soil × Host (df = 2) |
| Mycorrhizal colonization | | | |
| *G. mosseae* | 0.716 | 0.068 | <0.001 |
| *H. leucosarx* | 0.022 | 0.002 | 0.321 |
| *P. involutus* | <0.001 | 0.464 | 0.068 |
| Plant parameters | | | |
| Control | | | |
| Shoot length | 0.008 | <0.001 | <0.001 |
| Shoot biomass | 0.534 | <0.001 | <0.001 |
| Root length | 0.429 | 0.090 | <0.001 |
| Shoot N concentration | 0.567 | 0.009 | 0.364 |
| Shoot N content | 0.887 | <0.001 | <0.001 |
| Shoot P concentration | 0.180 | <0.001 | 0.090 |
| Shoot P content | 0.237 | 0.002 | <0.001 |
| *G. mosseae* | | | |
| Shoot length | 0.060 | 0.006 | 0.031 |
| Shoot biomass | <0.001 | <0.001 | <0.001 |
| Root length | <0.001 | 0.008 | 0.013 |
| Shoot N concentration | 0.729 | 0.024 | 0.083 |
| Shoot N content | <0.001 | <0.001 | <0.001 |
| Shoot P concentration | <0.001 | <0.001 | <0.001 |
| Shoot P content | 0.029 | <0.001 | <0.001 |
| *H. leucosarx* | | | |
| Shoot length | 0.063 | 0.012 | 0.203 |
| Shoot biomass | 0.012 | <0.001 | 0.112 |
| Root length | <0.001 | <0.001 | 0.694 |
| Shoot N concentration | <0.001 | <0.001 | <0.001 |
| Shoot N content | <0.001 | 0.001 | <0.001 |
| Shoot P concentration | <0.001 | <0.001 | 0.001 |
| Shoot P content | 0.545 | <0.001 | 0.022 |
| *P. involutus* | | | |
| Shoot length | 0.134 | 0.028 | <0.001 |
| Shoot biomass | <0.001 | <0.001 | 0.006 |
| Root length | 0.027 | <0.001 | 0.314 |
| Shoot N concentration | 0.618 | 0.011 | <0.001 |
| Shoot N content | <0.001 | 0.002 | 0.580 |
| Shoot P concentration | 0.611 | 0.037 | 0.222 |
| Shoot P content | 0.001 | 0.075 | 0.048 |

* df Error = 45.

## RESULTS

*Experiment 1: One fungus origin and three different host plant origins.*

*Survival* - Only 50 % of the control plants survived, whereas from cuttings inoculated with each of the mycorrhizal fungi survival was over 80 %, irrespective of origin.

*Mycorrhizal colonization* - Host origin affected mycorrhizal colonization of *H. leucosarx*. Soil type affected mycorrhizal colonization of cuttings with *H. leucosarx* and *P. involutus*, whereas mycorrhizal colonization of cuttings with *G. mosseae* was only affected by the origin × soil interaction (TABLE 9-2). Colonization by *G. mosseae* was lower on cuttings from Vledder in soil I, but not in soil II. Colonization by *H. leucosarx* was highest on cuttings from Vledder, irrespective of soil type, and higher on cuttings from Schoenus in soil I than in soil II. Mycorrhizal colonization by *P. involutus* was higher in soil I on cuttings from Schoenus and Vledder than in soil II, and in soil I lower on cuttings from Oosterend than on those from the other origins (TABLE 9-3).

*Plant growth* - *Salix repens* collected from different field sites, showed differences with regard to soil and to mycorrhizal colonization (FIG. 9-1). Cuttings from Schoenus grew better in soil I than in soil II, whereas cuttings from Vledder showed the opposite trend (FIGS. 9-1a,c). Cuttings from Oosterend grew equally well on both soils (FIG. 9-1b). Host origin affected shoot length, shoot biomass and root length for all three fungal species, whereas for non-mycorrhizal plants it affected only shoot growth and biomass increment. For all three fungal species soil type affected root length and shoot biomass, but not shoot length (TABLE 9-2).

Differences between origins were much larger on soil II than on soil I (TABLE 9-3). In general, cuttings from Schoenus were smaller in shoot length or weight than those from Vledder on soil II. Non-mycorrhizal cuttings from Schoenus had longer shoots on soil I than on soil II, whereas the opposite pattern was observed for cuttings from Vledder. Cuttings inoculated with *P. involutus* from Schoenus also produced longer shoots on soil I than on soil II, but cuttings inoculated with *G. mosseae* and *H. leucosarx* from Vledder showed the opposite effect. Dry weight showed a similar pattern. Root length in soil II was highest for mycorrhizal cuttings from Schoenus, however, non-mycorrhizal cuttings had lowest root length on cuttings from Schoenus. Irrespective of their origin, cuttings inoculated with *G. mosseae* and *H. leucosarx* had higher root length in soil II compared to soil I, but for cuttings inoculated with *P. involutus* root length was similar between both soil types. Root length of non-mycorrhizal plants from Schoenus was higher in soil I than in soil II, but those of Vledder lower in soil I (TABLE 9-3).

**TABLE 9-3.** Mycorrhizal colonization, shoot length, shoot dry weight, and root length (means ± 1 SE, $n$ = 10) of *Salix repens* (Schoenus, Oosterend, Vledder) inoculated with *Glomus mosseae, Hebeloma leucosarx, Paxillus involutus* or not inoculated (control), harvested after 12 weeks (Soil I or II).

| Fungal treatment | Provenance | Mycorrhizal colonization (%) | | Shoot length (%$t_0$) | | Shoot dry weight (%$t_0$) | | Root length (%$t_0$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Soil I | Soil II | Soil I | Soil II | Soil I | Soil II | Soil I | Soil II |
| Control ($t_0$) | Schoenus | 0 | | 100% (47.0 ± 2.4 mm) | | 100% (97.3 ± 3.5 mg) | | 100% (0.9 ± 0.1 m) | |
| | Oosterend | 0 | | 100% (62.1 ± 1.9 mm) | | 100% (96.3 ± 4.1 mg) | | 100% (3.2 ± 0.2 m) | |
| | Vledder | 0 | | 100% (40.7 ± 1.9 mm) | | 100% (52.5 ± 5.1 mg) | | 100% (2.1 ± 0.2 m) | |
| Control | Schoenus | 0 | 0 | **377 ± 28** b | **111 ± 22** a | **146 ± 16** a | **50 ± 7** A | **380 ± 36** b | **178 ± 36** a |
| | Oosterend | 0 | 0 | 323 ± 20 a | 316 ± 40 b | 157 ± 11 a | 157 ± 23 B | 228 ± 27 a | 365 ± 84 b |
| | Vledder | 0 | 0 | **306 ± 40** a | **516 ± 22** c | **180 ± 29** a | **400 ± 24** C | **249 ± 40** a | **576 ± 38** c |
| *G. mosseae* | Schoenus | 3.8 ± 0.4 b* | 3.1 ± 0.4 a | 295 ± 42 a | 243 ± 22 a | 133 ± 11 a | 138 ± 9 A | **267 ± 23** b | **1743±168** b |
| | Oosterend | 3.9 ± 0.6 b | 2.8 ± 0.2 a | 286 ± 22 a | 373 ± 46 b | **142 ± 12** a | **207 ± 28** B | **452 ± 22** b | **728 ± 81** a |
| | Vledder | 1.8 ± 0.2 a | 3.6 ± 0.4 a | **338 ± 40** a | **484 ± 40** c | **168 ± 14** a | **404 ± 15** C | **191 ± 22** a | **932 ± 43** a |
| *H. leucosarx* | Schoenus | **87 ± 2.2** a | **79 ± 2.8** a | 387 ± 44 a | 420 ± 56 bc | 126 ± 15 a | 155 ± 28 A | **278 ± 54** b | **832 ±157** b |
| | Oosterend | 84 ± 1.9 a | 83 ± 2.8 ab | 327 ± 39 a | 330 ± 22 ab | 172 ± 13 b | 180 ± 15 A | **155 ± 14** a | **401 ± 53** a |
| | Vledder | 93 ± 1.6 b | 90 ± 1.7 b | **367 ± 21** a | **575 ± 43** c | **222 ± 11** b | **391 ± 34** B | **162 ± 15** a | **387 ± 15** a |
| *P. involutus* | Schoenus | **74 ± 4.8** b | **48 ± 8.5** a | **362 ± 24** b | **194± 28** a | **140 ± 10** a | **77 ± 7** A | 606 ± 56 b | 442 ± 39 b |
| | Oosterend | 60 ± 3.4 a | 53 ± 5.2 a | 248 ± 22 a | 266 ± 30 a | 129 ± 7 a | 107 ± 13 B | 259 ± 25 a | 194 ± 26 a |
| | Vledder | **71 ± 3.9** b | **40 ± 7.3** a | 286 ± 18 a | 367 ± 22 b | 153 ± 13 a | 141 ± 10 C | 225 ± 24 a | 217 ± 20 a |

* Values within a column (per fungal treatment) that are followed by a different letter indicate significant differences between host provenances and values printed in bold indicate significant differences between soil types, at $P < 0.05$ according to a LSD test, and a two-factor ANOVA (soil type: df = 1, provenance: df = 2; df Error = 45)

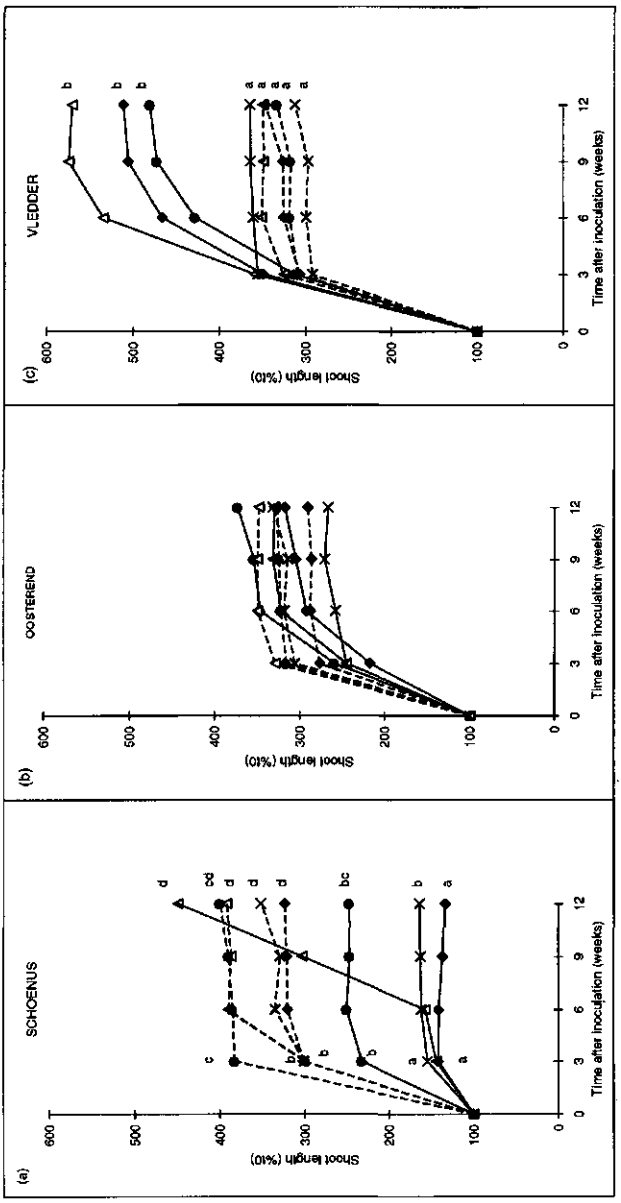



**FIGURE 9-1.** Mean shoot length of *Salix repens* inoculated with *Glomus mosseae* (●), *Hebeloma leucosarx* (○), *Paxillus involutus* (□) or non-inoculated agar plugs (control) (■) over 12 weeks. Cuttings originating from (a) Schoenus, (b) Oosterend, and (c) Vledder. Different letters next to points at 3, and 12 weeks after inoculation indicate different shoot length between mycorrhizal treatments and soil types (soil I: dotted line; soil II: continuous line) ($P < 0.05$) according to LSD tests.

**TABLE 9-4.** Shoot N concentration, shoot P concentration, shoot N content and shoot P content (means ± 1 SE, $n$ = 10) of *Salix repens* (Schoenus, Oosterend, Vledder) inoculated with *Glomus mosseae, Hebeloma leucosarx, Paxillus involutus* or not inoculated (control), harvested after 12 weeks (Soil I or II).

| Fungal treatment | | N conc. (%$t_0$) | | P conc. (%$t_0$) | | N content (%$t_0$) | | P content (%$t_0$) | |
|---|---|---|---|---|---|---|---|---|---|
| | Provenance | Soil I | Soil II | Soil I | Soil II | Soil I | Soil II | Soil I | Soil II |
| Control ($t_0$) | Schoenus | 100% (12.12 ± 0.34 g kg$^{-1}$) | | 100% (1.61 ± 0.04 g kg$^{-1}$) | | 100% (1.192 ± 0.075 mg) | | 100% (0.159 ± 0.011 mg) | |
| | Oosterend | 100% (10.53 ± 0.17 g kg$^{-1}$) | | 100% (1.75 ± 0.09 g kg$^{-1}$) | | 100% (1.008 ± 0.036 mg) | | 100% (0.170 ± 0.013 mg) | |
| | Vledder | 100% (10.91 ± 0.26 g kg$^{-1}$) | | 100% (1.94 ± 0.08 g kg$^{-1}$) | | 100% (0.565 ± 0.031 mg) | | 100% (0.101 ± 0.006 mg) | |
| Control | Schoenus | 60 ± 3 a* | 63 ± 2 b | 78 ± 3 a | 139 ± 53 B | **83 ± 8** a | **31 ± 4** a | **109 ± 10** a | **69 ± 30** a |
| | Oosterend | 61 ± 4 a | 58 ± 13 b | 76 ± 4 a | 62 ± 9 A | 94 ± 6 a | 97 ± 16 b | 115 ± 5 a | 86 ± 15 b |
| | Vledder | 55 ± 2 a | 51 ± 2 a | **60 ± 3** a | **39 ± 1** A | **100 ± 17** a | **205 ± 9** c | **103 ± 15** a | **159 ± 10** c |
| *G. mosseae* | Schoenus | 67 ± 5 b | 63 ± 2 b | **74 ± 5** b | **26 ± 2** A | 86 ± 7 ab | 86 ± 6 a | **94 ± 5** a | **35 ± 3** a |
| | Oosterend | 54 ± 4 a | 62 ± 8 ab | 70 ± 4 b | 62 ± 7 C | **74 ± 5** a | **119 ± 6** b | **95 ± 6** a | **120 ± 14** b |
| | Vledder | 58 ± 2 ab | 50 ± 2 a | **63 ± 2** a | **36 ± 1** B | **97 ± 6** b | **205 ± 8** c | **105 ± 8** a | **148 ± 7** c |
| *H. leucosarx* | Schoenus | **62 ± 5** b | **120 ± 11** c | 108 ± 6 b | 116 ± 7 C | **73 ± 6** a | **170 ± 25** b | 127 ± 12 a | 171 ± 29 a |
| | Oosterend | 61 ± 2 b | 60 ± 3 b | 113 ± 10 b | 79 ± 4 B | 104 ± 6 b | 106 ± 6 a | 184 ± 11 b | 140 ± 13 a |
| | Vledder | 51 ± 2 a | 48 ± 1 a | **90 ± 5** a | **62 ± 2** A | **114 ± 8** b | **188 ± 15** b | 203 ± 17 b | 240 ± 12 b |
| *P. involutus* | Schoenus | **53 ± 2** a | **72 ± 4** b | 84 ± 4 a | 96 ± 13 B | **74 ± 6** a | **54 ± 4** a | **114 ± 7** a | **69 ± 8** a |
| | Oosterend | 54 ± 3 a | 52 ± 2 a | 85 ± 6 a | 98 ± 10 B | **70 ± 4** a | **56 ± 6** a | 106 ± 7 a | 108 ± 25 b |
| | Vledder | **65 ± 4** b | **46 ± 1** a | 78 ± 3 a | 69 ± 2 A | **98 ± 7** b | **66 ± 6** a | 118 ± 7 a | 193 ± 7 b |

* Values within a column (per fungal treatment) that are followed by a different letter indicate significant differences between host provenances and values printed in bold indicate significant differences between soil types, at $P < 0.05$ according to a LSD test, and a two-factor ANOVA (soil type: df = 1, provenance: df = 2; df Error = 45).

*Plant nutrient uptake* - Host origin affected shoot N and P concentration and content for all fungal species (except shoot P content for cuttings inoculated with *P. involutus*). Soil type affected shoot N content for all three species and shoot P content for cuttings inoculated with *G. mosseae* and *P. involutus*. Non-mycorrhizal plants were affected by origin of the cuttings, but not by soil type (TABLE 9-2).

Effect of origin on nitrogen and phosphorus content was again stronger in soil II than in soil I (TABLE 9-4). For non-mycorrhizal cuttings and cuttings inoculated with *G. mosseae*, N and P content of soil II were lower for Schoenus and highest for Vledder. Shoot N content of cuttings with *P. involutus* was higher on soil I irrespective of their origin. Shoot N-content on the other hand was higher on soil II for cuttings with *G. mosseae* (Oosterend and Vledder) and *H. leucosarx* (Schoenus and Vledder). Shoot N content was always highest for cuttings of Vledder, irrespective of fungal species (TABLE 9-4).

**TABLE 9-5.** Mycorrhizal effectiveness based on shoot dry weight, shoot N-content and shoot P-content (means, $n = 10$) of *Salix repens* (Schoenus, Oosterend, Vledder) inoculated with *Glomus mosseae, Hebeloma leucosarx, Paxillus involutus* harvested after 12 weeks (Soil I or II).

| Fungal treatment | Provenance | Mycorrhizal effectiveness | | | | | |
|---|---|---|---|---|---|---|---|
| | | Shoot biomass | | Shoot N-content | | Shoot P-content | |
| | | Soil I | Soil II | Soil I | Soil II | Soil I | Soil II |
| *G. mosseae* | Schoenus | -0.10 | ***+0.64** | +0.04 | +0.64 | -0.16 | -0.97 |
| | Oosterend | -0.11 | +0.21 | **-0.21** | +0.18 | -0.21 | +0.28 |
| | Vledder | -0.07 | +0.01 | -0.03 | 0.00 | +0.02 | -0.07 |
| *H. leucosarx* | Schoenus | -0.16 | **+0.68** | -0.14 | +0.82 | +0.14 | +0.60 |
| | Oosterend | +0.09 | +0.13 | +0.10 | +0.08 | +0.38 | +0.39 |
| | Vledder | **+0.19** | -0.02 | **+0.14** | -0.09 | +0.49 | +0.34 |
| *P. involutus* | Schoenus | -0.04 | **+0.35** | -0.12 | +0.43 | +0.04 | 0.00 |
| | Oosterend | -0.22 | -0.47 | -0.34 | -0.73 | -0.08 | +0.20 |
| | Vledder | -0.18 | **-1.84** | -0.02 | -2.11 | +0.13 | +0.18 |

* Values within a column (per fungal treatment) that are printed in bold are significantly different from the control treatment in table 3 or 4 at $P < 0.05$ according to a LSD test, and a two-factor ANOVA (soil type: df = 1, provenance: df = 2; df Error = 45) detected differences amongst treatments.

*Symbiotic effectiveness* - Mycorrhizal effectiveness (biomass) was relatively constant and usually slightly negative in soil I (except for cuttings with *H. leucosarx* from Vledder), but showed large differences depending on plant origin and mycorrhizal fungal

species in soil II. Mycorrhizal effectiveness was highest for Schoenus and lowest for Vledder, irrespective of fungal species. Mycorrhizal effectiveness of cuttings with *G. mosseae* and *H. leucosarx* was similar and higher than of cuttings with *P. involutus*. Effectiveness was negative for cuttings from Oosterend and Vledder when inoculated with *P. involutus* (TABLE 9-5).

Mycorrhizal effectiveness (N-content) showed a similar pattern as mycorrhizal effectiveness (plant biomass) in soil I and soil II. However, mycorrhizal effectiveness (P-content) was low for cuttings from Schoenus inoculated with *G. mosseae* and for all origins mycorrhizal effectiveness was higher for cuttings inoculated with *H. leucosarx* than for cuttings with *G. mosseae*. Effectiveness of *P. involutus* was slightly positive for all origins.

*Experiment 2: One host origin and four different isolates of H. leucosarx.*

*Survival* - Only 50 % of the control plants survived, whereas from cuttings inoculated with *H. leucosarx* survival ranged from 75-92 % (isolates L1: 83 %, L143: 75 %, L144: 92 % and L73: 75 %).

*Plant performance and mycorrhizal colonization* - Shoot length, shoot biomass, root length, shoot P concentration, shoot N content and shoot P content were all significantly affected by the mycorrhizal treatment (FIG. 9-2, one factor ANOVA, $F_{4,39}$ = 5.04, 8.46, 2.76, 22.98, 5.94, 15.48, respectively ; $P < 0.05$), whereas none of the *H. leucosarx* isolates differed when the non-mycorrhizal plants were left out of the analysis (one factor ANOVA, $P > 0.05$).

When non-mycorrhizal plants were included in the analysis, isolate L1 showed the highest mycorrhizal colonization (LSD-tests, FIG. 9-2a). However, between fungal isolates of *H. leucosarx* effects on plant performance were small. Isolate L1 showed lower shoot length of *S.repens* than isolate L144 (FIG. 9-2b). Isolate L143 showed lower shoot biomass of *S. repens* compared to isolates L144 and L73 (FIG. 9-2c). Furthermore, isolate L144 showed longer root length of *S.repens* than isolates L1 and L73 (FIG. 9-2d). Within *H. leucosarx* isolates no effects were observed on shoot nutrient concentrations and contents (FIGS. 9-2e-h). Isolates (L143, L144) collected from the field site from which the cuttings originated did not perform better than the other two isolates.

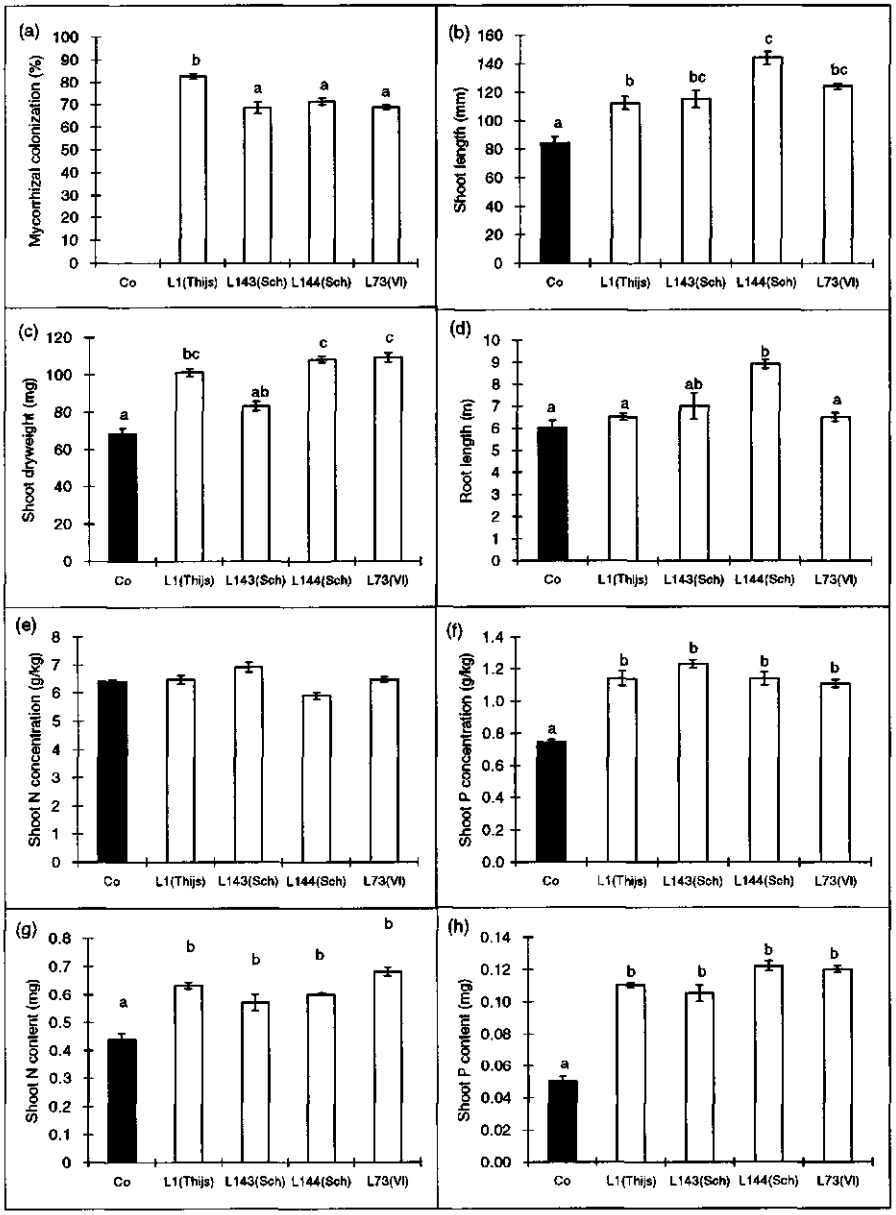



**FIGURE 9-2.** Mean (a) mycorrhizal colonization; (b) shoot length; (c) shoot dry weight; (d) root length; (e) shoot N concentration; (f) shoot P concentration; (g) shoot N content and (h) shoot P content of *Salix repens* inoculated with *Hebeloma leucosarx;* isolate L1 (Thijssensduin), L143, L144 (Schoenus), L73 (Vledder) or non-inoculated agar plugs (control), harvested after 12 weeks. Bars represent (±) 1 SE. Significant differences are indicated by different letters ($P < 0.05$) according to LSD tests.

## DISCUSSION

*Symbiotic effectiveness*

Symbiotic effectiveness (or mycorrhizal dependence) is expressed by comparing mycorrhizal to non-mycorrhizal plants. However, a number of drawbacks surround application of this concept. If non-mycorrhizal plants show lower survival than the mycorrhizal treatments (as in this study), mycorrhizal effectiveness is underestimated. As noted by Janos (1993) effectiveness furthermore depends on the interaction between mycorrhizal plant × mycorrhizal fungus × soil characteristics. This study addressed the relative contribution of mycorrhizal plant (by using genetically uniform clones of *S. repens*) and mycorrhizal fungus (by using genetically uniform material of two EcMF and one AMF). Because soil characteristics have a large impact on the outcome of the effectiveness calculation, it has been proposed to calculate response curves over a range of soil conditions, usually a gradient of phosphate availability (Brundrett *et al.*, 1996). In the first experiment therefore two different soil types were used. However, two further disadvantages with the calculation of effectiveness have less often been mentioned.

First, it is not straightforward which plant parameter should be used to measure effectiveness. Comparisons between mycorrhizal and non-mycorrhizal plants on the basis of above-ground biomass or shoot N-content or shoot P-content yielded (very) different outcomes in the present study. This is problematic when comparing different species of mycorrhizal fungi as the mycorrhizal symbiosis is multifunctional (Newsham *et al.*, 1995b). However, even in this study where one fungus with genetically different plant material was compared large differences were noted. Cuttings from different origins inoculated with *P. involutus* showed large differences in symbiotic effectiveness based on biomass, and shoot N-content, but were similar in effectiveness based on shoot P-content.

Second, different species of mycorrhizal fungi show large differences in response time and response duration, so that harvest time greatly affects the calculated effectiveness. In earlier studies (CHAPTERS 4 AND 5) it was shown that *G. mosseae* had a very rapid, positive effect (cf the growth curves of cuttings from Schoenus inoculated with *G. mosseae* in FIG. 9-1a), whereas *H. leucosarx* responded more slowly (cf large shoot length increment in cuttings from Schoenus in FIG. 9-1a).

Mycorrhizal effectiveness data can therefore not be used to compare fungal species, but for the purpose of assessing the relative contribution of plant and fungal origin to symbiotic effectiveness such data have some usefulness.

*Host plant origin*

In agreement with the first hypothesis, symbiotic effectiveness, measured on the basis of shoot biomass and shoot N-content was highest for cuttings from Schoenus (the poorest site) and lowest for cuttings from Vledder (the richest site), at least on the less nutrient-poor soil (soil II). It is unlikely that these differences were due to initial differences in shoot weight or N-content, as cuttings from Schoenus and Oosterend were largely similar at the start of the experiment, but showed large differences in both parameters at the end of the experiment. This difference was also not due to differences in colonization per se as on soil II mycorrhizal colonization of cuttings inoculated with *G. mosseae* or *P. involutus* was similar between Schoenus and Vledder, whereas for cuttings inoculated with *H. leucosarx* the origin from the poorer sites had even lower colonization. However, at the end of the experiment mycorrhizal cuttings from Schoenus had a larger root length increment than those of Vledder, irrespective of soil nutrient availability. It seems therefore possible that genetical variation in root characteristics explains the differences in symbiotic effectiveness. Genetical variation in plants for mycorrhizal colonization have been reported for groundnut (Kesava Rao *et al.*, 1980), cowpea (Mercy *et al.*, 1990), pearl millet (Krishna *et al.*, 1985) and loblolly pine (Dixon *et al.*, 1987).

Fitter (1985) noted that specific root length is highly plastic and generally increases on poorer soils. Non-mycorhizal cuttings from Schoenus showed higher root length on the poorest soil (soil I), whereas the non-mycorrhizal cuttings from Vledder showed higher root length on the less poor soil. This suggests that both phenotypic plasticity and genetic differences play a large role in this respect. Plant root response to soil conditions is furthermore influenced by mycorrhizal fungus, as root length was higher on the less poor soil irrespective of origin for both *G. mosseae* and *H. leucosarx*.

Effects of host plant origin have also been investigated by Tonkin *et al.* (1989). They studied five clones of *Eucalyptus marginata* Don ex. Sm., inoculated with two strains of *Pisolithus tinctorius*. Plant origin was relevant in that one strain of *P. tinctorius* formed only ectomycorrhizas with four clones from mature trees but not with a clone from a 4-month old seedling. However, from these four clones derived from mature trees no effect of host origin was reported. Effects of plant origin is usually investigated with seeds of different origins. Effects would then depend on the relative contribution of within-origin variation and between-origin variation. As mycorrhizal colonization has been shown to reduce variation in plant performance (Burgess & Malajczuk, 1989), such experiments

might underestimate the importance of genetic variation due to plant origin. This might explain the small differences in EcM development among seed origins in *Picea mariana* (Thomson *et al.*, 1990).

Contrary to the second hypothesis, symbiotic effectiveness was usually lower on the poorest soil (soil I), irrespective of origin and fungal species. This could be due to the fact that soil I was effectively too poor as a consequence of which the mycorrhizal symbiosis itself was hardly beneficial. The observation that most calculations of effectiveness (based on shoot biomass and shoot N-content, but not shoot P-content) were (slightly) negative on soil I (i.e. that non-mycorrhizal plants outperformed mycorrhizal plants) would be consistent with this explanation.

Differences in effectiveness as assessed by shoot biomass and shoot N-content on the one hand and shoot P-content on the other might have been caused by the low N/P-ratio of cuttings in this study at the start of the experiment (ranging from 5.5 to 7.5 and hence indicating N limitation but not P limitation).

Symbiotic effectiveness was also influenced by the interaction between plant origin and soil type. The growth curves (FIG. 9-1) indicate that, irrespective of mycorrhizal fungal partner, plants from Schoenus generally grew better on soil I than on soil II, whereas cuttings from Vledder grew better on soil II than on soil I. Cuttings from Oosterend were almost always intermediate in their reactions. Interestingly, the growth curves did not indicate any differential growth depending on soil type or fungal species.

*Fungal origin*

Differences in symbiotic effectiveness were absent for fungal isolates from different origins. The largest difference in shoot weight was even noted between isolates from the same origin (FIG. 9-2). The uniformity of response contrasts with observations by Burgess *et al.* (1994) who noted a large variation in the extent of mycorrhizal colonization by different isolates of *Pisolithus tinctorius* inoculated on *Eucalyptus grandis* W. Hill. They also established a highly significant positive correlation between the extent of mycorrhizal development and biomass for the plant. However, their results could have been due to the fact that four of their isolates were not associated with eucalypts (two from Australia, one from Europe, one from North America). Variation in the other 16 isolates, all from eucalypts, was much lower. A recent publication (Martin *et al.*, 1998) indicates that the name *P. tinctorius* is used for a species aggregate of which strains from various

continents, associated with different trees, show substantial molecular differences. Apparently, geographic differentiation has led to ecological differentiation, even in this EcMF with its broad host range.

Large variation in plant response has also been observed for loblolly pine inoculated with three different isolates of *P. tinctorius* (Dixon *et al.*, 1987). Lack of variation in response of various isolates of *H. leucosarx* might be related to the fact that the four isolates were isolated from one host species, i.e. *S. repens*. *Hebeloma leucosarx* is only associated with members of the genus *Salix*. Crossing experiments and molecular data indicate that the species consists of 2 InterCompatibilityGroups that have recently diverged (Aanen & Kuyper, 1999). Even in cases of limited effects of fungal origin there might be substantial genetic variation. A dikaryon was produced in the lab from monospore progeny of another strain of *H. leucosarx* and this reconstituted dikaryon completely failed to form mycorrhizas with cuttings of *S. repens*, even though the parent strain was found in association with *S. repens* (E.W. van der Heijden, unpubl. observation). Inability of (some) reconstituted dikaryons to form mycorrhizas has also been noted with *P. tinctorius* (Lamhamedi *et al.*, 1990) and *Laccaria bicolor* (Kropp, 1997). Apparently there must be strong selection in the field for the ability to form mycorrhizas and to establish an efficient symbiotic relationship. This strong selection will likely reduce variation in plant effects in compatible combinations in the field.

Variation in fungal response with genetically uniform plant material was much lower than variation in plant response with genetically uniform fungal material, both for EcMF and AMF, in agreement with hypothesis iv. This suggests that more attention should be devoted to understanding the role of plant genetic variation in establishing and maintaining a compatible and effective mycorrhizal symbiosis (cf Smith *et al.*, 1992). Specifity phenomena in mycorrhizal symbiosis might have been approached too much from a mycocentric perspective. Studies considering the ecological significance of mycorrhizal symbiosis should be more explicit about the selection of both mycorrhizal plant and fungus.

# 10

## When a low amount of mycorrhiza is enough: plant limits to colonization by arbuscular mycorrhizal fungi.

**ABSTRACT**

*Arbuscular mycorrhizal (AM) inoculum potential in* Salix repens *was studied in two experiments. In the first experiment with a naturally high AM and low ectomycorrhizal (EcM) inoculum potential, AM colonization was low in* S. repens. *AM and EcM did not show a negative correlation. A large P-uptake was noted that coincided with the establishment of the AM symbiosis. These data therefore suggest that the plant controls the amount of AM colonization.*

*In the second experiment in two field plots, relatively dry or extremely wet, AM increased and EcM decreased with moisture content. The higher AM colonization intensity after waterlogging was due to intraradical spore formation. EcM was reduced after waterlogging, in particular the EcM sheath, but fractions of the Hartig net survived. Changes in colonization of both mycorrhizal types after waterlogging are best explained as differential expressions of their survival strategies.*

*Short-term and long-term benefits of a high amount of AM for* Salix repens *are discussed.*

**KEY WORDS**: Arbuscular mycorrhiza, ectomycorrhiza, *Salix repens*, dual infection, inoculum potential, waterlogging.

## INTRODUCTION

Most plants form only one type of mycorrhiza, a mutualistic association between fungi and plant roots. Only few plant species are known that form both arbuscular mycorrhiza (AM) and ectomycorrhiza (EcM). In these plants, field observations have sometimes shown dominance by AM fungi, sometimes by EcM fungi (Lodge, 1989). The factors that favour dominance by AM or EcM have yet to be determined. Most mature soils harbour extensive mycelia of both AM and EcM fungi (AMF and EcMF), which do not only function as a nutrient foraging system, but also as a source of inoculum for uncolonized roots (Brundrett *et al.*, 1985; Smith & Read, 1997).

In several of these dual mycorrhizal plant species, AM fungi colonize seedlings first due to their initially higher inoculum potential (Read *et al.*, 1977; Chilvers *et al.*, 1987). However, they are rapidly replaced by EcM fungi (Chilvers *et al.*, 1987; Lodge, 1989). Lodge (1989) noted that many dual mycorrhizal plant species typically grow in flood plains and suggested that plants with the ability to form both types of mycorrhiza might have a selective advantage in such habitats.

*Salix repens* L. is one of the dual mycorrhizal plant species (Harley & Harley, 1987), widespread in a great variety of plant communities in The Netherlands. Field observations of *S. repens* roots, both in coastal dune ecosystems (16 sites comprising a natural range of highly different environmental conditions) and in inland ecosystems (4 sites), have indicated that EcM colonization ranges from (70-) 80-100 %. This high EcM colonization was observed in all sites: 1) even in sites where no EcM fungal sporocarps have been observed, 2) in primary successional sites, and 3) in regularly flooded sites where AM fungi were hypothesized to dominate (CHAPTER 3). AM colonization in these plots was very low. Colonization varied between habitat types and seasons, but mycorrhizal colonization intensity was always less than 11% (CHAPTER 3). Thus, in different habitats and geographical locations the root colonization pattern of *S. repens* was similar. AM colonization was mainly observed on and within ectomycorrhizal root fragments, suggesting that both types of mycorrhiza did not interact negatively. Moreover, mycorrhizal syntheses in the laboratory have shown similar colonization to the field by both types of mycorrhiza in cuttings of *S. repens* in single inoculations (CHAPTERS 4, 5, 7, 8, AND 9).

Four explanations have been forwarded for low colonization. Low AM colonization in the laboratory could be caused by specificity of the fungal species

(McGonigle & Fitter, 1990; Dhillion, 1992) or by a low level of inoculum applied. Low AM colonization in the field might be explained by the high inoculum pressure of EcM fungi. Finally, low colonization by AM fungi might reflect plant control over colonization in relation to plant nutrient demand. I tested these possibilities by baiting *S. repens* in the laboratory in a field soil with high AM inoculum potential and low EcM inoculum potential. I hypothesized that, if under these conditions AM colonization was still low, the plant limits the amount of colonization by AM fungi.

Amount of colonization could also depend on the water availability of sites. Lodge (1989) hypothesized that in a moisture gradient from relatively dry to wet conditions AM colonization will increase and EcM colonization will decline. I tested this hypothesis by baiting *S. repens* in a soil moisture gradient, connected with a vegetation shift from both EcM and AM plants (30 versus 70 % cover) to only AM plant species.

## MATERIAL AND METHODS

### *Plant material*

Cuttings (shoot tops) of *Salix repens* used in experiment 1 and 2 were collected in December 1995 and March 1995, respectively, from the field site 'Schoenus I' on the isle of Terschelling. All cuttings were collected from the same plant in order to obtain genetic homogeneity. After storage of the cuttings at 4 °C for one month, cuttings were trimmed to 4 cm and surface-sterilized twice in (freshly prepared) 6 % $H_2O_2$ for 1 min (in series of 20 in 300 mL) and rinsed three times in (fresh) demineralized water (1 L) for 10 min. Each cutting was placed individually in a culture tube containing 20 ml sterile water agar (1 %). After a 10 weeks of rooting period of the cuttings in a climate chamber (photon flux density 120 $\mu E.m^{-2}.s^{-1}$ (11,000 lux), daylight 16 h at 20 °C and darkness 8 h at 16 °C, relative air humidity 70 %) equally developed cuttings were selected for the experiments.

### *Experiment 1: Laboratory experiment in a field soil with high AM and low EcM potential*

In March 1996, an intact sod (40*80*10 cm) of grassland was collected from a nutrient-poor field site in Wijster, Drenthe. In this grassland *S. repens* occurs locally. The selected sod, however, only contained AM plant species. No EcM plant species occurred within 10 m distance from the sod. This sod was placed in a climate chamber (photon flux density 350 $\mu E.m^{-2}.s^{-1}$ (20,000 lux), daylight 16 h at 20 °C and darkness 8 h at 16 °C, relative air humidity 60 %). After two weeks of equilibration, 50 rooted cuttings of *S.*

*repens* (*c.* 100 mg shoot weight, 10 cm root length) were introduced as bait plants. The plants were watered with demineralized water. Another 20 cuttings were harvested at the moment of planting (t = 0). Eight cuttings of *S. repens* were harvested randomly, after 2, 4, 6, 8 and 10 weeks successively.

Both at the start and at the end of the experiment, three soil samples were taken randomly (t = 0 and 10). For each sampling date soil samples were mixed and dried separately at 40 °C for four days. Total amounts of N and P in soil were determined after digestion in sulphuric acid, salicylic acid, and 30 % hydrogen peroxide. Plant-available nutrients in soil were analysed according to the $CaCl_2$ method (Houba *et al.* 1990). The acid digests and the $CaCl_2$ extracts were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard.

*Experiment 2: Field experiment in a soil moisture gradient*

This experiment was conducted in Wijster, Drenthe at the nature reserve 'IJsbaan'. This field site comprises a natural gradient from relatively dry soil conditions to a fen. Each year the water table is artificially increased in winter. *Salix repens* has established in the relatively dry part of this moisture gradient and is associated with AM (unidentified) and EcM fungi. Sporocarps of e.g. *Cortinarius uliginosus* Berk., *Hebeloma leucosarx* P.D. Orton, and *Lactarius theiogalus* (Bull.: Fr.) S.F. Gray have been identified over at least three years, EcM morphotypes have been traced from sporocarps and were described for below-ground identification.

Three plots, each measuring 4 $m^2$, were selected. Plot 1 was located in the established *S. repens* community at 30 m distance from the fen, water level on average 20 cm below surface in mid-summer. Plot 2 was located in a moist part near the fen at 15 m distance from the fen, water level on average 5 cm below surface, where no *S. repens* and no sporocarps of EcMF were found. Plot 3 was located just near the fen and completely waterlogged. In each plot 100 rooted cuttings of *S. repens* (*c.* 90 mg shoot weight, 150 cm root length) were planted at equal distance from each other at the end of June 1995. Due to the extremely wet summer in 1995 (since June 1995) plot 3 was never within reach for harvesting and therefore excluded from the experiment. Another 20 cuttings were harvested at the moment of planting (t = 0). After 7 (August) and 13 weeks (Sept./Oct.), 15 cuttings were harvested randomly from each plot. During winter all plots were completely flooded and covered with ice during three months. After this winter it was not possible to

enter the plots before the end of May (1996), due to the high water level. From the 70 bait plants remaining before winter, just 15 per plot could be traced. Therefore, after 48 weeks (May) and 61 weeks (September), 5 and 8 cuttings, respectively, were harvested randomly in each plot.

*Plant performance, AM and EcM colonization*

Shoot length was measured after harvesting. Shoot weight was determined after 72 hours at 70 °C. The plants were individually digested in sulphuric acid, salicylic acid and 30 % hydrogen peroxide. Total N and P were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard (Novozamsky *et al.*, 1988).

The roots were immersed in water over a 2 mm sieve to remove most of the soil and organic matter, and rinsed gently to avoid damage of the mycorrhizas. All root systems were split, in order to store cleaned roots for AM assessment in 50 % alcohol, and for EcM assessment in glutaraldehyde buffer (Alexander & Bigg, 1981), until they could be processed. In each sample root length was determined according to Newman (1966) (expt 1: 100 $cm^3$ sample volume; expt 2: 1200 $cm^3$ sample volume). For AM assessment roots were cleared with 10 % KOH for three hours in a water bath at 90 °C, bleached in 10 % $H_2O_2$ for 1 hour, acidified in 1 % HCl for 15 min and stained with trypan blue (Phillips & Hayman, 1970; APPENDIX C) in lactophenol for 30 min. AM colonization was estimated by a modified line intersect method (McGonigle *et al.*, 1990), where a minimum of 100 intersections per root subsample (replicated three times per sample) was scored for the presence of AM structures. AM root length colonization (RLC) times intensity (I) (RLCI = RLC * % of the cross section of the colonized root) was calculated. For EcM assessment the numbers of EcM root tips and total numbers of root tips were counted. EcM frequencies were calculated (100 % * numbers of EcM root tips/total numbers of root tips). EcM mantles and Hartig nets were visible on long stretches of lateral roots and not only on the root tips, similar to colonization by AMF. Previous studies of mycorrhizal *S. repens* root systems showed that, due to the high colonization of *Salix repens* roots by EcM fungi, EcM frequency (%) was similar to EcM root length colonized (%) (CHAPTERS 3, 5, 6, 7, AND 8). Therefore, EcM root length was considered equal to EcM frequency.

*Statistical analysis*

Data were analysed by Analysis of Variance using the statistical package STATISTICA (StatSoft). Prior to analysis proportional mycorrhizal colonizations were arcsine square root transformed and other data were logarithmically transformed. Bartlett's test was used to determine whether variances were equal between treatments. Differences among means were evaluated with the LSD-test (Sokal & Rohlf, 1995). Data not normally distributed (even after transformation) were tested with Kruskal-Wallis and Mann-Whitney U-tests (Siegel & Castellan, 1988).

Correlation between AM colonization and EcM colonization of all mycorrhizal root sytems was tested using Spearman's rank correlation test (Siegel & Castellan, 1988).

## RESULTS

*Experiment 1: Laboratory experiment in a field soil with high AM and low EcM potential*

Shoot biomass of *S. repens* increased over time, but just not significantly ($F_{5,43}$ = 2.35, $P$ = 0.055, data not shown), whereas root length significantly increased over time ($F_{5,43}$ = 2.58, $P$ = 0.040, FIG. 10-1h). Both AM and EcM colonization changed significantly over time ($F_{4,35}$ = 2.41, $P$ = 0.043, $F_{4,35}$ = 41.40, $P$ < 0.001, respectively, FIGS. 10-1a,b). AM colonization of *S. repens* reached its highest intensity after 8 weeks, but between harvests after 2, 4, 6 and 10 weeks differences were not significant. An increase in EcM colonization was observed from week 6 to 10, but the EcM sheath was poorly developed. Spearman's rank correlation test indicated a significantly positive correlation between EcM (%RLC) and AM (%RLCI) ($R$ = 0.37, $P$ = 0.016), whereas no significant correlation was found between EcM (%RLC) and AM (%RLC) ($R$ = 0.27, $P$ = 0.086). A final check on root colonization (n = 5) by AM and EcM after 16 weeks showed 3.2 % (AM RLCI) and 64 % (EcM RLC). Even though EcM colonization was significantly higher ($P$ < 0.001), AM colonization was not significantly different from the previous sampling dates ($P$ = 0.18).

Different AM structures showed significant differences. After 6 and 8 weeks %RLC by hyphae was higher than at the other harvests ($F_{4,35}$ = 6.99, $P$ = 0.002). Also %RLC by vesicles was significantly higher after 4, 8 and 10 weeks ($F_{4,35}$ = 3.64, $P$ = 0.029). Hyphal coils and arbuscules hardly occurred and showed no differences (TABLE 10-1).

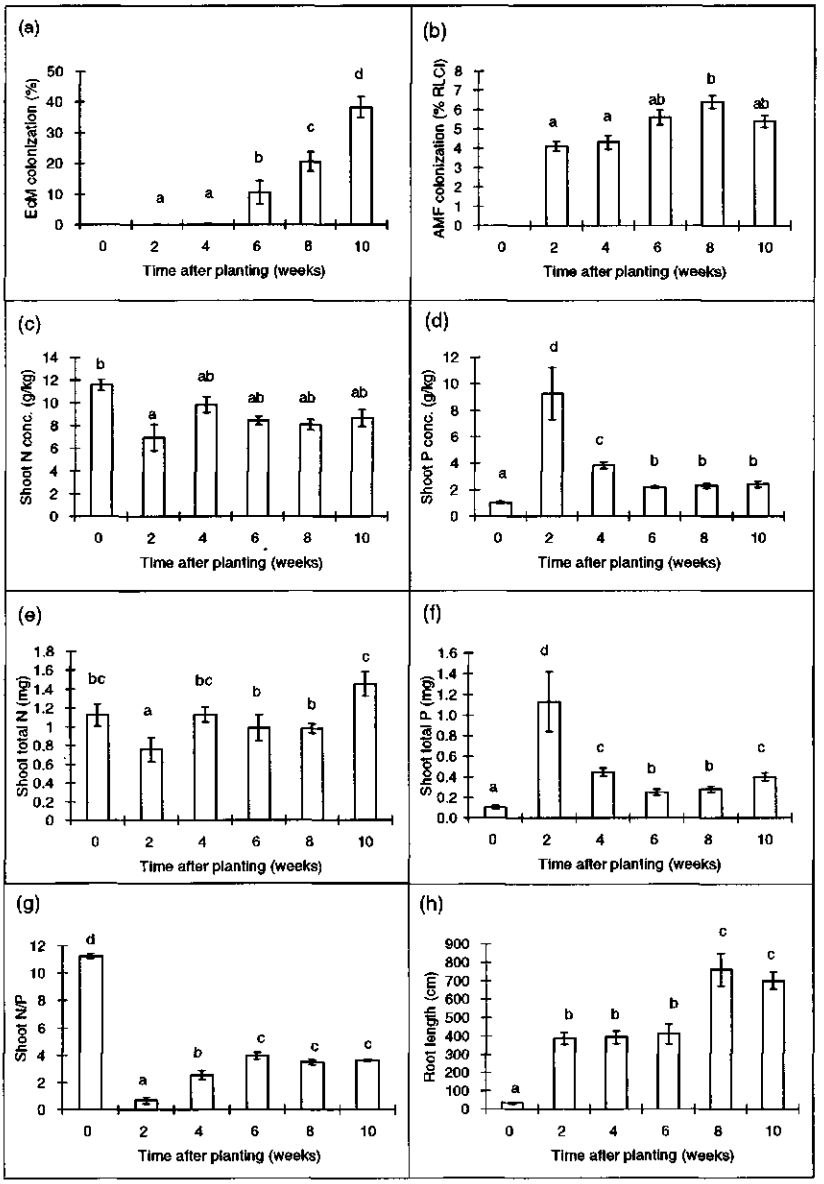



**FIGURE 10-1.** Mean ectomycorrhizal colonization (a), arbuscular mycorrhizal colonization (b), shoot N concentration (c), shoot P concentration (d), shoot N content (e), shoot P content (f), shoot N/P ratio (g) and root length (h) of *Salix repens* during 10 weeks. Error bars indicate (±) 1 SE ($n$ = 8). Bars represented by different letters are significantly different according to LSD or MWU ($P < 0.05$), and a one-factor ANOVA (df = 4, 35) or Kruskal-Wallis test.

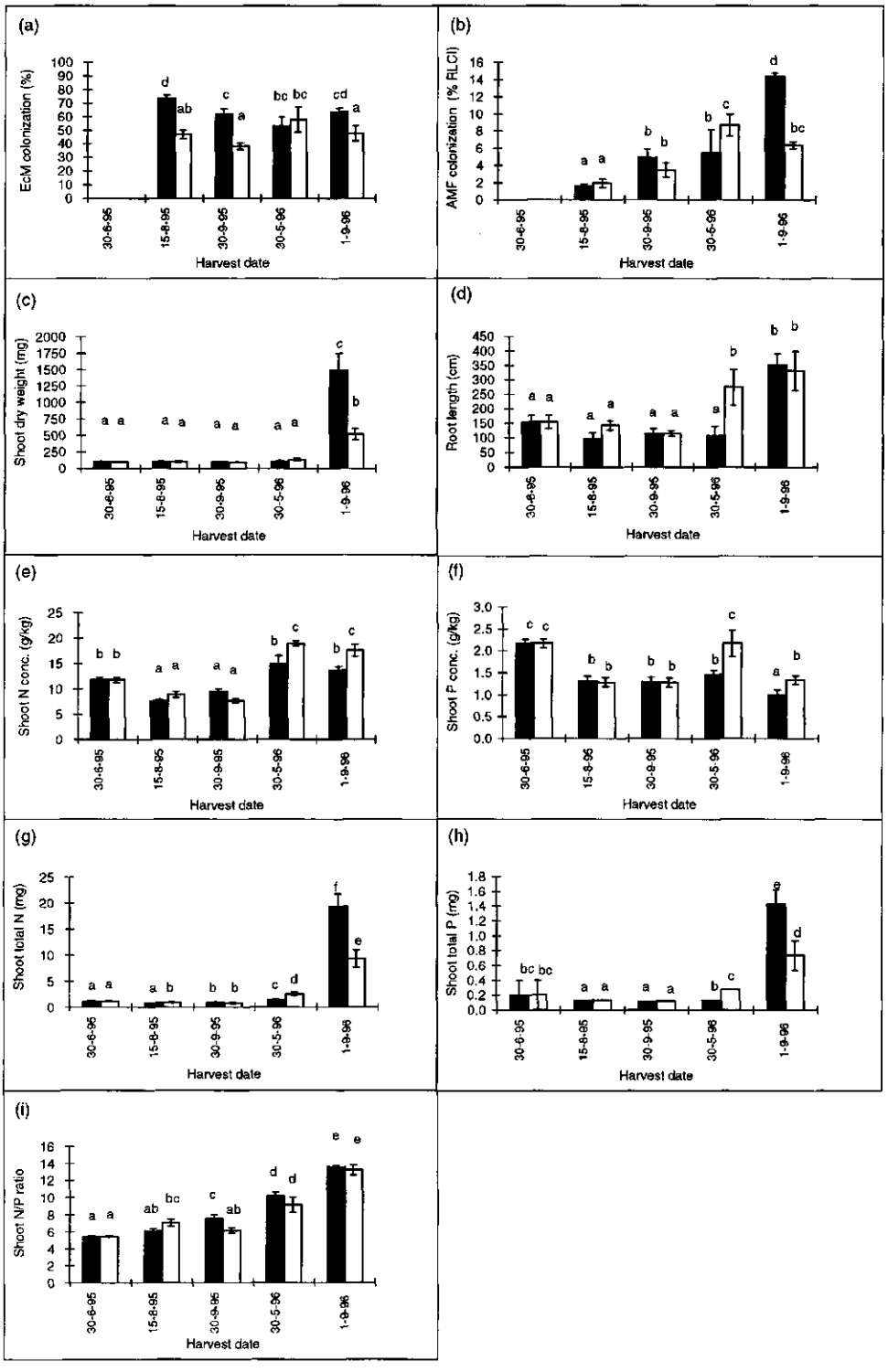
Within the first two weeks a large P uptake was noted, coinciding with the establishment of the AM symbiosis. P concentration of shoots changed significantly over time (LSD between t = 0 and t = 2 weeks, $P < 0.001$, FIG. 10-1d). In contrast, a decrease in shoot N concentration was observed in the first two weeks (LSD between t = 0 and t = 2 weeks, $P < 0.001$, respectively, FIG. 10-1c). Shoot P and N content showed corresponding changes. This resulted in a N/P ratio of 0.8 after two weeks (FIG. 10-1g), whereas analysis showed that soil $(N/P)_{total}$ ratio was similar to the original shoot N/P ratio, i.e. 5.3, and the soil $(N/P)_{diss.}$ ratio was even higher, i.e. 43.3 (Average nutrient contents at t = 0 weeks: $N_{tot}$ 2.24 $mg.g^{-1}$, $P_{tot}$ 0.42 $mg.g^{-1}$, $N_{ts}$ 0.013 $mg.g^{-1}$, and $P_s$ 0.0003 $mg.g^{-1}$; and at t = 10 weeks: $N_{tot}$ 2.17 $mg.g^{-1}$, $P_{tot}$ 0.39 $mg.g^{-1}$, $N_{ts}$ 0.017 $mg.g^{-1}$, and $P_s$ 0.0008 $mg.g^{-1}$. No significant differences were found in soil nutrient status between samplings at the start and the end of the experiment). Recovery of shoot N content was shown after four weeks together with the first development of EcM (FIGS. 10-1e, a).

**TABLE 10-1.** Incidence of different arbuscular mycorrhizal fungal structures (means) in *Salix repens* during 10 weeks.

| | Arbuscular mycorrhizal colonization | | | | | |
|---|---|---|---|---|---|---|
| Harvest (weeks) | AM Intensity (%RLCI) | Total AM (%RLC) | Hyphae (%RLC) | Hyphal coils (%RLC) | Vesicles (%RLC) | Arbuscules (%RLC) |
| 2 | 4.1 a* | 19.9 a | 19.1 a | 0 a | 0.4 a | 0.4 a |
| 4 | 4.3 a | 17.9 a | 14.1 a | 0.4 a | 3.5 b | 0 a |
| 6 | 5.6 ab | 26.8 a | 23.9 b | 0.7 a | 2.1 a | 0 a |
| 8 | 6.4 b | 29.6 a | 24.3 b | 0.4 a | 4.0 b | 0.4 a |
| 10 | 5.4 ab | 23.2 a | 18.2 a | 0 a | 4.6 b | 0.4 a |

* Different letters within a column indicate significant differences between harvests according to LSD or MWU ($P < 0.05$), and a one-factor ANOVA (df = 4, 35) or Kruskal-Wallis test.

**FIGURE 10-2.** Mean ectomycorrhizal colonization (%) (a), arbuscular mycorrhizal colonization (b), plant dry weight (c), root length (d), shoot N concentration (e), shoot P concentration (f), shoot N content (g), shoot P content (h), shoot N/P ratio (i) of *Salix repens* in two plots (■ plot 1 (dry); □ plot 2 (wet)) in a soil moisture gradient. Error bars indicate (±) 1 SE. Bars represented by different letters are significantly different according to LSD or MWU ($P < 0.05$), and a two-factor ANOVA (df = 3, 86) or Kruskal-Wallis test (see opposite page).


(a)
EcM colonization (%)
Harvest date
(b)
AMF colonization (% RLCI)
Harvest date
(c)
Shoot dry weight (mg)
Harvest date
(d)
Root length (cm)
Harvest date
(e)
Shoot N conc. (g/kg)
Harvest date
(f)
Shoot P conc. (g/kg)
Harvest date
(g)
Shoot total N (mg)
Harvest date
(h)
Shoot total P (mg)
Harvest date
(i)
Shoot N/P ratio
Harvest date
30-6-95
15-8-95
30-9-95
30-5-96
1-9-96

*Experiment 2: Field experiment in a soil moisture gradient*

In the first 7 weeks after planting EcM frequency increased to 72 % (FIG. 10-2a). In the first year (1995) no difference in colonization by AMF occurred between plots 1 and 2 (FIG. 10-2b, plot effect: $F_{1,86}$ = 0.67, $P$ = 0.415), whereas EcM colonization was significantly lower in the wet plot (plot effect: $F_{1,86}$ = 20.94, $P$ < 0.001).

In the first growth season (1995) AM colonization significantly increased over time while EcM colonization tended to decrease over time (before the water table was raised artificially) (FIG. 10-2a, harvest effect; $F_{3,86}$ = 2.70, $P$ = 0.053). Not only EcM %RLC in plot 2 was lower, but in particular development of EcM root tips differed between plots 1 and 2. Mycorrhizas of *C. uliginosus* and *H. leucosarx* could easily be identified after 13 weeks in plot 1, whereas in plot 2 ectomycorrhizas could not be identified, as external structures (mantle, mycelium) were hardly developed.

**TABLE 10-2.** Incidence of different arbuscular mycorrhizal fungal structures (means) in *Salix repens* in two plots in a soil moisture gradient (1: dry, 2: wet).

| | Arbuscular mycorrhizal colonization | | | | | | |
|---|---|---|---|---|---|---|---|
| Harvest date | AM Intensity (%RLCI) | AM Total (%RLC) | Hyphae (%RLC) | Hyphal coils (%RLC) | Vesicles (%RLC) | Int. spores (%RLC) | Arbuscules (%RLC) |
| Plot 1 | | | | | | | |
| 15/8/95 | 1.8 a* | 9.1 a | 6.3 a | 0 a | 1.4 a | 0 a | 0 a |
| 30/9/95 | 4.3 b | 5.0 a | 3.0 a | 0.1 a | 0.5 a | 2.1 b | 0 a |
| 30/5/96 | 5.0 b | 7.2 a | 6.2 a | 0.4 a | 0.2 a | 0 a | 0.4 a |
| 1/9/96 | 14.2 d | 26.5 a | 20.6 a | 2.7 a | 3.8 a | 5.2 b | 0.4 a |
| Plot 2 | | | | | | | |
| 15/8/95 | 2.0 a | 3.0 a | 2.1 a | 0 a | 0.6 a | 0 a | 0.3 a |
| 30/9/95 | 3.8 b | 3.7 a | 2.5 a | 0 a | 1.2 a | 0 a | 0 a |
| 30/5/96 | 7.2 c | 15.0 a | 10.7 a | 2.6 a | 1.3 a | 0 a | 0.4 a |
| 1/9/96 | 6.0 bc | 22.2 a | 17.4 a | 0 a | 4.1 a | 0 a | 0.7 a |

* Different letters within a column indicate significant differences according to LSD or MWU ($P$ < 0.05), and a two-factor ANOVA (df = 3, 86) or Kruskal-Wallis test.

The relatively high AM colonization after the period of waterlogging in winter was a consequence of the relatively high RLCI of intraradical spores (i.e. spores: 5 %RLC ≈ 5 %RLCI, whereas hyphae: 30 %RLC ≈ 2-3 %RLCI). Colonization by hyphae and vesicles (hyphal coil and arbuscule formation were very low) was similar to previous harvest periods (interaction between plot × harvest: $P$ = 0.641 and $P$ = 0.230, respectively). However, the intensity of colonization was increased due to intraradical sporulation of AM

fungi (Kruskal-Wallis test, $P = 0.025$) (TABLE 10-2). AM (both %RLCI and %RLC) and EcM (%RLC) were not significantly correlated ($R = 0.08$, $P = 0.45$; $R = 0.05$, $P = 0.65$, respectively).

No differences in plant performance between plots within one year after planting were observed (FIG. 10-2c) (no damage due to grazing was observed in the present experiment). At the final harvest in September 1996 biomass of *S. repens* in the drier plot was significantly higher than in the wetter plot 2 (FIG. 10-2c, interaction between harvest × plot: $P < 0.001$), concomitant with higher N and P contents (FIG. 10-2g, plot: $F_{1,86} = 8.02$, $P = 0.006$, interaction between harvest × plot, $F_{4,86} = 5.27$, $P < 0.001$, and FIG. 10-2h, plot: $F_{1,86} = 8.82$, $P = 0.004$, interaction between harvest × plot, $F_{4,86} = 9.17$, $P < 0.001$, respectively). Shoot N and P concentration showed a significant decrease during the first year. In the second growth season, shoot N and P concentrations were significantly higher in the wetter plot (FIGS. 10-2e,f). Shoot N/P ratio significantly increased over time and only significantly differed between plots at t = 13 weeks (FIG. 10-2i, interaction between harvest × plot, $F_{4,86} = 2.81$, $P = 0.030$).

**DISCUSSION**

In experiment 1 the associated grassland plant species showed on average 60 % RLCI AM colonization (data not shown). This indicated a high potential of AMF inoculum. AM colonization in *S. repens* roots, however, was surprisingly low after 10 and also after 16 weeks. EcM inoculum potential in this sod was very low, suggesting that this low AM colonization cannot be explained by competition with EcM for root space as reported by Chilvers *et al.* (1987). Moreover, within the first two weeks colonization by AMF was similar to colonization in field collected root samples of *S. repens*, indicating rapid colonization by AM fungi. In 16 dune sites, *S. repens* was also consistently slightly AM and highly EcM (CHAPTER 3). In addition, in another laboratory experiment in which different amounts of inoculum of *Glomus mosseae* were supplied no difference in colonization was observed (CHAPTER 5). Mown grassland can be very rich in AMF species. Bever *et al.* (1996) recorded 23 distinct species and indicated that further sampling revealed several additional species. Hettrick & Bloom (1983) recorded 21 species in a tall grass prairie and Johnson *et al.* (1991) 25 species in an old field succession. It is therefore likely that a diverse plant community, as in the vegetation (grass sod) used, harbours a diverse AM community. Even though *S. repens* showed mycorrhizal specificity (CHAPTER 3) it is unlikely that this specificity is responsible for low AM

colonization in *S. repens*. I therefore conclude that low AM colonization is best explained by plant control.

This low AM colonization coincided with a high shoot P uptake compared with N uptake (even though arbuscules were lacking), contrary to both the relatively high availability of N (versus a low availability of P in the soil ($N/P_{dissolved}$ = 43)) and N limitation in the cuttings (shoot N/P = 5.3). There is ample evidence that a major benefit of AMF to plants is improved P nutrition (e.g. Jakobsen *et al.*, 1994; Smith & Read, 1997). Low AM colonization (less than 5 %) has generally been considered negligible. However, in single inoculations with *Glomus mosseae* similarly low AM colonization appeared very effective in enhancing the short term growth response, P uptake, and root foraging plasticity of *S. repens* when compared to non-mycorrhizal plants (CHAPTERS 4 AND 5).

In these experiments, cuttings with a very high shoot to root ratio have been used. In such plants only the shoot can initially act as a sink for mineral nutrients. When, as in this case uncolonized cuttings are planted in an existing mycorrhizal network, the phosphorus will be rapidly translocated to the shoot. Under such conditions of an extensive mycelial network, to which these cuttings with a very small root system are integrated, it is likely that root length, commonly considered as a reliable predictor of P-inflow (Tinker *et al.*, 1992) is not a good parameter for assessing nutrient uptake. In my study P-inflow in the first two weeks was very high, viz. $6.2*10^{-12}$ moles $P.m^{-1}.s^{-1}$. This is about an order of magnitude higher than commonly reported, and two times higher than inflow by early stage infection of EcM in *Salix*. Jones *et al.* (1991) reported $3.2*10^{-12}$ moles $P.m^{-1}.s^{-1}$ inflow in EcM *Salix* cuttings over the first 50 days of mycorrhizal infection.

In the absence of an extensive mycorrhizal network initial nutrient uptake will be substantially lower as the formation of networks will take some time. It is therefore not surprising that in laboratory experiments P inflow was much smaller, though still two times higher than non-mycorrhizal plants both at three and 15 weeks after inoculation, but also much higher in the first interval over three weeks than in the interval over 15 weeks ($4.1*10^{-13}$ and $1.1*10^{-14}$ moles $P.m^{-1}.s^{-1}$, for three and 15 weeks respectively, E.W. van der Heijden, unpubl.). After outplanting cuttings adjust a functional equilibrium as root length increases more rapidly than shoot length. This functional readjustment results in a nutrient readjustment as well. The decline in P content after week two (or lower average P inflow over 15 weeks compared to three weeks in the laboratory experiment) is consistent with this readjustment.

Jones *et al.* (1991) suggested that EcM fungi increase P uptake in *Salix* in early stages of EcM development. Though, as in their study after 50 days, in previous experiments (CHAPTERS 4 AND 5) I also noted a higher P uptake in single inoculations with EcM fungi (after about seven weeks), but no high P uptake was noted by EcM fungi after 2 weeks (CHAPTERS 4, 5 AND 6). My data suggest that the initial P inflow is due to AMF as no EcM colonization was observed at two weeks.

The data from the soil moisture gradient experiment generally confirmed results of Lodge (1989) that flooding negatively affected EcM formation. However, in this study, in particular the development of the EcM mantle was strongly reduced, even though EcM root length colonized was only slightly inhibited. It is likely that, next to the lower EcM inoculum availability, low oxygen availability was responsible for the reduction of EcM formation in the wet plot, compared with the relatively dry plot. Flooding in winter also negatively affected EcM development in both plots. In May 1996, external structures of EcM were hardly developed in both plots. Thus external development of EcM was negatively affected by flooding. Contrary to several studies that have reported that flooded trees are generally non-mycorrhizal (e.g. Lorio *et al.*, 1972), *S. repens* was consistently colonized by EcM fungi throughout the growth season. Marshall & Patullo (1981) also reported their wetland willow to be ectomycorrhizal throughout the growth season. EcM colonization was reduced under waterlogged conditions but had not completely disappeared. This might indicate that EcM fungi survive through fragments of the Hartig net in root systems of *S. repens*. Penetration of cortical cell walls may also have occurred, as previously observed for *Cenococcum* (Mikola, 1948). The EcM sheath may disappear but after the water table has dropped, development may start from fractions of the Hartig net (Smith & Read, 1997).

Inhibition of AM formation has also been observed under waterlogged conditions (Keeley, 1980; Smith & Read, 1997). In this study AM colonization was raised by flooding. This increase, however, was not an increase in mycorrhizal root length colonization but an increase in mycorrhizal intensity due to intraradical spore formation. This higher colonization intensity is better explained by AMF sheltering in roots in this period of waterlogging. The root system of *S. repens* will probably contain more oxygen than the surrounding waterlogged soil and AM fungi may use this oxygen (Grosse *et al.*, 1996; Jackson & Attwood, 1996). Spore formation was only observed in plot 1 and this may be explained by plot 2 containing wetland plant species, known to transport oxygen to their roots (Grosse *et al.*, 1996; Weisner & Strand, 1996). These roots may be better

supplied with oxygen and therefore AM fungi may have selected root systems of these plants to retreat in for survival. Intraradical spore formation in roots of *S. repens* was observed previously in some dune field sites that were temporarily partly flooded, whereas this was not found in the drier parts of these sites or during samplings when these sites were drained (CHAPTER 3).

Data from 1995 suggest a negative association between AM and EcM, but the data from 1996 showed that the pattern was not consistent. A comparison between both years serves as a warning that in ecosystems with a dynamic water table short term observations might capture only part of mycorrhizal behaviour. Lodge & Wentworth (1990) observed a negative correlation between AM and EcM and explained this by a negative interaction between these mycorrhizal types. Although my data of relatively high colonization intensity by AM fungi (14 %) versus the rather low colonization by EcM fungi (50 %) are consistent with such an explanation, they are better explained as the consequence of different expressions of an internal survival strategy of both mycorrhizal types (intraradical spores, Hartig net). However, quantification of AM is based on internal structures and of EcM on external structures. Therefore, internal survival is manifested by higher AM colonization and lower EcM colonization and consequently misinterpreted as a negative interaction. This shift was previously interpreted as a shift in functional relevance of both mycorrhizal types under these circumstances as well.

Unlike the negative correlation between EcM and AM reported by Dhillion (1994), both experiments showed no significant negative correlations between observed AM and EcM proportional colonization within the same root system (as the positive correlation in experiment 1 reflects mycorrhizal establishment of both types). More importantly, one should not draw conclusions on negative or positive interactions of mycorrhizal types by correlations between AM and EcM colonization from static data alone.

*Salix repens* is always slightly arbuscular mycorrhizal and highly ectomycorrhizal. Both mycorrhizal types colonize roots of *S. repens* independently. Low AM colonization in *S. repens* is very effective early in the season but has lower net benefits over a longer period than EcM fungi (CHAPTERS 3, 4 AND 5). Therefore, I suggest that *S. repens* limits AM colonization. Consequently, the functional significance of the particularly high colonization intensity with AM spores for the plant is debatable, while the benefit for the fungus is obvious. Plant investments for harbouring AM spores under waterlogged conditions might be an unavoidable consequence for a plant that is dependent on AM early in the season or early in plant development.

# 11

## Arbuscular mycorrhizal fungi suppress subsequent colonization by ectomycorrhizal fungi in a dual mycorrhizal plant.

*The functioning of plants is influenced to a large extent by the presence of mutualistic and pathogenic organisms (van der Putten et al., 1993; Gange & Brown, 1997; Bever et al., 1997; van der Heijden et al., 1998). Below-ground organs of plants cannot both maximize benefit from root mutualistic mycorrhizal fungi and minimize costs resulting from colonization by pathogenic fungi. Direct interactions between mycorrhizal fungi and root pathogens can suppress the latter group, as described for both arbuscular and ectomycorrhizal symbioses (Smith & Read, 1997). In dual mycorrhizal plants, which form associations with both arbuscular and ectomycorrhizal fungi, interactions might also negatively affect mycorrhizal functioning. Here is shown that arbuscular mycorrhizal fungi suppress subsequent colonization of ectomycorrhizal fungi in the dual mycorrhizal plant* Salix repens *in laboratory and field experiments. Suppression of arbuscular mycorrhizal fungi by ectomycorrhizal fungi was absent. These results raise questions about additional benefits and costs of being dual mycorrhizal and emphasizes the need to explicitly consider the role of plant and fungal control over mycorrhizal colonization.*

Arbuscular mycorrhizal fungi (AMF), which are as old as, and causally implicated in, the evolution of land plants (Simon *et al.*, 1993), must have dealt very early on with plant defence. Their ability to colonize the roots of a large majority of an extant plant species must be due to specific mutual communication and recognition as a result of which the plant defence system is overcome (Gianinazzi-Pearson *et al.*, 1996). Evidently, there must be limits in suppressing defence mechanisms as mycorrhizal plants would otherwise be more prone to root pathogen attack. A number of reports indicate that mycorrhizal fungi can reduce disease incidence and severity caused by fungal pathogens. However, this suppressive effect is variable and influenced by plant nutritional status, relative density of the inoculum of pathogenic and mycorrhizal fungi, and whether or not the plants are mycorrhizal before being challenged with propagules of the pathogen (Graham, 1988; Linderman, 1992; Fitter & Garbaye, 1994).

The present study showed for the first time that colonization by AMF restricts subsequent colonization by another functional group of mycorrhizal fungi, viz. ectomycorrhizal fungi (EcMF) both in laboratory and field experiments.

In a range of sixteen field sites in the Netherlands with different environmental conditions (pH, moisture), *Salix repens*, a dual mycorrhizal plant, had consistently low colonization by AMF (1-10 %) and high colonization by EcMF (usually over 85 %). Cuttings (vegetative shoots), which were inoculated in the laboratory with only AMF (*Glomus mosseae*) or only EcMF (either *Hebeloma leucosarx, Inocybe vulpinella,* or *Paxillus involutus*) showed low levels of AM and high levels of EcM colonization, respectively (FIG. 11-1a). In simultaneous inoculations in the laboratory (*Glomus* with one of the three EcMF), *Glomus, Hebeloma* and *Inocybe* colonized similar proportions of the root system as when inoculated individually; however, *Paxillus* was significantly suppressed (FIG. 11-1b). In sequential inoculations, where EcMF followed inoculation with AMF (*Glomus*), all three EcMF were strongly suppressed (colonization ranging between 10 and 15 %, FIG. 11-1c) whereas *Glomus* was not affected (data not shown). Similar results were obtained when EcMF followed inoculation with *Acaulospora laevis* (AMF) (data not shown as the experiment did not include *Acaulospora* followed to inoculation with EcMF). In the sequential inoculations, where *Glomus* followed inoculation with EcMF, none of the EcMF were suppressed (data not shown), and more importantly *Glomus* was also not suppressed (FIG. 11-1d).

Under controlled conditions in two different field soils (a calcareous, humus-poor and an acidic, humus-rich site) where *Salix repens* grew, the outcome was similar. *Glomus* suppressed subsequent colonization by EcMF, but *Hebeloma* did not affect subsequent colonization by AMF (TABLE 11-1). Finally, in a field experiment at the two sites referred to above, again the same interaction was observed, viz. with *Glomus* suppressing subsequent colonization by EcMF, but *Hebeloma* not affecting subsequent colonization by AMF. These effects were highly significant at t = 8 weeks, but the second harvest (at t = 24 weeks) indicated that this suppression was only transient (TABLES 11-1 and 11-2).

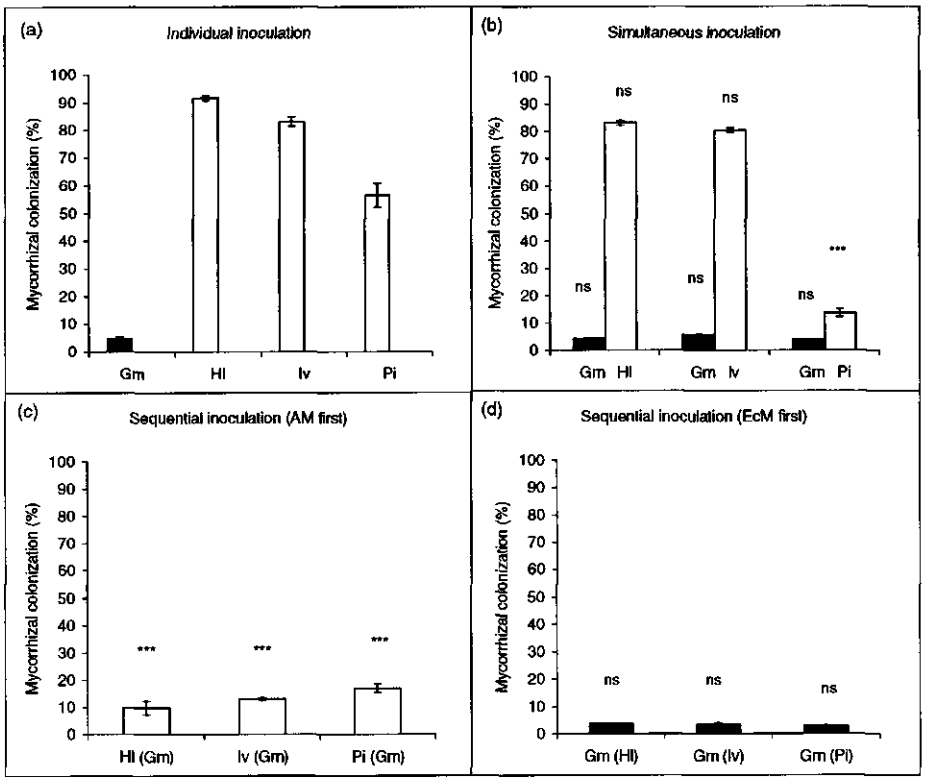



**FIGURE 11- 1.** Mean mycorrhizal colonization (± 1 SE) (AM ■; EcM □) of *Salix repens* cuttings 15 weeks after inoculation either (a) individually with *Glomus mosseae* (Gm), *Hebeloma leucosarx* (Hl), *Inocybe vulpinella* (Iv) or *Paxillus involutus* (Pi), (b) simultaneously with *Glomus* & *Hebeloma* (Gm Hl), *Glomus* & *Inocybe* (Gm Iv) or *Glomus* & *Paxillus* (Gm Pi), (c) sequentially (AMF inoculated previously) with *Hebeloma* (Hl (Gm)), *Inocybe* (Iv (Gm)) or *Paxillus* (Pi (Gm)) or (d) sequentially (EcMF inoculated previously) with *Glomus* (Gm (Hl, Iv or Pi)). Significant differences in the colonization by the four fungi compared to their colonization (individual inoculation) when simultaneously or sequentially inoculated are indicated with asterisks ($P < 0.001$: *** or not significant: ns).

**TABLE 11-1.** Mycorrhizal colonization (± SE) of *Salix repens* cuttings inoculated with *Glomus mosseae*, *Hebeloma leucosarx* or uninoculated (control). Cuttings were planted in non sterile soil collected from Schoenus (relatively young, calcareous site) or from Groene strand (relatively old, acidic site) after 8 weeks and 24 weeks ($n = 8$).

| | Control (simultaneous) | | *Glomus mosseae* (sequential) | | *Hebeloma leucosarx* (sequential) | |
|---|---|---|---|---|---|---|
| Harvest (weeks) | AM | EcM | AM | EcM | AM | EcM |
| Field sites | (%) | (%) | (%) | (%) | (%) | (%) |
| t = 0 weeks 'start' | 0 | 0 | 3.5 b* (0.1) | 0 | 0 | 87.1 E* (1.3) |
| t = 8 weeks | | | | | | |
| 'young-calcareous' | 4.7 c (0.4) | 62.2 B (1.1) | 5.2 c (0.4) | **37.7 A (1.9)** | 4.4 c (0.4) | 82.3 D (2.5) |
| 'old-acidic' | 3.5 b (0.3) | 54.4 B (1.5) | 3.4 ab (0.2) | **43.6 A (1.3)** | 3.1 a (0.1) | 71.4 CD (2.8) |
| t = 24 weeks | | | | | | |
| 'young-calcareous' | 3.1 ab (0.2) | 56.6 BC (6.5) | 4.1 bc (0.3) | 62.3 BC (5.4) | 3.8 b (0.4) | 81.8 D (1.4) |
| 'old-acidic' | 2.9 ab (0.2) | 73.6 D (2.2) | 4.0 bc (0.2) | 69.2 CD (3.5) | 2.2 a (0.2) | 77.8 D (1.9) |

* Significant differences (tested by a three-way factorial ANOVA and LSD values; $P < 0.01$) within functional types of mycorrhiza are indicated by different letters in small letters for AM and in capitals for EcM.

**TABLE 11-2.** Mycorrhizal colonization (± SE) of *Salix repens* inoculated with *Glomus mosseae, Hebeloma leucosarx* or uninoculated (control). Cuttings were planted in Schoenus (relatively young, calcareous site) or in Groene strand (relatively old, acidic site) after 8 weeks and 24 weeks ($n = 12$).

| | Control (simultaneous) | | *G. mosseae* (sequential) | | *H. leucosarx* (sequential) | |
|---|---|---|---|---|---|---|
| Harvest (weeks) | AM | EcM | AM | EcM | AM | EcM |
| Field sites | (%) | (%) | (%) | (%) | (%) | (%) |
| t = 0 weeks 'start' | 0 | 0 | 3.5 ab* (0.1) | 0 | 0 | 87.1 E* (1.3) |
| t = 8 weeks | | | | | | |
| 'young-calcareous' | 5.0 cd (0.5) | 85.4 EF (2.1) | 6.1 d (0.9) | **62.1 B (2.5)** | 4.1 bc (0.5) | 79.4 D (2.1) |
| 'old-acidic' | 3.4 a (0.3) | 71.0 CD (1.3) | 3.9 ab (0.2) | **47.8 A (3.4)** | 3.0 a (0.2) | 69.1 C (2.3) |
| t = 24 weeks | | | | | | |
| 'young-calcareous' | 3.5 ab (0.2) | 93.2 FG (1.5) | 3.7 ab (0.3) | 87.7 EF (2.7) | 3.7 ab (0.2) | 96.9 G (0.8) |
| 'old-acidic' | 3.3 a (0.2) | 80.1 DE (2.8) | 3.0 a (0.2) | 77.6 D (2.5) | 2.9 a (0.1) | 77.3 D (2.4) |

* Significant differences (tested by a three-way factorial ANOVA and LSD values; $P < 0.01$) within functional types of mycorrhiza are indicated by different letters in small letters for AM and in capitals for EcM.

Earlier investigations (Chilvers *et al.*, 1987; Lodge, 1989) have also shown a low AMF and high EcMF colonization in dual mycorrhizal plants. The current interpretation that EcMF prevent colonization by AMF on newly formed roots due to the barrier that the ectomycorrhizal sheath provides (Chilvers *et al.*, 1987), is based on circumstantial evidence and not supported by results in the present study.

Various hypotheses have been put forward to explain the suppressive effect of AMF on fungal pathogens and these mechanisms might also explain suppression of EcMF by AMF: (1) Both mycorrhizal fungi could compete for photosynthates with the earlier colonist restricting access to carbon by the second. This explanation is considered unlikely as such costs would be symmetrical (i.e. irrespective of inoculation sequence); (2) Improved nutrient status, due to the primary mycorrhizal fungus, could enhance increased resistance to other root invaders. This is unlikely as an improved P-status (the primary nutritional benefit of AMF infection) does not seem to repress the formation of ectomycorrhizas (Smith & Read, 1997); (3) Alternatively, both mycorrhizal types could compete for colonization sites. However, *Glomus* colonized only a (very) minor portion of the root system, leaving a large part of it uncolonized. Furthermore, the root length of *S. repens* after colonization by AMF was much larger than that after EcMF (AMF: 71 m opposed to EcMF: 14 - 34 m), and it would be difficult to reconcile competition for root space with such an effect on root architecture; (4) Production of antimicrobial compounds might also be involved in the interference of both mycorrhizal types. This mechanism is known for several ectomycorrhizal fungi amongst which is *Paxillus involutus* (Duchesne *et al.*, 1989). Antimicrobial compounds have not yet been reported for AMF; (5) Colonization by AMF mobilizes plant defence mechanisms even though the reaction of plants after initial colonization by AMF has been reported as weak, transient, uncoordinated and localized (Gianinazzi-Pearson *et al.*, 1996). This last point is considered as the most likely explanation for the observations in this study.

The present results raise further questions about dual mycorrhizas. First, how common are dual mycorrhizas in plants that have previously been considered as ectomycorrhizal? Dual mycorrhizas have recently been reported in several EcM tree genera (Smith *et al.*, 1998; Moyersoen & Fitter, 1999), suggesting that the ability to maintain AM has been primitively retained by many EcM trees. Secondly, do such plants derive benefit from being dual mycorrhizal? The benefits of being dual mycorrhizal are known for *Alnus, Salix, Populus* and *Eucalyptus*, but have also been shown for ectomycorrhizal *Pinus* when growing in the presence of an arbuscular mycorrhizal plant

(Smith *et al.*, 1998). The absence of complete resistence to AM fungi suggests that there must be alternative benefits conferred by the association.

Finally, if the suppression of EcMF by AMF is only transient, does the same mechanism occur in pathogen suppression by AMF in AM plant species? This would seem likely, as it has been reported that AMF protected plants from a fungal pathogen after 60 days, but not after 90 days (Newsham *et al.*, 1995a).

## METHODS

*Experiment 1: Laboratory*

One AM fungus and three EcM fungi were inoculated either individually, simultaneously or sequentially on *Salix repens*. The substrate used was a sand-perlite mixture 1:3 (v/v). This was autoclaved twice (1 h, 121 °C, 1 atm.) with a 48 h interval and left for one week. Root growth chambers (vertically placed Petri dishes 15 cm diameter, with a slit in the edge) were filled with the sterile substrate. Sterile root cuttings of *Salix repens* were individually inoculated (each fungus in 30 replicates) with one AM fungus, *Glomus mosseae* (BEG 12), and three cultures of EcM fungi; *Hebeloma leucosarx* (L1), *Inocybe vulpinella* (L17) and *Paxillus involutus* (L37) all collected from the Dutch Wadden isle Terschelling. Inoculated cuttings were grown for 25 weeks in a climate chamber (photon flux density 120 $\mu E.m^{-2}.s^{-1}$, day 16 h at 20 °C and night 8 h at 16 °C, relative air humidity 70 %). At t = 15 weeks *S. repens* cuttings inoculated both individually (8 replicates) and simultaneously (15 replicates) were harvested. *Salix repens* cuttings inoculated with AM were subsequently inoculated with *H. leucosarx*, *I. vulpinella* and *P. involutus* (*G. mosseae*: 6 replicates, *A. laevis*: 4 replicates). *Salix repens* cuttings inoculated with EcM were subsequently inoculated with *G. mosseae* (8 replicates). These combinations and the individually inoculated plants (8 replicates) were harvested at t = 25 weeks. The plants were arranged in a complete randomized block design (within the growth cabinet), and the blocks were randomized every three weeks. The plants were watered every three weeks with 10 mL demineralized water. After harvesting at t = 15 and 25 weeks, root colonization by AM and EcM was measured (Giovannetti & Mosse, 1980; McGonigle *et al.*, 1990). AM root length colonization times intensity (RLCI = RLC * % cover by AM of the cross section of the root colonized) was calculated.

*Experiment 2: Field soil*

*Salix repens* cuttings were inoculated with either *G. mosseae*, *H. leucosarx* or remained uninoculated (control). Ten weeks after inoculation colonized cuttings (*G. mosseae*: 3.5 %; *H. leucosarx*: 87.1 %) and non-mycorrhizal control cuttings were used in this experiment. The control cuttings were at t = 10 weeks (start of outplanting) simultaneously exposed to both AMF and EcMF, cuttings previously inoculated with *G. mosseae* were subsequently exposed to EcMF (and AMF), and cuttings previously inoculated with *H. leucosarx* were subsequently exposed to AMF (and EcMF). The latter

two treatments are comparable to the sequential inoculations as in experiment 1. Two *S. repens* field sites (500 $m^2$) on the Wadden isle of Terschelling were selected, Schoenus (a relatively young, calcareous, humus-poor dune successional stage) and Groene strand (a relatively old, acidic, humus-rich dune successional stage). Soil was collected from these field sites. Soil from each field site was very well mixed, left for one week and divided over 56 pots (0.2 L). *Salix repens* cuttings inoculated with *G. mosseae*, *H. leucosarx* or uninoculated (control) (each with 16 replicates) were planted individually in these field soils and grown for 24 weeks in a climate chamber (see expt 1). At t = 8 and 24 weeks, 8 plants (per fungal treatment/field soil combination) were harvested randomly. Mycorrhizal colonization was estimated as in expt 1.

*Experiment 3: Field*

*Salix repens* was inoculated as in expt 2 (simultaneously run with expt 2). In both field sites (Schoenus, Groene strand) *S. repens* inoculated with *G. mosseae*, *H. leucosarx* or uninoculated (control) (each 100 replicates per field site) were randomly planted in four plots of 1 m * 1 m (25 cuttings of each fungal treatment per plot). At t = 8 and 24 weeks 12 plants (three per plot per fungal treatment/field site combination) were harvested randomly. Mycorrhizal colonization was estimated as in expt 1.

*Statistical analysis*

Data were tested using Analysis of Variance. Differences between means were evaluated using LSD-values ($P < 0.01$) (Sokal & Rohlf, 1995).

# 12

## Radial growth and biomass increment of ectomycorrhizal fungi as affected by water soluble foliar extracts of dominant plant species from *Salix repens* scrub.

**ABSTRACT**

Salix repens *is widespread in a great variety of dune plant communities, ranging from early successional sites on calcareous, humus-poor soils to late successional sites on acidic, humus-rich soils. Vegetation type and ectomycorrhizal species composition vary along the same successional gradient. Foliar aqueous extracts of* Salix repens *(from early and late successional sites), and the co-dominant vegetation,* Oxycoccus macrocarpos, Myrica gale, *and* Calamagrostis epigejos *were investigated for their effects on the growth of eight isolates of ectomycorrhizal fungi on calcareous (AMN) and acidic (MMN) media, to test whether occurrence or lack of a fungal species can be explained by stimulatory or inhibitory effects of litters of the co-dominant vegetation. Growth rates of ectomycorrhizal fungi were generally negatively affected by litter extract of* Salix *on AMN. Inocybe lacera was inhibited by all foliar extracts, but also by the acidic MMN without plant extracts. Radial growth and biomass increment of* Lactarius controversus *and* Paxillus involutus *were stimulated by extracts of* Myrica *and* Calamagrostis *on both media, while sporocarps were lacking in these field sites. On MMN* Scleroderma verrucosum *grew only on extracts of* Myrica *and* Calamagrostis, *which were also co-dominant in the field site from which the isolate originated. On calcareous media fungi generally performed better. Furthermore, these ectomycorrhizal fungi showed no relationship between radial growth and biomass increment.*

**KEY WORDS**: Ectomycorrhizal fungi, *Salix repens*, litter, dune ecosystems

## INTRODUCTION

Coastal dune ecosystems, in which *Salix repens* L. occurs, can be rich in ectomycorrhizal fungi. The ectomycorrhizal flora in the various vegetation types is quite diverse and a one-year (1992) inventory of 16 permanent plots on the Wadden Isle of Terschelling, each measuring 500 $m^2$, yielded 50 ectomycorrhizal fungi associated with *Salix repens* (Kuyper *et al.*, 1994; CHAPTER 2). These field sites ranged from dry to wet, and from calcareous, humus-poor (early successional; pH($CaCl_2$) 6.1-7.3) to acidic, humus-rich (late successional; pH($CaCl_2$) 3.6-5.1) soils. These sites also varied in their co-dominant vegetation. Number of species and sporocarps varied enormously between these field sites, and species richness and sporocarp abundance were not correlated (CHAPTER 2). Few fruitbodies were found in *Salix repens* scrub in which *Myrica gale* L. and *Oxycoccus macrocarpos* (Aiton) Pursh. co-dominated, and no sporocarps were observed where *Calamagrostis epigejos* (L.) Roth. co-dominated.

Several studies indicate a negative effect of litter on occurrence of ectomycorrhizal fungi. Baar & Kuyper (1993) found an increase of ectomycorrhizal species in old Scots pine (*Pinus sylvestris* L.) forests where litter and humus (and in some plots also the herbaceous vegetation) had been removed. Negative effects of litter on ectomycorrhizal fungi have been established by Melin (1946), Olsen *et al.* (1971), Rose *et al.* (1983), and Baar *et al.* (1994). Perry and Choquette (1987) suggested that sensitivity of ectomycorrhizal fungi to litter could be associated with their successional status. During succession litter accumulates causing a change in types and amount of potentially allelopathic substances released from decomposing litter, to which late successional mycorrhizal fungi might be better adapted.

In this study the effects of water soluble foliar extracts of *Salix repens*, both from a relatively early (calcareous, humus-poor), and a relatively late (acidic, humus-rich) successional stage, and of three important co-dominant plant species, viz. *Myrica gale*, *Oxycoccus macrocarpos*, and *Calamagrostis epigejos*, on both radial growth and biomass increment of eight ectomycorrhizal fungi were examined. As some of these species are characteristic for young or old stands of *Salix repens*, the composition and the pH of the growth medium were also varied to simulate successional differences.

## MATERIAL AND METHODS

### *Fungal cultures*

In the autumn of 1994 fungal cultures were obtained from sporocarps occurring in *Salix repens* communities. An attempt was made to isolate fungi from young, middle and older successional stages. Species composition from the younger successional stages comprised the taxa *Hebeloma, Inocybe, Cortinarius* and *Tricholoma*. Unfortunately, mycelia of *Inocybe, Cortinarius* and *Tricholoma* were very hard to be obtained from sporocarps, and I also failed to isolate *Russula* species representative for the older successional field sites. Therefore, only *Hebeloma leucosarx* P.D. Orton (L2) was cultured from sporocarps that were collected in a successionally young *Salix* scrub. Another isolate of *H. leucosarx* (L1) and *Inocybe agardhii* (Lund) P.D. Orton (L11) were collected in an old, regularly mown *Salix* scrub. *Scleroderma verrucosum* (Bull.: Pers.) Pers. (L69) was collected in a middle-aged scrub, grazed by horses; *Inocybe lacera* (Fr.: Fr.) Kumm. (L15), *Lactarius controversus* (Pers.: Fr.) Fr. (L14), and *Tomentella* sp. (L71) were collected in a successionally old *Salix repens* scrub. All these fungi originated from the Wadden Isle of Terschelling. *Paxillus involutus* (Batsch: Fr.) Fr. (L27) originated from an old scrub near Vledder (prov. Drenthe). The ecology of the fungal species tested is described in TABLE 12-1.

**TABLE 12-1.** Fungal isolates, codes, origin, habitat, ecology of the fungi and litter origin.

| | Code | Origin | Habitat | Ecology of the fungi |
|---|---|---|---|---|
| Fungal isolates: | | | | |
| *Hebeloma leucosarx* | L2 | Primaire vallei | Calcareous-Dry | All habitats, but high numbers of sporocarps in |
| *H. leucosarx* | L1 | Thijssensduin | Acidic-Wet | calcareous sites. |
| *Inocybe agardhii* | L11 | Thijssensduin | Acidic-Wet | All habitats, but high numbers of sporocarps in calcareous sites |
| *I. lacera* | L15 | Douwesplak | Acidic-Dry | Acidic habitats, on bare sand. |
| *Lactarius controversus* | L14 | Douwesplak | Acidic-Dry | Acidic habitats |
| *Paxillus involutus* | L27 | Vledder | Acidic-Wet | All habitats |
| *Scleroderma verrucosum* | L69 | Paardenwei | Acidic-Dry | Acidic habitats |
| *Tomentella* sp. | L71 | Douwesplak | Acidic-Dry | Unknown |
| Litters: | | | | |
| *Salix repens* | SRyoung | Schoenus | Calcareous-Wet | |
| *Salix repens* | SRold | West aan Zee | Acidic-Dry | |
| *Calamagrostis epigejos* | CE | Oosterend | Acidic-Dry | |
| *Myrica gale* | MG | Myrica | Acidic-Wet | |
| *Oxycoccum macrocarpos* | OM | Cranberry | Calcareous-Wet | |

*Leaf extracts and solid media*

Leaves of *Salix repens* (from an early and late successional stand), *Myrica gale*, *Oxycoccus macrocarpos*, and *Calamagrostis epigejos* were collected from Terschelling on October 27th, 1994. The leaves were immediately dried (50 °C, 48 h) and stored for subsequent use. For preparation of extracts 50 g of each litter type was ground and added to 450 ml distilled water (for *Salix* extracts 25 g in 450 ml distilled water). Extracts were made by shaking (22 °C, 24 h), autoclaved for 20 min (1 atm, 121 °C) and added to modified Melin-Norkrans (MMN) and to a medium based on MMN but with a higher pH (AMN). Basal MMN medium contained (in $g.l^{-1}$): agar (15), malt extract (3), glucose.$H_2O$ (10), $(NH_4)_2HPO_4$ (0.25), $KH_2PO_4$ (0.5), $MgSO_4.7H_2O$ (0.15), $CaCl_2.H_2O$ (0.067), NaCl (0.025), $FeCl_3$ (0.0012) and thiamine.HCl (100 µg) (Marx, 1969); pH ($H_2O$) was set to 5.6. The AMN was similar to MMN, but contained 10 times the amount of $CaCl_2$; and an additional N source, $KNO_3$ (0.5 $g.l^{-1}$), was provided. pH ($H_2O$) was set to 7.0. AMN is considered to reflect conditions in early successional sites (calcareous, higher pH and $NH_4$ and $NO_3$ nitrogen source), MMN those in late successional sites (acidic, $NH_4$ nitrogen source). The agar medium consisted of 15 ml extract in 100 ml MMN or AMN [1.7% (w/v)].

Mycelial plugs of five mm diameter were cut from the edges of three week old fungal colonies and transferred to Petri dishes (9 cm) containing 12 ml of MMN or AMN media with the extracts and control media (no extracts added). The agar plates were kept at 20 °C in dark. A complete set of uninoculated Petri dishes was included as a further control (for fungal biomass correction, and for comparison of the differences in pH and nutrient analyses after fungal growth). The experiments were ended after two months. Each treatment was replicated six times.

The sensitivity of the fungal isolates for extracts was assessed on the basis of radial growth rate and biomass. Colony radius was measured daily for two months for each fungus. Fungal growth rates were estimated from linear regression of colony radius against time during the linear phase of growth. Fungal dry weight was determined (three replicates per treatment) by dissolving the agar in hot distilled water, filtering the solutions, and drying (105 °C, 24 h) and weighing the filtrates (Jongbloed & Borst-Pauwels, 1990). According to Oort (1981) boiling will cause a loss of water-soluble compounds amounting to approximately 35 % of the total biomass with little variation between isolates and no effect of the age of cultures on the amount of loss. Data have not been corrected for this loss. The remaining replicates were used to determine pH($CaCl_2$), and N($NO_3^-$), N($NH_4^+$) and P($PO_4^{3-}$)

concentrations of the media after $CaCl_2$ extraction. The agar plates were cut in small fragments and extracted in 0.01M $CaCl_2$ (10 v/w, 20 °C, 2 h).

As the majority of the data were not normally distributed, data of growth rates and biomass of the fungi were analysed non-parametrically by Kruskal-Wallis test. After Kruskal-Wallis tests indicated signigicant effects, differences among means were evaluated with Mann Whitney U-tests (Siegel & Castellan, 1988). Difference in performance (radial growth and biomass production) of all fungi between substrates (media) was tested using a sign test (Sokal & Rohlf, 1995) and correlations between radial growth and biomass increment were analysed with Spearman's rank correlation tests for each fungus on both media (Sokal & Rohlf, 1995).

## RESULTS

### *Fungal growth*

Growth rates of *Hebeloma leucosarx* L1 and L2 (FIGS. 12-1a,b) were negatively affected by respectively, both *Salix* litter and *Salix* (old) litter extracts on AMN, while on MMN no effect was observed. *Calamagrostis* and *Myrica* stimulated growth rate of isolate L1 on AMN. *Oxycoccus* negatively affected growth rate of both isolates on AMN, and on MMN for L1 only. Generally, within a specific litter treatment, both isolates had a higher growth rate on AMN, but only L1 showed a significantly higher biomass on AMN than on MMN (TABLE 12-2). *Inocybe agardhii* was not affected by the litter extracts on AMN (FIG. 12-1c, TABLE 12-2), but its growth rate was completely inhibited by both *Salix* extracts on MMN. *Calamagrostis, Oxycoccus*, and *Myrica* stimulated the growth rate of *I. agardhii* on MMN. *Inocybe lacera* was inhibited by all litter extracts, and also by MMN (FIG. 12-1d). On both media growth rate of *Lactarius controversus* (FIG. 12-1e) was negatively affected by both *Salix* litter extracts. Biomass of *L. controversus* was increased by *Calamagrostis* and *Myrica* on both media (TABLE 12-2), and although *Oxycoccus* had a negative effect on radial growth rate of *L. controversus* on MMN, biomass production was significantly increased on MMN (TABLE 12-2). Radial growth rate and biomass production of *Paxillus involutus* on both media was depressed by the *Salix* litter extracts and *Oxycoccus*, but stimulated by *Calamagrostis* and *Myrica* (FIG. 12-1f, TABLE 12-2). *Scleroderma verrucosum* seemed inhibited by most of the extracts on both media, except for *Calamagrostis* and *Myrica* on MMN (FIG. 12-1g, and TABLE 12-2). *Tomentella* sp. was negatively affected by both *Salix* litters in growth rate and biomass on AMN (FIG. 12-1h, TABLE 12-2).

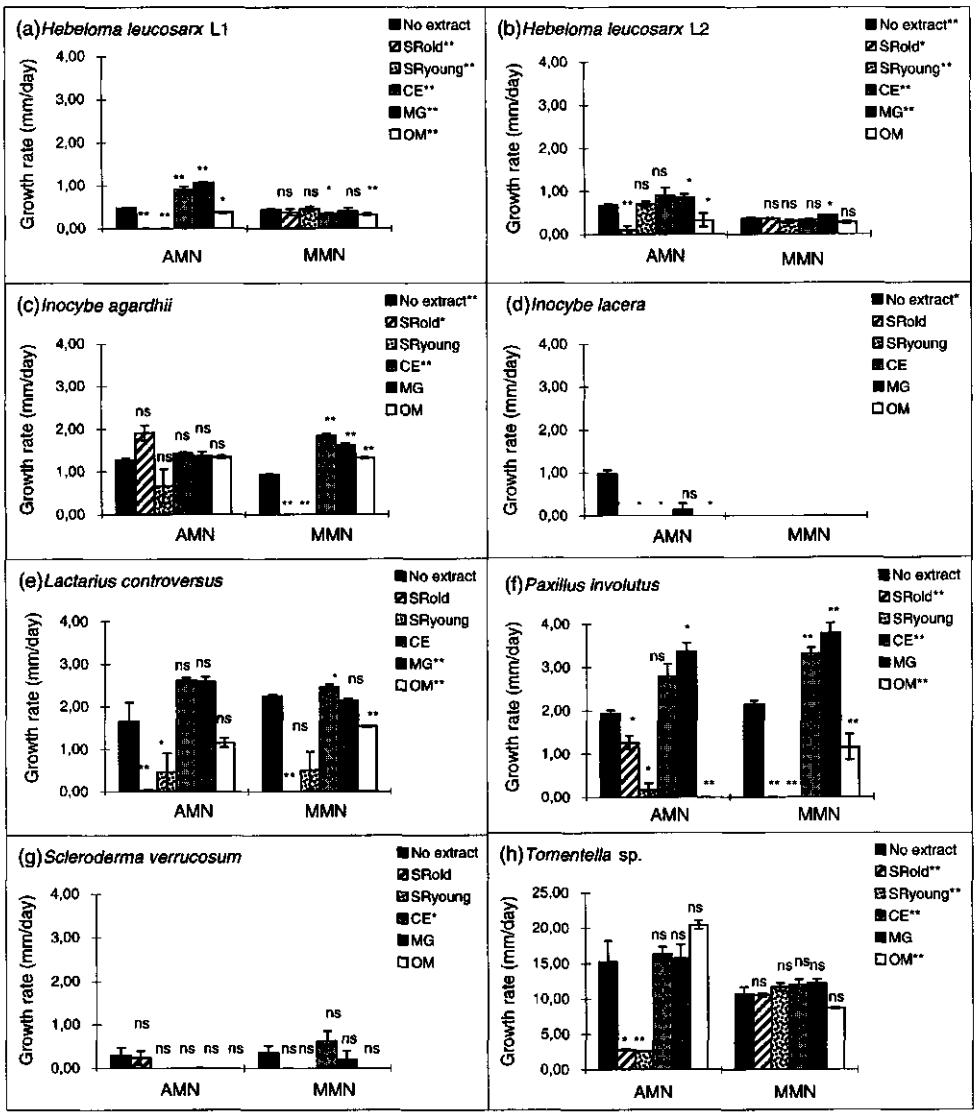



**FIGURE 12-1.** Average growth rate (mm/day) (±) 1 SE of *H. leucosarx* L1 (a), *H. leucosarx* L2 (b), *I. agardhii* (c), *I. lacera* (d), *L. controversus* (e), *P. involutus* (f), *S. verrucosum* (g) and *Tomentella sp.* (h) on AMN and MMN agar with extracts [1.7% (w/v)] of *S. repens* old (SRold), *S. repens* young (SRyoung), *C. epigejos* (CE), *M. gale* (MG), and *O. macrocarpos* (OM). Significant differences in Pairwise comparisons between growth rates are indicated by asterisks ($P < 0.05$* or $P < 0.01$**, Mann-Whitney U-tests). Asterisks above bars indicate difference between control and a given leaf extract (within substrates). Asterisks near leaf extract names indicate difference between the substrates (for a given leaf extract).

**TABLE 12-2.** Average fungal dry weight production (mg) and SE of *H. leucosarx* L1, *H. leucosarx* L2, *I. agardhii*, *I. lacera*, *L. controversus*, *P. involutus*, *S. verrucosum* and *Tomentella sp.* as affected by water soluble leaf extracts [1.7% (w/v)] of *S. repens* old, *S. repens* young, *C. epigejos*, *M. gale*, and *O. macrocarpos* on AMN and MMN media (means ± SE).

| Fungi n = 3 | Fungal dry weight production (mg) | | | | | |
|---|---|---|---|---|---|---|
| | No extract | *Salix repens* old | *Salix repens* young | *Calamagrostis epigejos* | *Myrica gale* | *Oxycoccus macrocarpos* |
| AMN | | | | | | |
| *H.leucosarx* L1 | **31.0± 3.2** | 0*** | 0*** | **46.9± 5.9**** | **46.2±6.9*** | 14.2± 1.6** |
| *H.leucosarx* L2 | 18.1± 5.8 | 16.7± 6.9 | **29.4± 6.1*** | 29.8± 5.5* | 39.5±8.8*** | 21.7± 6.1 |
| *I.agardhii* | 46.8± 8.8 | **39.9± 20.0** | **70.7± 21.1** | 58.6±25.0 | 40.2±10.9 | 72.7±15.2 |
| *I.lacera* | 45.5±21.7 | 0 | 0 | 0 | 7.7±6.6 | 0 |
| *L.controversus* | 19.7± 8.5 | 4.8± 1.7 | 11.6± 8.1 | 90.8±14.5* | **35.2±7.8*** | **8.4± 1.5** |
| *P.involutus* | 28.2± 0.7 | **19.8± 3.5*** | 12.1±9.0* | 37.4± 2.2* | 40.3±4.1* | **0*** |
| *S.verrucosum* | 18.3± 7.5 | 12.1± 4.4 | 0 | **0** | 0 | 0 |
| *Tomentella sp.* | **78.3± 9.4** | **40.9± 1.1*** | **39.9± 12.7*** | **84.8± 9.7** | **75.7±5.2** | **66.9± 6.3** |
| MMN | | | | | | |
| *H.leucosarx* L1 | **10.5± 3.3** | 14.1± 5.0 | 11.0± 3.6 | **22.5± 6.2*** | **28.9±9.1*** | 18.8± 2.5 |
| *H.leucosarx* L2 | 14.7± 4.6 | 8.7± 2.8 | **8.2± 2.7** | 26.5± 8.3* | 29.3±9.2** | 10.9± 3.5 |
| *I.agardhii* | 40.8±13.0 | **2.2± 0.5*** | **0*** | 53.6±17.0 | 66.0±21.5 | 78.6±25.1 |
| *I.lacera* | 0 | 0 | 0 | 0 | 0 | 0 |
| *L.controversus* | 24.8± 5.8 | 0* | 6.5± 1.5* | 76.1±24.0* | **72.6±23.1*** | **38.4± 8.6*** |
| *P.involutus* | 25.7± 6.2 | **0*** | 0* | 36.8± 8.6* | 33.4±8.0 | **19.6± 6.5** |
| *S.verrucosum* | 22.5± 2.8 | 0* | 0 | **30.1± 2.4** | 15.3±7.8 | 0* |
| *Tomentella sp.* | **21.8± 7.0** | **16.4± 5.1*** | **20.0± 6.3** | **32.3±10.4** | **29.5±9.5** | **29.2± 9.3** |

* Significant difference from the control in Pairwise comparisons (within fungus and substrate) are indicated by aserisks ($P < 0.05$*, $P < 0.01$** or $P < 0.001$***, Mann-Whitney-U tests). Data printed in bold indicate significant difference ($P < 0.05$) between the two substrates (within fungus and extract).

Wherever different from the control, extracts of *Calamagrostis* and *Myrica* increased performance of ectomycorrhizal fungi, whereas both *Salix* extracts generally showed inhibitory effects. Performance on AMN was significantly better than on MMN (Signtest for biomass $P < 0.008$). Growth rate and biomass increment were not necessarily correlated (TABLE 12-3).

**TABLE 12-3.** Correlation between average fungal growth rate and fungal biomass production of *H. leucosarx* L1, *H. leucosarx* L2, *I. agardhii*, *I. lacera*, *L. controversus*, *P. involutus*, *S. verrucosum*, and *Tomentella sp.* on AMN and MMN agar as affected by litter extracts [1.7% (w/v)] of *S. repens* old, *S. repens* young, *C. epigejos*, *M. gale*, and *O. macrocarpos*.

| | Substrates | | | | | |
|---|---|---|---|---|---|---|
| | AMN | | | MMN | | |
| Fungi | n | *R* | P-level | n | *R* | P-level |
| *Hebeloma leucosarx* L1 | 4 | 0.80 | ns | 6 | -0.54 | ns |
| *Hebeloma leucosarx* L2 | 6 | 0.88 | * | 6 | 0.48 | ns |
| *Inocybe agardhii* | 6 | -0.60 | ns | 4 | 0.20 | ns |
| *Inocybe lacera* | 2 | 1.00 | -- | 0 | -- | -- |
| *Lactarius controversus* | 6 | 0.94 | ** | 5 | 0.70 | ns |
| *Paxillus involutus* | 5 | 1.00 | ns | 4 | 0.80 | ns |
| *Scleroderma verrucosum* | 2 | -- | -- | 3 | 1.00 | -- |
| *Tomentella* sp. | 6 | 0.60 | ns | 6 | 0.54 | ns |

*($P < 0.05$* or $P < 0.01$**, Spearman Rank correlations) n is number of replicates.

**TABLE 12-4.** pH($H_2O$) of agar media as affected by water soluble leaf extracts [1.7% (w/v)] of *S. repens* (old), *S. repens* (young), *C. epigejos*, *M.gale*, and *O. macrocarpos*, and $N(NO_3^-)$, $N(NH_4^+)$, and $P(PO_4^{3-})$ (in mg $l^{-1}$) in water soluble plant extracts.

| | pH($H_2O$) | | $N(NO_3^-)$ | $N(NH_4^+)$ | $P(PO_4^{3-})$ |
|---|---|---|---|---|---|
| Litter extracts | AMN | MMN | ($mg.l^{-1}$) | ($mg.l^{-1}$) | ($mg.l^{-1}$) |
| No extract | 7.0 | 5.6 | | | |
| *S.repens* (old) | 6.9 | 6.0 | 4.51 | 5.04 | 4.87 |
| *S.repens* (young) | 6.9 | 5.9 | 5.63 | 4.51 | 4.81 |
| *Calamagrostis epigejos* | 7.0 | 6.1 | 4.90 | 126.82 | 4.38 |
| *Myrica gale* | 6.8 | 5.8 | 3.71 | 85.45 | 4.15 |
| *Oxycoccus macrocarpos* | 6.8 | 6.0 | 2.93 | 20.01 | 1.40 |

**TABLE 12-5.** pH and nutrient concentrations $N(NO_3^-)$, $N(NH_4^+)$ and $P(PO_4^{3-})$ ($mg.l^{-1}$) of the agarplates ($CaCl_2$ extraction). Initial pH($CaCl_2$) and concentration (Initial concentration), and after two months with or without fungal growth as affected by water soluble leaf extracts [1.7% (w/v)] of *Salix repens* (old), *Salix repens* (young), *Calamagrostis epigejos*, *Myrica gale*, and *Oxycoccus macrocarpos* ($n$ = 1 - 3, and . = not determined).

| | Water soluble litter extracts | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No extract | | | | *Salix repens* (old) | | | | *Salix repens* (young) | | | | *Calamagrostis epigejos* | | | | *Myrica gale* | | | | *Oxycoccus macrocarpos* | | | |
| Species | pH | $NO_3^-$ | $NH_4^+$ | $PO_4^{3-}$ | pH | $NO_3^-$ | $NH_4^+$ | $PO_4^{3-}$ | pH | $NO_3^-$ | $NH_4^+$ | $PO_4^{3-}$ | pH | $NO_3^-$ | $NH_4^+$ | $PO_4^{3-}$ | pH | $NO_3^-$ | $NH_4^+$ | $PO_4^{3-}$ | pH | $NO_3^-$ | $NH_4^+$ | $PO_4^{3-}$ |
| AMN | | | | | | | | | | | | | | | | | | | | | | | | |
| Initial concentration | 6.0 | 70 | 53 | 114 | 5.9 | 71 | 54 | 115 | 5.9 | 71 | 54 | 114 | 6.0 | 71 | 72 | 115 | 5.8 | 71 | 66 | 115 | 5.8 | 70 | 56 | 114 |
| No fungus | 5.6 | 69 | 52 | 114 | 5.6 | 66 | 49 | 114 | 5.5 | 62 | 63 | 114 | 5.7 | 71 | 72 | 115 | 5.0 | 55 | 76 | 113 | 5.6 | 72 | 40 | 115 |
| *H. leucosarx* 1 | 4.8 | 71 | 36 | 112 | . | . | . | . | . | . | . | . | 5.3 | 67 | 34 | 114 | 5.9 | 60 | 33 | 113 | 4.9 | 72 | 35 | 113 |
| *H. leucosarx* 2 | 5.0 | 82 | 4 | 109 | . | . | . | . | 5.6 | 54 | 6 | 114 | 5.6 | 48 | 10 | 113 | 5.5 | 67 | 10 | 114 | . | . | . | . |
| *I. agardhii* | 3.8 | 62 | 30 | 114 | 4.2 | 57 | 14 | 115 | . | . | . | . | 4.1 | 72 | 44 | 115 | 3.9 | 84 | 40 | 115 | 3.8 | 69 | 40 | 114 |
| *I. lacera* | 5.1 | 47 | 37 | 111 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *L. controversus* | 4.9 | 69 | 45 | 113 | . | . | . | . | . | . | . | . | 5.0 | 76 | 36 | 115 | 4.9 | 79 | 35 | 115 | 4.7 | 70 | 38 | 117 |
| *P. involutus* | . | . | . | . | 4.8 | 49 | 17 | 114 | . | . | . | . | 4.3 | 0 | 39 | 113 | 4.5 | 0 | 41 | 114 | . | . | . | . |
| *S. verrucosum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Tomentella sp* | 6.3 | 0 | 35 | 89 | 6.9 | 1 | 93 | 112 | 6.9 | 0 | 86 | 110 | 6.3 | 0 | 43 | 49 | 6.5 | 0 | 34 | 48 | 6.3 | 0 | 37 | 80 |
| MMN | | | | | | | | | | | | | | | | | | | | | | | | |
| Initial concentration | 4.6 | 0 | 53 | 114 | 5.0 | 0 | 54 | 114 | 4.9 | 0 | 54 | 114 | 5.1 | 0 | 72 | 115 | 4.8 | 0 | 66 | 115 | 5.0 | 0 | 56 | 114 |
| No fungus | 4.9 | 0 | 53 | 114 | 5.1 | 0 | 57 | 114 | 5.2 | 0 | 57 | 114 | 5.3 | 0 | 71 | 115 | 3.7 | 0 | 66 | 115 | 5.2 | 0 | 56 | 114 |
| *H. leucosarx* 1 | 3.6 | 0 | 17 | 114 | 4.6 | 0 | 39 | 114 | 4.2 | 0 | 27 | 114 | 3.8 | 0 | 7 | 115 | 3.8 | 0 | 8 | 115 | 3.8 | 0 | 16 | 116 |
| *H. leucosarx* 2 | 3.5 | 0 | 12 | 116 | 4.1 | 0 | 13 | 114 | 3.9 | 0 | 15 | 114 | 4.0 | 0 | 8 | 114 | 3.8 | 0 | 9 | 113 | 3.7 | 0 | 8 | 114 |
| *I. agardhii* | 3.4 | 0 | 5 | 114 | . | . | . | . | . | . | . | . | 3.8 | 0 | 8 | 115 | 3.8 | 0 | 8 | 115 | 3.7 | 0 | 8 | 115 |
| *I. lacera* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *L. controversus* | 3.4 | 0 | 8 | 113 | . | . | . | . | . | . | . | . | 3.7 | 0 | 6 | 107 | 3.7 | 0 | 9 | 104 | 3.6 | 0 | 6 | 101 |
| *P. involutus* | 3.3 | 0 | 6 | 115 | . | . | . | . | . | . | . | . | 3.4 | 0 | 7 | 115 | 3.5 | 0 | 9 | 114 | 3.5 | 0 | 11 | 113 |
| *S. verrucosum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Tomentella* sp | 3.5 | 0 | 6 | 115 | 4.7 | 0 | 35 | 106 | 4.5 | 0 | 40 | 114 | 5.7 | 0 | 53 | 115 | 5.2 | 0 | 41 | 114 | 4.1 | 0 | 12 | 114 |

*Chemical composition of leaf extracts and media*

pH of the media and available concentrations of $N(NO_3^-)$, $N(NH_4^+)$ and $P(PO_4^{3-})$ in the water soluble leaf extracts are presented in TABLE 12-4. The leaf extracts showed a slight acidifying initial effect on substrate pH of AMN, with the exception of *Calamagrostis*. The pH of MMN was raised by the extracts. Both *Salix* leaf extracts had similar extractable nutrient concentrations. Addition of these *Salix* extracts did hardly increase nutrient availability. *Calamagrostis* leaves released 25 times and *Myrica* 17 times more $NH_4^+$ than the control. *Oxycoccus* leaf extract contained the lowest $N(NO_3^-)$, and $P(PO_4^{3-})$ concentration, but four times the $N(NH_4^+)$ concentration of *Salix*.

$pH(CaCl_2)$ and nutrient concentrations of the agar plates at the start of the experiment and after 2 months are presented in TABLE 12-5. pH of the control decreased for AMN from 6.0 to 5.6, but increased for MMN substrate from 4.6 to 4.9. Addition of leaf extracts decreased pH on AMN, but increased pH on MMN (except for *Myrica* where a substantial decrease from 4.8 to 3.7 was noted).

In the absence of leaf extracts pH after fungal growth on AMN ranged from 3.8 (*I. agardhii*, which showed preferential uptake of $NH_4^+$) to 6.3 (*Tomentella* sp.: referential uptake of $NO_3^-$). On MMN changes were much smaller and ranged from 3.3 (*P. involutus*; highest $NH_4^+$ consumption) to 3.6 (*H. leucosarx* L1; lowest $NH_4^+$ consumption). The leaf litters hardly showed any additional effects on pH. Final pH after growth with leaf litter extracts was generally higher than after growth in the control on both AMN and MMN. On AMN with litter extracts *Tomentella* sp. and to a lesser degree *P. involutus* showed prefential uptake of $NO_3^-$, whereas *L. controversus* was unable to utilize nitrate. On MMN a large part of the ammonium was usually taken up, except by *Tomentella* sp. in 4 out of 5 cases with extracts. Substantial immobilisation of phosphorus was only noted for *Tomentella* sp. on AMN, whereas *L. controversus* immobilized some P on MMN.

## DISCUSSION

In this experiment only water soluble foliar extracts were tested. There is evidence that, besides the mechanical effects of litter and herbs (Sydes & Grime, 1981; Facelli & Pickett, 1991), litters influence plants by releasing certain organic compounds (Blaschke, 1979; Kuiters, 1987). Among these water soluble organic compounds, polyphenols and phenolic acids form important fractions. Although in the present study no attempt was made to analyse these phenolics, earlier studies have shown that freshly fallen litter of *Salix spp.*

had high leaching rates of simple phenolics and polyphenols (Kuiters & Sarink, 1986). Differences between litter types resulted mainly from the relative amounts in which these various phenolics occurred (Kuiters & Sarink, 1986). As contamination had to be avoided in this experiment, autoclaving of the litters was inevitable. This could have changed the nature of the phenolics. However, in a study of pine needle litter and shoots and roots of the grass *Deschampsia flexuosa* (L.) Trin., Baar *et al.* (1994) noted that autoclaving did only result in a small shift of the molecular weight distribution of the organic components. Autoclaving also resulted in minor changes of the concentration of water-soluble phenolic acids. The alternative sterilization method of these extracts, i.e. filter sterilization, was considered not appropriate in this study as filtration of an aqueous fungal extract resulted in significant retention of phenolics (M. Kotterman, pers. comm.). These phenolics were more retained when more non-polar. The phenolics in litter extract are not well defined, so it was thought conceivable that retention of some specific phenolic could occur.

Inhibitory effects of *S. repens* extracts are possibly related to high amounts (the concentrations used were only half of those from the other plant species investigated) of salicylic acids (Olsen *et al.*, 1971; Kuiters & Sarink, 1986). Production of aromatic substances is not only dependent on the identity of the plant species, but also shows variability between years (Nilsson *et al.*, 1998) and sites. Litter from *Salix* from an early and late successional site affected radial growth and biomass production on AMN differently for *H. leucosarx* L2 (isolated from a primary dune, performing better on litter from the young stand) and *P. involutus* (performing better on litter from a late successional stand). Moreover, foliar litter extracts contain more inhibitory compounds than these simple phenolics. After aromatic substances from extracts of *Populus* were removed by treatment with active charcoal, inhibitory effects still occurred (Olsen *et al.*, 1971).

The evergreen *O. macrocarpos* should, according to its leaf lifespan, contain fairly large amounts of aromatic substances for defence against herbivory. This may explain the inhibitory effects of the *O. macrocarpos* extract and the low number of ectomycorrhizal species and sporocarps occurring in this field site. Besides inhibitory substances of leaf litter extracts, stimulatory effects may also occur, e.g. by additional nutrients such as nitrogen. This provides an explanation for better performance (higher biomass) of fungi on both media in the presence of extracts from *Calamagrostis* and *Myrica*, which both contain very large amounts of $NH_4^+$. Although few or no sporocarps of ectomycorrhizal fungi were observed in the field sites in which *Calamagrostis* or *Myrica* dominated, leaf litter extracts did stimulate

growth rate and biomass of various fungi, especially *L. controversus* and *P. involutus*. Such stimulatory effects could easily be explained by the high concentrations of $NH_4^+$ in these extracts (Boxman *et al.*, 1986; Jongbloed & Borst-Pauwels, 1990; Littke *et al.*, 1984; Bending & Read, 1996), i.e. *Paxillus involutus* also increased biomass when cultured on media in which the amount of $NH_4^+$ was raised (E.W. van der Heijden, unpubl. data). The stimulatory effect of *Calamagrostis* litter on ectomycorrhizal fungal growth and the fact that no sporocarps occurred in the *Calamagrostis* co-dominated field site may be better explained by the impenetratable thick root zone than by negative effects of accumulating litter. The scarcity of ectomycorhizal fungi in *Myrica* dominated field sites seems inconsistent with the stimulatory effects of the foliar extracts. This discrepancy can possibly be explained by a negative interaction between Frankia and ectomycorrhizal fungi, as some Frankia strains are known to exude anti-fungal compounds (Akkermans *et al.*, 1989).

The effects on fungal isolates used in this experiment do not necessarily hold for those species in general. Earlier investigations have shown that different isolates of one species can react differently to the same treatments (Marx, 1981; Kieliszewska-Rokicka, 1992; Arnebrant, 1994). Two isolates of *H. leucosarx* from two contrasting field sites were chosen, L2 from a primary successional stage, where sporocarps were very abundant, L1 from an old successional stage, where only low numbers of sporocarps were observed (Kuyper *et al.*, 1994; Chapter 2). In both field sites sporocarp number and mycorrhizal colonization by that species were correlated (CHAPTER 3). On AMN L2 was able to grow on both *Salix* leaf extracts, whereas L1 was not, suggesting that L1 might not be adapted to alkaline conditions. In the absence of litter and in the presence of litter of *Calamagrostis* and *Myrica* L2 immobilized more ammonium, but attained a lower mycelial weight. On MMN, however, both isolates performed similarly.

*Inocybe agardhii* and *I. lacera* were both used to check a possible general effect within a genus, but such an effect was not observed. Both isolates originated from successionally old field sites, where sporocarps of *I. agardhii* were frequent and those of *I. lacera* rare (Kuyper *et al.*, 1994; CHAPTER 2). Growth rate of *I. agardhii* was not affected by *Salix* litter on AMN, but was inhibited on the substrate MMN. Dry weight production of *I. agardhii* in the presence of *Salix* litter was also significantly higher on AMN than on MMN. *Salix* litter (from the young stand) tended to increase dry weight production compared to the AMN control (no extract). Although this isolate of *I. agardhii* was collected from an acidic field site, field observations showed that it is more common on the calcareous field sites

(unpubl. data). *Lactarius controversus, P. involutus* and *S. verrucosum* were more common in middle-aged and older successional field sites (CHAPTER 2). All of them, however, were negatively affected by the *Salix* litters on both media suggesting that tolerance to leaf litter extracts is not always higher in late successional species, contrary to the hypothesis by Perry & Choquette (1987). However, *S. verrucosum* grew well on the extracts from *Calamagrostis* and *Myrica*, both plants being common at the site where the isolate used had been collected.

Both biomass increment and radial growth were determined independently because of the different significance of these parameters for the functioning of the mycorrhizal association. Mycorrhizal fungi increase the nutrient uptake of trees both by increasing the absorbing area and by providing efficient cellular transport mechanisms (Smith & Read, 1997). Radial growth will contribute to the expansion of the absorbing area in the soil, making nutrients with a low mobility more easily available, while increase in biomass may enlarge the capacity for accumulation of nutrients (and may also reflect carbon costs for the plant).

Extrapolation from laboratory experiments with leaf litter extracts to field conditions should be executed cautiously. Pre-treatment of the material such as grinding and drying may have large effects on experimental results. The use of freshly fallen or aged litter might also affect experimental outcomes (Gallet & Lebreton, 1995). Under field conditions, the allelopathic effects will also depend on local soil conditions. Sandy, humus-poor soils do not generally concentrate toxins to high levels, since they lack colloidal material, but humus will have a concentrating effect on organic substances released from litter. For most fungal species behaviour on both media (AMN and MMN) was different for most foliar extracts. Better performance on AMN than on MMN suggests that the low pH, possibly as a consequence of ammonium uptake, might exert a negative influence. A similar conclusion was reached by Jongbloed & Borst-Pauwels (1990). It is therefore important that the pH of the medium is comparable to that of the soil where the fungus commonly grows.

The question may also be raised whether the behaviour of the ectomycorrhizal fungi in vitro is sufficiently similar to that of the extramatrical mycelium in vivo. Timonen & Sen (1998) noted polyphenol oxidase activity of the extramatrical mycelium of *Suillus bovinus* and *Paxillus involutus*, but where unable to detect these enzymes in pure culture mycelium. Giltrap (1982), however, noted polyphenol oxidase activity in both *S. bovinus* and *P. involutus* in pure culture. Clearly, enzyme expression of mycorrhizal fungi under different environmental conditions demands further study.

Nevertheless, the results in the present experiment indicate that leaf litter extracts affect performance of ectomycorrhizal fungi (and hence their functional significance), and

may qualitatively and quantitatively influence ectomycorrhizal species composition in *Salix repens* scrubs that differ in age, soil type and co-dominant vegetation.

# 13

# GENERAL DISCUSSION

*"General ecological patterns emerge most clearly from the glorious diversity of life when systems are no too complicated, that is when the contingencies are manageable (as in the population of a single species, or very small numbers of interacting species), and at very large scales, when a kind of statistical order emerges from the scrum. The middle ground is a mess. It is fascinating to study, but do not expect universal rules, even simple contingent general rules, to emerge. If and when they do, treasure them"* (Lawton, 1999).

## INTRODUCTION

The major aim of this thesis was to investigate the functional significance of different mycorrhizal symbionts of *Salix repens*. How does mycorrhizal fungal diversity contribute to broad ecological tolerance of *S. repens*? How do environmental conditions affect cost and benefits of the mycorrhizal symbiosis? How does internal plant nutrient status affect mycorrhizal functioning? How do mycorrhizal fungi interact on the root system? A question that derives from my investigation is: to what extent can my results be generalized over other mycorrhizal plants and mycorrhizal fungi?

Ecologists have always been impressed much about biological diversity. In the face of this diversity they have come to divergent views about the generality of ecological explanations. Therefore the question is timely in how far my results can be generalized. Is generalization possible because the system is not too complicated? Or is generalization possible because a statistical order is emerging? Of course, one could always increase number of fungal or plant taxa investigated, but in the face of limited personnel and financial resources such pleas are gratuitous. Furthermore, such choices need to be evaluated against the need for addressing new questions: Can plants exert control over mycorrhizal colonization? If so, could plant control constrain evolution of these mycorrhizal fungi? How should plant benefit be realistically assessed? How should mycorrhiza be quantified?

**MYCORRHIZAL DIVERSITY**

For ectomycorrhizal fungi, species diversity is estimated to be around 15,000 (Brussaard *et al.*, 1997) of which in my study about 20 have been included. This number compares favourably with the 78 EcM taxa noted in 16 *Salix repens* communities (CHAPTER 2). With regard to other EcM tree species, EcM fungal richness of *S. repens* might not be particularly high. Trappe (1977) claimed that at least 2000 species could associate with Douglas fir (*Pseudotsuga menziesii*). The number of species associated with various tree genera in Europe is also substantial; according to Newton & Haigh (1998) about 233 species are associated with oak (*Quercus* spp.), 223 with beech (*Fagus sylvatica*), and 201 with pine (*Pinus sylvestris*). However, morphological species diversity is only part of the picture. Morphospecies might consist of several biological species. Aanen (1999) noted that the morphospecies complex of *Hebeloma crustuliniforme* in northwestern Europe consists of at least 17 biological species that are all associated with members of the Salicaceae. Finally, there might be genetic diversity within (morphological or biological) species (Cairney, 1999). However, provenance of the ectomycorrhizal fungus *Hebeloma leucosarx* did not make a difference in plant responses (CHAPTER 9).

Species diversity of arbuscular mycorrhizal fungi is low, with less than 160 species described to date. Many of these species seem to have a large or even world wide distribution. Species diversity can be relatively high both in monospecific stands and in highly diverse plant communities (Morton *et al.*, 1995). Ecological specificity has also been demonstrated on two levels: fungi selectively colonizing roots of certain plant species, and fungi exerting specific effects on certain plant species. It remains to be demonstrated that the fungi that show specific beneficial effects on certain plant species are also preferentially associated with these plant species. However, discussions on species richness of AMF should take the possibility into account that low richness might in fact be an artefact, due to our inability to recognize more than 160 or so morphotaxa based on spore characters only.

Next to being able to morphotype these 160 described species, assessment of AMF species diversity will involve a number of spore trappings either directly from the field soil or by using trap cultures. Species number increased with number of traps (CHAPTER 3). Furthermore, host-dependent sporulation rates indicate that the AMF diversity detected using trap cultures may depend upon the species of host plant used in the trap. Therefore, a single trap or trap plant species is unlikely to capture all AMF species present in a soil sample, and a diversity of approaches is necessary for more complete discriptions of AM fungal species diversity at a given location (Bever *et al.*, 1996). The importance of trapping procedures is

evident from a comparison of data on AMF species composition as indicated in CHAPTER 3. However, it is questionable whether the effort of using a variety of different trapping procedures is worthwhile, because genetic diversity within species of AMF can be very high (Sanders *et al.*, 1996; Clapp *et al.*, 1995) and this may coincide with physiologically different activities.

Assessing species diversity on the basis of sporocarps (EcMF) or individual spores (AMF) needs to be complemented by assessing species diversity in or around roots. Below-ground 15 EcM morphotypes have been described and these morphotypes generally correspond to genus (CHAPTERS 2 AND 3). Accuracy of below-ground EcM typing will be increased with the help of molecular methods. However, it has not been convincingly shown that the trade-off between accuracy of identification and number of root tips investigated (see discussion CHAPTER 2) really makes molecular identification imperative (depends on the research question). Unfortunately, no differences in colonization structures of AMF could be observed in *S. repens*, thus identification of different fungal taxa in the roots was not possible. Moreover, AMF, identified on the basis of morphotyping of fungal spores trapped from the field soil collected in the *S. repens* communities, may not have formed mycorrhizas with *S. repens*, but with its co-dominant vegetation. Molecular methods could be helpful for species identification of the AMF associated with the host plant of interest (Helgason *et al.*, 1998).

Such molecular identifications should explicitly address the question of host specificity of AMF of *S. repens* in relation to that of plants of the co-dominant vegetation. Evolution of specificity of AMF associated with *S. repens* might coincide with specific behaviours as *S. repens* is consistently only slightly AM (CHAPTERS 3, 4, 5 AND 8). Similarly, other supposedly ectomycorrhizal plants might be dual mycorrhizal as suggested in CHAPTER 11, and, considering the specific nature of the AMF – EcMF interaction, this might constrain co-evolution with (specific) AMF (species).

### CONSEQUENCES OF MYCORRHIZAL DIVERSITY

Two further sets of questions derive from observations of high mycorrhizal fungal diversity, viz. questions about factors that regulate diversity, and questions about the functional importance of diversity.

High species and genetic diversity of mycorrhizal fungi might be related to niche breadth of the plant. Niche differentiation via resource partitioning is one explanation for mycorrhizal diversity (Bruns, 1995). Resources could be partitioned in time or space. Two

obvious time gradients are related to season and to age of the host or stand. Spatial heterogeneity of resources might be coupled to the clumped distribution of mycorrhizal species. However, niche dimensions of mycorrhizal fungi do not only include the soil where the external mycelium forages but also the plant-fungus interface. If different mycorrhizal fungi have differential effects on plants and if plants can control mycorrhizal colonization to some extent, coexistence of different mycorrhizal fungi might be explained by different plant benefits. Evidence for plant control over arbuscular mycorrhizal colonization has been reported in CHAPTERS 5, 7, 8 AND 10. The different proportions of colonizations by ectomycorrhizal fungi as reported in CHAPTERS 6 AND 7 might indicate some sort of plant control over EcM colonization as well. Progress in this respect will be likely forthcoming from studies on the physiological mechanisms of plant control. As plant control over mycorrhizal colonization probably will affect interactions with other rhizosphere organisms (e.g. fungal pathogens), such mechanistic studies might have wider applications.

Data from field observations and all subsequent experiments confirm that *S. repens* is slightly AM and highly EcM. While these observations are not new, I propose a new interpretation. Usually, low AM and high EcM have been interpreted as evidence for AM suppression by EcM. However, when such static data were put under the scrutiny of an experimental investigation of interactions, a different pattern emerged: EcM did not suppress AM, but AM temporarily did suppress EcM (CHAPTERS 4 AND 8)! These observations also emphasize both the need for studying interactions directly and the need for studying underlying mechanisms. However, one of the three ectomycorrhizal fungi under examination suppressed subsequent colonization by AMF (but to a far lesser extent) suggesting that a diversity of mechanisms regulates interaction between EcMF and AMF. Apparently such interactions cannot be interpreted in the general framework of functional types, but need to take into account the specific characteristics of the fungi. It remains to be investigated whether general patterns emerge from groupings of fungal species according to their different strategies (CHAPTER 6).

The functional importance of mycorrhizal diversity has been addressed in CHAPTERS 4, 6, 7 AND 8. My results indicate that no mycorrhizal fungus exerts the full suite of mycorrhizal benefits [see below] and therefore provide circumstantial evidence that mycorrhizal diversity both between and within mycorrhizal types (AM and EcM) contributes to niche breadth of *S. repens*.

**QUANTIFICATION OF MYCORRHIZA UNIT**

Whereas many investigations have addressed questions about the measurement of diversity, far less attention has been devoted to the quantification of a specific 'mycorrhiza unit'. A number of parameters have been proposed, such as proportional colonization, absolute root length colonized, number of root tips (only for EcM fungi), and intensity of colonization (only for AM fungi). These different parameters have been compared in CHAPTERS 3, 5 AND 10, and show that none of these parameters performed best consistently. Two problems need special emphasis; (1) flooding (CHAPTER 10) affects estimates of colonization by AMF and EcMF differently, thereby falsely suggesting a negative interaction; (2) mycorrhizal activity might well precede visible mycorrhizal structures (arbuscules or fungal sheath), as suggested for AMF (CHAPTER 5) and demonstrated for EcMF (CHAPTER 6). Finally, comparability of colonization data needs further attention. It has been common practice to apply different methods for assessment of colonization and express both as proportional (fractional) colonization. However, even if both are expressed similarly it is by no means clear if such colonization data in dual mycorrhizal plants are additive or not. The same question may be applied to additivity of proportional colonization of different ectomycorrhizal or arbuscular mycorrhizal fungi.

The large difference between AM and EcM colonization in *S. repens* also raises the question about the relationship between amount of colonization and magnitude of effect. My data are straightforward: contrary to what is commonly assumed, there is no such relationship! High infection by EcMF (sometimes without concomitant high plant response) might be a consequence of the EcMF maximizing their own fitness. Changes in mycorrhizal morphology as affected by pH and N/P availability without changes in plant response, as described in CHAPTER 7, support this conclusion. If so, an intriguing question follows. In field samplings only part of the mycorrhizas seemed to be well developed (CHAPTERS 2 AND 3). Are those well developed mycorrhizas more important for plant nutrition than the undeveloped mycorrhizas? Or do they only reflect maximum fungal fitness (and possibly higher plant costs)?

**ASSESSMENT OF MYCORRHIZAL BENEFITS**

Similar problems to the assessment of a certain 'mycorrhiza unit' surround the assessment of mycorrhizal benefit from a phytocentric perspective. Growth (biomass increment) has usually been applied as a surrogate measure for plant fitness. However, decreased performance combined with increased survival, as demonstrated for *S. repens*

inoculated with *Acaulospora laevis* (CHAPTER 4), clearly shows that plant growth cannot be equated with plant fitness. In fact, mycorrhizal benefit might be manifested only in certain life stages such as establishment (where incorporation in a mycorrhizal network might result in a large nutritional benefit, cf. CHAPTER 10) or flowering and initial fruit setting (which might explain relatively high AM colonization in springtime, cf. CHAPTER 3). Why does *S. repens* not downregulate its AM colonization in life stages when there is no apparent benefit? Maybe, our conception of mycorrhizal benefit has been too limited. Increased attention for temporal benefits of mycorrhiza can result in an appreciation that different mycorrhizal fungi might be functional at different time periods (CHAPTERS 3 AND 5). Awareness of temporal benefits will also result in a broader range of parameters by which this benefit is assessed (carbon gain, nutrient gain, non-nutritional benefits). I have compared these different parameters and expanded on the view of non-nutritional benefits, such as changes in root architecture, which have both been reported for EcMF (CHAPTER 6) and AMF (CHAPTER 5). Such non-nutritional benefits are maybe less restricted to certain life stages.

These changes in root architecture, caused by mycorrhizal fungi, have large implications for calculations of nutrient inflow and uptake. My results indicated that nutrient inflow (calculated per unit root length) and nutrient uptake (calculated as total shoot nutrient content) do not necessarily correlate (CHAPTER 6). In fact my results raised a more fundamental question, whether nutrient inflow in mycorrhizal plants should be based on root length, and, if not, which other parameter(s) is (are) more suitable.

## MYCORRHIZAL STRATEGIES

My results also led to the hypothesis that two strategies of mycorrhizal fungi exist, viz. root replacement strategy and root manipulation strategy (CHAPTER 6). It should be emphasized that classification of these strategies relates to fungal behaviour and not to the fungi per se. Expression of these strategies depends also on the abiotic environment (CHAPTER 7). Evidence for the root manipulation strategy has not been reported before. This could be due to the fact that it is specific for certain mycorrhizal fungi only (limited generality) or that the environments under which other experiments were executed, were not conducive to expression of this strategy. Therefore, applicability of classifications of fungal strategies needs more attention. Such classifications should not be restricted to comparisons within functional types of mycorrhiza, as evidence of this root manipulation strategy was noted for both functional types. Comparison of both strategies also indicated

differential behaviour with regard to nitrogen and phosphorus. Apparently, N/P ratios are not only important for plants, but also for the mycorrhizal fungi. This topic warrants further study.

### *SALIX REPENS* AS A MODEL ORGANISM?

In my thesis *S. repens* has been used as a model organism. However, for what other plant species is *S. repens* indeed a model? It may be too early to give a final answer to this question, but for the time being the following suggestions are offered.

One of the advantages of *S. repens* as a model was that it could be multiplied vegetatively and that genetically homogeneous cuttings could be used. Another unexpected advantage became apparent as well. Cuttings, with their high shoot to root ratio, clearly showed nutritional benefits of initial mycorrhizal colonization. In addition, the effects might have been enhanced by placing the cuttings in an intact mycorrhizal network (CHAPTER 10). Next to that, variation in internal nutrient (phosphorus) status of winter cuttings, depending on collecting date (CHAPTER 5), offered more opportunities to study arbuscular mycorrhizal functioning. The use of cuttings collected in December with a low P status (and a low root to shoot ratio), showed that even low AM colonization could be very effective in P supply to the shoot.

The interactions between EcMF and AMF (CHAPTERS 4 AND 8) led to a number of new hypotheses on dual mycorrhizas (CHAPTER 11). How common are dual mycorrhizas in plants that have been formerly considered ectomycorrhizal? The screening of pines, birches and beeches for the presence of AM is advocated. If these are found, do such plants derive benefits from being dual mycorrhizal? Do the same interactions between EcMF and AMF occur on these other trees and does that constrain the evolution of AMF associated with those trees? I suspect that the same patterns (AMF showing low colonization, substantial benefits, and suppression of EcMF) occur with other ectomycorrhizal tree species. If so, *S. repens* can be seen as a model organism However, the use of the term model organism harbours the risk of overgeneralization. Just as every mycorrhizal fungus of *S. repens* showed its own unique behaviour each mycorrhizal plant will also show its uniqueness. Or, as stated by McIntosh (1985):

*"Seek simplicity, and distrust it"*

# REFERENCES

**Aanen, D.K. (1999)** Species and speciation in the *Hebeloma crustuliniforme* complex. Ph.D. thesis, Wageningen Agricultural University, The Netherlands.

**Aanen, D.K., Kuyper, Th.W. (1999)** Intercompatibility tests in the *Hebeloma crustuliniforme* complex in northwestern Europe. *Mycologia* **91**: 783-795

**Aanen, D.K., Kuyper, Th.W., Boekhout, T., Hoekstra, R.F. (2000)** Phylogenetic relationships in the genus *Hebeloma* based on ITS 1 and 2 sequences, with special emphasis on the *Hebeloma crustuliniforme* complex. *Mycologia* (accepted for publ.).

**Abuzinadah, R.A., Read, D.J. (1986)** The role of proteins in nitrogen nutrition of ectomycorrhizal plants. I. Utilization of peptides and proteins by ectomycorrhizal fungi. *New Phytol.* **103**: 481-493.

**Agerer, R. (1992)** Ectomycorrhizal rhizomorphs: Organs of contact. *In*: Mycorrhizas in Ecosystems (*Edited by* D.J. Read, D.H. Lewis, A.H. Fitter, I.J. Alexander) pp. 303-310. University Press, Cambridge.

**Agerer, R. (1987-1998)** Colour atlas of ectomycorrhizae. Einhorn Verlag, Schwäbisch Gmünd.

**Akkermans, A., Hahn, D., Zoon, F. (1989)** Interactions between root symbionts, root pathogens and actinorhizal plants. *Ann. Sci. For.* **46** (suppl.): 765S-771S.

**Alexander, I.J. Bigg, W.L. (1981)** Light microscopy of ectomycorrhizas using glycol methacrylate. *Trans. Br. Mycol. Soc.* **77**: 425-429.

**Allen, E.B., Allen, M.F., Helm, D.J., Trappe, J.M., Molina, R., Rincon, E. (1995)** Patterns and regulation of mycorrhizal plant and fungal diversity. *Plant Soil* **170**: 47-62.

**APG (1998)** An ordinal classification for the families of flowering plants. *Ann. Miss. bot. Gdn* **85**: 531-553.

**Arnebrant, K. (1994)** Nitrogen amendments reduce the growth of extramatrical ectomycorrhizal mycelium. *Mycorrhiza* **5**: 7-15.

**Arnolds, E. (1992)** The analysis and classification of fungal communities with special reference to macrofungi. *In*: Fungi in vegetation science (*Edited by* W. Winterhoff) pp. 7-47. Kluwer Academic Publishers, Dordrecht.

**Arnolds, E., Kuyper, Th.W. (1995)** *Cortinarius* species associated with *Salix repens* in the Netherlands. *Beih. Sydowia* **8**: 5-27.

**Baar, J., Kuyper, Th.W. (1993)** Litter removal in forests and effect on mycorrhizal fungi. *In*: Fungi of Europe: Investigation, Recording, and Mapping (*Edited by* D.N. Pegler, L. Boddy, B. Ing, P. Kirk) pp. 275-286. Royal Botanic Gardens, Kew.

**Baar, J., Ozinga, W.A., Sweers, I.L., Kuyper, Th.W. (1994)** Stimulatory and inhibitory effects of needle litter and grass extracts on the growth of some ectomycorrhizal fungi. *Soil Biol. Biochem.* **26**: 1073-1079.

**Baar, J. (1995)** Ectomycorrhizal fungi of Scots pine as affected by litter and humus. Ph.D. thesis, Wageningen Agricultural University, The Netherlands.

**Baar, J., Oude Elferink, M. (1996)** Ectomycorrhizal development on Scots pine (*Pinus sylvestris* L.) seedlings in different soils. *Plant Soil* **179**: 287-292

**Baar, J., Comini, B., Oude Elferink, M., Kuyper, Th. W. (1997)** Performance of four ectomycorrhizal fungi on organic and inorganic nitrogen sources. *Mycol. Res.* **101**: 523-529.

**Baser, C.M., Garrett, H.E., Mitchell, R.J., Cox, G.S., Starbuck, C.J. (1987)** Indolebutyric acid and ectomycorrhizal inoculation increase lateral root initiation and development of container-grown black oak seedlings. *Can. J. For. Res.* **17**: 36-39.

**Bending, G.D., Read, D.J. (1996)** Nitrogen mobilization from protein-polyphenol complex by ericoid and ectomycorrhizal fungi. *Soil Biol. Biochem.* **28**: 1603-1612.

**Blaschke, H. (1979)** Leaching of water-soluble organic substances from coniferous needle litter. *Soil Biol. Biochem.* **11**: 581-584.

**Boxman, A.W., Sinke, R.J., Roelofs, J.G.M. (1986)** Effects of $NH^+_4$ on the growth and $K^+$ ($^{86}$ Rb) uptake of various ectomycorrhizal fungi in pure culture. *Water, Air Soil Poll.* **31**: 517-522.

**Bever, J.D., Morton, J.B., Antonovics, J., Schultz, P.A. (1996)** Host-dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. *J. Ecol.* **84**: 71-82.

**Bever, J.D., Westover, K.M., Antonovics, J. (1997)** Incorporating the soil community into plant population dynamics: the utility of the feedback approach. *J. Ecol.* **85**: 561-571.

**Boucher, D.H., James, S., Keeler, K.H. (1982)** The ecology of mutualism. *Ann.Rev.Ecol.Syst.* **13**: 315-347.

**Bowen, G.D. (1980)** Misconceptions, concepts and approaches in rhizosphere biology. *In*: Contemporary microbial ecology (*Edited by* D.C.Ellwood, J.N. Hedger, M.J. Latham, J.M. Lynch, J.M. Slater) pp. 283-304. Academic Press, London.

**Bowen, G.D. (1984)** Tree roots and the use of soil nutrients. *In*: Nutrition of plantation forests (*Edited by* G.D. Bowen, K.S. Nambiar) pp. 147-179. Academic Press, London.

**Braak, C.J.F. ter (1990)** Update notes: CANOCO version 3.10. Agricult. Math. Group, Wageningen.

**Brandes, B., Godbold, D.L., Kuhn, A.J., Jentschke, G. (1998)** Nitrogen and phosphorus acquisition by the mycelium of the ectomycorrhizal fungus *Paxillus involutus* and its effects on host nutrition. *New Phytol.* **140**: 735-743.

**Brewster, J.L., Tinker, P.B. (1972)** Nutrient flow rates into roots. *Soils and fertilizers* **35**: 355-359.

**Bronstein, J.L. (1994)** Conditional outcomes in mutualistic interactions. *Trends Ecol.Evol.* **9**: 214-217.

**Brundrett, M.C., Piché, Y., Peterson, R.L. (1985)** A developmental study of the early stages in vesicular-arbuscular mycorrhiza formation. *Can. J. Bot.* **63**: 184-194.

**Brundrett, M., Bougher, N., Dell, B., Grove, T., Malajczuk, N. (1996)** Working with mycorrhizas in Forestry and Agriculture. ACIAR Monograph 32. Pirie printers, Canberra, Australia.

**Bruns, T.D. (1995)** Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. *Plant Soil* **170**: 63-73.

**Bruns, T.D, Szaro, T.M., Gardes, M., Cullings, K.W., Pan, J.J., Taylor, D.L., Horton, T.R., Kretzer, A., Garbelotto, M., Li, K. (1998)** A sequence database for the identification of ectomycorrhizal Basidiomycetes by phylogenetic analysis. *Molec.Ecol.* **7**: 257-272.

**Brussaard, L., Behan-Pelletier, V.M., Biquell, D.E., Brown, V.K., Didden, W., Folgarait, P., Fragoso, C. Freckman, D.W., Gupta, V.V.S.R., Hattori, T., Hawksworth, D.L., Klopatek, C., Lavelle, P., Malloch, D.W., Rusek, J., Söderström, B., Tiedje, J.M., Virginia, R.A. (1997)** Biodiversity and ecosystem functioning in soil. *Ambio* **26**: 563-570.

**Burgess, T., Malajczuk, N. (1989)** The effect of ectomycorrhizal fungi on reducing the variation of seedling growth of *Eucalyptus globulus*. *Agric. Ecosyst. Environm.* **28**: 41-46.

**Burgess, T., Dell, B., Malajczuk, N. (1994)** Variation in mycorrhizal development and growth stimulation by 20 *Pisolithus* isolates inoculated on to *Eucalyptus grandis* W. Hill ex Maiden. *New Phytol.* **127**: 731-739.

**Cairney, J.W.G. (1999)** Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. *Mycorrhiza* **9**: 125-135.

**Cázares, E., Trappe, J. (1993)** Vesicular endophytes in roots of the Pinaceae. *Mycorrhiza* **2**: 153-156.

**Cázares, E., Smith, J.E. (1996)** Occurrence of vesicular-arbuscular mycorrhizae in *Pseudotsuga menziesii* and *Tsuga heterophylla* seedlings grown in Oregon Coast Range soils. *Mycorrhiza* **6**: 65-67.

**Chapin, F.S. (1980)** The mineral nutrition of wild plants. *Ann. Rev. Ecol.Syst.* **11**: 233-260.

**Chilvers, G.A., Lapeyrie, F.F., Horan, D.P. (1987)** Ectomycorrhizal vs endomycorrhizal fungi within the same root system. *New Phytol.* **107**: 441-448.

**Clapp, J.P., Young, J.P.W., Merryweather, J.W., Fitter, A.H. (1995)** Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytol.* **130**: 259-265.

**Clarkson, D.T., Hanson, J.B. (1980)** The mineral nutrition of higher plants. *Ann. Rev. Plant Physiol.* **31**: 231-298.

**Connell, J.H., Lowman, M. (1989)** Low diversity tropical rain forests: some possible mechanisms for their existence. *Am. Nat.* **134**: 88-119.

**Courtecuisse, R. (1984)** Transect mycologique dunaire sur la Côte d'Opale (France) I: Les groupements héliophiles et arbustifs de la xérosère. *Doc. mycol.* **15** (57-58): 1-115.

**Courtecuisse, R. (1986)** Transect mycologique dunaire sur la Côte d'Opale (France) II: Les groupements de l'hygrosère. *Doc. mycol.* **17** (66): 1-70.

**Chu-Chou, M., Grace, L.J. (1987)** Mycorrhizal fungi of *Pseudotsuga menziesii* in the South islands of New Zealand. *Soil Biol. Biochem.* **20**: 883-886.

**Dahlberg, A., Jonsson, L., Nylund, J-E. (1997)** Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old growth Norway spruce forest in South Sweden. *Can. J. Bot.* **75**: 1323-1335.

**Danielson, R.M. (1984)** Ectomycorrhizal associations of jack pine in northeastern Alberta. *Can. J. Bot.* **62**: 932-939.

**Deacon, J.W., Donaldson, S.J., Last, F.T. (1983)** Sequences and interactions of mycorrhizal fungi on birch. *Plant Soil* **71**: 257-262.

**Deacon, J.W., Fleming, L.V. (1992)** Interactions of ectomycorrhizal fungi. *In*: Mycorrhizal Functioning (*Edited by* M.F. Allen) pp. 249-300. Chapman and Hall, New York.

**De Bary, A. (1887)** Comparative morphology and biology of the Fungi, Mycetezoa and Bacteria [English translation of 1884 edition]. Clarendon Press, Oxford, UK.

**Dhillion, S.S. (1992)** Evidence for host-mycorrhizal preference in native grassland species. *Mycol. Res.* **96**: 359-362.

**Dhillion, S.S. (1994)** Ectomycorrhizae, Arbuscular Mycorrhizae, and *Rhizoctonia* sp. of Alpine and Boreal *Salix* spp. in Norway. *Arctic Alp. Res.* **26**: 304-307.

**Dighton, J., Mason, P.A. (1985)** Mycorrhizal dynamics during forest tree development. *In*: Developmental biology of higher fungi (*Edited by* D. Moore, L.A. Casselton, D.A. Woods, J.C. Frankland) pp. 117-139. University Press, Cambridge.

**Dixon, R.K., Garrett, H.E., Stelzer, H.E. (1987)** Growth and ectomycorrhizal development of loblolly pine progenies inoculated with three isolates of *Pisolithus tinctorius*. *Silvae Genetica* **36**: 240-245.

**Dodd, J.C., Rosendahl, S., Giovanetti, M., Broome, A., Lanfranco, L., Walker, C. (1996)** Inter and intraspecific variation within the morphologically -similar arbuscular mycorrhizal fungi *Glomus mosseae* and *Glomus coronatum*. *New Phytol.* **113**: 113-122.

**Dosskey, M.G., Linderman, R.G., Boersma, L. (1990)** Carbon-sink stimulation of photosynthesis in Douglas fir seedlings by some ectomycorrhizae. *New Phytol.* **115**: 269-274.

**Douds, D.D., Chaney, W.R. (1982)** Correlation of fungal morphology and development to host growth in a green ash mycorrhiza. *New Phytol.* **92**: 519-526.

**Downes, G.M., Alexander, I.J., Cairney, W.G. (1992)** A study of aging spruce [*Picea sitchensis* (Bong.) Carr.] ectomycorrhizas. I. Morphological and cellular changes in mycorrhizas formed by *Tylospora fibrillosa* (Burt.) Donk and *Paxillus involutus* (Batsch ex Fr.) Fr. *New Phytol.* **122**: 141-152.

**Doyle, J.A. (1998)** Phylogeny of vascular plants. *Ann. Rev. Ecol. Syst.* **29**: 567-599.

**Duchesne, L.C., Peterson, R.L., Ellis, B.E. (1988)** Pine root exudate stimulates the synthesis of antifungal compounds by ectomycorrhizal fungus *Paxillus involutus*. *New Phytol.* **108**: 471-476.

**Duchesne, L.C., Peterson, R.L., Ellis, B.E. (1989)** The time course of disease suppression and antibiosis by the ectomycorrhizal fungus *Paxillus involutus*. *New Phytol.* **111**: 693-698.

**Dunne, M.J., Fitter, A.H. (1989)** The phosphorus budget of a field-grown strawberry (*Fragaria* × *ananassa* cv. Hapil) crop: evidence for a mycorrhizal contribution. *Ann. Appl. Biol.* **114**: 185-193.

**Ek, H., Anderson, S., Arnebrant, K., Söderström, B. (1994)** Growth and assimilation of $NH_4^+$ and $NO_3^-$ by *Paxillus involutus* in association with *Betula pendula* and *Picea abies* as affected by substrate pH. *New Phytol.* **128**: 629-637.

**Ekblad, A., Wallander, H., Carlsson, R., Huss-Danell, K. (1995)** Fungal biomass in roots and extramatrical mycelium in relation to macronutrients and plant biomass of ectomycorrhizal *Pinus sylvestris* and *Alnus incana*. *New Phytol.* **131**: 443-451.

**Ellenberg, H. (1974)** Zeigerwerte der Gefässpflanzen Mitteleuropas. Verlag Erich Goltze KG, Göttingen.

**Ericsson, T. (1981)** Effects of varied nitrogen stress on growth and nutrition in three *Salix* clones. *Physiol. Plant.* **51**: 423-429.

**Erland, S., Söderström, B. (1990)** Effects of liming on ectomycorrhizal fungi infecting *Pinus sylvestris* L.I. Mycorrhizal infection in limed humus in the laboratory and isolation of fungi from mycorrhizal roots. *New Phytol.* **115**: 675-682.

**Erland, S., Söderström, B. (1991)** Effects of liming on ectomycorrhizal fungi infecting *Pinus sylvestris* L. III. Saprophytic growth and host plant infection at different pH values in unsterile humus. *New Phytol.* **117**: 405-411.

**Favre, J. (1955)** Les champions supérieurs de la zone alpine du Parc National Suisse. *Ergebn. wiss. Unters. schweiz. Nat. Parks* **5** (N.F.): 1-212.

**Favre, J. (1960)** Catalogue descriptif des champions supérieurs de la zone alpine du Parc National Suisse. *Ergebn. wiss. Unters. schweiz. Nat. Parks* **6** (N.F.): 323-610.

**Facelli, J.M., Pickett, S.T.A. (1991)** Plant litter: its dynamics and effects on plant community structure. *Bot. Rev.* **57**: 1-32.

**Finlay, R., Söderström, B. (1992)** Mycorrhiza and carbon flow to the soil. *In*: Mycorrhizal functioning (*Edited by* M.J. Allen) pp. 134-160. Academic Press, London.

**Fitter, A.H. (1977)** Influence of mycorrhizal infection on competition for phosphorus and potassium by two grasses. *New Phytol.* **79**: 119-125.

**Fitter, A.H. (1985)** Functional significance of root morphology and root system architechture. *In*: Ecological interactions in soil; plants, microbes and animals (*Edited by* A.H. Fitter, D. Atkinson, D.J. Read, M.B. Usher) pp. 87-106. Blackwell Scientific Publications, Oxford.

**Fitter, A.H., Garbaye, J. (1994)** Interactions between mycorrhizal fungi and other soil organisms. *Plant Soil* **159**: 123-132.

**Fitter, A.H., Moyersoen, B. (1996)** Evolutionary trends in root-microbe symbioses. *Phil. Trans. R. Soc. London B.* **351**: 1367-1375.

**Fontana, A. (1962)** Ricerche sulle micorrhize del genere *Salix*. *Allionia* **8**: 67-85.

**Frankland, J.C., Harrison, A.F. (1985)** Mycorrhizal infection of *Betula pendula* and *Acer pseudoplatanus*: relationship with seedling growth and soil factors. *New Phytol.* **101**: 133-151.

**Fries, N. (1985)** Intersterility groups in *Paxillus involutus*. *Mycotaxon* **24**: 403-409

**Gallet, C., Lebreton, P. (1995)** Evolution of phenolic patterns in plants and associated litters and humus of a mountain forest ecosystem. *Soil Biol. Biochem.* **27**: 157-165.

**Gange, A.C., Brown, V.K. (1997)** Multitrophic interactions in terrestrial systems. Blackwell Science, Oxford.

**Gange, A.C., Bower, A., Stagg, P.G., Aplin, D.M., Gillam, A.E., Bracken, M. (1999)** A comparison of visualization techniques for recording arbuscular mycorrhizal colonization. *New Phytol.* **142**: 123-132.

**Gardes, M., Bruns, T.D. (1996)** Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above and below-ground views. *Can. J. Bot.* **74**: 1572-1583.

**Gay, G., Normand, L., Marmeisse, R., Sotta, B., Debaud, J.C. (1994)** Auxin overproducer mutants of *Hebeloma cylindrosporum* Romagnesi have increased mycorrhizal activity. *New Phytol.* **128**: 645-657.

**George, E., Marschner, H., Jakobsen, I. (1995)** Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. *Critical Rev. Biotechnology* **15** (3/4): 257-270.

**Gianinazzi-Pearson, V., Dumas-Gaudot, E., Golotte, A., Tahiri-Alaoui, A., Gianinazzi, S. (1996)** Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. *New Phytol.* **133**: 45-57.

**Gibson, F., Deacon, J.W. (1990)** Establishment of ectomycorrhizas in asceptic culture: effects of glucose, nitrogen and phosphorus in relation to successions. *Mycol. Res.* **94**: 166-172.

**Giltrap, N.J. (1982)** Production of polyphenol oxidases by ectomycorrhizal fungi with special relevance to *Lactarius* spp. *Trans. Br. Mycol. Soc.* **78**: 75-81.

**Giovannetti, M., Mosse, B. (1980)** An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.* **84**: 489-500.

**Graf, F. (1994)** Ecology and sociology of macromycetes in snowbeds with *Salix herbacea* L. in the alpine Vally of Radönt (Grisons, Switzerland). *Dissertationes Botanicae* **235**: 1-242.

**Graham, J.H. (1988)** Interactions of mycorrhizal fungi with soil borne plant pathogens and other organisms: and introduction. *Phytopathology* **78**: 365-366.

**Grosse, W., Jovy, K., Tiebel, H. (1996)** Influence of plants on redox potential and methane production in water-saturated soil. *Hydrobiol.* **340**: 93-99.

**Habte, M., Byappanahalli, M.N. (1994)** Dependency of cassava (*Manihot esculenta*) on vesicular-arbuscular mycorrhizal fungi. *Mycorrhiza* **4**: 241-245.

**Harley, J.L., Harley, E.L. (1987)** A checklist of mycorrhiza in the British flora. *NewPhytol.* **105** (suppl.): 1-102.

**Harley, J.L., Smith, S.E. (1983)** Mycorrhizal symbiosis. Academic Press, London.

**Heijden, M. van der, Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R. (1998)** Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* **396**: 69-72.

**Helgason, T., Daniell, T.J., Husband, R., Fitter, A.H., Young, J.P.W. (1998)** Ploughing up the wood-wide web? *Nature* **394**: 431.

**Hetrick, B.A.D., Bloom, D. (1983)** Vesicular-arbuscular mycorrhizal fungi associated with native tall grass prairie and cultivated winter wheat. *Can. J. Bot.* **61**: 2140-2146.

**Hibbett, D.S., Pine, E.M., Langer, E., Langer, G., Donoghue, M.J. (1997)** Evolution of gilled mushrooms and puffballs inferred from ribosomal DNA sequences. *Proc. nat. Acad. Sci. USA* **94**: 12002-12006.

**Hooker, J.E., Munro, M., Atkinson, D. (1992)** Vesicular-arbuscular mycorrhizal fungi induced alteration in poplar root system morphology. *Plant Soil* **145**: 207-214.

**Hooker, J.E., Atkinson, D. (1996)** Arbuscular mycorrhizal fungi-induced alteration of tree-root architecture and longevity. *Z. Pflanzenernähr. Bodenk.* **159**: 229-234.

**Houba, V.J.G., van der Lee, J.J., Novozamsky, I. (1995)** *In* Soil Analysis Procedures, Other Procedures, syllabus Soil and Plant Analysis, Part 5B.

**Houba, V.J.G., Novozamsky, I., Lexmond, Th.M., van der Lee, J.J. (1990)** Applicability of 0.01*M* $CaCl_2$ as a single extraction solution for the assessment of the nutrient status of soils and other diagnostic purposes. *Comm. Soil Sci. Plant Anal.* **21**: 2281-2290.

**Ingleby, K. Mason, P.A., Last, F.T., Fleming, L.V. (1990)** Identification of ectomycorrhizas. *ITE Res. Publ.* **5**: 1-112. London.

**Jackson, M.B., Attwood, P.A. (1996)** Roots of willow (*Salix viminalis* L.) show marked tolerance to oxygen shortage in flooded soils and in solution culture. *Plant Soil* **187**: 37-45.

**Jakobsen, I., Abbot, L.K., Robson A.D. (1992a)** External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 1. Spread of hyphae and phosphorus inflow to the roots. *New Phytol.* **120**: 371-380.

**Jakobsen, I., Abbot, L.K., Robson A.D. (1992b)** External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 2. Hyphal transport of $^{32}P$ over defined distances. *New Phytol.* **120**: 509-516.

**Jakobsen, I., Joner, E.J., Larsen, J. (1994)** Hyphal phosporus transport, a key stone to mycorrhizal enhancement of plant growth. *In*: Impact of Arbuscular Mycorrhizas on sustainable Agriculture and Natural Ecosystems (*Edited by* S. Gianinazzi, H. Schuepp) pp. 133-146. Basel, Switzerland: Birkhauser Verlag.

**Janos, D.P. (1993)** Vesicular-arbuscular mycorrhizae of epiphytes. *Mycorrhiza* **4**: 1-4.

**Jansen, A.E. (1991)** The mycorrhizal status of Douglas fir in The Netherlands: its relation with stand age, regional factors, atmospheric pollutants and tree vitality. *Agric. Ecosyst. Environm.* **35**: 191-208.

**Johnson, N.C., Zak, D.R., Tilman, D., Pfleger, F.L. (1991)** Dynamics of vesicular-arbuscular mycorrhizae during old field succession. *Oecologia* **86**: 349-358.

**Johnson, N.C., Tilman, D., Wedin, D. (1992)** Plant and soil controls on mycorrhizal fungal communities. *Ecology* **73**: 2034-2042.

**Johnson, N.C., Graham, J.H., Smith, F.A. (1997)** Functioning of mycorrhizal interactions along the mutualism-parasitism continuum. *New Phytol.* **135**: 575-585.

**Jones, M.D., Durall, D.M., Tinker, P.B. (1990)** Phosphorus relationships and production of extramatrical hyphae by two types of willow ectomycorrhizas at different soil phosphorus levels. *New Phytol.* **115**: 259-267.

**Jones, M., Durall, D.M., Tinker, P.B. (1991)** Fluxes of carbon and phosphorus between symbionts in willow ectomycorrhizas and their changes with time. *New Phytol.* **119**: 99-106.

**Jones, M.D., Durall, D.M., Tinker, P.B. (1998)** A comparison of arbuscular and ectomycorrhizal *Eucalyptus coccifera*: growth response, phosphorus uptake efficiency and external hyphal production. *New Phytol.* **140**: 125-134.

**Jong, T.J. de, Klinkhamer, P.G.L., de Heiden, J.L.H. (1995)** The effect of water and mycorrhizal infection on the distribution of *Carlina vulgaris* on sand dunes. *Ecography* **18**: 384-389.

**Jongbloed, R.H., Borst-Pauwels, G.W.F.H. (1990)** Effects of ammonium and pH on growth of some ectomycorrhizal fungi in vitro. *Acta Bot. Neerl.* **39**: 49-358.

**Jongman, R.H.G., ter Braak, C.J.F., van Tongeren, O.F.R. (1987)** Data analysis in community and landscape ecology. Pudoc, Wageningen.

**Jonsson, L., Dahlberg, A., Nilsson, M.C., Kårén, O., Zackrisson, O. (1999)** Continuity of ectomycorrhizal fungi in self-regenerating boreal forests of *Pinus sylvestris*: comparison between seedlings and old trees in terms of mycobiont diversity. *New Phytol.* **142**: 151-162.

**Kårén, O., Nylund, J-E. (1997)** Effect of ammonium sulphate on the community structure and biomass of ectomycorrhizal fungi in a Norway spruce stand in southwestern Sweden. *Can. J. Bot.* **75**: 1628-1642.

**Keeley, J.E. (1980)** Endomycorrhizae influence growth of blackgum seedling in flooded soils. *Am. J. Bot.* **67**: 6-9.

**Keizer, P.J., Arnolds, E. (1994)** Succession of ectomycorrhizal fungi in roadside verges planted with common oak (*Quercus robur* L.) in Drenthe, The Netherlands. *Mycorrhiza* **4**: 147-159.

**Kesava Rao, P.S., Tilak, K.V.B.R., Arunachalam, V. (1990)** Genetic variation for VA mycorrhizal-dependent phospate mobilization in groundnut (*Arachis hypogaea* L.). *Plant Soil* **122**: 137-142.

**Kieliszewska-Rokicka, B. (1992)** Effect of nitrogen level on acid phosphatase activity of eight isolates of ectomycorrhizal fungus *Paxillus involutus* cultured in vitro. *Plant Soil* **139**: 229-238.

**Koide, R.T., Li, M. (1989)** Appropriate controls for vesicular-arbuscular mycorrhizal research. *New Phytol.* **111**: 35-44.

**Kooijman, A.M., Dopheide, J.C.R., Sevink, J., Takken, I., Verstraten, J.M. (1998)** Nutrient limitations and their implications on the effects of atmospheric deposition in coastal dunes; lime-poor and lime-rich sites in the Netherlands. *J. Ecol.* **86**: 511-526.

**Krishna, K.R., Shetty, K.G., Dart, P.J., Andrews, D.J. (1985)** Genotype dependent variation in mycorrhizal colonization and response to inoculation to pearl millet. *Plant Soil* **86**: 113-125.

**Kropp, B.R. (1997)** Inheritance of the ability for ectomycorrhizal colonization of *Pinus strobus* by *Laccaria bicolor*. *Mycologia* **89**: 578-585.

**Kuiters, A.T. (1987)** Phenolic Acids and Plant Growth in Forest Ecosystems. Ph.D. thesis, Free University Amsterdam, The Netherlands.

**Kuiters, A.T. Sarink, H.M. (1986)** Leaching of phenolic compounds from leaves and needles of several deciduous and coniferous trees. *Soil Biol. Biochem.* **18**: 475-480.

**Kuyper, Th.W., Mekenkamp, E., Verbeek, M. (1994)** Die Pilzflora der Kriechweidengebüsche auf der Watteninsel Terschelling. *Z. Mykol.* **60**: 305-316.

**Laiho, O. (1970)** *Paxillus involutus* as a mycorrhizal symbiont of forest trees. *Act. Forest. Fenn.* **106**: 1-65.

**Lamhamedi, M.S., Fortin, J.A., Kope, H.H., Kropp, B.R. (1990)** Genetic variation in ectomycorrhiza formation by *Pisolithus arhizus* on *Pinus banksiana*. *New Phytol.* **115**: 689-697.

**Lapeyrie, F.F., Chilvers, G.A. (1985)** An endomycorrhiza-ectomycorrhiza succession associated with enhanced growth of *Eucalyptus dumosa* seedlings planted in a calcareous soil. *New Phytol.* **100**: 93-104.

**Last, F.T., Mason, P.A., Wilson, J., Deacon, J.W. (1983)** Fine roots and sheating mycorrhizas: their formation, function and dynamics. *Plant Soil* **71**: 9-21.

**Last, F.T., Dighton, J., Mason, P.A. (1987)** Successions of sheathing mycorrhizal fungi. *Trends Ecol. Evol.* **2**: 157-161.

**Lawton, J.H. (1999)** Are there general laws in ecology. *Oikos* **84**: 177-192.

**Lindermann, R.G. (1992)** Vesicular-arbuscular mycorrhizae and soil microbial interactions. *In*: Mycorrhizae in Sustainable Agriculture (*Edited by* G. J. Bethlenfalvay, R.G. Lindermann) pp. 45-70. ASA Special publication No. 54. Madison, Wisconsin, USA.

**Littke, W.R., Bledsoe, C.S., Edmonds, R.L. (1984)** Nitrogen uptake and growth in vitro by *Hebeloma crustuliniforme* and other Pacific Northwest mycorrhizal fungi. *Can. J. Bot.* **62**: 647-652.

**Lodge, D.J. (1989)** The influence of soil moisture and flooding on formation of VA- endo- and ectomycorrhizae in *Populus* and *Salix*. *Plant Soil* **117**: 255-262.

**Lodge, D.J., Wentworth, Th.R. (1990)** Negative associations among VA-mycorrhizal fungi and some ectomycorrhizal fungi inhabiting the same root system. *Oikos* **57**: 347-356.

**Lorio, P.L., Howe, V.K., Martin, C.N. (1972)** Loblolly pine rooting varies with microrelief on wet sites. *Ecology* **53**: 1134-1140.

**Lussenhop, J., Fogel, R. (1999)** Seasonal change in phosphorus content of *Pinus strobus* – *Cenococcum geophilum* ectomycorrhizae. *Mycologia* **91**: 742-746.

**Magurran, A.E. (1988)** Ecological diversity and its measurement. Chapman and Hall, London.

**Marschner, H. (1995)** Mineral nutrition of higher plants, 2nd edition, Academic Press, London.

**Marshall, P.E., Pattullo, N. (1981)** Mycorrhizal occurrence in willows in a northern freshwater wetland. *Plant Soil* **59**: 465-471.

**Martin, F., Delaruelle, C., Ivory, M. (1998)** Genetic variability in intergenic spacers of ribosomal DNA in *Pisolithus* isolates associated with pine, eucalypts and *Afzelia* in lowland Kenyan forests. *New Phytol.* **139**: 341-352.

**Marx, D.H. (1969)** The influence of ectotrophic mycorrhizal fungi on resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathol.* **59**: 153-163.

**Marx, D.H. (1981)** Variability in ectomycorrhizal development and growth among isolates of *Pisolithus tinctorius* as affected by source, age and reisolation. *Can. J. For. Res.* **11**: 168-174.

**Marx, D.H. (1990)** Soil pH and nitrogen influence *Pisolithus* ectomycorrhizal development and growth of loblolly pine seedlings. *For. Sci.* **36**: 224-245.

**McGonigle, T.P., Fitter, A.H. (1988)** Growth and phosphorus inflows of *Trifolium repens* L. with a range of indigenous vesicular-arbuscular mycorrhizal infection levels under field conditions. *New Phytol.* **108**: 59-65.

**McGonigle, T.P., Fitter, A.H. (1990)** Ecological specificity of vesicular arbuscular mycorrhizal associations. *Mycol. Res.* **94**: 120-122.

**McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, D.L., Swan, G.A. (1990)** A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytol.* **115**: 495-501.

**McIntosh, R.P. (1985)** The background of ecology – concept and theory. University Press, Cambridge.

**Melin, E. (1946)** Der Einfluss von Waldstreuextrakten auf das Wachstum von Bodenpilzen, mit besonderer Berücksichtigung der Wurzelpilze von Baümen. *Symbolae bot. Upsalienses* **8**: 1-116.

**Mercy, M.A., Shivashankar, G., Bagyaraj, D.J. (1990)** Mycorrhizal colonization in cowpea is host dependent and heritable. *Plant Soil* **121**: 292-294.

**Merryweather, J., Fitter, A.H. (1995)** Arbuscular mycorrhiza and phosphorus as controlling factors in the life history of *Hyacinthoides non-scripta* (L.) Chouard ex Rothm. *New Phytol.* **129**: 619-627.

**Mikola, P. (1948)** On the physiology and ecology of *Cenococcum graniforme*. *Comm. Instit. Forest. Fenn.* **36**: 1-104.

**Miller, D.D., Domoto, P.A., Walker, C. (1985)** Colonization and efficacy of different endomycorrhizal fungi with apple seedlings at two phosphorus levels. *New Phytol.* **100**: 393-402.

**Molina, R. (1979)** Ectomycorrhizal colonization of containerized Douglas-fir and Lodgepole pine seedlings with six isolates of *Pisolithus tinctorius*. *For. Sci.* **25**: 585-590.

**Molina, R., Massicotte, H.B., Trappe, J.M. (1992)** Specificity phenomena in mycorrhizal symbioses: community ecological consequences and practical implications. *In*: Mycorrhizal functioning (*Edited by* M.F. Allen) pp. 357-423. Chapman and Hall, New York.

**Molina, R., Myrold, D., Li, C.Y. (1994)** Root symbiosis of red alder: technological opportunities for enhanced regeneration and soil improvement. *In*: The biology and management of red alder (*Edited by* D.E. Hibbs, D.S. DeBell, R.F. Tarrant) pp. 23-46. Oregon State University Press, Corvallis, Oregon.

**Morton, J.B., Bentivenga, S.P., Bever, J.D. (1995)** Discovery, measurement, and interpretation of diversity in arbuscular endomycorrhizal fungi (Glomales, Zygomycetes). *Can. J. Bot.* **73** (Suppl. 1): S25-S32.

**Moser, M. (1959)** Beiträge zur Kenntnis der Wuchsstoffbeziehungen im Bereich ectotropher Mykorrhizen I. *Arch. Mikrobiol.* **34**: 251-269.

**Moyersoen, B., Fitter, A.H. (1999)** Presence of arbuscular mycorrhizas in typically ectomycorrhizal host species from Cameroon and New Zealand. *Mycorrhiza* **8**: 247-253.

**Mullen, R.B., Schmidt, S.K. (1994)** Mycorrhizal infection, phosphorus uptake, and phenology in *Ranunculus adoneus*: implications for the functioning of mycorrhizae in alpine systems. *Oecologia* **94**: 229-234.

**Natajaran, K., Mohan, V., Ingleby, K. (1992)** Correlation between basidiomata production and ectomycorrhizal formation in *Pinus patula* plantations. *Soil Biol. Biochem.* **24**: 279-280.

**Newbery, D.M., Alexander, I.J., Rother, J.A. (1997)** Phosphorus dynamics in lowland african rainforest: The influence of ectomycorrhizal trees. *Ecol. Mon.* **67**: 367-409.

**Newbery, D.M., Songwe, N.C., Chuyong, G.B. (1998)** Phenology and dynamics of an african rainforest at Korup, Cameroon. *In*: Dynamics of tropical communities (*Edited by* D.M. Newbery, H.H.T. Prins, N.D. Brown) pp. 267-308. Blackwell, Oxford.

**Newman, E.I. (1966)** A method of estimating the total length in a root sample. *J. Appl. Ecol.* **3**: 139-145.

**Newman, E.I., Eason, W.R. (1989)** Cycling of nutrients from dying roots to living plants, including the role of mycorrhizas. *Plant Soil* **115**: 211-215.

**Newsham, K.K., Fitter, A.H., Watkinson, A.R. (1995a)** Arbuscular mycorrhiza protect an annual grass from root pathogenic fungi in the field. *J. Ecol.* **83**: 991-1000.

**Newsham, K.K., Fitter, A.H., Watkinson, A.R. (1995b)** Multi-functionality and biodiversity in arbuscular mycorrhizas. *Trends Ecol. Evol.* **10**: 407-411.

**Newton, A.C. (1992)** Towards a functional classification of ectomycorrhizal fungi. *Mycorrhiza* **2**: 75-79.

**Newton, A.C., Haigh, J.M. (1998)** Diversity of ectomycorrhizal fungi in Britain: a test of the species-area relationship, and the role of host specificity. *New Phytol.* **138**: 619-627.

**Nicolson, T.H. (1975)** Evolution of vesicular arbuscular mycorrhiza. *In*: Endomycorrhizas (*Edited by* F.E. Sanders, B. Mosse, P.B. Tinker) pp. 25-34. Academic Press, London.

**Nilsson, M.C., Gallet, C., Wallstedt, A. (1998)** Temporal variability of phenolics and batasin-III in *Empetrum hermaphroditum* leaves over an eight-year period: interpretations of ecological function. *Oikos* **81**: 6-16.

**Novozamsky, I., Houba, V.J.G., Van Eck, R., Van Vark, W. (1988)** A novel digestion technique for multi-element plant analysis. *Comm. Soil Sci. Plant Anal.* **14**: 239-248.

**Nyland, J-E. (1988)** The regulation of mycorrhiza formation – carbohydrate and hormone theories reviewed. *Scand. J. For. Res.* **3**: 465-479.

**Oberdorfer, E. (1994)** Pflanzensoziologische Exkursionsflora für Süddeutschland und die angrenzenden Gebiete. Eugen Ulmer, Stuttgart.

**Økland, R.H., Eilertsen, O. (1994)** Canonical Correspondence Analysis with variation partitioning: some comments and application. *J. Veg. Sci.* **5**: 117-126.

**Olsen, R.A., Odham, G., Lindeberg, G. (1971)** Aromatic substances in leaves of *Populus tremula* as inhibitors of mycorrhizal fungi. *Physiol. Plant.* **25**: 122-129.

**Oort, A.J.P. (1981)** Nutritional requirements of *Lactarius* species, and cultural characters in relation to taxonomy. *Verh. Kon. Ned. Ak. Wet., Afd. Nat.kunde*, 2e Reeks **76**: 1-87.

**Pearson, J.N., Jakobsen, I. (1993)** Symbiotic exchange of carbon and phosphorus between cucumber and three arbuscular mycorrhizal fungi. *New Phytol.* **124**: 481-488.

**Perry, D.A., Choquette, C. (1987)** Allelopathic effects on mycorrhizae. *In*: Allelochemicals: role in agriculture and forestry (*Edited by* G.R. Waller) pp. 185-194. American Chemical Society, Washington DC.

**Phillips, J.M., Hayman, D.S. (1970)** Improved procedure for clearing roots and staining parasitic and vesicular-arbuscular fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* **55**: 158-161.

**Plenchette, C., Fortin, J.A., Furlan, V. (1983)** Growth response of several plant species to mycorrhizae in a soil of moderate P-fertility I. Mycorrhizal dependency under field conditions. *Plant Soil* **70**: 199-209.

**Putten, W.H. van der, Van Dijk, C., Peters, B.A.M. (1993)** Plant-specific soil-borne diseases contribute to succession in foredune vegetation. *Nature* **362**: 53-56.

**Read, D.J., Kianmehr, H., Malibari, A. (1977)** The biology of mycorrhiza in *Helianthemum* Mill. *New Phytol.* **78**: 305-312.

**Read, D.J., Francis, R., Finlay, R.D. (1985)** Mycorrhizal mycelia and nutrient cycling in plant communities. *In*: Ecological interactions in soil (*Edited by* A.H. Fitter) pp. 193-217. Blackwell Scientific Publications, Oxford, London.

**Read, D.J. (1989)** Mycorrhizas and nutrient cycling in sand dune ecosystems. *Proc. R. Soc.Edinburgh* **96B**: 89-110.

**Read, D.J. (1990)** Mycorrhizas in ecosystems - Nature's response to the 'law of the minimum'. *In*: Frontiers in Mycology (*Edited by* D.L. Hawksworth) pp. 101-130. C.A.B. International, Wallingford.

**Read, D.J. (1991)** Mycorrhizas in ecosystems. *Experientia* **47**: 376-391.

**Rechinger, K.H. (1964)** *Salix* L. *In*: Flora europaea Vol. 1 (*Edited by* T.G. Tutin, V.H. Heywood, N.A. Burges, D.H. Valentine, S.M. Walters, D.A. Webb) pp. 43-54. University Press, Cambridge.

**Rose, S.L., Perry, D.A., Pilz, D., Schoeneberger (1983)** Allelopathic effects of litter on the growth and colonization of mycorrhizal fungi. *J. Chem. Ecol.* **9**: 1153-1162.

**Rotheroe, M. (1993)** The macrofungi of British sand dunes. *In*: Fungi of Europe: Investigation, recording and conservation (*Edited by* D.N. Pegler, L. Boddy, B. Ing, P.H. Kirk) pp. 121-137. Royal Botanic Gardens, Kew.

**Rygiewicz, P.T., Andersen, C.P. (1994)** Mycorrhizae alter quality and quantity of carbon allocated below ground. *Nature* **369**: 58-60.

**Sanders, I.R., Clapp, J.P., Wiemken, A. (1996)** The genetic diversity of arbuscular mycorrhizal fungi in natural ecosystems – a key to understanding the ecology and functioning of the mycorrhizal symbiosis. *New Phytol.* **133**: 123-134.

**Schenk, N.C., Perez, Y. (1990)** Manual for the identification of VA mycorrhizal fungi, Second edition. International Culture Collection of VA Mycorrhizal Fungi, Gainesville, Florida.

**Sen, R. (1990)** Isozymic identification of individual ectomycorrhizas synthesized between Scots pine (*Pinus sylvstris* L.) and isolates of two species of *Suillus*. *New Phytol.* **114**: 617-626

**Sharpe, R.R., Marx, D.H. (1986)** Influence of pH and *Pisolithus tinctorius* ectomycorrhizae on growth and nutrient uptake of pecan seedlings. *Hort. Science* **21**: 1388-1390.

**Siegel, S., Castellan, N.J. (1988)** Nonparametric statistics for the behavorial sciences. McGraw-Hill, New York.

**Simon, L., Bousquet, R.C., Levesque, C., Lalonde, M. (1993)** Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. *Nature* **363**: 67-69.

**Smith, J.E., Johnson, K.A., Cázares, E. (1998)** Vesicular mycorrhizal colonization of seedlings of Pinaceae and Betulaceae after spore inoculation with *Glomus intraradices*. *Mycorrhiza* **7**: 279-285.

**Smith, S.E., Robson, A.D., Abbott, L.K. (1992)** The involvement of mycorrhizas in assessment of genetically dependent efficiency of nutrient uptake and use. *Plant Soil* **146**: 169-179.

**Smith, S.E., Read, D.J. (1997)** Mycorrhizal symbiosis. Academic Press. London, New York.

**Sokal, R.R., Rohlf, F.J. (1995)** Biometry, 3rd edition. Freeman and Company, San Francisco, CA.

**Stribley, D.P., Tinker, P.B., Rayner, J.H. (1980)** Relation of internal phosphorus concentration and plant weight in plants infected by vesicular-arbuscular mycorrhizas. *New Phytol.* **86**: 261-266.

**Stürmer, S.L. (1998)** Characterization of diversity of fungi forming arbuscular endomycorrhizae in selected plant communties. Ph.D thesis, West Virginia University, USA.

**Sydes, C., Grime, J.P. (1981)** Effects of tree leaf litter on herbaceous vegetation in deciduous woodland I. Field investigations. *J. Ecol.* **69**: 237-248.

**Taylor, T.N., Remy, W., Hass, H., Kerp, H. (1995)** Fossil arbuscular mycorrhizae from the early Devonian. *Mycologia* **87**: 560-573.

**Termorshuizen, A.J., Schaffers, A.P. (1989)** The relation in the field between fruitbodies of mycorrhizal fungi and their mycorrhizas. *Agric. Ecosystems Environ.* **28**: 509-512.

**Termorshuizen, A.J. (1991)** Succession of mycorrhizal fungi in stands of *Pinus sylvestris* in The Netherlands. *J. Veg. Sc.* **2**: 555-564.

**Termorshuizen, A.J., Ket, P.C. (1991)** Effects of ammonium and nitrate on mycorrhizal seedlings of *Pinus sylvestris*. *Eur. J. For. Path.* **21**: 404-413

**Tester, M., Smith, F.A., Smith, S.E. (1987)** The phenomenon of 'nonmycorrhizal' plants. *Can. J. Bot.* **65**: 419-431.

**Thomson, J., Matthes-Sears, U., Peterson, R.L. (1990)** Effects of provenance and mycorrhizal fungi on early seedling growth in *Picea mariana*. *Can. J. For. Res.* **20**: 1739-1745.

**Timonen, S., Sen, R. (1998)** Heterogeneity of fungal and plant enzyme expression in intact Scots pine –*Suillus bovinus* and -*Paxillus involutus* mycorrhizospheres developed in natural forest humus. *New Phytol.* **138**: 355-366.

**Tinker, P.B. (1975)** The soil chemistry of phosphorus and mycorrhizal effects on plant growth. *In*: Endomycorrhizas (*Edited by* F.E. Sanders, B. Mosse, P.B. Tinker) pp. 253-272. Academic Press, London.

**Tinker, P.B., Jones, M.D., Durall, D.M. (1992)** A Functional comparison of Ecto- and Endomycorrhizas. *In*: Mycorrhizas in Ecosystems (*Edited by* D.J. Read, D.H. Lewis, A.H. Fitter, I.J. Alexander) pp. 303-310. University Press, Cambridge.

**Tonkin, C.M., Malajczuk, N., McComb, J.A. (1989)** Ectomycorrhizal formation by micropropagated clones of *Eucalyptus marginata* inoculated with isolates of *Pisolithus tinctorius*. *New Phytol.* **111**: 209-214.

**Trappe, J.M. (1977)** Selection of fungi for ectomycorrhizal inoculation in nurseries. *Ann. Rev. Phytopath.* **15**: 203-222.

**Vesterholt, J. (1989)** A revision of *Hebeloma* sect. *Indusiata* in the Nordic countries. *Nord. J. Bot.* **9**: 289-319.

**Vogt, K., Bloomfield, J., Ammirati, J.F., Ammirati, S.R. (1992)** Sporocarp production by basidiomycetes, with emphasis on forest ecosystems. *In*: The fungal community: its organization and role in the ecosystem (*Edited by* G.C. Caroll, D.T. Wicklow) pp. 563-582. Marcel Dekker Inc. New York.

**Vozzo, J.A., Hacskaylo, E. (1974)** Endo- and ectomycorrhizal associations in five *Populus* species. *Bull. Torrey Bot. Club* **101**: 182-186.

**Walker, C. (1992)** Systematics and taxonomy of the arbuscular endomycorrhizal fungi (Glomales) – a possible way forward. *Agronomie* **112**: 887-897.

**Wallander, H., Nylund, J-E. (1991)** Effects of excess nitrogen on carbohydrate concentration and mycorrhizal development of *Pinus sylvestris* L. seedlings. *New Phytol.* **119**: 405-411.

**Wallander, H., Nylund, J-E. (1992)** Effects of excess nitrogen and phosphorus starvation on the extramatrical mycelium of ectomycorrhizal *Pinus sylvestris* L. *New Phytol.* **120**: 495-503.

**Watling, R., Rotheroe, M. (1989)** Macrofungi of sand dunes. *Proc. R. Soc.Edinburgh* **96B**: 111-126.

**Watling, R. (1992)** Relationships between macromycetes and the development of higher plant communities. *In*: The fungal community: its organization and role in the ecosystem (*Edited by* G.C. Caroll, D.T. Wicklow) pp. 427-458. Marcel Dekker Inc. New York.

**Weeda, E.J., Westra, R., Westra, Ch., Westra, T. (1985)** Nederlandse oecologische flora, wilde planten en hun relaties I. I.V.N. in collaboration with V.A.R.A. and V.E.W.I.N. The Netherlands.

**Weisner, S.E.B., Strand, J.A. (1996)** Rhizome architecture in *Phragmites autralis* in relation to water depth: implications for within-plant oxygen transport distances. *Folia Geobot. Phytotax.* **31**: 91-97.

**Westhoff, V., Van Oosten, M.F. (1991)** De Plantengroei van de Waddeneilanden. KNNV, Pirola publications, Schoorl, The Netherlands.

**Willis, A.J. (1963)** Braunton Burrows: the effects on the vegetation of the addition of mineral nutrients to the dune soils. *J. Ecol.* **51**: 353-374.

**Wilson, J.M., Tommerup, I.C. (1992)** Interactions between fungal symbionts: VA mycorrhizae. *In*: Mycorrhizal functioning (*Edited by* M.F. Allen) pp. 199-248. Chapman & Hall, New York.

## APPENDIX A

**Type of habitat, geographical location, plant dominants and subdominants, vegetation vs. moss cover (vpc/mc), vegetation height (vh) and average root depth (rd) in 16 *Salix repens* field plots on dune ecosystems in Dutch Wadden Isles.**

| Type | | Site/Location | Conditions | Dominant plants | Subdominant plants (<5%) | vpc/mc(%) | vh(cm) | rd(cm) |
|---|---|---|---|---|---|---|---|---|
| Calcareous Dry | 1 | Primaire valley<br>53° 23'55"N<br>5° 13'25"E | Windy valley close to the beach, drifting sand | *Salix repens* L.<br>*Carex panicea* L.<br>*Hydrocotyle vulgaris* L. | *Agrostis stolonifera* L.<br>*Carex oederi* Retz.subsp. *oederi* | 45/3 | 5-25 | 100 |
| | 2 | Tafelduin<br>53° 23'55"N<br>5° 13'55"E | Drifting sand | *Salix repens* | | 70/0 | 40-100 | 100 |
| | 3 | Hobbelduin<br>53° 23'55"N<br>5° 13'45"E | Drifting sand | *Salix repens* | *Ammophila arenaria* (L.) Link<br>*Cerastium diffusum* Pers.<br>*Festuca arundinacea* Schreber<br>*Hieracium umbellatum* L. | 60/10 | 20-50 | 100 |
| Calcareous Wet | 4 | Schoenus I<br>53° 23'50"N<br>5° 13'45"E | Valley separated from sea by dunes, high groundwatertab. | *Salix repens*<br>*Hydrocotyle vulgaris* | *Epipactis palustris* (L.) Crantz<br>*Linum catharticum* L. | 55/60 | 60 | 25 |
| | 5 | Schoenus II<br>53° 23'50"N<br>5° 13'45"E | Valley separated from sea by dunes, high ground water table | *Salix repens*<br>*Hydrocotyle vulgaris*<br>*Schoenus nigricans* L. | *Agrostis stolonifera* L.<br>*Calamagrostis epigejos* (L.) Roth<br>*Carex oederi, Carex panicea*<br>*Epipactis palustris*<br>*Mentha aquatica* L. | 70/60 | 75 | 20 |
| | 6 | Cranberry<br>53° 24'05"N<br>5° 14'55"E | The same valley, later succession stage as plot 4 and 5 | *Salix repens*<br>*Oxycoccus macrocarpos* (Aiton) Purs | *Calamagrostis epigejos*<br>*Carex nigra* (L.) Reichard<br>*Hydrocotyle vulgaris*<br>*Potentilla palustris* (L.) Scop | 90/60 | 30 | 25 |

**APPENDIX A** continued

| Type | | Site/Location | Conditions | Dominant plants | Subdominant plants (<5%) | vpc/mc(%) | vh(cm) | rd(cm) |
|---|---|---|---|---|---|---|---|---|
| Acidic Dry | 8 | Douwesplak<br>53° 24'45''N<br>5° 21'35''E | Steep dune slope, high burrowing pressure by rabbits | *Salix repens*<br>*Empetrum nigrum* L.<br>*Polypodium vulgare* L. | | 65/75 | 30-80 | 20 |
| | 9 | Noord helling WAZ<br>53° 23'55''N<br>5° 15'30''E | Steep dune slope | *Salix repens*<br>*Empetrum nigrum* | *Ammophila arenaria*<br>*Carex arenaria*<br>*Polypodium vulgare* | 55/65 | 30-100 | 20 |
| | 10 | Oosterend<br>53° 24'30''N<br>5° 22'55''E | Inland site on old dunes, grazing by horses and goats | *Salix repens*<br>*Agrostis capillaris*<br>*Poa pratensis* | *Ammophila arenaria*<br>*Carex arenaria*<br>*Festuca ovina* L.subsp. *tenuifolia* (Sibth.) Celak<br>*F. rubra* L. subsp. *commutata*<br>*Hypochaeris radicata* | 60/5 | 30-60 | 25 |
| | 11 | Paardenwei<br>53° 24'40''N<br>5° 24'00''E | Inland site on middle aged dunes, grazing by horses | *Salix repens*<br>*Myrica gale* L.<br>*Agrostis capillaris*<br>*Festuca ovina* subsp. *tenuifolia* | | 55/35 | 10-30 | 15 |

**APPENDIX A** continued

| Type | | Site/Location | Conditions | Dominant plants | Subdominant plants (<5%) | vpc/mc(%) | vh(cm) | rd(cm) |
|---|---|---|---|---|---|---|---|---|
| Acidic Wet | 12 | Dazenplak<br>53° 24'55''N<br>5° 24'25''E | Inland site, high ground water table | *Salix repens*<br>*Erica tetralix* L.<br>*Empetrum nigrum* | *Agrostis capillaris*<br>*Ammophila arenaria*<br>*Poa pratensis* | 55/35 | 40-100 | 20 |
| | 13 | Groene strand<br>53° 23'55''N<br>5° 16'20''E | Inland steep dune slope,high ground water table | *Salix repens*<br>*Erica teralix*<br>*Empetrum nigrum* | | 75/25 | 40-100 | 20 |
| | 14 | Myrica<br>53° 23'50''N<br>5° 15'40''E | Inland site, high ground water table | *Salix repens*<br>*Myrica gale* | *Carex nigra* | 90/1 | 80-100 | 30 |
| | 15 | Telefooncellen<br>53° 24'15''N<br>5° 17'30''E | Inland site, very high ground water table | *Salix repens*<br>*Lythrum salicaria* L.<br>*Carex nigra* | *Calamagrostis epigejos*<br>*Carex trinervis* Degland<br>*Eleocharis palustris*<br>*Galium palustre* L.<br>*Hydrocotyle vulgaris* | 60/25 | 100 | 35 |
| | 16 | Thijssensduin<br>53° 24'55''N<br>5° 24'25''E | Inland site, high ground water table, mown with removal in August | *Salix repens* | *Carex oederi*<br>*Holcus lanatus* L.<br>*Leontodon autumnalis* L.<br>*Lotus corniculatus* subsp. *corniculatus*<br>*Potentilla anserina* L.<br>*Prunella vulgaris* L.<br>*Sagina procumbens* L. | 40/5 | 5-10 | 20 |

## APPENDIX B

**Characteristics of mycorrhizas formed by indigenous fungi on roots of *Salix repens* in 16 field sites on Terschelling.**

| Mycorrhizal Morphotypes | Key morphological features |
|---|---|
| *Cenococcum* | Black mycorrhizas. The mantle has an angular stellate arrangement. Thick, black septated hyphae radiating from the mantle surface. |
| *Cortinarius* | Slightly tortuous hairy mycorrhizas with rhizomorphs, enclosed air between mantle hyphae. Rhizomorphs almost parallelly emanating from the mantle, with clamp connections. Colour: white to yellow |
| *Hebeloma* | Fairly long, slender, and infrequently branched mycorrhizas. Dense, abundant hyphae, with clamp connections are invariably found surrounding the mantle. Mantle surface: a net synenchyma composed of parallel bands of diverging hyphae. Colour: silver-white. |
| *Inocybe* | Short mycorrhizas with a frequent short branching habit. The mantle surface is smooth and shiny, with loose hyphae, with clamp connections emanating from the mantle surface. Mantle surface: a net prosenchyma of distinctly arranged, shortened, dichotomously branched hyphae (distinct septa). Colour: white, browning with age. |
| ITE4 | Short mycorrhizas, with bluntly rounded tips and frequent, irregularly spaced branches. The mantle surface is reticulate and shiny. Loose, straggly hyphae emanating from the mantle surface. Mantle surface: broad hyphal elements forming a net synenchyma or a non-interlocked irregular synenchyma. Colour: white to light-brown. |
| ITE5 | Short, stubby and blunt-ended mycorrhizas, with a frequent, sometimes pinnate, branching pattern. The mantle surface is distinctly granular in appearance. Mantle surface: a regular synenchyma of thick-walled, angular, often rounded cells. Colour: blackish-brown. |
| ITE6 | Fairly long and sinuous mycorrhizas, with frequent irregular spaced branches. Dense abundant white hyphae are found surrounding the mantle. Mantle surface: a net synenchyma of distinctly shaped hyphal elements. Colour: white-yellow to brown. |

**APPENDIX B** CONTINUED

| Mycorrhizal morphotypes | Key morphological features |
|---|---|
| *Laccaria* | Fairly long and sinuous mycorrhizas, with frequent, irregular spaces, short branches. Loose, straggly hyphae close to the mantle surface. Mantle surface: a net prosenchyma composed of woolly interwoven hyphae, with occasional clamp connections. Colour: various shades of orange to brown. |
| *Lactarius* | Fairly long mycorrhizas, with a frequent, sometimes pinnate, branching pattern. The mantle surface is smooth and shiny. Mantle surface: a net synenchyma of hyphae, covered by a widely spaced network of granular, laticiferous hyphae. Colour: yellowish-brown. |
| *Paxillus* | Very long and tortuous mycorrhizas, with numerous irregularly spaced branches typically occurring along the main axis. Globose sclerotia are frequently found loosely connected to the mycorrhiza by means of the dense wefts of hyphae or abundant simple strands. Mantle surface: a net prosenchyma of loosely interwoven hyphae, with clamp connections. Colour: silver-white. |
| *Tuber* | Short and stubby mycorrhizas, with frequent, irregular, often short branches. The mantle is smooth and shiny, obscured by a dense covering of setae. The mantle surface: irregular, interlocking synenchyma. Colour: light-brownish. |
| nr.1 | Short mycorrhizas, with bluntly rounded tips. Radiating hyphae, with clamp connections emanating from mantle surface. Colour: light-brown. |
| nr.2 | Short, stubby and blunt-ended mycorrhizas. The mantle is distinctly granular in appearance, with many emanating hyphae with clamp connections. Colour: dark, almost black. |
| nr.3 | Short and smooth mycorrhizas. Hyphae radiating (without clamp connections) (spines) from mantle surface. Colour: dark-brown. |
| nr.4 | Short and smooth mycorrhizas. Loose hyphae around the mantle surface. Mantle surface: a felt prosenchyma. Colour: light-brownish. |

# APPENDIX C

## Staining procedure for arbuscular mycorrhizal colonization in *Salix repens*

**INTRODUCTION:** A variety of stains are available for assessing arbuscular mycorrhizal colonization. Stains differ in effectiveness (Gange *et al.*, 1999) and in toxicity as many of these dyes are mutagenic or carcinogenic. I tried several of these stains.

Method as described in CHAPTERS 3 to 11: Field collected *S. repens* roots and *S. repens* roots inoculated with *Glomus mosseae* in the laboratory were stained* with Acid Fuchsin (0.01 % in lactoglycerol), Cotton blue (0.05 % in lactoglycerol), Aniline blue (0.01 % in lactoglycerol) or Trypan blue (0.05 % in lactoglycerol or lactophenol) for 30 minutes.

*Prior to the staining treatment, clearing method was best when 3 hours in 10% KOH in a waterbath at 90 °C, bleaching for 1 hour in 10% $H_2O_2$, and acidifying for 15 minutes in 1% HCl.

Arbuscular mycorrhizal colonization of *Salix repens* in field collected roots (n = 25) or artificially inoculated roots ($n$ = 10) with *Glomus mosseae* when stained with Acid Fuchsin, Cotton blue, Aniline blue or Trypan blue.

| | Field roots | | | Laboratory roots | | |
|---|---|---|---|---|---|---|
| Stain | % RLC | % RLCI | structures | % RLC | % RLCI | structures |
| Acid Fuchsin | 0 | 0 | - | 0 | 0 | - |
| Cotton blue | 0 | 0 | - | 0 | 0 | - |
| Aniline blue | 0 | 0 | - | 0 | 0 | - |
| Trypan blue in lactoglycerol | 0 | 0 | - | 0 | 0 | - |
| Trypan blue in lactophenol | ~5-20 | ~5-10 | hyphae, vesicles, coils, spores | ~10 | ~5 | hyphae, vesicles |

**DISCUSSION AND CONCLUSION:** Stains are listed from least hazardous towards more hazardous. This resulted in a staining method according to Phillips & Hayman (1970), with Trypan blue in lactophenol, that seemed necessary for AM staining in *Salix repens*.

# APPENDIX D

**Collection of ectomycorrhizal fungi isolated from *Salix repens* (of which mycorrhiza syntheses were succesful).**

| Ectomycorrhizal fungus | Code [a] | Provenance | Collecting date | CBS Code [b] |
|---|---|---|---|---|
| *Cortinarius pauperculus* | L110 | Douwesplak | 26 Sept. 1995 | CBS 668.97 |
| *Cortinarius trivialis* | L89 | Jan Thijssensduin | 25 Sept. 1995 | |
| *Hebeloma leucosarx* | L1 | Jan Thijssensduin | 7 Sept. 1994 | CBS 100143 |
| *H. leucosarx* | L73 | Vledder (Drenthe) | 16 Sept. 1995 | CBS 100142 |
| *H. leucosarx* | L143 | Schoenus | 11 Oct. 1995 | |
| *H. leucosarx* | L144 | Schoenus | 11 Oct. 1995 | |
| *H. psammophilum* | L129 | Primaire vallei | 11 Oct. 1995 | |
| *H. psammophilum* | L130 | Schoenus | 11 Oct. 1995 | |
| *Inocybe lacera* | L15 | Douwesplak | 7 Sept. 1994 | |
| *I. vulpinella* | L17 | Primaire vallei | 8 Sept. 1994 | |
| *Laccaria bicolor* | L34 | Diever (Drenthe) | 27 Sept. 1994 | CBS 669.97 |
| *L. laccata* | L153 | Schiermonnikoog | 7 Nov. 1995 | CBS 670.97 |
| *L. proxima* | L70 | Paardenwei | 27 Oct. 1994 | |
| *L. proxima* | L80 | Paardenwei | 25 Sept. 1995 | |
| *Lactarius controversus* | L14 | Douwesplak | 7 Sept. 1994 | CBS 671.97 |
| *L. controversus* | L45 | Schiermonnikoog | 5 Oct. 1995 | CBS 672.97 |
| *L. controversus* | L118 | Douwesplak | 26 sept. 1995 | CBS 673.97 |
| *L. helvus* | L12 | Dazenplak | 7 Sept. 1994 | |
| *L. helvus* | L115 | Douwesplak | 26 Sept. 1995 | |
| *L. helvus* | L117 | Douwesplak | 26 Sept. 1995 | |
| *Paxillus involutus* | L16 | Tafelduin | 8 Sept. 1994 | |
| *P. involutus* | L27 | Vledder (Drenthe) | 27 Sept. 1994 | CBS 674.97 |
| *P. involutus* | L37 | Diever (Drenthe) | 27 Sept. 1994 | CBS 675.97 |
| *P. involutus* | L82 | Paardenwei | 25 Sept. 1995 | |
| *P. involutus* | L83 | Paardenwei | 25 Sept. 1995 | CBS 100140 |
| *P. involutus* | L101 | Dazenplak | 25 Sept. 1995 | CBS 676.97 |
| *Scleroderma verrucosum* | L69 | Paardenwei | 27 Oct. 1994 | |
| *Xerocomus rubellus* | L113 | Douwesplak | 26 Sept. 1995 | |
| *X. rubellus* | L114 | Douwesplak | 26 Sept. 1995 | CBS 100141 |

[a] I isolated and maintained these fungal cultures on artificial media (AMN and transferred to new media every three month, some fungi had special requirements and also needed transfer to new media each month) until mycorrhiza syntheses were successful in my last experiments (CHAPTERS 5 AND 6) in 1997.

[b] In 1997 the collection was sent to the Centraalbureau voor Schimmelcultures (P.O.Box 273, 3740 AG in Baarn, The Netherlands). Fungal cultures that could be maintained under their standardized culture conditions are now available under the CBS code.

# APPENDIX E

**Effects of addition of non-mycorrhizal clover roots to control and EcM treatments.**

**INTRODUCTION:** Experiments with EcMF and AMF differ in the way in which fungal inoculum is added. With AMF inoculum is added together with plant roots. I assessed whether addition of non-mycorrhizal roots could also affect plant performance.

Methods as described in CHAPTER 4: In addition half ($n$ = 5) of the control plants and the EcM treatment (*Hebeloma leucosarx*) were supplied with the same amount (same as arbuscular mycorrhizal clover roots used for inoculation of the *Glomus mosseae* or *Acaulospora laevis* treatment) of non-mycorrhizal clover roots. One harvest after 12 weeks. Data were analysed after logaritmic transformation (mycorrhizal colonization was arcsine square root transformed) with a 1 factor ANOVA

Results of ANOVA ($P$ values) executed for shoot biomass, root length, shoot N and P content and mycorrhizal colonization of *Salix repens* ($n$ = 5) inoculated with *Hebeloma leucosarx* or not inoculated. The factor 'roots' represents addition or no addition of 0.67 g fw. non-mycorrhizal clover roots.

| | Source of variation 'roots' (df = 1, df Error = 8) | |
|---|---|---|
| Response variable | *Hebeloma leucosarx* | Control |
| Shoot biomass | 0.412 | 0.087 |
| Root length | 0.494 | 0.718 |
| Shoot N content | 0.469 | 0.069 |
| Shoot P content | 0.200 | 0.064 |
| Mycorrhizal colonization | 0.978 | |

**CONCLUSION:** Addition of non-mycorrhizal clover roots has no effects on plant and mycorrhizal performance (in 12 weeks).

# APPENDIX F

**Effects of addition of washings of AM clover roots to control and EcM treatments.**

**INTRODUCTION:** The question has been repeatedly brought up about appropriate non-mycorrhizal controls. For a general discussion I refer to Koide & Li (1989). I tested whether addition of washings of AM clover roots affected plants and mycorrhizal performance.

Methods as described in CHAPTER 4: In addition the control plants and the EcM treatment (*Hebeloma leucosarx*) were supplied without or with washings (Koide & Li, 1989) of each of the arbuscular mycorrhizal clover roots used for inoculation of the *Glomus mosseae* or *Acaulospora laevis* treatment, each in 5 replicates Moreover, the *G. mosseae* treatment was supplied with a washing of *Acaulospora laevis* inoculum and vice versa. One harvest after 12 weeks. Data were analysed after logaritmic transformation (mycorrhizal colonization was arcsine square root transformed) with a 1-factor ANOVA.

Results of ANOVA (*P* values) executed for shoot biomass, root length, shoot N and P content and mycorrhizal colonization of *Salix repens* ($n = 5$) inoculated with *Hebeloma leucosarx* or not inoculated. The factor 'washing' represents no washing, *G. mosseae* washing or *A. laevis* washing for control and *Hebeloma leucosarx* (df = 2, df Error = 12). The factor 'washing' represents no washing or washing of the other AMF inoculum for *Glomus mosseae* and *Acaulospora laevis* (df = 1, df Error = 8).

| | Source of variation 'washing' | | | |
|---|---|---|---|---|
| Response variable | Control | *Hebeloma leucosarx* | *Glomus mosseae* | *Acaulospora laevis* |
| Shoot biomass | 0.268 | 0.535 | 0.089 | 0.100 |
| Root length | 0.390 | 0.182 | 0.362 | 0.144 |
| Shoot N content | 0.236 | 0.297 | 0.665 | 0.209 |
| Shoot P content | 0.071 | 0.327 | 0.857 | 0.373 |
| Mycorrhizal colonization | | 0.530 | 0.398 | 0.607 |

**CONCLUSION:** Addition of washings in this study had no effects on plant and mycorrhizal performance.

# APPENDIX G

**Murphy's law** *'If anything can go wrong, it will'*

This is the original Law of Murphy which has some derivations:

- If there is a possibility of several things going wrong, the one that will cost most to repair will be the one to go wrong.
- If you anticipate three different ways in which a procedure can go wrong, and circumvent these, then a fourth way will promptly develop.
- Every solution brings about new problems.
- No matter what goes wrong, it will probably look right.

CONCLUSION: Having been working for years now to out-manoeuvre Murphy it looks like a lost cause. It is virtually impossible to keep ahead of Murphy; so remember my philosophy:

*'Smile, in Drenthe things will be worse.....'*

## SUMMARY

Mycorrhizas are associations between fungi and plant roots. From this association both plant and fungus derive benefit and both partners have no or only limited ability to complete their life cycle under natural conditions without the association. The fungus receives carbohydrates from the plant, whereas the plant benefits through increased nutrient and water uptake. Mycorrhizal fungi can also alleviate biotic and abiotic stress. There is a considerable diversity in structure and function of mycorrhizas, and this diversity is important for the functioning of mycorrhizas in ecosystems. On the basis of structure a number of different types of mycorrhiza are recognized, of which the most important are arbuscular mycorrhiza and ectomycorrhiza. Most plants form only one type of mycorrhiza, but several plants, which includes members of the Salicaceae (willows and poplars) are able to simultaneously form arbuscular mycorrhiza and ectomycorrhiza. Such plants are called dual mycorrhizal. The occurrence of dual mycorrhizal plants leads to a number of research questions. Does a plant benefit from being dual mycorrhizal? Or are such plants less able to control colonization by mycorrhizal fungi? How differently do arbuscular and ectomycorrhizas function? How do arbuscular and ectomycorrhizal fungi interact on the same root system?

These questions about the significance of different mycorrhizal fungi that belong to different functional types (AM and EcM) have been investigated in *Salix repens* (creeping willow), a small shrub that is common in the dunes in the Netherlands. The species has a wide ecological amplitude and occurs from dry to wet, and from calcareous, humus-poor to acidic, humus-rich soils. As a model organism for mycorrhizal studies *S. repens* has a number of advantages such as its ability to grow under a wide range of environmental conditions and its ability to form both arbuscular and ectomycorrhizas, enabling an unbiased comparison between functional differences between both types. Finally, the plant can be multiplied vegetatively resulting in relatively strong plants that are genetically identical. The present research involved a combination of field studies and field experiments (both on the Isle of Terschelling) and laboratory experiments (at the Biological Station Wijster).

The diversity of ectomycorrhizal fungi (EcMF) occurring in 16 stands of *S. repens* was studied during a period of three years. The stands included dry to wet, and calcareous to acidic conditions. On the basis of sporocarps 78 taxa of EcMF were found, belonging to 12 genera. Members of the Cortinariaceae were well represented. Most species were host-specific to the Salicaceae. Ordination on the basis of fungal species composition showed that pH and the concentration of plant available phosphorus significantly contributed to the

variation explained, while ordination on the basis of fungal genera indicated that pH and moisture significantly contributed to the variation explained. Fifteen EcM morphotypes could be recognized, but morphotype composition was different at two sampling dates. Numbers of sporocarps were not correlated with numbers of EcM tips and diversity above-ground (species or genus level) was not correlated with diversity below-ground (morphotype level). I therefore conclude that neither diversity nor abundance above-ground can be used to estimate below-ground diversity or abundance (CHAPTER 2). Proportional EcM colonization was high in all plots (usually more than 80%), independent of habitat. Intensity of AM colonization was low in all plots (usually less than 5%). Lack of differences in either EcM or AM colonization between habitats indicates that the relative importance of both mycorrhizal types does not change during succession. Differences in habitat preference of EcMF and AMF suggest that mycorrhizal diversity contributes to the wide ecological amplitude of *S. repens*. Plant nutritional status indicates that the relative importance of nitrogen and phosphorus does not change during succession but during season. Arbuscular mycorrhizal colonization was significantly higher in spring than at later sampling dates. Although AM colonization was low even in spring time, these colonization levels were nevertheless covering phosphorus demands of the plant. Decline of AM colonization at the end of the season coincided with a decrease in AM inoculum potential. From these observations I conclude that changes in AM colonization are likely caused by changes in plant demand or soil nutrient availability. My data do not support the hypothesis that EcM colonization interferes with AM colonization (CHAPTER 3).

Interactions between EcMF and AMF have been studied under controlled conditions in the laboratory. Cuttings of *S. repens* were inoculated with either a species of AMF (*Glomus mosseae, Acaulospora laevis*) or EcMF (*Hebeloma leucosarx, Inocybe vulpinella, Paxillus involutus*) and with one AMF and one EcMF. These latter treatments included simultaneous inoculations and sequential inoculations. Inoculation with a single species led to proportional colonization similar to that observed in the field: less than 5% for both AMF and more than 80% for EcMF. In simultaneous inoculations both species colonized the same amount as when inoculated singly, except the combination *P. involutus* and *G. mosseae*, where mutual suppression was observed. In sequential inoculations colonization by AMF suppressed subsequent colonization by EcMF. *Glomus mosseae* was only inhibited *by P. involutus*, not by the other two species of EcMF. On the basis of these data I conclude that EcM colonization does not interfere with AM colonization. Plant response to inoculation was not correlated with proportional colonization. *Glomus mosseae* had a rapid positive

effect on plant performance but *A. laevis* depressed plant performance. However, survival of plants inoculated with *A. laevis* was twice that of control plants. Ectomycorrhizal fungi had effects that became manifested after a longer period. Compared to the non-mycorrhizal controls, plants inoculated with *G. mosseae* had larger root length, whereas plants inoculated with EcMF had smaller root length (CHAPTER 4).

Competition and coexistence of AMF and EcMF was also investigated in two field sites and in the lab in the same field soils. Initial inoculation with the AMF *G. mosseae* affected subsequent colonization by EcMF negatively, but initial inoculation with the EcMF *H. leucosarx* did not affect subsequent colonization by AMF. The negative effect of AMF on EcMF was only transient and was hardly noticeable after 24 weeks. Plants simultaneously exposed to AMF and EcMF did not show negative interactions between both mycorrhizal types (CHAPTER 8).

Apparently, AMF transiently mobilize plant-defence reactions. This interaction between both functional types of mycorrhiza raises a number of questions about dual mycorrhizas that are discussed in CHAPTER 11. How common are dual mycorrhizas? Recent reports suggest that a number of trees, hitherto considered as EcM, are dual mycorrhizal and that they might derive benefits from this dual mycorrhiza. The results from CHAPTERS 4 AND 8 also might provide a more general framework for studying fungus-fungus interactions in the mycorrhizosphere, such as mycorrhizal fungi - pathogenic fungi interactions.

Differences in AM colonization in spring and summer, related to differences in leaf phosphorus content, raise the question whether functional significance of mycorrhizas in cuttings might be dependent on their internal nutrient concentration. Cuttings collected in December with a low shoot phosphorus concentration were compared with cuttings collected in March with a high shoot phosphorus concentration. Non-mycorrhizal control and cuttings with the EcMF *H. leucosarx* showed similar responses, whereas inoculation with the AMF *G. mosseae* showed a much more positive response on cuttings with a low internal phosphorus status than cuttings with a high internal phosphorus status. However, in both cases there was a positive short-term effect on shoot growth and root length. As in other experiments benefits from inoculation with the EcMF *H. leucosarx* occurred over a longer term than benefits from inoculation with *G. mosseae* (CHAPTER 5).

In view of the diversity of EcMF associated with *S. repens* an experiment was executed under controlled conditions in which 11 different EcMF were compared. None of these species exerted the full range of mycorrhizal benefits. Two strategies of ectomycorrhizas were recognized, viz. root manipulation strategy and root replacement

strategy. Species with the root manipulation strategy increased root length and had a more effective nitrogen economy than species with the root replacement strategy, which more conformed to the traditional model of mycorrhizal functioning. A consequence of these differences in mycorrhizal strategies is that nutrient inflow per unit root length (uptake efficiency) did not correlate with total shoot nutrient uptake (uptake effectiveness). Different species of EcMF furthermore differed in proportional colonization and magnitude of response. However, low colonization did not imply low reponse, and high response did not imply high colonization. Effects of *Cortinarius trivialis* were partly independent of the formation of ectomycorrhizas as they also occurred with aqueous extracts of the fungal mycelium (CHAPTER 6; aqueous extracts of other EcMF were not tested).

Expression of plant benefits to inoculation depends on the environmental conditions. In order to investigate conditionality of mycorrhizal benefits for *S. repens*, two EcMF (*H. leucosarx, P. involutus*), one AMF (*G. mosseae*) and a non-mycorrhizal control were grown in a full factorial experiment with pH and nitrogen-to-phosphorus-ratio as independent factors. Almost all plant parameters were significantly affected by pH, nitrogen-to-phosphorus ratio, and the interaction between both factors, providing ample evidence for the conditionality of mycorrhizal responses. In general, EcMF were more beneficial than the AMF. Benefits were also larger after 26 weeks than after 12 weeks (CHAPTER 7).

Mycorrhizal functioning is affected by compatibility of plant and fungus. Genetic variation in plant and fungal species could therefore influence the effectiveness of the symbiosis. Both the importance of genetic variation of *S. repens* and the importance of genetic variation of the EcMF *H. leucosarx* were investigated. Plant origin had a large effect on symbiotic effectiveness; this effect was probably mediated through genetic differences in root parameters. There was also a large plant origin $\times$ soil type interaction. Plants from the nutrient-poor habitat performed better on the nutrient-poor soils, and plants from the richer habitat performed better on the richer soil. Effects of plant origin were also dependent on the fungal species. Fungal species origin had only a minor effect on symbiotic effectiveness. These results highlight the need to be explicit about the provenance and compatibility of both mycorrhizal plant and fungus (CHAPTER 9).

As in all field observations and laboratory experiments a low colonization of AMF was observed, colonization by AMF was investigated in a grass sod with a high AM inoculum potential and a low EcM inoculum potential. Again, AM colonization was low, while it was not affected by EcM colonization. A large phosphorus uptake was noted after establishment of the AM symbiosis, probably due to the fact that the cuttings were rapidly

integrated in the mycorrhizal network in the grass sod. All observations of low AM colonization combined with a substantial positive plant response suggest that AM colonization is under control by *S. repens*. High internal AM colonization intensity was observed after water-logging and coincided with a reduced EcM development. Although such changes in colonization of both mycorrhizal types after water-logging might suggest interference of both types of mycorrhizal fungi, it is likely that these changes are better explained as differential expressions of their survival strategies (CHAPTER 10).

In dune plant communities with *S. repens* no other EcM trees or shrubs occur. Species composition of these communities might still be determined by stimulatory and inhibitory substances released by decomposing litter of co-dominant plants in these communities. Effects of aqueous extracts of 4 plant species on the growth and biomass of 8 isolates of EcMF were investigated under axenic conditions on artificial media with low and high pH. Litter extracts of *Myrica gale* and *Calamagrostis epigejos* increased growth and biomass of most EcMF; however, in the field no sporocarps of EcMF were found in such plots. Growth of fungi on aqueous extracts of *S. repens* was poor. On the medium with a higher pH the EcMF generally performed better. It was finally noted that these EcMF showed no relationship between radial growth and biomass increment (CHAPTER 12).

Finally in CHAPTER 13, the question is addressed whether these results can be generalized over other mycorrhizal plants and mycorrhizal fungi. Attention is given to factors that regulate mycorrhizal fungal diversity and the need to consider plant control over mycorrhizal colonization is emphasized. Although my results clearly indicate the functional importance of mycorrhizal species diversity for performance of *S. repens*, they also warn against a priori grouping of mycorrhizal fungi in functional types, viz. arbuscular mycorrhiza and ectomycorrhiza. Classification of fungal strategies, such as the root manipulation strategy, which was observed in both ectomycorrhizas and arbuscular mycorrhizas, might provide alternatives that need to be further explored. A number of questions about mycorrhizal quantification and assessment of mycorrhizal benefits are also addressed. I conclude that high mycorrhizal fungal diversity, both in taxonomic and functional terms, is both determining and determined by the wide ecological amplitude of *S. repens*.

## SAMENVATTING

Mycorrhizas zijn samenlevingsvormen tussen schimmels en plantenwortels. Zowel de plant als de schimmel hebben baat bij deze verbinding, en beiden hebben niet of nauwelijks het vermogen om onder natuurlijke omstandigheden hun levenscyclus te voltooien wanneer de andere partner afwezig is. Voor de schimmel is het voordeel van deze samenlevingsvorm gelegen in het verkrijgen van energierijke koolhydraten, terwijl voor de plant het voordeel is gelegen in een betere opname van water en plantenvoedende stoffen. Mycorrhizaschimmels kunnen ook de effekten van ongunstige milieu-omstandigheden verminderen; dit betreft zowel abiotische (bijvoorbeeld zware metalen) als biotische (bijvoorbeeld plantenziektenverwekkers) stress-faktoren. Er bestaat een aanzienlijke variatie in struktuur en functie van mycorrhizas. Deze variatie is van belang voor de rol van mycorrhizas in ecosystemen. Op basis van struktuur kan een aantal verschillende mycorrhizatypen worden onderscheiden. De belangrijkste daarvan zijn arbusculaire mycorrhiza en ectomycorrhiza. De meeste planten vormen slechts één mycorrhizatype, maar sommige planten zoals bijvoorbeeld de vertegenwoordigers van de Salicaceae (de wilgenfamilie, waartoe wilgen en populieren behoren) kunnen gelijktijdig arbusculaire mycorrhiza en ectomycorrhiza vormen. Dit gelijktijdig optreden van twee mycorrhizatypen wordt duale mycorrhiza genoemd. Het voorkomen van planten met duale mycorrhiza leidt tot een aantal onderzoeksvragen. Heeft een plant voordeel van duale mycorrhiza? Of zijn zulke planten juist minder goed in staat om de kolonisatie van hun wortels door mycorrhizaschimmels te controleren? In welke mate hebben ectomycorrhiza en arbusculaire mycorrhiza een verschillende functie? Op welke manier vindt er interactie plaats tussen ectomycorrhiza en arbusculaire mycorrhiza op hetzelfde wortelsysteem?

Vragen over de betekenis van verscheidene mycorrhizaschimmels die tot verschillende functionele groepen behoren (arbusculaire mycorrhiza, ectomycorrhiza) zijn onderzocht aan *Salix repens* (kruipwilg), een lage struik die algemeen in de Nederlandse duinen voorkomt. Kruipwilg lijkt weinig kieskeurig in haar standplaatskeuze en komt voor op zowel droge als natte, en op zowel kalkrijke, humusarme als zure, humusrijke standplaatsen voor. Als een modelplant voor onderzoek aan mycorrhiza heeft kruipwilg een aantal voordelen, zoals het vermogen om op een groot aantal verschillende standplaatsen te groeien en de mogelijkheid om zowel ectomycorrhiza als arbusculaire mycorrhiza te vormen. Hierdoor is een eerlijker vergelijking tussen de functionele verschillen tussen beide typen mogelijk. (Vergelijking tussen een langzaam groeiende boom met ectomycorrhiza en een

snelgroeiend kruid met arbusculaire mycorrhiza is in dit opzicht oneerlijk.) Tot slot kan kruipwilg gemakkelijk via stekken vermeerderd worden zodat relatief krachtige planten ontstaan die ook alle dezelfde erfelijke samenstelling hebben. Het onderzoek omvatte een combinatie van veldwaarnemingen en veldproeven op het Waddeneiland Terschelling en laboratoriumexperimenten op het Biologisch Station Wijster.

De soortenrijkdom van ectomycorrhizaschimmels, welke voorkwamen in 16 proefvlakken met kruipwilg, werd gedurend een periode van drie jaar bestudeerd. Deze proefvlakken kwamen voor op droge tot natte, en van kalkrijke tot zure standplaatsen. Op basis van de vruchtlichamen (paddestoelen) werden 78 soorten ectomycorrhizapaddestoelen gerapporteerd; deze behoorden tot 12 geslachten. Met name vertegenwoordigers van de Cortinariaceae (de familie van de gordijnzwammen) waren goed vertegenwoordigd. De meeste soorten hadden een voorkeur voor vertegenwoordigers van de wilgenfamilie als gastheer. Een ordinatie op basis van deze soortensamenstelling gaf aan dat de zuurgraad en voor de plant beschikbare hoeveelheid fosfor een significante bijdrage leverden tot de verklaarde variatie, terwijl een ordinatie op basis van paddestoelengeslachten aangaf dat zuurgraad en vochtgehalte een significante bijdrage leverden tot de verklaarde variatie. Vijftien morfologische typen van ectomycorrhizas konden worden onderscheiden. De samenstelling van deze morphotypen was echter verschillend op twee verschillende oogsttijdstippen. Het aantal paddestoelen toonde geen verband met het aantal mycorrhizas dat werd gevonden en de rijkdom bovengronds (op basis van soorten of geslachten) toonde geen verband met de ondergrondse rijkdom aan morphotypen. Op grond van deze waarnemingen concludeer ik dat noch soortenrijkdom noch talrijkheid bovengronds bruikbaar is als schatter voor de rijkdom of talrijkheid ondergronds (HOOFDSTUK 2). De procentuele kolonisatie door ectomycorrhizaschimmels was hoog in alle proefvlakken (gewoonlijk meer dan 80%), onafhankelijk van de standplaats. De intensiteit van kolonisatie door arbusculaire-mycorrhizaschimmels was overal laag (gewoonlijk minder dan 5%). De afwezigheid van verschillen tussen standplaatsen wat betreft kolonisatie door ectomycorrhiza- or arbusculaire-mycorrhizaschimmels geeft aan dat het relatieve belang van beide mycorrhizatypen tijdens de vegetatiesuccessie niet verandert. Standplaatsvoorkeuren van verschillende ectomycorrhiza- en arbusculaire-mycorrhizaschimmels maken aannemelijk dat de mycorrhizasoortenrijkdom een belangrijke bijdrage levert aan de grote ecologische amplitude van kruipwilg. De voedingstoestand van de plant wijst erop dat de relatieve betekenis van stikstof en fosfor eveneens niet verandert in de loop van de successie. Daarentegen werd wel waargenomen dat de relatieve betekenis van beide plantenvoedende

stoffen gedurende het seizoen verandert. Kolonisatie door arbusculaire-mycorrhizaschimmels was in het voorjaar aanmerkelijk hoger dan in de zomer of najaar. Hoewel de intensiteit van kolonisatie ook in het voorjaar laag was, was deze voldoende om aan de fosforbehoefte van kruipwilg te voldoen. De afname in kolonisatie door arbusculaire-mycorrhizaschimmels gedurende het groeiseizoen valt samen met een afname in het inoculumpotentiaal van deze schimmels. Op basis van deze waarnemingen kom ik tot de conclusie dat de veranderingen in kolonisatie door arbusculaire-mycorrhizaschimmels het gevolg zijn van veranderingen in de behoefte van de plant of in de beschikbaarheid van plantenvoedende stoffen in de bodem. Mijn waarnemingen leveren geen steun voor de hypothese dat kolonisatie door ectomycorrhizaschimmels een remmende invloed heeft op kolonisatie door arbusculaire-mycorrhizaschimmels (HOOFDSTUK 3).

Interacties tussen ectomycorrhiza- en arbusculaire-mycorrhizaschimmels werden bestudeerd in het laboratorium onder gecontroleerde omstandigheden. Stekken van kruipwilg werden beënt met ofwel een schimmelsoort die arbusculaire mycorrhiza vormt (*Glomus mosseae*, *Acaulospora laevis* - voor beide soorten bestaan geen Nederlandse namen) ofwel een schimmelsoort die ectomycorrhiza vormt (*Hebeloma leucosarx* - vleeskleurge vaalhoed; *Inocybe vulpinella* - kleine duinvezelkop; *Paxillus involutus* - gewone krulzoom). Ook werden planten beënt met zowel een ectomycorrhiza- als arbusculaire-mycorrhizavormende schimmel. Deze laatste behandelingen bestonden zowel uit een gelijktijdige beënting als een elkaar opvolgende beënting. Wanneer stekken met één soort werden beënt, leidde dat tot kolonisatie die niet verschillend was van de kolonisatie zoals die in het veld was waargenomen: minder dan 5% kolonisatie voor beide arbusculaire-mycorrhizaschimmels en meer dan 80% voor de drie ectomycorrhizaschimmels. Bij gelijktijdige beënting koloniseerden beide soorten dezelfde hoeveelheid wortels als in de afzonderlijke beënting, behalve in de combinatie gewone krulzoom - Glomus. In dat geval werd wederzijdse remming waargenomen. In opeenvolgende beëntingen remde de kolonisatie door arbusculaire-mycorrhiza-schimmels de daarop volgende kolonisatie door ectomycorrhizaschimmels. In het omgekeerde geval werd Glomus door de gewone krulzoom geremd, maar niet door de twee andere soorten ectomycorrhizaschimmels. Op basis van deze uitkomsten stel ik vast dat kolonisatie door ectomycorrhizaschimmels geen remming van kolonisatie door arbusculaire-mycorrhizaschimmels tot gevolg heeft. Deze conclusie weerlegt een eerdere hypothese waarin juist wel een remmende invloed gesuggereerd werd. Er bestond geen verband tussen de mate van kolonisatie en het effect op plantengroei. Glomus had een snel en positief effect op de groei van kruipwilg, maar Acaulospora had een negatief effect. Opgemerkt moet

worden dat planten beënt met Acaulospora een tweemaal zo hoge overleving toonden dan niet-beënte planten. De effecten van ectomycorrhizaschimmels werden pas na wat langere tijd zichtbaar. In vergelijking met de niet-beënte controleplanten hadden planten beënt met Glomus duidelijk langere wortels en planten beënt met ectomycorrhizaschimmels kortere wortels (HOOFDSTUK 4).

Concurrentie en coëxistentie van arbusculaire-mycorrhiza- en ectomycorrhiza-schimmels is ook onderzocht in twee proefvlakken en in het laboratorium met grond uit deze beide proefvlakken. Wanneer kruipwilg eerst was beënt met Glomus had dit een negatief effekt op de latere kolonisatie door ectomycorrhizaschimmels, maar wanneer kruipwilg eerst was beënt met de vleeskleurige vaalhoed had dit geen effect op de latere kolonisatie door arbusculaire-mycorrhizaschimmels. Dit negatieve effekt van arbusculaire-mycorrhizaschimmels op ectomycorrhizaschimmels was van voorbijgaande aard en was nauwelijks meer aanwezig na 24 weken (ongeveer een groeiseizoen). Planten die gelijktijdig werden blootgesteld aan beide mycorrhizatypen lieten geen negatieve interactie tussen beide typen zien (HOOFDSTUK 8).

Blijkbaar schakelen arbusculaire-mycorrhizaschimmels het afweersysteem van de plant tijdelijk aan. De interactie tussen beide mycorrhizatypen roept een aantal vragen over duale mycorrhiza op die in HOOFDSTUK 11 worden besproken. Hoe algemeen komt duale mycorrhiza voor? Recente waarnemingen wekken de indruk dat een aantal boomsoorten, waarvan voorheen verondersteld werd dat ze uitsluitend ectomycorrhiza vormen, ook duale mycorrhiza heeft en aan die duale mycorrhiza voordelen ontleent. De resultaten van HOOFDSTUKKEN 4 EN 8 verschaffen mogelijk ook een meer algemeen kader waarbinnen interacties tussen schimmels kunnen worden bestudeerd in de onmiddellijke omgeving van de gemycorrhizeerde wortel, zoals de interactie tussen mycorrhizaschimmels en planten-ziektenverwekkende schimmels.

De verschillen in kolonisatie door arbusculaire-mycorrhizaschimmels tussen het voorjaar en het najaar in het veld, die samenhangen met verschillen in fosforgehalte van de bladeren en scheuten, roepen de vraag op in hoeverre de functionele betekenis van mycorrhiza voor de plant afhankelijk is van de voedingstoestand van de plant. Stekken met een laag fosforgehalte, die in december waren verzameld, werden vergeleken met stekken met een hoog fosforgehalte, die in maart waren verzameld. Terwijl niet-beënte controlestekken en stekken die beënt waren met de vleeskleurige vaalhoed geen verschillen toonden in de reactie van de plant, bleken decemberstekken beënt met Glomus een veel sterker positieve reactie van de plant op te roepen dan maartstekken. In beide gevallen was er

overigens wel sprake van een positief korte-termijneffect op de groei van de scheut en de wortellengte. Net als in andere experimenten bleek ook hier het voordeel van beënting met de vleeskleurige vaalhoed later op te treden en langer te duren dan het voordeel van beënting met Glomus (HOOFDSTUK 5).

Vanwege de hoge soortenrijkdom aan ectomycorrhizaschimmels die met kruipwilg waren verbonden, werd een proef onder gecontroleerde omstandigheden uitgevoerd waarin 11 verschillende soorten ectomycorrhizaschimmels werden vergeleken. Het bleek dat geen der schimmels alle voordelen van de mycorrhiza vertoonde. Twee mycorrhizastrategieën konden worden herkend, deze zijn wortelmanipulatie en wortelvervanging genoemd. Soorten die de strategie van wortelmanipulatie toonden hadden een gunstig effekt op de wortellengte van kruipwilg en gingen (vanuit het oogpunt van de plant) zuiniger met stikstof om dan schimmels die de strategie van wortelvervanging toonden. Deze laatste schimmels pasten ook beter in het standaardmodel waarop mycorrhizas functioneren. Een belangrijk gevolg van deze verschillen in mycorrhizastrategie is beschreven. Vanwege de grote effecten die de schimmelsoorten hadden op de wortellengte van kruipwilg bleek de opname van plantenvoedende stoffen per eenheid wortellengte (de klassieke maat voor de opname-efficiëntie) geen verband te tonen met de totale opname van plantenvoedende stoffen. Verschillende soorten ectomycorrhizaschimmels verschilden in procentuele kolonisatie en de grootte van het effect op de plant. Tussen beiden bleek echter geen verband te bestaan en lage kolonisatie ging niet samen met geringe effecten op de plant, of grote effecten op de plant met hoge kolonisatie. De effecten van *Cortinarius trivialis* (gegordelde gordijnzwam) waren zelfs gedeeltelijk onafhankelijk van de vorming van ectomycorrhizas; een waterig extract van de zwamvlok had eveneens een positief effect op kruipwilg (HOOFDSTUK 6; waterige extracten van de andere schimmels zijn niet getest).

De mate waarin de mogelijke voordelen voor de plant van kolonisatie door mycorrhizaschimmels ook tot uitdrukking komen, hangt af van de milieu-omstandigheden. Dit verschijnsel wordt conditionaliteit (voorwaardelijkheid) genoemd. Conditionaliteit van mycorrhizavoordelen voor kruipwilg werd onderzocht voor twee soorten ectomycorrhiza-schimmels (vleeskleurige vaalhoed, gewone krulzoom) en één soort arbusculaire-mycorrhizaschimmel (Glomus); ook niet-beënte controleplanten werden onderzocht. De milieuomstandigheden omvatten drie verschillende zuurgraden en drie verschillende relatieve beschikbaarheden van stikstof en fosfor. Beide faktoren werden onafhankelijk gevarieerd in een factoriële proef. Vrijwel alle gemeten planteneigenschappen werden significant beïnvloed door zuurgraad, stikstof-fosfor-verhouding, en de interactie tussen

beide factoren, zodat conditionaliteit werd aangetoond. Over het algemeen hadden ectomycorrhizaschimmels een groter positief effekt dan arbusculaire-mycorrhizaschimmels. Ook waren de voordelen van beënting voor de plant groter na 26 weken dan na 12 weken (HOOFDSTUK 7).

Het functioneren van de mycorrhizasymbiose hangt af van de specifieke plant-schimmel combinatie. Erfelijke variatie binnen een planten- of schimmelsoort kan daardoor ook de efficiëntie van de symbiose bepalen. Zowel het belang van erfelijke variatie in kruipwilg als in de vleeskleurige vaalhoed werd apart onderzocht. De herkomst van de plant bleek een groot effect te hebben op de efficiëntie van de symbiose; dit effect werd vermoedelijk indirect bepaald door erfelijke verschillen in worteleigenschappen. Bovendien bleek dat het effect van plantenherkomst afhangt van de omstandigheden waaronder de proef wordt uitgevoerd. Planten van een voedselarme standplaats deden het relatief beter op voedselarm substraat, planten van een voedselrijkere (hoewel nog steeds relatief arme) standplaats beter op een wat rijker substraat. Het effect van plantenherkomst bleek ook per schimmelsoort verschillend. Herkomst van de vleeskleurige vaalhoed bleek daarentegen nauwelijks verschil uit te maken. Deze uitkomsten geven aan dat het belangrijk is om meer aandacht te schenken aan de herkomst van plant en schimmel en de mate waarin beiden aan elkaar aangepast (kunnen) zijn (HOOFDSTUK 9).

Omdat al mijn veldwaarnemingen en laboratoriumproeven een (zeer) lage kolonisatie door arbusculaire-mycorrhizaschimmels lieten zien, onderzocht ik de kolonisatie van kruipwilg door arbusculaire-mycorrhizaschimmels in een graszode waarin arbusculare mycorrhiza talrijk voorkwam en ectomycorrhiza zeer schaars was. Opnieuw bleek dat de kolonisatie door arbusculaire-mycorrhizaschimmmels zeer laag was en opnieuw bleek er geen effect van de ectomycorrhizaschimmels op de kolonisatie door de arbusculaire-mycorrhizaschimmels. Deze stekken lieten een zeer hoge fosforopname in de scheut zien, hetgeen vermoedelijk samenhangt met een snelle integratie van deze stekken in het mycorrhizanetwerk dat in de bodem voorkomt. Mijn waarnemingen dat een (zeer) lage kolonisatie door arbusculaire-mycorrhizaschimmels gepaard gaat met een aanzienlijk positief effect op de plant, lijken er op te wijzen dat de kolonisatie door arbusculaire mycorrhiza door de plant gereguleerd kan worden. Een hoge inwendige kolonisatie werd slechts waargenomen na overstroming en was het gevolg van de vorming van sporen binnen de wortel. Door overstroming nam de externe kolonisatie door ectomycorrhizaschimmels af. Hoewel deze veranderingen in kolonisatie zouden kunnen wijzen op negatieve invloed van het ene type op het andere is een andere verklaring veel waarschijnlijker: de veranderingen

zijn het gevolg van de manier waarop overstroming de overlevingsstrategieën van beide typen mycorrhizaschimmels beïnvloedt (HOOFDSTUK 10).

In de plantengemeenschappen die in de duinen werden onderzocht bleek kruipwilg de enige plant met ectomycorrhiza te zijn. Dit sluit echter niet uit dat de soortensamenstelling van ectomycorrhizapaddestoelen ook beïnvloed wordt door positieve en negatieve effecten van stoffen die vrijkomen bij de afbraak van blad van andere plantensoorten die algemeen in die vegetaties voorkomen. Effecten van waterige extracten van vier plantensoorten werden onderzocht op de groei en gewichtstoename van 8 stammen van ectomycorrhizaschimmels in reincultuur op media met een hoge en lage pH. Strooiselextracten van *Myrica gale* (gagel) en *Calamagrostis epigejos* (duinriet) leidden tot een betere groei en gewichtstoename van de meeste soorten; echter, in de proefvlakken waarin deze planten talrijk voorkwamen werden nauwelijks paddestoelen aangetroffen. Groei van de schimmels op bladextracten van kruipwilg was daarentegen slecht. Het medium met de hogere pH bleek geschikter voor deze schimmels dan het medium met de lage pH. Er bleek geen verband te bestaan tussen de radiale groei en de toename van het gewicht (HOOFDSTUK 12).

Tot slot wordt in HOOFDSTUK 13 de vraag gesteld in welke mate mijn resultaten een algemener karakter hebben en ook toepasbaar zijn voor andere mycorrhizaplanten en mycorrhizaschimmels. Ik bediscussieer factoren die de rijkdom aan mycorrhizaschimmels kunnen reguleren en benadruk de noodzaak om aandacht te schenken aan de controle van de plant over de kolonisatie door mycorrhizaschimmels. Hoewel mijn resultaten aangeven dat een grote soortenrijkdom aan mycorrhizaschimmels van belang is voor kruipwilg, waarschuw ik tevens voor een te snel groeperen van soorten in functionele typen (dat wil zeggen ectomycorrhiza en arbusculaire mycorrhiza). Ik acht het mogelijk dat andere indelingen, die de indeling in functionele typen doorsnijden (zo komt bijvoorbeeld de strategie van wortelmanipulatie zowel voor bij ectomycorrhiza- als arbusculaire-mycorrhizaschimmels), betere mogelijkheden bieden. Een aantal vragen die te maken hebben met de manier waarop de eenheid van mycorrhiza wordt gekwantificeerd en de manier waarop voordeel voor de plant wordt bepaald, worden besproken. Ik concludeer dat de hoge mycorrhizarijkdom (zowel in taxonomisch als functioneel opzicht) van kruipwilg zowel de brede standplaatskeuze van kruipwilg bepaalt alsmede daardoor bepaald wordt.

## CURRICULUM VITAE

Elisabeth Wilhelmina (Liesbeth) van der Heijden werd geboren op 3 september 1967 te 's Gravenhage. Na het behalen van de diploma's MAVO-D (1983) en HAVO (1985) moest helaas worden afgezien van een opleiding aan de sportacademie (wegens een chronische sportblessure), maar een opleiding aan het Hoger Laboratorium Onderwijs in Delft (Polytechnische faculteit van de Hogeschool Rotterdam en Omstreken) bleek een verrassend leuk alternatief. Deze opleiding tot oecologisch/fytopathologisch analiste werd dan ook met plezier afgesloten met een stage en een afstudeervak Vegetatiekunde/Oecologie aan de Rijks Universiteit Utrecht, bij de vakgroep Botanische Oecologie (1989/1990). Zij verrichtte onderzoek naar de effecten van 'zure regen' op arbusculaire mycorrhiza in zaailingen van kruidachtige planten in heidevegetaties. Uit nieuwsgierigheid werd 'de studie' voortgezet aan de Landbouwuniversiteit in Wageningen (1990). De studie Biologie (specialisatie milieubiologie) werd qua vakkensamenstelling breed ingevuld en afgerond met twee zeer uiteenlopende afstudeervakken, Aquatische ecotoxicologie en Milieusysteemanalyse. Na haar afstuderen in 1993 werkte zij, tot andere baantjes zich aanboden, in de tuinbouw in het Westland (daar waar zij al sinds 1983 een centje bijverdiende). In tijdelijke dienst bij de ingenieursbureaus Aquasense in Amsterdam en Witteveen en Bos in Deventer ondekte zij dat 'wetenschappelijk' onderzoek haar voorkeur had. In juli 1994 werd zij aangesteld als Onderzoeker in Opleiding bij het Biologisch Station in Wijster van de Landbouwuniversiteit Wageningen. Het in dat kader verrichte onderzoek resulteerde in dit proefschrift.