Organic nitrogen uptake and endophytic, mutualistic fungi in Dutch heathland ecosystems

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In dulci Iubilo
In zoete vreugde

Voor mijn familie en vrienden

Abstract

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Dutch heathlands were formerly dominated by the evergreen dwarf shrubs *Calluna vulgaris* and *Erica tetralix,* but during the 1970s and 1980s both species were increasingly displaced by the grasses *Deschampsia flexuosa* and *Molinia caerulea*. The causes of these changes include the direct and indirect effects of increased deposition of atmospheric nitrogen. This thesis focuses on the role of organic nitrogen uptake and endophytic, mutualistic fungi in Dutch heathland ecosystems. I tested the hypothesis that tannin-rich plant species are able to monopolize the nutrient cycle by increasing the amounts of organic nitrogen forms relative to inorganic nitrogen forms. In addition, those species will be favored which absorb organic nitrogen compounds through their associations with ericoid or ectomycorrhizal fungi. A field inventory and the results of related experiments under controlled conditions showed that nitrogen addition and shading both negatively affect the concentration of tannins in *Calluna* plants. Only in the greenhouse experiment was the presence of mycorrhizal structures in roots negatively affected by the addition of nitrogen. In the field experiment, shading reduced the amount of mycorrhizal structures in roots. It is concluded that when ericaceous plants are shaded by grasses that have become dominant due to increased nitrogen supplies, these effects will be intensified and displacement by competition will be accelerated. To test the effect of tannin-rich litter on nitrogen mineralization rates, an incubation experiment was performed with different types of shrub litter and grass litter. It was found that litters with C:N ratios above 30 and high tannin concentrations (as found in *C. vulgaris* and *Vaccinium vitis-idaea*) decrease the amounts of inorganic nitrogen and concomitantly increase the amounts of dissolved organic nitrogen in soils. When searching for the root inhabitants of ericaceous plants there was a great diversity of endophytic fungal species present. Several new species of fungi were identified, one of which was published as *Cryptosporiopsis rhizophila* Verkley & Zijlstra. Synthesis trials and Bavendamm tests to elucidate the ecological role of this new fungal species revealed that *C. rhizophila* isolates are able to associate with roots of *C. vulgaris* and have the potential to fulfil the same ecological function to their ericoid host as well-known mycorrhiza formers. Surprisingly, it was discovered that fungi isolated from grass roots contained endophytic fungal species related to the *Helotiales*, the phylogenetic group to which most of the ericaceous fungal isolates isolated in this research belong. A competition experiment tested the effects of tannin-rich litter types on soil nitrogen and the outcome of the competition between grass and shrub species. In none of the treatments were *C. vulgaris* plants able to outcompete *D. flexuosa*. Grass plants were able to benefit more efficiently from the available soil nitrogen released from the types of litter added. Furthermore, in the treatments with low nutrient availability, there was hardly any competitive suppression of shrub plants by the grasses. This suggests that grasses from the heathland systems have adapted well to the high amounts of organic nitrogen – including adaptation to related ericaceous fungal symbionts. In conclusion, this thesis shows that being competitive for heathland plants by monopolizing the nutrient cycle with tannin-rich litter is not that simple if competitors have adopted the same strategy to use nutrients.

Contents

Page 11 Chapter 1

General introduction

Jantineke D. Zijlstra

Page 31 Chapter 2

The effect of nutrient supply and light intensity on tannins and mycorrhizal colonisation in Dutch heathland ecosystems

Jantineke D. Zijlstra & Frank Berendse

Submitted

Page 57 Chapter 3

Phenolic compounds regulate nitrogen cycling: results from an incubation experiment

Jantineke D. Zijlstra & Frank Berendse

Submitted

Page 77 Chapter 4

Diversity of symbiotic root endophytes of the *Helotiales* in ericaceous plants and the grass, *Deschampsia flexuosa*.

Jantineke D. Zijlstra, Pieter van 't Hof, Jacqueline Baar, Gerard J.M. Verkley, Richard S. Summerbell, Istvan Paradi, Wim G. Braakhekke & Frank Berendse.

Studies in Mycology (2005) 53:147-162.

Page 109 Chapter 5

Phylogeny and taxonomy of root-inhabiting *Cryptosporiopsis* species, and *C. rhizophila* sp. nov., a fungus inhabiting roots of several *Ericaceae*

Gerard J.M. Verkley, Jantineke D. Zijlstra, Richard C. Summerbell & Frank Berendse

Mycological Research (2003) 107: 689-698.

Page 129 Chapter 6

The ecological role of *Cryptosporiopsis rhizophila*

Jantineke D. Zijlstra, Gerard J.M. Verkley, Jacqueline Baar & Frank Berendse

Submitted

Page 147 Chapter 7

Effects of total phenolics in litter on the competition between *Calluna vulgaris* and *Deschampsia flexuosa*

Jantineke D. Zijlstra & Frank Berendse

Submitted

Page 167 Summary

Samenvatting

Page 183 Nawoord

Curriculum vitae

List of publications

Affiliations of the co-authors

Chapter 1

General introduction

Jantineke D. Zijlstra

Dutch heathland ecosystems

Dutch heathland ecosystems harbour an intriguing plant community, consisting of ericaceaous plants, grasses, carnivorous vascular plants, lichens and mosses. We distinghuish dry heathlands with *Calluna vulgaris* (L.) Hull and *Deschampsia flexuosa* (L.) Trin. as the key plant species and wet heathlands with *Erica tetralix* L. and *Molinia caerulea* (L.) Moench as co-occurring plant species (Schamineé *et al*. 1996). Heathlands are semi-natural ecosystems, which have developed when farmers about 4000 years ago created open spots in woodlands as browsing places for their cattle and sheep (Webb 1998). Heather was able to survive when forests had been burned and on eroded poor soils. From the Middle Ages heathlands became an integrated part of the agricultural farming system. Sheep grazed on the heather and their manure was mixed with heather turfs to fertilize the arable fields. Since the introduction of artificial fertilizers in the '50s, this extensive farming system was no longer needed. As a result the area of heathlands rapidly declined. In 1988 the estimated area was 42.000 ha, about 7% of the heathland area, which had been present in 1833 (De Smidt 1975). The remaining heathland ecosystems in the Netherlands form an important part of the Western-European heathlands with a high conservation value (Pitcairn *et al*. 1995, Webb 1998).

Several factors threaten the conservation of Dutch heathlands. Currently, 35% of heathlands have become dominated by the grasses *Deschampsia flexuosa* and *Molinia cearulea* (Bobbink *et al*. 1998). This shift of plant dominance occurred largely due to effects of increased atmospheric nitrogen deposition (Berendse & Aerts 1984, Aerts 1993ab, Berendse 1994) and nutrient enrichment of the soil due to artificial fertilizer and manure (RIVM). Additionally, several outbreaks of heather beetle, *Lochmaea suturalis* (Thomson) in the '80s and at the Strabrechtse heide in 2004, caused severe mortality of heather plants (Heil & Diemont 1983, Bobbink & Heil 1993, Power *et al*. 1998, pers. comm. State Forestry). Finally, increased nitrogen deposition has affected important plant factors such as drought and frost resistance of the dwarf shrubs (Power *et al*. 1998).

Effects of atmospheric nitrogen deposition on heather survival

There appeared to be a strong correlation between the increased grass dominance and the increased atmospheric nitrogen deposition (Berendse & Aerts 1984, Heil & Bruggink 1987). So, during the 1980s and 1990s, Dutch researchers have put a lot of effort in elucidating the

effects of increased nitrogen on heather vegetation and the effects on the competition with dominant grasses (Berendse & Aerts 1984, Aerts & Berendse 1988, Aerts *et al*. 1990, 1991, Van den Eerden *et al*. 1991, Dueck *et al*. 1991, Berendse & Elberse 1990, Berendse 1994). Additionally, the amount of phosphorus was found to be a limiting factor in heather growth (Aerts & Berendse 1988, Diemont 1996). In general, expansion of grass species during later succesional phases was explained by their high maximum growth rate, which enabled them to profit much more rapidly from the increased nutrient supply. Also heather plants were found to respond positively to nitrogen additions, but with much lower growth rates than dominant grasses. In addition, shrub plants seem to survive and compete best under acid and nutrient-poor conditions, in which grasses do not perform equallyl (Berendse & Aerts 1984). In competition experiments with heathland plants, primarily above-ground biomass and vegetation cover characteristics were measured (Berendse & Aerts 1984, Aerts & Berendse 1988, Dueck *et al*. 1991), although Aerts *et al*. (1991) showed that below-ground competition for nutrients can be equally important.

In the analysis of the effects of increased atmospheric nitrogen on the competition between shrubs and grasses it was more and more appreciated that not only the amount of produced biomass can create a positive feedback, but that also the chemical composition of plant tissue can play an essential role (Lipson & Näsholm 2001). This new insight started when Northup *et al*. (1995) proposed that especially the role of high phenolic concentrations in litter can increase the plant fitness due to the effects of these compounds on the ratio between the release of dissolved organic nitrogen compounds (DON) and inorganic nitrogen (IN) from soil organic matter. Northup found in a soil fertility gradient a clear negative correlation between the phenolic concentrations in *Pinus muricata* litter and the release of inorganic nitrogen. Simultaneously they reported of a positive correlation with the release of dissolved organic N. Higher DON:IN ratios would favour those species that are able to absorb organic nitrogen compounds through their associations with ericoid or ectomycorrhizal fungi. Northup proposed his ideas to explain the convergent evolution of tannin-rich plant communities on highly leached soils. The unconventional ideas of Northup enrolled a large amount of publications from several disciplines (165 citations, 01-11-2006) which supported or opposed his views (e.g. Hooper 1998, Peñuelas & Estiarte 1998, Michelsen *et al*. 1998, Eckstein *et al*. 1999, Lipson *et al*. 1999, Campbell *et al*. 2000, Hättenschwiler & Vitousek 2000, Kalbitz *et al*. 2000, Wardle *et al*. 1998, Cornelissen *et al*. 2001, Kraus *et al*. 2003, Güsewell 2004). The

evolution of tannin-rich plant communities on highly acidic and infertile soils has been confirmed throughout the world in recent years (Schimel & Chapin 1996, Bradley *et al*. 1997, Northup 1995, 1998, Inderjit and Mallik 1999, Fierer *et al*. 2001).

In discussions about N deposition effects on the vegetation, the role of changes in plant-available organic nitrogen has not been properly addressed, although some attention has been paid to the importance of shifts in the relative abundance of organic versus inorganic nitrogen for species compositions in plant communities (Diekmann & Falkengren-Grerup 1998, Näsholm *et al*. 1998). However, nitrogen deposition could change the organic/inorganic nitrogen balance both directly by adding inorganic nitrogen, and indirectly by the inhibitory effect of inorganic nitrogen on proteolytic activity in soils (Smith *et al*. 1989). Moreover, additions of nitrogen can also have negative effects on the concentration of carbon-based secondary compounds in ericaceous leaves (Bryant *et al*. 1983) and indirectly influence the production of dissolved organic nitrogen. It is common knowledge that nitrogen addition increases nitrogen concentrations in heather (*Calluna vulgaris* (L.) Hull) (Berdowski and Siepel 1988, Iason and Hester 1993, Duncan *et al*. 1994, Hartley *et al*. 1995, Carrol *et al*. 1999, Gordon *et al*. 1999) and other ericaceous plants (Prescott *et al*. 1993, Mallik 1996, Nordin *et al*. 1998), but the expected decrease in total phenolics or condensed tannin levels due to nitrogen additions has not yet been empirically shown either in the greenhouse or in the field (Iason and Hester 1993, Iason *et al*. 1993, Hartley *et al*. 1995, Kerslake *et al*. 1998, Bradley *et al*. 2000, Alonso *et al*. 2001).

Dissolved organic nitrogen, a key nutrient in heathland ecosystems

Dwarf shrubs are able to sustain on acidic, nutrient poor soils. In these ecosystems, the release of inorganic nitrogen as ammonium (NH_4^+) and nitrate (NO_3^-) is in general low compared to other ecosystems, but can vary between 1 – 13 g N m^{-2} yr^{-1} (Berendse & Elberse 1990, Van Vuuren 1992). Additional supplies of phosphorus can also disturb the vulnerable heathland ecosystem, as phosphorus can be an important limiting factor for grass growth (Aerts & Berendse 1988, Diemont 1996). However, the effects of phosphorus limitations will not be addressed in this thesis. It has long been recognized that the mineralization of organic nitrogen to ammonium and its subsequent oxidation to nitrate are the major bottlenecks restricting supply of nutrients to plants (Chapin 1995). In infertile ecosystems, however, measured rates of net microbial production of inorganic nitrogen are often less than half the

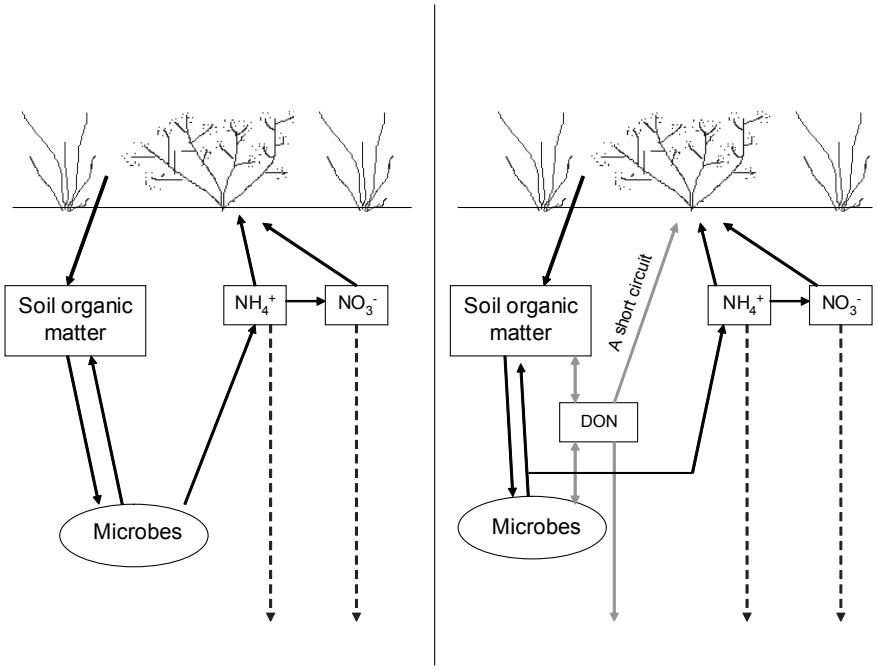

Figure 1. Established (left) and developing (right) views of the terrestrial nitrogen cycle. Dashed lines indicate losses that can be regulated by plant demand. Black lines indicate biological transformations of nitrogen where microbes control the conversion of solid soil organic nitrogen to inorganic nitrogen. Grey lines indicate a mixture of biological and physical processes involved in DON cycling. Note the possibility of direct plant uptake of DON without microbial intervention. DON may also leach from ecosystems despite high plant demand for N. (From Neff *et al.* 2003).

observed rates of nitrogen acquisition by plants (Fisk & Schmidt 1995, Kaye & Hart 1997). This suggest, that plants tap some pool of organic nitrogen in addition to the uptake of the inorganic nitrogen released by mineralization (Northup *et al*. 1995, 1998). See also Figure 1.

Mycologists have early recognized that organic nitrogen was an important nitrogen source for plants with ericoid or ectomycorrhizal symbioses (Handley 1954, 1961, Stribley & Read 1980, Abuzinadah & Read 1988). By now, a number of laboratory and field studies have shown that a broad range of plants may have access to organic nitrogen sources with or without support of mycorrhizal fungi (Chapin *et al*. 1993, Jones & Darrah 1994, Kielland 1994, Turnbull *et al*. 1995, Raab *et al*. 1996, Schimel & Chapin 1996, Schmidt & Stewart

1997, Näsholm *et al*.1998, Lipson *et al*. 1999, Raab *et al*. 1999, Persson *et al*. 2003). Especially, the experiments of Näsholm *et al.* (1998) with applied dual-labeled glycine and labeled ammonium to trees, grasses and dwarf shrubs in a boreal forest, provided convincing evidence that plants of different functional groups are able to absorb simple organic N compounds under field conditions. Nevertheless, although the idea that plants can directly use organic nitrogen sources by plant species is widely accepted, the extent of the role of mutualistic, endophytic fungi in this organic nitrogen uptake is a current theme of debate (Hodge *et al*. 2001, Persson *et al*. 2003, Persson & Näsholm 2001). Heathland soils are found to be rich in dissolved organic nitrogen (Gimingham 1972, Abuarghub & Read 1988). Most of this DON is, however, not comprised of free amino acids or proteins but consists of humic substances such as protein-tannin complexes (Qualls *et al*. 1991). Ericaceous plants are supposed to have a competitive advantage as they have an exclusive symbiosis with mutualistic, endophytic fungi, which are able to produce enzymes that degrade protein-tannin complexes.

Interactions with tannins

The ericaceous plant have developed strategies to conserve nutrients very efficiently (Grime, 1979). Therefore, most evergreen ericaceous plants are characterized by thick, sclerophyllous leaves, which are low in nitrogen, but contain high amounts of carbon-based secondary compounds such as tannins. Leaves and bark may contain up to 40% tannin by dry weight (Kuiters 1990, Matthews *et al*. 1997). Tannins (also referred as polyphenolics) can be subdivided into hydrolysable tannins (sugar molecules esterified by a number of gallic acid moieties) and condensed tannins, also referred to as proanthocyanidins (polymers of flavan-3-ols) (Harborne 1997). Hydrolysable tannins occur universally in higher plants, whereas condensed tannins are the most abundant polyphenols in woody plants, but are usually absent in herbaceous plants (Swain 1979, Haslam 1989). Tannin production can vary widely between and within plants due to biotic and abiotic effects on the short term (Jones and Hartley 1999, Hättenschwiler & Vitousek 2000). Additionally, on the long term genotypic selection can occur (Findlay *et al*. 1996). The main ecological function of these tannin compounds is seen as chemical defense against herbivores (Bryant *et al*. 1983), although it remains difficult to provide unequivocal evidence for this assumption (see references in Kraus *et al*. 2003). Nevertheless, there are important side-effects of plants producing litter with high tannin

contents. In plant material protein-tannin complexes can be formed before the leaves are shedded, or during the process of senescence tannins can mix with cell contents to form additional recalcitrant complexes (Gallet *et al*. 1999). Furthermore, tannins which leach into the soil may form complexes with proteins in the soil. In this way, nitrogen losses in infertile ecosystems can be reduced.

Tannins in litter have been suggested to influence soil nitrogen production in several ways (Kuiters 1990, Appel 1993, Inderjit *et al*. 1999, Hättenschwiler & Vitousek 2000, Kraus *et al*. 2003, 2004). Most important, they bind with proteins through hydrogen-bonding and hydrophobic effects, of which the resulting protein-tannin complexes are difficult to degrade by micro-organisms (Schimel *et al*. 1996, Hagerman *et al*. 1998, Fierer *et al*. 2001). Other reported negative effects of tannins on nutrient cycling include the inhibition of decomposition (Handayanto *et al*. 1997), mineralization (Schimel *et al*. 1996, 1998, Bradley *et al*. 2000, Fierer *et al*. 2001), nitrification (Rice & Pancholy 1973, Baldwin *et al*. 1983, Olson & Reiners 1983), nitrogen fixation (Schimel *et al*. 1998), microbial activity and enzyme activity (Scalbert 1991, Field & Lettinga 1992). The effect of decreased N mineralization by additions of tannins to soils was shown not only under laboratory conditions (Schimel *et al*. 1996, 1998, Bradley *et al.* 2000, Fierer *et al*. 2001), but also in Alaska taiga soils (Schimel *et al*. 1996, 1998). In addition, when poplar tannins are fractionated in high and low molecular weight, it appeared that high-molecular-weight tannins bound N-containing substrates and reduced mineral N pools, whereas lower- molecular-weight tannins appeared to act as substrates or as toxins for microbial organisms thereby respectively increasing or decreasing immobilization (Fierer *et al*. 2001, Kraus 2002).

Role of endophytic mutualistic fungi in ericaceous plant roots

Traditionally, the ericoid mycorrhizal association is described as a symbiosis between mutualistic root-endophytic ascomycetous fungi and ericaceous plant roots (see Table 1 for overview of endophytic root fungi). Ericoid mycorrhizal fungi (ERM) form characteristic hyphal coils in epidermal root cells (Smith & Read 1997). The strains of the *Rhizoscyphus ericae* (formerly referred to as *Hymenoscyphus ericae)* - complex (*Helotiales*) and *Oidiodendron maius* (G.L. Barron) are the most widely distributed and investigated ERM fungi (Read 1996, Straker 1996, Smith & Read 1997). Recently, molecular identification

showed that the diversity of ERM fungi is much larger than once assumed (Monreal *et al*. 1999, Vrålstad 2002a, Perotto *et al*. 2002, Allen *et al*. 2003, Bergero *et al*. 2003). In addition, the host range of ERM fungi appears to include some non-ericaceous plants as well (Duckett & Read 1995, Bergero *et al.* 2000, Vrålstad *et al.* 2002).

Ericoid endophytic fungi can facilitate the uptake by plants of organic nitrogen. This is due to their saprotrophic abilities. Chalot & Brun (1998) showed that ericoid mycorrhizal fungi may facilitate acces, to N from amino sugars and nucleic acids. Proteins and amino acids are released from protein-tannin complexes by the activity of a range of hydrolytic and oxidative enzymes (Bending & Read 1996, 1997, Cairney & Burke 1998). For example, Sokolovski *et al*. (2001) showed that *C. vulgaris* (L.) Hull root cells increased amino acid uptake when root cells were colonized with *R. ericae*. Among the dark septate endophyte species, *Phialocephala fortinii* and *Cadophora* (*Phialophora*) *finlandica* also have the ability to hydrolyse organic nitrogen sources such as proteins, but their precise role in organic nitrogen uptake is not clear (Jumpponen & Trappe 1998, Jumpponen *et al.* 1998, Caldwell *et al.* 2000, Mandyam & Jumpponen 2005).

Table 1. Overview of endophytic root fungi occurring in heathland ecosystems in ericaceous plant species.

Fungi group	Taxonomic position	Host plants	References
Ericoid mutualistic fungi (with nutrient benefit for the host):			
Rhizoscyphus ericae - aggregate (≡ *Hymenoscyphus ericae*)	*Helotiales, Leotiomycetes*	*Calluna vulgaris*, *Erica tetralix*	Read 1996, Straker 1996, Smith & Read 1997
Oidiodendron maius G.L. Barron	*Onygenales , Myxotrichaceae*	*Vaccinium vitis-idaea*, *Oxycoccus* sp., *Rhododendron* sp.	Rice & Currah 2005
Ericoid endophytic fungi (non-pathogenic presence of intracellular structures in roots):			
Cryptosporiopsis rhizophila Verkley & Zijlstra	*Helotiales Dermataceae*	*Calluna vulgaris*, *Erica tetralix*, *Vaccinium vitis-idaea*, *Vaccinium myrtillus*	Verkley *et al.* 2003
Cryptosporiopsis brunnea Sigler	*Helotiales Dermataceae*	*Gaultheria shallon*	Sigler *et al.* 2005
Cryptosporiopsis ericae Sigler	*Helotiales Dermataceae*	*Gaultheria shallon* *Vaccinium membranaceum* *Vaccinium ovalifolium*	Sigler *et al.* 2005
Oidiodendron griseum Robak	*Onygenales, Myxotrichaceae*	*Vaccinium* sp.	Rice & Currah 2005
Phialocephala fortinii Wang & Wilcox	*Helotiales Helotiaceae*	*Calluna vulgaris*, *Empetrum nigrum* *Vaccinium vitis-idaea*, *Vaccinium myrtillus*	Jumpponen & Trappe 1998
Meliniomyces variabilis (variable white taxon, *Scytalidium vaccinii*) Hambleton & Sigler	*Helotiales, Leotiomycetes*	*Rhododendron albiflorum* *Vaccinium membranaceum* *Phyllodoce empetriformis* *Empetrum nigrum*	Hambleton & Sigler 2005
Sebacinaceae	*Sebacinaceae*	*Gaultheria shallon*	Allen *et al.* 2003, Weiss *et al.* 2004

Outline of the thesis

In this dissertation I have tried to answer the following questions:

- Which ericoid mycorrhizal fungi species are present in plant roots from Dutch heathland ecosystems?
- Has the increased nitrogen deposition in The Netherlands negatively affected colonization of ericoid mycorrhizal fungi in heather roots?
- Has the increased nitrogen deposition in The Netherlands negatively affected amounts of carbon-based secondary chemicals in ericaceous plants? And to which extent are tannins in ericaceous plants and dominant grasses affected by nutrients and varying intensities of light.
- Do tannins in litter inhibit soil mineralisation rates and increase the ratio DON: IN (dissolved organic N: inorganic N)?
- To which extent regulate tannins in plant litter the nitrogen uptake in plants?
- Does litter with different amounts of tannins alter the competition between grass and heather?

Chapter 2 focuses on the effects of nutrient supply and light intensity on tannins and mycorrhizal colonization. It presents a combination of a descriptive field inventory performed at different heathland sites, a field experiment conducted during three years and a pot experiment in the greenhouse. In **Chapter 3** we have tried to elucidate the effects of tannins in litter and plant extracts on N mineralization and the ratio DON: IN. The diversity of symbiotic root endophytes of the *Helotiales* in ericaceous plants and the grass *Deschampsia flexuosa* is discussed in **Chapter 4**. Studying the surprising variety in endophytic fungi from ericaceous roots resulted in several new fungal species and one of these, *Cryptosporiopsis rhizophila*, is described in **Chapter 5**. **Chapter 6** wants to give an insight in the ecological role of several different isolates of *C. rhizophila* in comparison with well-known ericoid mycorrhiza formers. In **Chapter 7**, we show the results of a competition experiment with *Calluna vulgaris* and *Deschampsia flexuosa* growing on different litter treatments with varying amounts of tannins.

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Chapter 2

The effect of nutrient supply and light intensity on tannins and mycorrhizal colonisation in Dutch heathland ecosystems

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Abstract

Increased atmospheric nitrogen deposition has shifted plant dominance from ericaceous plants to grass species. To elucidate the reduced competitiveness of heather, we tested the hypothesis that additions of nitrogen reduce the concentrations of phenolics and condensed tannins in ericaceous leaves and retard mycorrhizal colonisation in ericaceous plants. We also tested the negative effects of reduced light intensity on carbon-based secondary compounds and mycorrhizal colonisation in ericaceous plants.

We performed a field inventory at three heathland sites in the Netherlands varying in nutrient supply and light intensity. Leaves of ericaceous plants and grasses were collected and analysed for concentrations of tannins, phenolics, and nutrients. Similarly, we took root samples to record mycorrhizal colonisation and soil samples to measure the soil mineralisation. In addition, we conducted two-factorial experiments with *C. vulgaris* plants, in which we varied fertiliser and shade levels under greenhouse and field conditions.

The field inventory revealed that nitrogen addition and shading both negatively affected the concentration of total phenolics. The total phenolics and condensed tannin concentrations were positively correlated ($P<0.001$), but in the field experiment the condensed tannins were not significantly affected by the treatments. Our results provide the first evidence that the carbon nutrient balance can be used to predict the amount of total phenolics in the dwarf shrub *Calluna vulgaris*. In the field experiments, shading of plants resulted in significantly less mycorrhizal colonisation. Only in the greenhouse experiment did addition of nitrogen negatively affect mycorrhizal colonisation.

Our results imply that increased atmospheric nitrogen deposition can depress the tannin concentrations in ericaceous plants and the mycorrhizal colonisation in roots, thereby reducing the plants' competitiveness with respect to grasses. Additionally, if ericaceous plants are shaded by grasses that have become dominant due to increased nitrogen supply, these effects will be intensified and competitive replacement will be accelerated.

Key words: *Calluna vulgaris*, carbon-nutrient balance, dominant grasses, *Ericaceae*, ericoid mycorrhiza, nitrogen deposition.

Introduction

In the Netherlands, increased atmospheric nitrogen deposition has shifted plant dominance in heathlands from ericaceous plants towards grass species (Berendse and Aerts 1984, Aerts 1993, Berendse *et al.* 1994). It is estimated that around 35% of the heathland area has become grass-dominated (Bobbink *et al.*1998). The increased dominance of grasses has been attributed to their higher growth rate potential than that of dwarf shrubs (Berendse and Elberse 1990). Furthermore, outbreaks of heather beetle on nitrogen-fertilised vegetation have also strongly accelerated the expansion of grasses (Heil and Diemont 1983, Bobbink and Heil 1993, Power *et al.* 1998). Other factors (e.g. drought- and frost resistance of dwarf shrubs) can also be affected by changes in nitrogen deposition (Power *et al.* 1998).

An alternative hypothesis, proposed by Northup et al. (1995, 1998) is that the dominance of plant species in nutrient-poor ecosystems is the outcome of the production of high levels of carbon-based secondary compounds, e.g. tannins. Mineralisation has traditionally been considered to be the critical factor in nitrogen cycling, the decisive factor in nutrient-poor ecosystems (Chapin 1995, Northup *et al.* 1995). However, evidence is increasing that the production of large amounts of tannins and the concomitant utilisation of organic nitrogen enables plants in nutrient-poor ecosystems to short-cut the nitrogen cycle, so reducing their dependence on soil nitrogen mineralisation (Bradley *et al.* 2000, Fierer *et al.* 2001, Schimel *et al.* 1996, 1998). So, within these ecosystems plant species which are able to use organic nitrogen are expected to have a competitive advantage over plants which are not able to use these nitrogen sources.

Tannins can be subdivided into the hydrolysable tannins (sugar molecules esterified by a number of gallic acid moieties) and the condensed tannins, also referred to as proanthocyanidins (polymers of flavan-3-ols) (Harborne 1997). Both types of tannins can affect nutrient availability in the soil. They interact with proteins during the decomposition of litter material (Schimel *et al.* 1996, Fierer *et al.* 2001, Haslam 1998). Tannin–protein complexes are difficult to mineralise, and subsequently they determine the proportions of nitrogen released in dissolved organic and inorganic forms (NH_4^+, NO_3^-) (Handley 1961, Northup *et al.* 1995)

Ericaceous plants can use organic nitrogen more efficiently due to symbiosis with ericoid mycorrhizal fungi (Bending and Read 1997). Via the exudative enzymes, these fungi are able

to solubilise tannin-protein complexes (Bending and Read 1997). Sokolovski *et al.* (2002) showed that *Calluna vulgaris* (L.) Hull roots are able to use more organic nitrogen when colonised with these fungal symbionts. Therefore, at low mineralisation rates, ericaceous plants are thought to outcompete plant species that form arbuscular mycorrhiza, or non-mycorrhizal plants unable or less able to absorb organic nitrogen (Northup *et al.* 1998, Read *et al.* 2004).

Additions of nitrogen can have negative effects on the carbon-based secondary compounds in ericaceous leaves (Bryant *et al.* 1983). According to the carbon nutrient balance (CNB) hypothesis (Bryant *et al.* 1983), an increase in nitrogen concentration – as a result of increased nitrogen deposition – would reduce the production of carbon-based secondary compounds. In this case, the available carbon is invested in growth rather than in defence. It is well established that nitrogen addition increases nitrogen concentrations in heather (*Calluna vulgaris* (Berdowski and Siepel 1988, Iason *et al.* 1993, Duncan *et al.* 1994, Hartley *et al.* 1995, Carrol *et al.* 1999, Gordon *et al.* 1999) and other ericaceous plants (Mallik 1996, Prescott *et al.* 1993, Nordin *et al.* 1998). However, the expected decrease in total phenolics or condensed tannin levels due to nitrogen additions has not yet been empirically shown neither in the greenhouse nor in the field (Iason and Hester 1993, Iason *et al.* 1993, Hartley *et al.* 1995, Kerslake *et al.* 1998, Bradley *et al.* 2000, Alonso *et al.* 2001).

Increased levels of atmospheric nitrogen can also lead to decreases in ericoid mycorrhizal colonisation of ericaceous roots. With increased inorganic nitrogen supply, one would expect that the plant invests, less carbon to symbiosis with root-associated fungi and so ericaceous dwarf shrubs would hardly be able to compete with grasses. Two greenhouse experiments have shown that ammonium addition can negatively affect the extent of ericoid mycorrhizal colonisation in *C. vulgaris* roots (Mickel *et al*. 1991, Yesmin *et al*. 1996). However, there is no indication from field experiments that adding nitrogen reduces mycorrhizal colonisation (Lee *et al*. 1992, Caporn *et al*. 1995, Johannson 2000).

Light intensity can also influence the amounts of carbon-based secondary compounds and mycorrhizal colonisation in ericaceous plants. However, conflicting results have been published. Iason and Hester (1993) showed in a field experiment that shading reduced the concentration of total phenolics in *C. vulgaris*. In contrast, Hartley *et al.* (1995) found no effects of shading on the concentration of total phenolics and condensed tannins in *C. vulgaris*

plants. In non-ericaceous plants, shading usually reduces the levels of carbon-based secondary chemicals (Hartley *et al.* 1997, Hendriksson *et al.* 2003, Ruohamaki *et al.* 1996, Iason *et al.* 1996). However, the effect of shading on mycorrhizal colonisation in ericaceous plants has not been tested. We therefore decided to test the hypothesis that shaded plants will invest less in mycorrhizal symbiosis and mycorrhizal colonisation will be reduced.

The relation between phenolic levels in plant material and mycorrhizal colonisation has been hypothetical, but it was never thoroughly examined. In this research we asked which abiotic factors (nitrogen and light) would significantly influence the levels of both phenolic compounds and of mycorrhizal colonisation. Initially, we carried out a field inventory on several heathland plants, to study the natural variation of phenolics, condensed tannins and mycorrhizal colonisation in the field. We selected four heathland sites and four *Quercus-Vaccinium* forest sites in the centre and north of the Netherlands. The sites differed in soil nitrogen supply (soil subjected to recent sod removal vs. soil with a thick organic layer) and light intensity (shaded vs. non-shaded communities). Additionally, at one of the non-shaded sites we collected four ericaceous plant species and two dominant grass species, to analyse the interspecific variation in phenolic and condensed tannin levels. Subsequently, we conducted fertiliser experiments, with *C. vulgaris* in the greenhouse and under field conditions. In these experiments we also applied shading to test the negative feedback of reduced light intensity on phenolic compounds and mycorrhizal colonisation.

Material & Methods

Field inventory

Field sites and selected species

The field locations were selected at three sites in the Netherlands. In the north: Dwingelderveld National Park (A, 52º47'N, 6º25'E). In the centre: De Hoge Veluwe National Park (B, 52º4'N, 5º50'E) and Hoog Buurlo (C, 52º10'N, 5º54'E). Table 1 shows the collected plant species and relevant soil data for each site. To compare variation in plant chemistry as affected by soil nutrient supply, we selected two dwarf shrub vegetation sites (A and B) with *Calluna vulgaris* and *Deschampsia flexuosa* (L.) Trin. Plots in recently turf-stripped sites were chosen adjacent to older heathland sites. At the stripped sites the turf layer had been

removed up to four years prior to the moment of plant collection. The groundwater levels of the sites were similar. The effects of light intensity on *Vaccinium* and *Deschampsia* were investigated by comparing shaded (50% incident light) and non-shaded sites at Dwingelderveld National Park and Hoog Buurlo. In this comparison we included *Quercus robur* L. woodlands with a herbaceous layer dominated by the ericaceous dwarf shrubs *Vaccinium vitis-idaea* L. (A) or *V. myrtillus* L. (C). On these sites the grass *D. flexuosa* was present. The non-shaded site at Hoog Buurlo was a mixed heathland with *C. vulgaris*, *Erica tetralix* L., *V. myrtillus*, *V. vitis-idaea* L., *Molinia caerulea* (L.) Moench and *D. flexuosa*. The field sites were roughly 25 x 50 m. Within each site, five plots of 1 m^2 were chosen 10-25 m apart.

Leaf measurements

Plant material was collected on 5 and 6 June and 7 and 8 September 2000. Only the first year growth of green leaves was sampled. The leaf material was kept cool and brought to the laboratory where it was frozen immediately (-18ºC) until handled for analyses. Leaves were dried for two days at 38 ºC. The leaves were separated from stems and flowers by sieving (2 mm) before grinding. For extraction 20 ml of 50% (v/v) methanol was added to 0.19 g dry leaf material. The mixture was covered and placed in a water bath (75ºC) for 1 h. The sample was then filtered through a glass filter and the extract adjusted to 50 ml with 50% (v/v) methanol. Total phenolics were determined following the Folin-Ciocalteu method (Waterman and Mole 1994). Condensed tannins were analysed following the butanol-HCl method of Porter, Hrstich and Chan (1986). All analyses were performed in duplicate. Given the problems and complexities of applying an appropriate standard for the proanthocyanidin method (Waterman and Mole 1994), the data are presented as final absorbance at 550 nm. Another portion of dried leaves (70^{o}C) was pulverised and C and N concentrations were measured using an elemental analyser (Fisons Instruments, EA 1108).

Soil measurements

Soil cores (10 cm deep, 5 cm diameter) were taken on 5 and 6 June 2000 and stored at 4º C overnight. After removing any coarse roots and stones, the extractable NH_4-N and NO_3-N were determined in 10 g fresh soil extracted in 25 ml 1M KCl. The extracts were filtered through filter paper (Schleicher and Schüll no. 589^3). Concentrations of the extractable ions in the soil were calculated from the concentrations in the extract using the soil water content. The soil pH was also measured in the same soil extract.

To estimate the net mineralisation rate, a subsample of 10 g soil was incubated for 6 weeks at 20ºC and then the extractable NH_4-N and NO_3-N were measured. Net mineralisation rates were calculated from the difference between the amount of NH_4^+ and NO_3^- before and after incubation. To measure the soil water content a subsample of 5 g soil was dried (105ºC) overnight. Organic matter content was determined after combustion at 550ºC. The C and N concentrations were measured using an elemental analyser (Fisons Instruments, EA 1108).

Collection of plant roots and mycorrhizal analyses

For each species, roots of one individual plant were collected in each plot on 7 and 8 September 2000 with a soil auger of 20 cm (10 cm diam). The roots were kept moist in a plastic bag (25 x 10 cm). The soil was removed by washing the roots over a 2 mm sieve. In the laboratory, root tips were further cleaned from organic material with forceps, and stored in 50% ethanol. They were then stained in 0.2% solution of trypan blue in lactic acid: glycerol: water (3.25:3:4 by vol.) and transferred to a storage solution of lactic acid: glycerol: water (1:2:1 by vol.). From each root, 30 healthy root tips were then randomly selected, mounted on a microscopic slide and, using a light microscope, were examined at 40× magnification for the presence of mycorrhizal structures. Colonisation of cortical cells was estimated as percentage of colonized cells in the superficial cell layers in 1 cm root.

Greenhouse experiment

From May 2001 to March 2002 we conducted a two-factorial experiment with shading and fertilisation in a greenhouse with controlled climatic conditions (light/dark: 14/10 h., light intensity 50 $W.m^{-2}$, temperature day/night: 20 ºC/ 15ºC, 70% R.H.). The treatments were: no shade or fertiliser (S-F-), fertiliser (S-F+), shade (S+F-), shade and fertiliser (S+F+). Each treatment was replicated five times. Twenty plastic pots (14 cm diam) were filled with 2.5 kg sand (sand mixed with organic-rich soil, 5:1, v/v). We added one part organic-rich soil to provide the control treatments with a basic level of nutrients. *C. vulgaris* seedlings (approx. 2.5 cm tall) were collected from the De Hoge Veluwe National Park and placed three in each pot. Given the results of the field study, we assumed that all roots from the collected *C. vulgaris* seedlings were colonised by mycorrhizal fungi. The shade treatment involved excluding 50% of incident light using shade netting around each individual pot. The pots were spaced widely to avoid the shade netting constructions shading other plants. Fertilised plants received amounts equivalent to 75 kg N, 25 kg P and 50 kg K ha^{-1} yr^{-1}. The fertiliser was

applied in June and August. In this experiment, we chose a compound fertiliser, to prevent phosphate limitations. The pots were placed randomly. The plants were watered regularly to keep the soil moisture at 60 % of water saturation; for this purpose, the pots were weighed twice per week. After 12 months the above-ground plant parts were clipped off at soil level and dried at 38ºC, to measure the dry weight. The roots were gently removed from the soil, then washed to remove any adhering soil and also dried at 38ºC. Total phenolics, condensed tannins, nitrogen and carbon content and mycorrhizal colonisation were measured as described in the previous section. The amounts of inorganic nitrogen and pH KCl of the soil were determined.

Field experiment

At a site in De Hoge Veluwe National Park a field experiment was carried out from September 2001 to March 2003. The field site (50 x 60 m) was a heathland dominated by *C. vulgaris.* Other plant species were *E. tetralix*, *M. caerulea* and *D. flexuosa*. The field treatments were similar to the treatments in the greenhouse experiment. Each treatment was replicated at five sites in this area, following a randomised block design. Within a site, individual plots of 1 m^2 were chosen 5-10 m apart and the sites were spaced at 40-50 m. Each plot was fenced with fine-meshed wire to exclude large herbivores. To reduce the light by 50% in the shade treatments, shade nets were put around and above the enclosures (1 m height). The fertilised plots received 50 kg N ha^{-1} yr^{-1} (NH_4NO_3) in a single treatment. Initial amounts of soil nitrogen were measured before the enclosures were erected and again at harvest. Inorganic nitrogen was measured as described earlier and the total dissolved nitrogen (DON + inorganic N) was determined conductimetrically after persulfate oxidation of the extract (Yu *et al.*, 1993). DON was calculated by subtracting inorganic nitrogen from the total dissolved nitrogen. At the end of the experimental period, the first year's growth of green leaves were harvested. Per plot we collected one root sample. Shoots and roots were analysed as described for the field inventory.

Statistical analyses

Field inventory

The effects of sod removal or light intensity on soil chemistry parameters was analysed using ANOVA with sod removal and site, or light intensity and site as the respective fixed factors

(P<0.05). The effects of sod removal on plant characteristics were analysed using ANOVA with sod removal and site as the fixed factors (P<0.05). The effects of light intensity on plant characteristics was analysed by ANOVA with shade and plant species as the fixed factors (P<0.05). If the assumption of heterogeneity of variance was violated, the data were log-transformed. To compare total phenolic levels between plants under different treatments at one site, one-way ANOVA was carried out followed by the Tukey post hoc test (P<0.05).

Field and greenhouse experiment

The results of the greenhouse and field experiments were analysed using two-way ANOVA with shade and fertiliser as fixed factors (P<0.05).

Results

Field inventory

On sites where the turf layer had recently been stripped, the net mineralisation of the soil was lower than on older heathland sites with thicker organic layers (Table 1). No differences in net mineralisation were found between the shaded and non-shaded sites. The sites with *V. myrtillus* showed higher mineralisation rates compared to the sites with *V. vitis-idaea* (Table 1). The concentration of total phenolics in the plant ranged from 50-437 mg tannic acid equivalents /g dry weight (Fig.1A-C). Differences between the amounts of phenolics were related to plant species, growth conditions and site (Table 2). Ericaceous leaves contained larger amounts of total phenolics than the grasses (Fig. 1C, P<0.001). There was also considerable variation among the ericaceous species. At the species-rich site at Hoog Buurlo, *V. myrtillus* leaves contained the highest amounts of total phenolics, *V. vitis-idaea* and *C. vulgaris* were intermediate, while levels were lowest in *E. tetralix* (Fig. 1C, P<0.05). Soil nutrient supply significantly decreased the concentration of total phenolics and condensed tannins. Leaves of *C. vulgaris* showed higher levels of total phenolics and condensed tannins when grown on humus-poor soils compared to humus-rich soils (Fig. 1A, Table 2). Shade also decreased the levels of phenolics and tannins (Table 2). Plants growing under shaded conditions produced remarkably less total phenolics and condensed tannins, not only in the ericaceous plants, but also in the grass *D. flexuosa* (Fig. 1 B,E, Table 2). There was a strong positive relationship between the amount of total phenolics and the amount of condensed

Table 1. Field inventory overview of the investigated site pairs contrasting with respect to soil nutrients (A) or light intensity (B), showing growth conditions, the plant species collected at each site, and related soil nutrient factors (mean ± SE, d.f. = 4). Sites: A = Dwingelderveld National Park; B= De Hoge Veluwe National Park; C = Hoog Buurlo. Plant species: Cv = *Calluna vulgaris*; Vvi = *Vaccinium vitis-idaea*; Vm = *V. myrtillus*. Significance levels for the main effects and the interaction between them are given. **P*<0.05, ***P*<0.01, ****P*<0.001, and NS = non-significant.

A - Soil nutrients					Sod removed	Site	Interaction
Site	A	A	B	B			
Growth conditions	Sod removal (N-)	Thick organic layer (N+)	Sod removal (N-)	Thick organic layer (N+)			
Plant species	Cv	Cv	Cv	Cv			
N mineralisation (mg N ha^{-1} yr^{-1})	25 (21)	46 (31)	8 (2)	120 (42)	***	*	**
C:N soil	34.5 (1.9)	28.7 (0.6)	24.6 (0.4)	25.9 (0.6)	NS	***	**
pH-KCl	3.1 (0.1)	2.9 (0.0)	3.4 (0.1)	2.9 (0.0)	***	NS	NS
Org. matter (%)	0.10 (0.0)	0.13 (0.02)	0.06 (0.0)	0.12 (0.01)	*	NS	NS
B – Light intensity					**Shade**	**Site**	**Interaction**
Site	A	A	C	C			
Growth conditions	No shade (S-)	Shade (S+)	No shade (S-)	Shade (S+)			
Plant species	Vvi	Vvi	Vm	Vm			
N mineralisation (mg N ha^{-1} yr^{-1})	52 (72)	34 (46)	107 (63)	159 (60)	NS	**	NS
C:N soil	25.9 (1.5)	24.3 (0.7)	25.6 (0.3)	20.1 (0.3)	***	*	*
pH-KCl	2.8 (0.2)	2.9 (0.1)	2.8 (0.0)	2.9 (0.0)	NS	NS	NS
Org. matter (%)	0.17 (0.02)	0.11 (0.04)	0.16 (0.01)	0.08 (0.01)	***	NS	NS

Table 2. Analysis of variation in plant chemistry: amount of total phenolics, condensed tannins, mycorrhizal colonisation, concentration of nitrogen, concentration of carbon, and leaf C:N ratio. F values and significance levels for the main effects of the factors sod removal, site, shading and plant species, and the interaction between them are given: **P*<0.05, ***P*<0.01 and ****P*<0.001.

	Total phenolics	Condensed tannins	Mycorrhizal colonization	% C	% N	C:N ratio
Soil nutrients:						
Sod removal	14.0**	16.6***	0.0	0.2	230.8***	314.2***
Site	7.2*	2.8	1.9	16.6***	31.9***	23.2***
Interaction	0.6	0.2	12.9**	0.5	2.5	0.0
Light intensity:						
Shading	980.3***	217.5***	16.1***	3.8	211.7***	225.8***
Plant species	1016.1***	123.5***	8.6**	80.8***	153.9***	300.2***
Interaction	461.1***	129.5***	2.1	6.5**	9.8***	0.1

tannins (Table 2, r^2= 0.72, *P*<0.001), especially in the ericaceous species. In grasses there were negligible amounts of condensed tannins. The nitrogen concentration and C:N ratio in plant leaves depended on plant species, growth condition and site (Table 2). The level of mycorrhizal colonisation depended on growth conditions and plant species (Fig. 2, Table 2). We did not find a significant effect of sod removal on the level of mycorrhizal colonisation at the *C. vulgaris* sites. In contrast, shading had a clear negative effect on mycorrizal colonisation in roots of *Vaccinium* plants. Shading also reduced mycorrhizal colonisation in grass roots, but to a lesser extent compared to the ericaceous plants.

Greenhouse experiment

Total biomass of the *C. vulgaris* plants was affected by both shade and fertiliser treatments (Table 3). With nitrogen fertilisation, the plants produced more shoots and roots. Shading induced a reduction of the root biomass: the related shoot:root ratios were increased by more than 100%. Flower production was increased by fertilisation and decreased by shade. Also the amount of total phenolics decreased significantly when plants were shaded. Whereas, shading plus fertilisation resulted in the lowest amounts of total phenolics. As expected, the C:N ratios in the leaves decreased due to fertilisation and shading. In this experiment, fertilisation had a significant negative effect on the amount of mycorrhizal colonisation in the *C. vulgaris* roots. The mycorrhizal colonisation was positively related to the amount of inorganic nitrogen (linear regression, r^2= 0.34, *P*=0.007), but negatively related to total biomass (linear regression, r^2= 0.23, *P*=0.03) and root biomass (linear regression, r^2= 0.29, *P*=0.02).

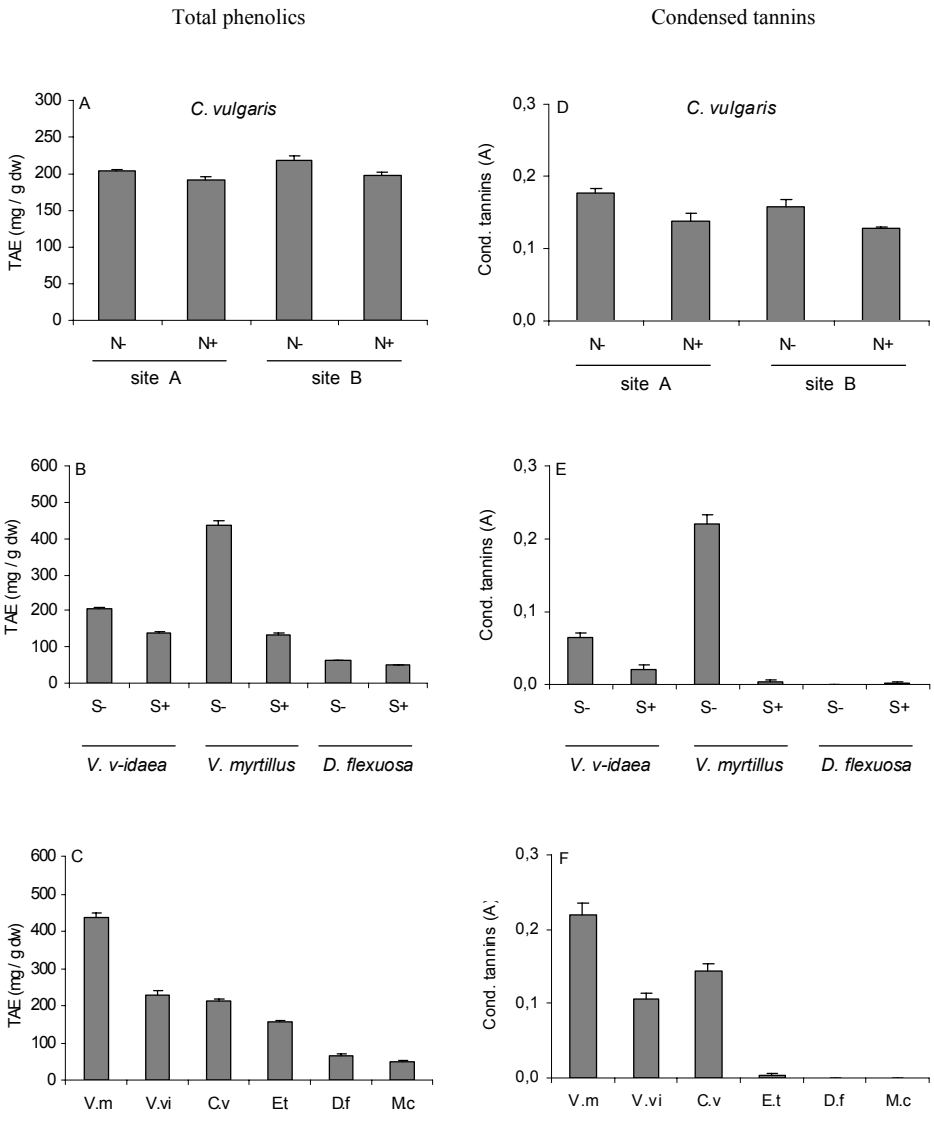

Figure 1. Results of the field inventory. Mean values of total phenolics (TAE = tannic acid eq. mg / g dw), A-C and condensed tannins (absorption value), D-F in ericaceous plants and grasses at contrasting growth conditions and sites. A & D. Nutrient contrasts: N- = sod removed, N+ = thick organic layer. Site A = Dwingelderveld National Park; site B = De Hoge Veluwe National Park. B & E. Light intensity contrasts within *Vaccinium vitis-idaea* (site A), *V. myrtillus* (site C) and *D. flexuosa* (site C): S+ = shaded, S- = non-shaded. C & F, non-shaded site at Hoog Buurlo (site C) with four ericaceous plants and two dominant grasses: C.v, *Calluna vulgaris*; E.t, *Erica tetralix*; D.f, *Deschampsia flexuosa*; M.c, *Molinia caerulea*; V.vi, *Vaccinium vitis-idaea*; V.m, *V. myrtillus*. Data are ± SE (*n*=5).

Field experiment

In the field experiment the amount of total phenolics and C:N ratio in ericaceous plants were negatively affected by both shading and fertilisation (Table 5). In contrast, the amount of condensed tannins was not affected. As expected, the fertilised plants had higher nitrogen concentrations than unfertilised plants. Mycorrhizal colonisation was only affected by shading. The shade treatment increased the amount of inorganic nitrogen and DON in the soil, but reduced the ratio DON: inorganic nitrogen. Mycorrhizal colonisation was not related to any of the measured plant or soil parameters. The amount of inorganic nitrogen was negatively correlated with the amount of total phenolics (linear regression, $r^2 = 0.29$, P=0.02) and condensed tannins in the plants (linear regression, $r^2 = 0.20$, P=0.06).

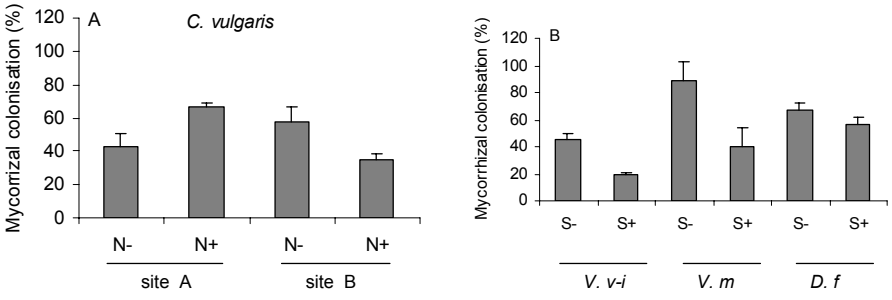

Figure 2. Mycorrhizal colonisation (%) in roots of *Calluna vulgaris*, *Vaccinium vitis-idaea*, *V. myrtillus* and *Deschampsia flexuosa* under contrasting growth conditions. A, nutrient contrasts: N- = sod removed, N+ = thick organic layer. Site A = Dwingelderveld National Park; site B = De Hoge Veluwe National Park. B, light intensity contrasts within *Vaccinium vitis-idaea* (site A), *V. myrtillus* (site C) and *D. flexuosa* (site C): S+ = shaded, S- = non-shaded. Data are means ± SE (n=5).

Table 3. Results of the greenhouse experiment with *Calluna vulgaris* seedlings. Average values (± SE) are shown for the plant and soil characteristics. Significance levels for the main effects and the interaction between them are given. **P*<0.05, ***P*<0.01, ****P*<0.001, and NS= non-significant. S+ = with shade, S- = no shade; F+ = with fertiliser, F- = no fertiliser.

	S-F-	S-F+	S+F-	S+F+	S	F	S x F
Plant:							
Total biomass (g dw)	2.24 (0.07)	5.68 (0.40)	2.05 (0.31)	3.82 (0.72)	*	***	NS
Shoots (g dw)	1.48 (0.10)	3.54 (0.25)	1.56 (0.28)	2.51 (0.61)	NS	***	NS
Roots (g dw)	0.76 (0.18)	1.71 (0.30)	0.33 (0.08)	0.42 (0.03)	***	*	*
Shoot:root ratio	2.31 (0.47)	2.47 (0.61)	5.06 (0.44)	5.99 (1.30)	***	NS	NS
Flowers (g dw)	0.25 (0.04)	0.99 (0.11)	0.21 (0.04)	0.34 (0.05)	***	***	***
Total phenolics (mg tae/ g dw)	158 (4)	180 (5)	165 (8)	131 (6)	**	NS	***
%C	47.60 (0.36)	48.44 (0.19)	49.07 (1.15)	48.11 (0.36)	NS	NS	NS
%N	1.17 (0.05)	1.30 (0.04)	1.57 (0.26)	1.76 (0.06)	**	NS	NS
C: N ratio	41.0 (1.9)	37.3 (1.1)	33.6 (3.6)	27.4 (0.9)	***	*	NS
Total N shoots (mg)	26.0 (1.4)	74.0 (5.2)	31.0 (4.3)	66.0 (1.1)	NS	***	NS
Mycorrhizal colonisation (%)	27.0 (4.4)	14.4 (1.2)	23.3 (2.5)	22.6 (2.6)	NS	*	NS
Soil:							
Inorganic N (mg N/ kg dw)	3.66 (0.16)	0.52 (0.09)	2.72 (0.48)	1.61 (0.27)	NS	***	**
pH KCl	7.12 (0.04)	7.24 (0.04)	7.16 (0.05)	7.22 (0.02)	NS	**	NS

Table 4. Results of the field experiment at De HogeVeluwe National Park with *Calluna vulgaris* plants. Average values (± 1 SE) are shown for the plant and soil characteristics. Significance levels for the main effects between them are given. **P*<0.05, ***P*<0.01, ****P*<0.001, and NS= non-significant. There were no significant interactions between the effects of the two treatments. S+ = with shade, S- = no shade; F+ = with fertiliser, F- = no fertiliser.

	S-F-	S-F+	S+F-	S+F+	S	F
Plant:						
Total phenolics (mg tae/ g dw)	707 (86)	462 (56)	443 (51)	383 (52)	*	*
Cond. tannins (A_{550}/ 0.19 g dw)	0.042 (0.006)	0.021 (0.007)	0.021 (0.008)	0.016 (0.008)	NS	NS
% C	52.04 (0.47)	52.62 (0.27)	52.04 (0.23)	51.85 (0.99)	NS	NS
% N	1.61 (0.06)	1.83 (0.08)	1.80 (0.07)	1.99 (0.13)	NS	*
C: N ratio	32.49 (1.09)	28.94 (1.11)	29.03 (0.95)	26.32 (1.41)	*	*
Mycorrhizal colonisation (%)	20.92 (0.67)	20.12 (0.45)	11.36 (0.36)	10.70 (0.04)	*	NS
Soil:						
Inorganic N (mg N/ kg dw)	5.78 (1.10)	8.25 (1.23)	13.46 (1.18)	14.35 (1.61)	***	NS
DON (mg N/ kg dw)	19.34 (1.09)	23.39 (2.04)	27.21 (1.61)	27.69 (2.21)	**	NS
DON: inorg. N ratio	3.94 (0.90)	3.03 (0.34)	2.04 (0.08)	1.97 (0.09)	**	NS
pH KCl	2.83 (0.03)	2.84 (0.03)	2.84 (0.02)	2.84 (0.03)	NS	NS
pH H_2O	3.79 (0.03)	3.85 (0.05)	3.79 (0.07)	3.88 (0.08)	NS	NS

Discussion

Responses of mycorrhizal colonisation to N supply and light intensity

Our study shows that nitrogen supply negatively affects mycorrhizal colonisation of *C. vulgaris* roots under greenhouse conditions. This finding is in line with the results reported by Yesmin *et al.* (1996) and Mickel *et al.* (1991). It suggests that effects of nitrogen deposition are detrimental for the mycorrhizal colonisation of the dwarf shrub roots – and related to this – for the capacity of these plants to use the soluble organic nitrogen. However, in the field experiment we found no detrimental effects of nutrient addition on colonisation rates. This is in agreement with the results of Lee *et al.* (1992), Caporn *et al.* (1995) and Johansson (2000), who also found no effects of nutrient addition on mycorrhizal colonisation in field experiments. Apparently, the soil litter layer in the field containing large amounts of organic acids stimulate more microorganisms than the sand medium used in the greenhouse experiment with alkaline pH (Table 1 and 3). It seems that additional NH_4^+- N inputs become immobilised in the heath mor layer (Adams 1986, Whitehead *et al.* 1997, Kristensen and Hendriksen 1998). Furthermore, the difference between the response to nutrient supply in terms of the amount of mycorrhizal colonisation of heather roots in the field and greenhouse experiments might be explained by the fact that only in the greenhouse was it possible to establish strong nitrogen-limited conditions.

In the Dutch heathlands, nitrogen deposition has increased soil nutrient supply significantly and heather plants are now less nitrogen-limited than several decades ago (Bobbink *et al.* 1998). The nitrogen concentrations in the plant can be used as estimators of the atmospheric nitrogen deposition (Hicks *et al.* 2000). In the control plots of our field experiment, the nitrogen concentration in the leaves of *C. vulgaris* was, on average 1.61, compared with 1.17 in the greenhouse (Tables 3 and 4). Therefore it is not surprising that the long-term effects of nitrogen supply on mycorrhizal colonisation under our field conditions were less pronounced than the effects observed in the greenhouse. We conclude that the negative effects of increased atmospheric nitrogen on mycorrhizal colonisation of heathland plants still remain hypothetical. More experiments under field conditions are needed to investigate the effects of increased atmospheric nitrogen on the reduction of mycorrhizal colonisation and the possible consequences for organic nitrogen uptake.

In the field inventory, we found in general a positive correlation between the amount

of mycorrhizal colonisation in ericaceous plants and the concentration of total phenolics in the leaves. In contrast, neither of the experiments with *C. vulgaris* showed such a relationship. Although Sokolovski *et al.* (2002) also showed an increased organic nitrogen uptake by *C. vulgaris* root cells colonised by a mycorrhizal symbiont, the actual importance of the role of mycorrhiza for amino acid uptake in the field is still being debated (Persson and Näsholm 2001).

Our finding that shading reduced the amount of mycorrhizal colonisation in ericaceous plants under field conditions seems to be consistent with predictions of the carbon nutrient balance hypothesis and the protein competition model (Bryant *et al.* 1983, Jones and Hartley 1999). Simard *et al.* (2002) report that mycorrhizal fungi may receive carbon (e.g. sugars) amounting to 15-30% of the net photosynthate of their host plants. Also other studies (Bryant *et al.* 1983, Smith and Read 1997, Jones and Hartley 1999) have reported that shading reduced the amount of photosynthesis assimilates and therefore limited the amount of carbon translocated to the mycorrhizal symbiosis.

Responses of tannins to N supply and light intensity

Our results show that the carbon nutrient balance hypothesis can be very useful when predicting the amount of total phenolics in *C. vulgaris*. Both experiments confirmed our hypothesis that shade negatively affects the foliar concentration of total phenolics – a result previously found by Iason and Hester (1993). Ours is the first report that the addition of fertiliser results in lower concentrations of total phenolics under field conditions. The reason for the failure in previous field experiments with *C. vulgaris* to detect a significant fertiliser effect on the foliar content of total phenolics (Iason and Hester 1993, Iason *et al.* 1993, Hartley *et al.* 1995, Alonso *et al.* 2001) is probably that these experiments were too short (most lasted less than one year). We found that the amount of condensed tannins was not significantly affected by the addition of fertiliser. This is in accordance with the results of Iason and Hester (1993) and Bradley *et al.* (2000). Shading also had no detectable effect on the tannin levels. Concentrations of tannins in *C. vulgaris* plants do not seem to vary strongly.

We conclude from our field study that there is much natural variation in total phenolic and condensed tannin contents within and among ericaceous plants and that this depends strongly on site characteristics (light, soil nutrients) and plant species. The deciduous species, *V. myrtillus* seemed to have more plastic leaf characteristics than the evergreen species. The variation within and between the two grass species was remarkably smaller. The dominant

grass species, which have higher growth rates than the dwarf shrubs, showed smaller concentrations of secondary plant compounds. So, it is not surprising that there is a strong negative correlation between the foliar concentration of nitrogen and the concentrations of total phenolics and condensed tannins among plant species. The protein competition model (Jones and Hartley 1999) also suggests that the regulation of protein and phenolic synthesis are tightly linked due to the use of the same precursor phenylalanine. Therefore, plant cells do not appear to be capable of simultaneously synthesising proteins and phenolics at the same rate (Haukioja *et al.* 1998).

Consequences for the competition between dwarf shrubs and grasses

Berendse and Elberse (1990) hypothesized that ericaceous plants growing in nutrient-poor ecosystems have a competitive advantage over grass species because they are rich in carbon-based secondary compounds that prolong their life span and reduce nitrogen losses. We formulate the additional hypothesis that the symbiosis of ericaceous plants with their ericoid mycorrhizal fungi that degrade protein-phenolic complexes in the soil enables them to use organic nitrogen sources not available to other plants like grasses with their arbuscular mycorrhizal fungi (Berendse and Elberse 1990, Northup *et al.* 1995, Hättenschwiler and Vitousek 2000, Hodge *et al.* 2001, Aerts 2002).

Increased nitrogen deposition can seriously hamper the competitive advantage of ericaceous plants, not only by increasing the nitrogen availability in the soil, but also as this study and others have shown, by enhancing the nitrogen concentration in ericaceous litter (e.g. Hartley *et al.* 1995). Thereby the carbon: nitrogen ratio is reduced in the litter, which accelerates its decomposition and finally accelerates the mineralisation of the soil nitrogen (Berendse *et al.* 1994, Bret-Harte *et al.* 2004). When nutrient-poor soils become enriched with nitrogen, the grasses have a competitive advantage over ericaceous plants as they are able to benefit faster from the increased nitrogen supplies (Berendse *et al.* 1994). For example, the competition experiment of Berendse and Aerts (1984) showed that the dwarf shrub *Erica tetralix* was only able to outcompete *Molinia cearulea* at the non-fertilised, nutrient-poor sites, while *Molinia* replaced *Erica* after nutrient addition.

Secondly, our study revealed that the levels of total phenolics in the ericaceous plants can decrease in response to nitrogen additions, thereby enhancing the degradation of the litter and accelerating N mineralisation (Schofield *et al.* 1998). Due to the reduced concentrations of phenolic compounds in the litter, the inorganic forms of soil nitrogen can increase relative

to the organic forms (Northup *et al.* 1995, 1998). The field experiment with *C. vulgaris* showed that organic nitrogen was the most important nitrogen source, exceeding the amount of inorganic nitrogen by approximately 2-4 times (Table 4).

Under nitrogen-poor condition the symbiosis of ericaceous plants with their mycorrhizal fungi, which are able to use complex organic nitrogen sources, supposedly gives them an advantage. So, when the organic nitrogen sources become relatively less important due to increased atmospheric nitrogen, the grasses - which can benefit more from the inorganic nitrogen sources - will outcompete the heathland shrubs. Additionally, our data from the greenhouse experiment show that increased nitrogen addition can reduce mycorrhizal colonisation, which can result in less organic nitrogen being available to the ericaceous species (Sokolovski *et al.* 2002).

Finally, our data unequivocally show a positive feedback in the competition between ericaceous plants and grasses as a result of reduced light intensities, when nitrogen inputs increase. Shaded heathland plants not only produce less phenolics and tannins and higher nitrogen concentrations in the leaves, but also show reduced levels of mycorrhizal colonisation. In this way, shading reduces the competitive ability of heathland plants by directly reducing their nitrogen uptake capacity so that the expansion of the grasses at the cost of the dwarf shrubs will be strongly accelerated.

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Chapter 3

Phenolic compounds regulate nitrogen cycling: results from an incubation experiment

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Abstract

Polyphenolic compounds are often assumed to influence nutrient cycling, but their regulatory role is still being debated. In this study we wanted to test the hypothesis that litter with high amounts of tannins decrease the amounts of inorganic nitrogen in incubated soils, and concomitantly increase the amounts of dissolved organic nitrogen. We performed an incubation experiment with different types of shrub litter (*Calluna vulgaris, Vaccinium vitis-idaea* and *V. myrtillus*), grass litter (*Deschampsia flexuosa*), and also tested plant extracts, pure phenolics and pure tannic acid. With intervals of two weeks, the inorganic (IN) and dissolved organic nitrogen (DON) was measured during 16 weeks. Most pure phenolics showed no effects on the amount of inorganic nitrogen or the ratio DON:IN. Adding tannic acid clearly inhibited the production of inorganic nitrogen; the amounts of DON also tended to decrease compared to the nitrogen treatments without tannic acid, but more slowly. Extracts of the shrub litter had no effect on the amount of inorganic nitrogen compared to the control with water. We conclude that the effects of pure phenolics have been overestimated, but low molecular weight phenolics and condensed tannins can decrease the amounts of inorganic nitrogen compounds and concomitantly increase the amounts of dissolved organic nitrogen compounds in soils incubated with litters characterized by C:N ratios above 30.

Keywords: nitrogen cycling, mineralization, tannin, phenolics, *Ericaceae, Deschampsia flexuosa*.

Introduction

Thanks to their protein-binding capacity, polyphenolic compounds are able to influence nutrient cycling by interfering with several processes of mineralization and immobilization (Schimel *et al*. 1996), but their precise regulatory role is still being debated (Northup 1995, 1998; Schimel *et al*. 1998, Hättenschwiler & Vitousek 2000). When *Pinus sylvestris* litter was incubated, Northup (1995) found that the concentration of total phenolics was strongly negatively correlated with the amount of inorganic nitrogen (NH_4^+, NO_3^-) produced, whereas the amount of dissolved organic nitrogen (DON) was positively correlated with the concentration of total phenolics. A high ratio of DON to inorganic N was considered to have several benefits: to reduce nitrogen losses by leaching and denitrification, to short-cut the mineralization and, above all, to impart a competitive advantage to plants on infertile soils, enabling those plants with mycorrhiza to compete more strongly for organic nitrogen.

The few experiments done to test the effects of phenolic compounds on soil mineralization rates have yielded conflicting results. In soil microcosms with balsam fir foliage, Baldwin *et al*. (1983) showed specific inhibition of nitrification by low molecular weight phenolics (LMP) and condensed tannins (CT), but found hardly any influence on net ammonification. Yet Schimel *et al.* (1996) found no effects of LMP and CT on nitrification. Instead, they showed that condensed tannins inhibited respiration, whereas LMP stimulated it, leading both to reduced net nitrogen mineralization.

It is also unclear how the amount of polyphenolic compounds found in plant litter is correlated with the amount of dissolved organic nitrogen in the soil. Inderjit & Mallik (1997) found that additions of protocatechuic, *p*-coumaric, *p*-hydroxybenzoic or ferulic acids or a mixture of these phenolic compounds (10^{-4} M) increased the amount of the total phenolic content of humo-ferric podzol soils, but levels of dissolved organic nitrogen remained equal in comparison to the control treatment. Although phenolic compounds were proven to inhibit the production of inorganic nitrogen it remains unclear to what extent these compounds in litter increase the amount of DON in the soil. DON has been identified as an important pool in soil–plant nitrogen cycling in forest systems (Qualls & Haines 1991), arctic tundra (Atkin 1996) and in subtropical wet heathland (Schmidt & Stewart 1997).

In studies with litter concentrations of phenolics and nitrogen are frequently inversely correlated, so that the effect of high phenolic content are hardly to be differentiated from the effect of low nitrogen concentrations. To circumvent this problem, in some studies plant extracts were used. Pure phenolic compounds have also been used. Their inhibitory effects on nitrogen cycling have often been overestimated, because the doses used were not representative for natural, nutrient-poor soils (Inderjit & Mallik 1997).

In this study we set out to test the effects of pure phenolic compounds at concentrations found in natural heathland soils (Jalal & Read 1983), water extracts of fresh plant material and intact plant material (litter) on the amount of inorganic nitrogen, dissolved organic nitrogen (DON) and the ratio of DON to inorganic N. We incubated the litter, extracts or phenolics mixed with peat soil in sealed bags. The treatments were compared with controls.

Materials & Methods

Design of the experiment

As incubation units we used audiothene bags, 17.5 x 8 cm (excl. seal area), thickness 100 µm, which are permeable to CO_2 and O_2. Peat (Fixet Retailgroup, Apeldoorn) was used as the soil medium, since it has a low pH (pH_{KCl} = 4), which enhances the effects of phenols on soil processes. Each bag contained 35 g dry peat; after mixing with the amendments, the peat was wetted to 60% of water saturation, in order to maximise microbial activity. Pure phenolics were added at a concentration of 100 µM, which is the concentration which has been found to occur in natural heathland soils (Jalal & Read, 1983). The compounds were benzoic acid (Aldrich 10.947-9), *p*-coumaric acid (Aldrich H2.320-1), ferulic acid (Aldrich 12.870-8), *p*-hydroxybenzoic acid (Aldrich H2.005-9), protocatechuic acid (Aldrich D10.980-0), salicylic acid (Merck 1.00631.1000), vanillic acid (Aldrich H3.600-1), and a mixture of these compounds. The phenolics were dissolved in acetone; a control treatment with acetone alone was included to correct for the effect of acetone. Also a treatment with glucose was added to compensate for the C-addition in the phenolic treatments.

Table 1. Main characteristics of litter and leaf extracts of *Calluna vulgaris, Vaccinium myrtillus, V. vitis-idaea* and *Deschampsia flexuosa.*

	total polyphenolics (TAE mg/g dw)		condensed tannins (A)	N (mg/g dw)		C:N ratio
	litter	leaf extract	litter	litter	leaf extract	litter
V. myrtillus	444	288	0.24	20	7	24
V. vitis-idaea	198	175	0.10	14	7	36
C. vulgaris	382	115	0.13	16	1	30
D. flexuosa	26	11	0.00	20	188	22

Two inorganic treatments (ammonium chloride and nitrate) and one organic nitrogen treatment (glutamine, Aldrich G320-2) were applied; all at a concentration of 0.01 mM. We expected these treatments to stimulate mineralisation rates as all compounds are easily accessable nitrogen sources for micro-organisms**.** To test the effect of pure tannic acid on nutrient cycling, we added a treatment in which each of the nitrogen sources was mixed with 50 mg / g dw soil tannic acid (Aldrich 21.671-2).

The plant extracts were freshly prepared from green leaves of *Calluna vulgaris, Vaccinium vitis-idaea, V. myrtillus* and *Deschampsia flexuosa.* The green leaves (500 g fresh weight) were incubated (placed on the shaker 150 rev min^{-1}) with 500 ml tap water overnight at room temperature in closed plastic litre bottles. The next day the liquid was filtered through Schleicher & Schüll filter paper (589^3) and 11.5 ml of the filtrate was added to 35 g dry weight peat. In the control tap water was used. The litter treatments consisted of adding leaves of *C. vulgaris, V. vitis-idaea, V. myrtillus* and *D. flexuosa.* Green leaves (1 g dw) were sieved (1 mm mesh size) and added to 35 g dry weight peat. Table 1 contains the main characteristics of the different litter types. After the peat had been mixed with the amendments (solution, plant extract or litter) the bags were sealed and placed in an incubator at 20ºC in the dark. Each treatment had four replicates. Soil nitrogen and pH were measured after: 2, 4, 6, 8 and 16 weeks.

Measurements

The NH_4-N and NO_3-N in the filtered plant extracts was measured with a segment flow analyser (SANplus system). Extractable NH_4-N and NO_3-N in the soil was determined in 10 g fresh soil and extracted in 25 ml 1 M KCl. The extracts were filtered through Schleicher & Schüll filter paper 589[3] and analysed with a segment flow analyser (SANplus system). The soil concentrations of the extractable ions were calculated from the concentrations in the extract and corrected for soil water content. The pH of the soil was measured in the same soil extract. Total dissolved nitrogen(DON + inorganic N) was determined conductimetrically after persulfate oxidation of the extract (Yu *et al.,* 1993). DON was calculated by subtracting inorganic nitrogen from the total dissolved nitrogen. To measure the soil water content a subsample of 5 g soil was dried (105ºC) overnight. Organic matter was determined after combustion at 550ºC. After 16 weeks the remaining soil was dried at 38 ^{o}C, pulverised and the C and N concentrations were measured using an elemental analyser (Fisions Instruments, EA 1108).

Statistical analyses

The effect of phenolics was analysed using repeated measures ANOVA with phenolics compounds as between subject factor, time as within subject factor and inorganic nitrogen, DON, DON:inorganic nitrogen and pH as measured variables. Differences in inorganic nitrogen en DON were determined using a one-way ANOVA (Tukey's test, $P<0.05$). The effects of litter treatment (litter vs. plant extracts) and plant species on the amount of inorganic nitrogen, DON, DON:inorganic nitrogen and pH values was analysed by two-way ANOVA (Tukey's test, $P<0.05$). Linear regressions were used in the leaf extract and litter treatments between the amount of inorganic nitrogen and the DON. We also used stepwise linear regressions of soil parameters against characteristics of litter and plant extracts.

Results

Effect of pure phenolics

The repeated measures analyses showed that there were no significant overall effects of phenolic compounds on the amount of inorganic N, DON or the ratio DON: inorganic N. Figure 1 shows that after 8 weeks only protocatechuic acid had reduced inorganic N rates. By week 6, this phenolic compound had increased the amount of DON slightly compared to the

control treatments. The amount of DON was significantly reduced by ferulic acid after 8 weeks. After 16 weeks no differences in inorganic N or DON were measured between the phenolic compounds. The repeated measures showed a significant effect of phenolic compounds on the pH ($P< 0.001$). After 6 weeks did salicylic and vanillic acids produce an increase of the pH compared to the control with acetone (data not shown). After 16 weeks, only ferulic acid showed a decrease of the pH. No effects on the C:N ratios were measured.

Effect of tannic acid

Adding tannic acid to the nitrogen treatments with glutamine and ammonium chloride inhibited the production of inorganic nitrogen; the amounts of DON were reduced compared to the nitrogen treatments without tannic acid, but more slowly (Figure 2). Tannic acid also reduced the pH in most cases, at least during the first 6 weeks (data not shown). No effects of tannic acid on C:N ratios were found.

Effects of plant extracts

Throughout the incubation period, plant extracts of *C. vulgaris, V. myrtillus* and *V. vitis-idaea* had no effect on the amount of inorganic nitrogen compared to the control with water (Figure 3). Only the *D. flexuosa* extract produced more inorganic N after 16 weeks in comparison with the other extracts and the control. After 4 and 8 weeks, all extracts contained more DON than the control. However, the inorganic nitrogen was the main nitrogen source in this system (Table 3). The inorganic nitrogen measured in the peat correlated stronger with the total amount of nitrogen present in the plant extracts than with the amount of total phenolics (Table 4). Between the leaf extracts there was a significant negative correlation between the inorganic nitrogen and the DON (Figure 4, $r^2 = -0.15$, $P<0.001$). Only the plant extracts of *C. vulgaris* consistently reduced the pH throughout the incubation period, whereas *D. flexuosa* extracts tended to increase the pH after 8 weeks (data not shown).

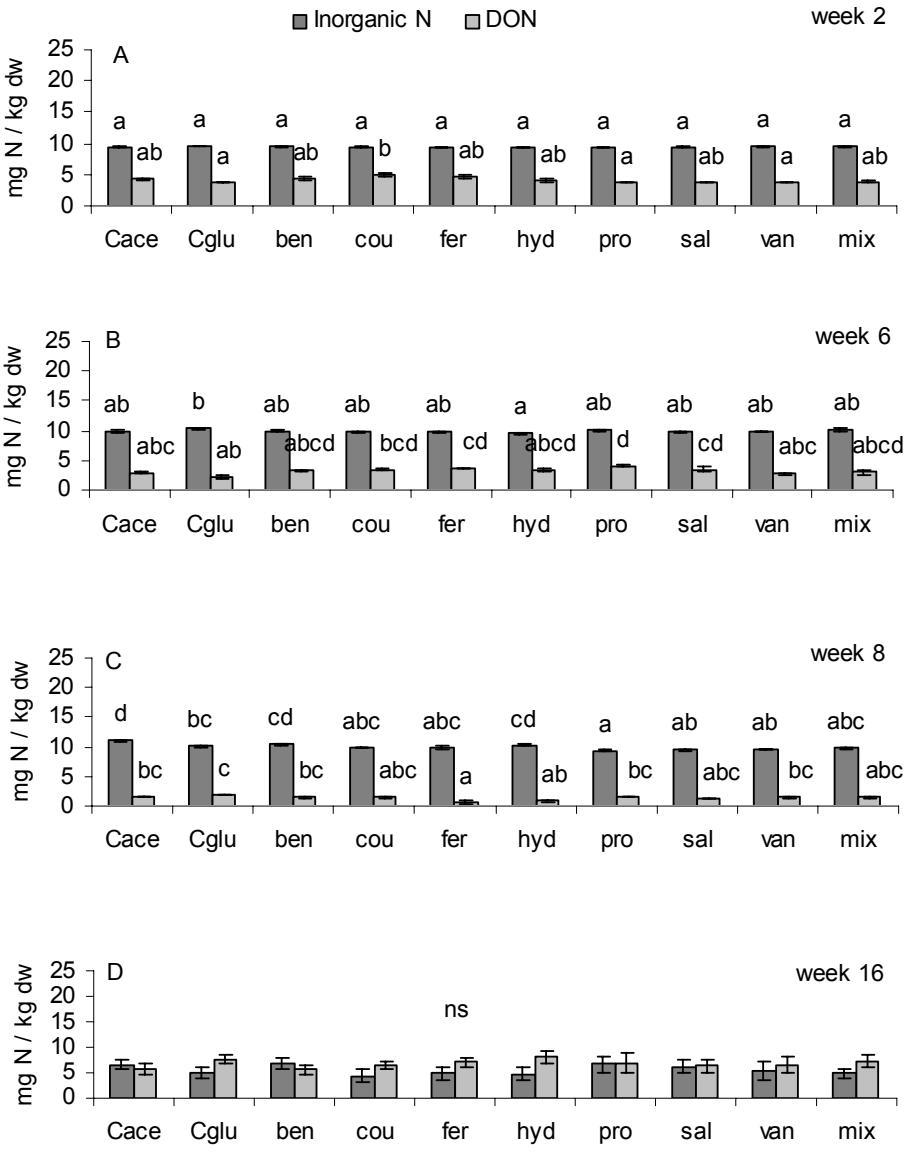

Figure 1. Effect of phenolic compounds on inorganic nitrogen and dissolved organic nitrogen (DON) after 2 (a), 6(b), 8(c) and 16 weeks (d), mean ±1 SE. Cace = acetone control, Cgluc = glucose control, Ben = benzoic acid, cou = p-coumaric acid, fer = ferulic acid, hyd = *p*- hydroxybenzoic acid, pro = protocatechuic acid, sal = salicylic acid, van = vanillic acid, mix = a mixture of these phenolics. One-way ANOVA (n = 4) was used and the multiple comparisons were done with the Tukey's test ($P<0.05$). Different letters indicate significant differences between phenolic acid treatments.

Table 2. A two-way ANOVA for effects of litter treatment (litter vs. plant extracts) and plant species on the amount of inorganic nitrogen, DON, DON:inorganic nitrogen and pH values. Shown are the degrees of freedom (df), *F* values and significant effects ($P<0.05$).

	Inorganic N			DON			DON:inorganic N			pH		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
species	3	50.2	0.001	3	26.7	0.001	3	17.4	0.001	3	35.3	0.001
litter	1	45.0	0.001	1	7.9	0.012	1	86.1	0.001	1	415.0	0.001
species x litter	2	32.3	0.001	2	46.6	0.001	2	23.6	0.001	2	14.9	0.001

Table 3. DON:inorganic nitrogen ratios in the soil in treatments with leaf extracts and litter of *Calluna vulgaris*, *Vaccinium myrtillus*, *V. vitis-idaea* and *Deschampsia flexuosa*. Numbers greater than 1 (bold figures) indicate a dominant amount of DON present in the nitrogen pool. * number is infinite large since the amount of inorganic nitrogen was reduced to almost zero.

week	2	4	6	8	16
Control	0.41	0.00	0.22	0.16	0.95
Leaf extracts:					
C. vulgaris	0.20	0.59	0.17	0.47	0.87
V. myrtillus	0.45	0.61	0.17	0.42	0.77
V. vitis-idaea	0.23	0.61	0.14	0.42	0.78
D. flexuosa	0.24	0.54	0.29	0.29	0.33
Litter:					
C. vulgaris	**5.54**	**1.00**	**4.39**	**1.83**	**>1***
V. myrtillus	**5.65**	**3.30**	0.78	0.46	0.39
V. vitis-idaea	**5.44**	**7.68**	**5.11**	**9.66**	**>1***
D. flexuosa	0.85	0.59	0.64	0.50	0.50

Table 4. Results of stepwise linear regressions of soil parameters against characteristics of litter and plant extracts of *Calluna vulgaris*, *Vaccinium myrtillus*, *V. vitis-idaea* and *Deschampsia flexuosa* (d.f.=15).

Dependent Variable	Step	Variable entered	Model r^2	*P*
Litter:				
Inorganic N	1	nitrogen	0.39	0.001
	2	total phenolics	0.53	0.001
DON	1	total phenolics	0.29	0.001
	2	nitrogen	0.50	0.001
DON:inorganic N	1	nitrogen	0.30	0.001
	2	carbon	0.34	0.001
pH	1	nitrogen	0.07	0.022
Leaf extracts:				
Inorganic N	1	nitrogen	0.31	0.001
	2	total phenolics	0.39	0.001

Effects of litter

In the litter treatments we found, in contrast with the leaf extracts, significant effects on the inorganic nitrogen, DON and inorganic nitrogen:DON (Table 2). All litter types showed clear immobilisation effects in the first two weeks (Figure 3). Whereas the litter of *D. flexuosa* released inorganic nitrogen after 8 weeks, the ericaceous litter types – especially *C. vulgaris* and *V. vitis-idaea* – continued to immobilise inorganic nitrogen. In the treatments with *C. vulgaris* and *V. vitis-idaea* litter, the amount of DON increased as the most important nitrogen source in the system, whereas concomitantly the amount of inorganic nitrogen declined to small amounts at the end of the incubation period (Table 3). The litter of *V. myrtillus* was a noteworthy exception, because after an immobilisation period of 8 weeks, it returned to the same levels of inorganic nitrogen as the controls with water and this was even increased after 16 weeks. This caused a remarkable shift in the ratio DON: inorganic nitrogen, towards inorganic nitrogen as the dominant nitrogen pool (Table 4). Litter of *D. flexuosa* increased the

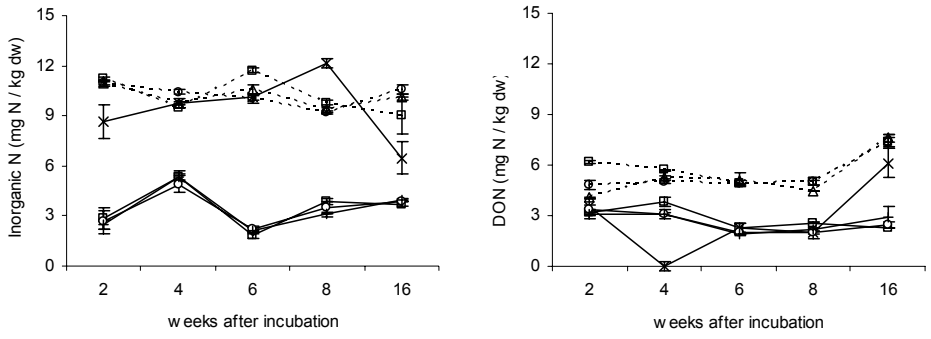

Figure 2. Effects of tannic acid addition in treatments with glutamine, NH_4 and NO_3 on the amount of inorganic nitrogen and dissolved organic nitrogen (DON). Broken lines indicate treatments with nitrogen addition alone and solid lines indicate treatments with nitrogen addition and tannic acid. Symbols: x Control, □ glutamine, Δ NH_4, ○ NO_3.

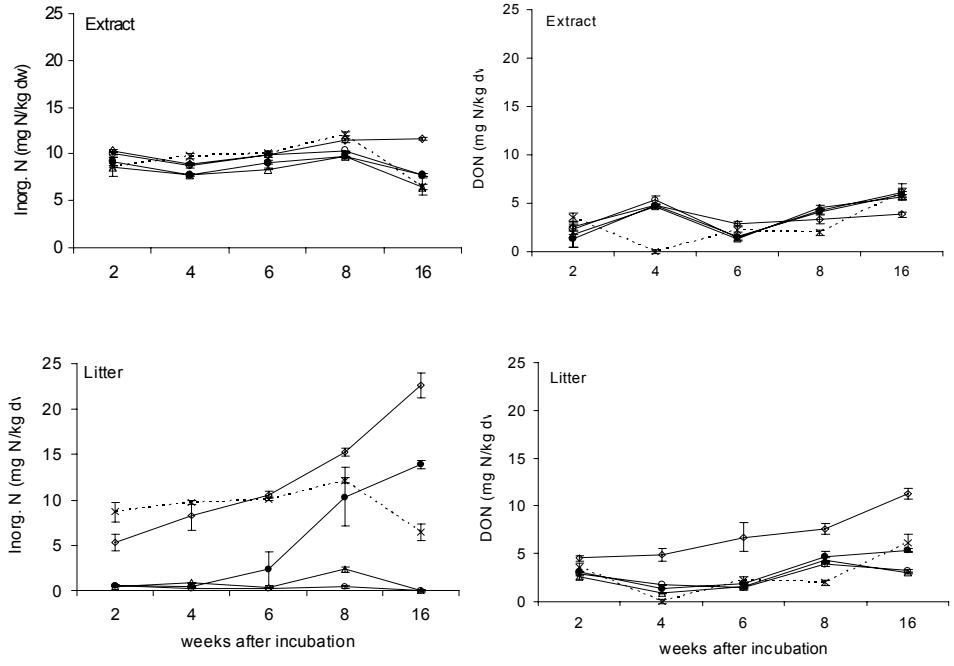

Figure 3. Effects of plant extracts and litter of *Calluna vulgaris, Vaccinium myrtillus, V. vitis-idaea* and *Deschampsia flexuosa* on inorganic nitrogen and on dissolved organic nitrogen (DON). Symbols: x Control, Δ *Calluna vulgaris*, ● *Vaccinium myrtillus*, ○ *V. vitis-idaea* and ◊ *Deschampsia flexuosa*.

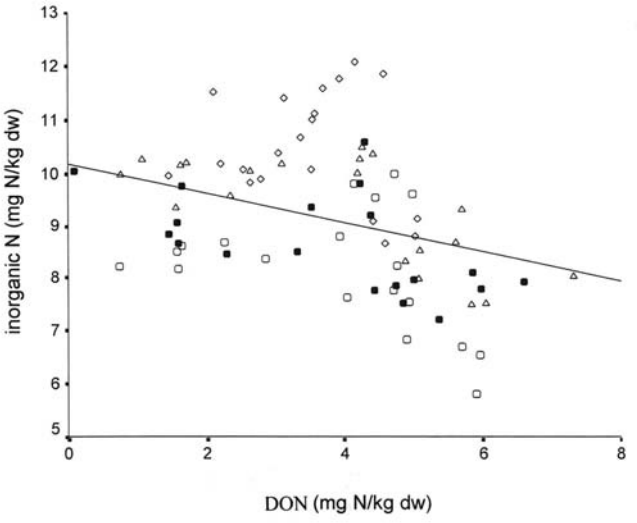

Figure 4. Correlation between the amount of inorganic nitrogen and the dissolved organic nitrogen (DON) among the leaf extract treatments. Symbols: Δ *Calluna vulgaris*, ● *Vaccinium myrtillus*, ○ *V. vitis-idaea* and ◊ *Deschampsia flexuosa*. Linear regression: $r^2 = -0.15$, $P < 0.001$.

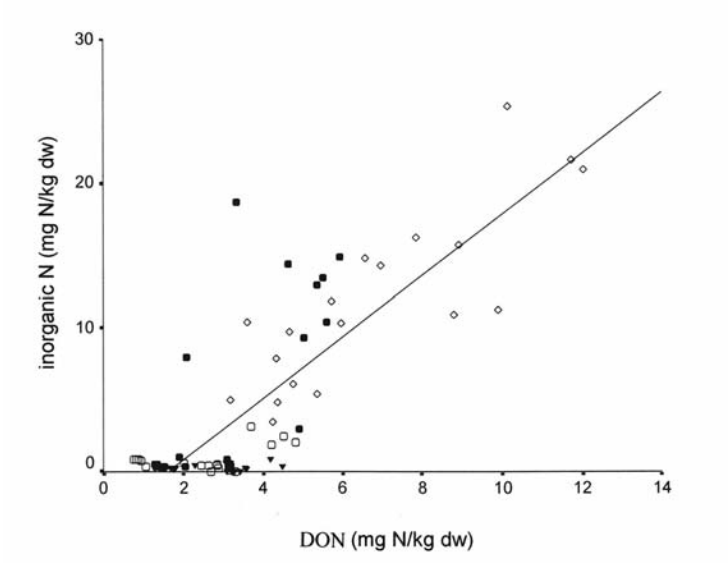

Figure 5. Correlation between the amount of inorganic nitrogen and the dissolved organic nitrogen (DON) among the litter treatments. Symbols: Δ *Calluna vulgaris*, ● *Vaccinium myrtillus*, ○ *V. vitis-idaea* and ◊ *Deschampsia flexuosa*. Linear regression: $r^2 = 0.69$, $P < 0.001$.

DON during the whole period, but nevertheless the main nitrogen pool consisted of inorganic nitrogen (Table 3). During the first four weeks of incubation, the limited amount of inorganic nitrogen measured could have been reduced by the concentration of total phenolics and condensed tannins, but after 8 weeks the percentage nitrogen in the litter regulated the inorganic nitrogen cycling (Table 4). On the measured amount of DON, the effects of the concentration of total phenolics and condensed tannins seem to last until 16 weeks of incubation. Due to the presence of *V. myrtillus* and *D. flexuosa* litter we found a significant positive relation in the litter between the amount of inorganic nitrogen and DON (Figure 5). The rank order in which the litter treatments increased the pH compared to the control with water was *C. vulgaris* < *V. vitis-idaea* < *V. myrtillus* < *D. flexuosa.*

Discussion

Effects of pure phenolics and tannic acid

In our experiment we found clear effects of tannic acid on the production of inorganic nitrogen, but no such effects for the pure phenolics. Rice & Pancholy (1974) were the first to discover a strong inhibition of oxidation of ammonia in soil suspensions was by nitrifying microorganisms due to e.g. ferulic acid at very low concentrations of 10^{-6} and 10^{-8} M. In contrast, McCarty & Bremner (1986) found no inhibition of nitrification in soils in their two-week incubation experiments in which they tested phenolic acids (e.g. *p*-hydroxybenzoic acid, *p*-coumaric acid, vanillic acid and ferulic acid) at concentrations of 10, 100 and 250 μg g^{-1} soil. Similarly, in later experiments (McCarty *et al*. 1991) with ferulic, caffeic and *p*-coumaric acids, ammonia oxidation by autotrophic microorganisms was not retarded, even when the phenolic concentrations in cultures of these microorganisms greatly exceeded their concentration in soils. Our experiment was much longer than the experiments described above, yet we also found no long-term effects. We therefore conclude that the effects of phenolic acids on nitrification and ammonification have been overestimated (see also Inderjit & Mallik 1997).

Our results did not reveal a clear relationship between the addition of phenolics and the amount of DON measured in the soil. We found variable effects of phenolics on DON: only the addition of protocatechuic acid led to an increase, whereas ferulic acid decreased the

amount of DON. Inderjit & Mallik (1997) also showed that an increase in total phenolics in the soil did not always result in more organic nitrogen. Only in their dark brown podzol horizons did protocatechuic, *p*-coumaric and *p*-hydroxybenzoic acids decrease the amount of organic nitrogen in the soil. The authors failed to show this response with ferulic acid. So, any effects of pure phenolic compounds remain doubtful. These compounds are breakdown products arising from the pathway of phenolics and these low molecular weight phenols may provide an added carbon source for soil microbes (Schimel *et al*. 1996, Inderjit & Mallik 1997, Souto *et al*. 2000, Fierer *et al*. 2001).

In contrast, when applied at a concentration of 50 mg / g soil, a heavy molecular weight polyphenolic compound like tannic acid effectively reduces the amount of inorganic nitrogen by approximately 50%. Additions of tannic acid increase the C:N ratio drastically, leading to strong immobilisation of inorganic nitrogen. In our experiments we had no cellulose control, so it cannot be ruled out that the high C:N ratio leading to immobilisation was the main reason for the effects measured (Vance & Chapin 2001). Although inorganic N was obviously reduced, we observed no reduction of DON.

Effect of plant extracts

In our experiment the nitrogen content in the leaf extract had the strongest regulatory effect, whereas the phenolic content correlated less stronger with mineralised nitrogen in the system. The large fraction of phenolics in the plant extracts probably consisted of low molecular weight phenolics. These water-soluble compounds have been shown to inhibit net mineralization rates, due to stimulation of microbial growth and immobilization (Schimel *et al*. 1996). Nevertheless, our plant extracts did not inhibit mineralization rates and DON was effected slightly after 4 and 8 weeks. Reason for this could be the relatively short preparation period of the extract. Northup (1995, 1998) incubated litter for three weeks, after which inorganic nitrogen and dissolved organic nitrogen were determined. With a longer incubation period it is likely that more phenolics will be extracted from the plant material, also fractions of higher molecular weight fractions.

Effects of litter

The main reason for the difference between the effect of the plant extracts and the litter is probably the condensed tannin present in the litter. Most condensed tannins are cell-bound

and are not present in water extracts (Waterman & Mole 1997). Schimel *et al*. (1996) showed that this group of phenolics is particularly capable of binding proteins. The amount of nitrogen in the litter was a good predictor of the mineralization and DON rates, whereas the amounts of total phenolics and condensed tannins only had predictive value during the first six weeks of incubation. In his early work with woodland plants, Kuiters (1987) concluded that the nutrient content of the litter was more important than the effect of released organic substrates. The predictive value of measured amounts of total phenolics and condensed tannins is still restricted, because all available analysing techniques have their shortcomings (Waterman & Mole 1997, Makkar *et al*. 1999, Yu & Dahlgren 2000). According to Yu & Dahlgren (2000), a solid-state ^{13}C NMR method yields the largest amounts of total condensed tannins, while other extraction/assay methods only yield 50-86% of the condensed tannin fraction. On the other hand, if we excluded the *V. myrtillus* litter from the Pearson correlation analyses, we found the amount of condensed tannins as being the most predictive value for the amount of inorganic nitrogen and DON during the whole incubation period (R^2 = ranging from 0.82-0.97, $p<0.001$).

A striking result from our experiment was the treatment with *V. myrtillus* litter. Initially, the litter immobilised inorganic nitrogen as did the other ericaceous litters, but after six weeks it released nitrogen at the same rates as *D. flexuosa* litters. At this turning point, the amount of DON – relative to the amount of inorganic nitrogen – changed from the major to the minor nitrogen component in the system. Though the two litter types started off with the same nitrogen content, the soil with *V. myrtillus* contained more low molecular weight phenolics (and thus had higher C:N ratios) and therefore the microbes needed more time to produce inorganic nitrogen.

It seems that *C. vulgaris* and *V. vitis-idaea* litters with high amounts of total phenolics and high C:N ratios (≥ 30) efficiently reduced the production of inorganic nitrogen over a prolonged period and concomitantly increased ratios of dissolved organic nitrogen. In these litter systems the organic nitrogen became the most important nitrogen pool, whereas the amount of inorganic nitrogen diminished. So, this observation serves to corroborate Northup's theory.

Conclusions

We conclude that the effects of pure phenolics have been overestimated. However, we can confirm Northup's theory, because in litters with C:N ratios above 30, we found low molecular weight phenolics and condensed tannins decreasing the amounts of inorganic nitrogen and concomitantly increasing the amounts of soluble organic nitrogen. More research needs to be done to study the importance of low molecular weight phenolics and condensed tannins for the nutrient cycling in the field.

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Chapter 4

Diversity of symbiotic root endophytes of the *Helotiales* in ericaceous plants and the grass, *Deschampsia flexuosa*

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Abstract

Root endophyte fungi of ericaceous plants were compared with those obtained from the dominant grass in Dutch heathlands, *Deschampsia flexuosa*. We investigated the phylogenetic relatedness of these fungi and their effects on nutrient uptake in both *Calluna vulgaris* and *D. flexuosa* seedlings in synthesis trials *in vitro*. Molecular analysis based on nuclear ribosomal internal transcribed spacer (ITS) region sequences revealed that four grass root endophytes belonged to the *Helotiales* (*Ascomycetes*). The majority of the ericaceous root isolates (68 %) also clustered within the *Helotiales* and showed a remarkably high diversity. Other important fungal groups included *Phialocephala fortinii*-like fungi, making up 22 % of isolates, and *Cryptosporiopsis* species, making up 8 %. Results of the synthesis trials showed that both grass root and ericaceous isolates colonized roots of both test host species successfully and could be seen to significantly enhance nitrogen uptake of inoculated *D. flexuosa* and *C. vulgaris* seedlings when these were compared to the uninoculated controls. We conclude that beneficial, helotialean fungi associate with roots of *D. flexuosa* and that these form a group potentially overlapping in phylogeny and function with endophytes from *Ericaceae*.

Key words: Calluna vulgaris, *Deschampsia flexuosa*, diversity, *Helotiales*, nitrogen uptake, root endophytes, synthesis trials.

Introduction

In the Netherlands, *Deschampsia flexuosa* has become a dominant grass species in heathlands due to high deposition rates of atmospheric nitrogen, derived substantially from industrial and agricultural sources and amounting to as much as 45 kg N ha^{-1} yr^{-1} (Berg & Verhoef 1998, Van Oene *et al*. 1999). The competitive success of this grass is thought to be due to a growth rate that is higher than that of ericaceous shrubs (Berendse & Elberse 1990, Berendse 1998). Recently it has been shown that this grass is also able to absorb considerable amounts of organic nitrogen. This enables it to use nitrogen forms other than ammonium and nitrate, thus reducing its dependence on nitrogen mineralization in the soil (Näsholm *et al*. 1998, Falkengren-Grerup *et al*. 2000, Persson *et al*. 2003). Although the type of mycorrhizal association seen in *D. flexuosa* has received some attention, the role of mutualistic endophytic fungi in organic nitrogen uptake by this species has not yet been elucidated (Persson & Näsholm 2001, Persson *et al*. 2003).

Deschampsia flexuosa can be colonized by multiple types of mutualistic fungi. The most common colonizers are arbuscular mycorrhizal fungi (AMF) in the *Glomeromycota*. Colonisation is seen in the production of vesicles and arbuscules in root epidermal cells (Harley & Harley 1987, Smith & Read 1997). *Deschampsia flexuosa* in alpine plant communities is also colonized by fungi with dark septate hyphae, and these fungi produce microsclerotia within and between epidermal cells (Read & Haselwandter 1981). Similar fungi frequently belong to a group known as dark septate endophytes (DSE), which are *Ascomycota*. Some members of this group appear to be mycorrhizal, at least in some hosts and habitats, including *Phialophora finlandica* Wang & Wilcox and *Phialocephala fortinii* Wang & Wilcox (Smith & Read 1997, Jumpponen 2001). *Phialocephala fortinii* is the most studied representative of the DSE complex, and seems to be distributed throughout the temperate Northern Hemisphere without showing apparent host specificity (Jumpponen & Trappe 1998, Ahlich *et al*. 1998, Addy *et al*. 2000, Grünig *et al*. 2002a). The mutualistic status of *P. fortinii* is debated, because no nutrient-exchange interfaces comparable to those of mycorrhizas have been identified (Jumpponen & Trappe 1998). Reports on the effects of *P. fortinii* on host plants reveal relationships that seem to range from parasitism to mutualism (Jumpponen & Trappe 1998, Jumpponen 2001). However, these differences can possibly be

attributed to the use of undefined isolates and to experimental designs that favour either *P. fortinii* or the host (Addy *et al*. 2000).

Read and Haselwandter (1981) estimated colonisation levels of AMF and dark septate hyphae in *D. flexuosa* roots collected from an Austrian alpine ecosystem. In *D. flexuosa* they found that AMF colonisation was on average 40 % and the percentage of dark septate hyphal colonisation was estimated at between 1–10 %. The identity of the dark septate fungal partner in the *D. flexuosa* roots remained unresolved. Vrålstad *et al*. (2002a) suggested that some *D. flexuosa* endophytes could belong to the *Helotiales* (*Ascomycota*). The *Helotiales* is a diverse fungal order in which the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf & Kornan (see note on this name, next paragraph) is classified along with the DSE species *P. fortinii* and *P. finlandica*. In a recent phylogenetic analysis, the *H. ericae* aggregate also appeared to include a group of closely related, more or less darkly pigmented root-associated ascomycetes (Vrålstad *et al*. 2002a). Further evaluation is needed, however, because the analysis in question used the *Rhytismatales* as an outgroup. Gernandt *et al*. (2001) showed with small subunit nuclear ribosomal DNA sequences that *Rhytismatales* and *Helotiales* belong to the same order. The affinities of the grass root endophytes are best tested with a different outgroup.

After this manuscript was written, we became aware of the reclassification of *H. ericae* as *Rhizoscyphus ericae* Zhuang & Korf (Zhuang & Zhuang 2004). We follow the reasons outlined by Hambleton and Sigler (2005–this volume) for changing our usage to this new correct name, while still referring to broad group of isolates related to this species as the *H. ericae* aggregate, and retaining the designation "*Hymenoscyphus* sp." for sequences downloaded from GenBank that are connected to isolates in the *H. ericae* aggregate but not identified at the species level.

The role of AMF in organic nitrogen uptake is negligible. Arbuscular mycorrhizal fungi (AMF) mainly promote plant growth by enhancing uptake of inorganic phosphate. They are not able to capture nutrients from organic nitrogen sources, e.g. glycine (Smith & Read 1997, Hodge 2001). In contrast, ericoid mycorrhizal (ERM) fungi facilitate the uptake of organic nitrogen. This is due to their saprotrophic abilities. Proteins and amino acids are released from protein polyphenol complexes by the activity of a range of hydrolytic and oxidative enzymes (Cairney & Burke 1998, Bending & Read 1996, 1997). For example, Sokolovski *et al*. (2002)

showed that *Calluna vulgaris* (L.) Hull root cells increased amino acid uptake when they were mycorrhizally associated with *R. ericae*. Among the DSE species, *P. fortinii* and *P. finlandica* also have the ability to hydrolyse organic nitrogen sources such as proteins, but their precise role in organic nitrogen uptake is not clear (Jumpponen *et al*. 1998, Caldwell *et al*. 2000).

The ericoid mycorrhizal association is described as a symbiosis between mutualistic, root-endophytic ascomycetous fungi and ericaceous plant roots. Ericoid mycorrhizal (ERM) fungi form characteristic hyphal coils in epidermal root cells (Smith & Read 1997). The strains of the *H. ericae–Scytalidium vaccinii* complex (*Helotiales*) and *Oidiodendron maius* G.L. Barron [uncertain ordinal classification, formerly *Onygenales* (Guarro & Cano 2002)] are the most widely dispersed and investigated ERM fungi (Read 1996, Straker 1996, Smith & Read 1997). Recently, molecular identification showed that the diversity of ERM fungi is much larger than was once assumed (Monreal *et al*. 1999, Perotto *et al*. 2002, Vrålstad 2002a, Allen *et al*. 2003, Bergero *et al*. 2003). In addition, the host range of ERM fungi appears to include some non-ericaceous plants (Duckett & Read 1995, Bergero *et al*. 2000, Vrålstad *et al*. 2002b).

Table 1. Overview of collection sites of *Deschampsia flexuosa* in The Netherlands: ecosystem classification and dominant plant species.

Nr.	Ecosystem	Location	Dominant species
1.	Heathland	Otterlo, Nat. Park De Hoge Veluwe	*Calluna vulgaris*
			Erica tetralix
			Molinia caerulea
			Deschampsia flexuosa
2.	Heathland	Bennekom, Gemeentebosch	*Vaccinium myrtillus*
			D. flexuosa
3.	Forest	Otterlo, Nat. Park De Hoge Veluwe	*Pinus sylvestris*
			D. flexuosa
4.	Forest	Ede, Hoekelum	*P. sylvestris*
			D. flexuosa
5.	Grass monoculture	Otterlo, Nat. Park De Hoge Veluwe	*D. flexuosa*
6.	Grass monoculture	Bennekom, Gemeentebosch	*D. flexuosa*

Our objective was to better understand the prevalence and function in *D. flexuosa* both of ERM fungi and of the poorly understood group of endophytes related to *R. ericae*. Therefore we investigated the phylogenetic relatedness of these fungi and their effects on nutrient uptake on both *Calluna vulgaris* and *D. flexuosa* seedlings in synthesis trials *in vitro*.

Materials & Methods

Root collection and isolation of fungi

Deschampsia flexuosa plants with intact roots were collected in spring 2003 from heathland, forest and grass monoculture ecosystems in the central region of the Netherlands (Table 1). For each ecosystem we selected two locations, each consisting of five replicate sites. Five plants were collected at each site for a total of 30 plants. Root systems from individual healthy plants (without dark-coloured, necrotic tissue) were cleaned to remove organic material as well as adhering ericaceous roots and other heterogeneous materials. Root tips were excised and surface-sterilized for 15 s with 4 % hypochlorite, followed by 30 s exposure to 70 % ethanol solution and three rinses in sterile water. Three sterilized root tips (1 cm) were placed in each Petri dish on malt extract agar [MEA; (Oxoid, Hampshire, U.K.) agar, 20 g; distilled water, 1000 mL] amended with 30 mg/L streptomycin sulphate. Plates were incubated at 20 °C and observed daily for hyphal emergence. Mycelia growing out of the root tips were transferred after about 7 d to 2 % MEA. Pure cultures were checked weekly for sporulation and the slow growing, nonsporulating isolates were divided into three different morphological groups. Cultures were roughly grouped based on colour and appearance. Morphotype 1 consisted of cultures with black colonies with white margins; morphotype 2 contained beige, velvety isolates, and morphotype 3 contained isolates with salmon-colored colonies. Above and beyond the characters mentioned, all isolates assigned to the same morphotype were highly similar to one another. Five isolates from different morphological groups and habitats were used for molecular analyses. The ericoid endophytic fungal isolates were collected in September 2000, from roots of whole plants of *C. vulgaris* (50 plants), *Erica tetralix* (35 plants), *Empetrum nigrum* (10 plants), *Vaccinium myrtillus* (10 plants) and *V. vitis-idaea* (15 plants) from three different heather locations and adjacent forests in The Netherlands. Localities were Dwingeloo, Dwingelderveld National Park; Hoog Buurlo, Hoog Buurlosche heath; and Otterlo, De Hoge Veluwe National Park. *Ericaceae* in De Hoge

Veluwe National Park could not be collected at precisely the same sites where *D. flexuosa* was collected. Ericaceous endophytic fungi were isolated using the same method as was used for endophytic fungi in *Deschampsia flexuosa*. Rapidly growing, heavily sporulating mould isolates such as *Penicillium* spp., *Verticillium* spp. *Fusarium* spp., and *Trichoderma* spp., were discarded, because our intent was to focus on isolates resembling ERM fungi.

Morphological identification

Cultures were roughly grouped based on colour and appearance. Some cultures sporulating as *Cryptosporiopsis* and related species could be identified in morphological or molecular analyses. Some DSE started to sporulate after nine months storage at 4 ºC and could be identified morphologically as *P. fortinii*. Our interest was focused on ERM fungi; therefore we performed no further molecular analyses on DSE from ericoid sources, though three *P. fortinii*-like isolates, one somewhat atypical with subglobose-square conidia, were deposited in the Centraalbureau voor Schimmelcultures (CBS; Utrecht, the Netherlands) as CBS 110240–110242.

DNA extraction and sequencing

Of the 154 slow-growing cultures from ericaceous roots, which made up 68 % of the total isolates, 40 isolates were randomly selected for phylogenetic analysis. Five isolates from *D. flexuosa* were analysed in comparison. Genomic DNA was extracted from young mycelium with the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Subsequently, PCR and restriction fragment length polymorphism (RFLP) were applied (Gardes & Bruns 1996). For approximate species-level clustering and identification, sequences were obtained for the internal transcribed spacer (ITS) region of the nuclear rRNA repeat using the fungus-specific primer pair ITS1-F and ITS-4 (White *et al*. 1990, Gardes & Bruns 1996, Bidartondo *et al*. 2001). The fungal ITS-RFLP patterns were produced using the restriction enzymes *Alu* I, *Hinf* I and *Mbo* I (MBI-Fermentas, Brunschwig Chemie BV, Amsterdam, the Netherlands). RFLP band sizes were estimated to within an error margin of no more than 10 %. Cloned sequences were compared to the sequences in GenBank using a Blast algorithm at the NCBI homepage, http://www.ncbi.nlm.nih.gov/.

Table 2. Included reference material and sequences of ascomycetes of *Helotiales* (ingroup taxa) and *Onygenales* (outgroup taxa).

GenBank accession no.	Taxon	Strain	Family or Order	Host	Geographic origin	Source
Ingroup taxa of *Helotiales*:						
AJ301960	Ascomycete sp.	BBA 71218	Unclassified	*Erica* sp.	Germany	Nirenberg *et al.* (2002)
AJ430227	Axenic ectomycorrhizal root isolate	ARON 3026	Helotiaceae	*Betula pubescens*	Norway	Vrålstad *et al.* (2002a)
AJ430207	Axenic grass root isolate	ARON 2927	anamorphic *Helotiales*	*Deschampsia flexuosa*	Norway	Vrålstad *et al.* (2002a)
AJ430208		ARON 2929	anamorphic *Helotiales*	*D. flexuosa*	Norway	Vrålstad *et al.* (2002a)
AJ430399		ARON 2959	unclassified *Helotiales*	*D. flexuosa*	Norway	Vrålstad *et al.* (2002a)
AJ430225		ARON 2889	*Dermateaceae*	*Festuca ovina*	Norway	Vrålstad *et al.* (2002a)
AJ430226		ARON 2892	*Dermateaceae*	*F. ovina*	Norway	Vrålstad *et al.* (2002a)
AF335454	*Bisporella citrina*	'F 140146 (UBC)'	*Helotiaceae*	*Unknown*	Unknown	Berbee *et al.* (unpubl.)
AF530462	*Cadophora hiberna*	GB5530	anamorphic *Helotiales*	*Robinia pseudoacacia*	Spain	Bills (unpubl.)
AY176753	*Cryptosporiopsis rhizophila*	CBS 109839	anamorphic *Dermateaceae*	*E. tetralix*	The Netherlands	Verkley *et al.* (2003)
AY176755	*C. rhizophila*	CBS 110603	anamorphic *Dermateaceae*	*Calluna vulgaris*	The Netherlands	Verkley *et al.* (2003)
AY279186	Epacrid root endophyte sp.	RK 2	unknown	*Epacris microphylla*	Australia	Williams *et al.* (unpubl.)
AF252840	Ericoid endophyte sp.	GU36	unclassified *Helotiales*	*C. vulgaris*	UK	Sharples *et al.* (2000)
AF252843		GU37	unclassified *Helotiales*	*C. vulgaris*	UK	Sharples *et al.* (2000)
AF252842		GU44	unclassified *Helotiales*	*C. vulgaris*	UK	Sharples *et al.* (2000)
AF252841		DGC25	unclassified *Helotiales*	*C. vulgaris*	UK	Sharples *et al.* (2000)
AF261659	Glacial ice euascomycete	GI307	unknown	Isolated from glacial ice	Greenland	Ma *et al.* (2000)
AF284122	*Rhizoscyphus ericae*	UAMH 6735	*Helotiaceae*	*Gaultheria shallon*	Canada	Allen *et al.* (unpubl.)
AY112921	*R. ericae*	UBCtraSeq1442.1	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)

Table 2. (continued)

AF081435	*Hymenoscyphus* sp.	UBCM8	*Helotiaceae*	*G. shallon*	Canada	Monreal *et al.* (1999)
AF081438		UBCM20	*Helotiaceae*	*G. shallon*	Canada	Monreal *et al.* (1999)
AF284123		UBCtra1302.5	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AF284124		UBCtra1302.11	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AY219881		UBCtra1436C	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AY219879		UBCtra1439C	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AY354269	*Mollisia* sp.	olrim132	*Dermateaceae*	*B. pendula*	Sweden	Lygis *et al.* (2004)
AJ430223	*M. minutella*	ARON 3129	*Dermateaceae*	*Epilobium angustifolium*	Norway	Vrålstad *et al.* (2002a)
AJ430222	*M. cinerea*	ARON 3139	*Dermateaceae*	*Picea abies*	Norway	Vrålstad *et al.* (2002a)
AY078151	Cf. *Phialocephala fortinii*	DSE-C	*Helotiaceae*	*Pinus sylvestris*	Germany	Grünig *et al.* (2002b)
AY078152		DSE-C	*Helotiaceae*	*P. abies*	Switzerland	Grünig *et al.* (2002b)
AY078141	*Phialocephala fortinii*	93-301	*Helotiaceae*	*Fagus sylvatica*	Switzerland	Grünig *et al.* (2002b)
AY078143		CBS 109313	*Helotiaceae*	*C. vulgaris*	Switzerland	Grünig *et al.* (2002b)
AF486126	*P. scopiformis*	CBS 468.94	*Helotiaceae*	*P. abies*	Germany	Grünig *et al.* (2002b)
AJ430224	*Pyrenopeziza revincta*	ARON 3150	*Dermateaceae*	*E. angustifolium*	Norway	Vrålstad *et al.* (2002a)
AF149074	*Cryptosporiopsis brunnea*	UBCtra 288	anamorphic *Dermateaceae*	*G. shallon*	Canada	Sigler *et al.* (2005)
AF284133		UBCtra 1522.5	unknown	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AJ430228	*Tapesia cinerella*	ARON 3188	*Dermateaceae*	Decaying twig/bark	Norway	Vrålstad *et al.* (2002a)
AJ430229	*T. fusca*	ARON 3154	*Dermateaceae*	Decaying twig/bark	Norway	Vrålstad *et al.* (2002a)
Outgroup taxa of *Onygenales*:						
U18363	*Ajellomyces capsulatus*	–	*Onygenaceae*	unknown	unknown	Berbee *et al.* (1995)
U18360	*Coccidioides posadasii*	–	anamorphic *Onygenales*	unknown	unknown	Berbee *et al.* (1995)

Table 3. Origins and GenBank accession numbers of representative root isolates collected from *Deschampsia flexuosa* and ericaceous plants in The Netherlands.

GenBank accession no.	CBS accession no.	Description	Host plant	Geographic origin
AY599235	115904	grass PPO-G1	*Deschampsia flexuosa* (heathland)	Otterlo, Nat. Park "De Hoge Veluwe"
AY599236	115905	grass PPO-G2	*D. flexuosa* (grass)	Otterlo, Nat. Park "De Hoge Veluwe"
AY599237	116049	grass PPO-G3	*D. flexuosa* (forest)	Otterlo, Nat. Park "De Hoge Veluwe"
AY599238	115906	grass PPO-G4	*D. flexuosa* (grass)	Bennekom, Gemeentebosch
AY599239	115907	ericaceous PPO-E1	*Vaccinium myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
AY599240*		ericaceous PPO-E2	*Calluna vulgaris*	Otterlo, Nat. Park "De Hoge Veluwe"
AY599241	115908	ericaceous PPO-E3	*C. vulgaris*	Otterlo, Nat. Park "De Hoge Veluwe"
AY599242	115909	ericaceous PPO-E4	*C. vulgaris*	Otterlo, Nat. Park "De Hoge Veluwe"
AY599243*		ericaceous PPO-E5	*C. vulgaris*	Otterlo, Nat. Park "De Hoge Veluwe"
AY599244	115910	ericaceous PPO-E6	*C. vulgaris*	Hoog Buurlo, Hoog Buurlosche heide
AY599245*		ericaceous PPO-E7	*C. vulgaris*	Dwingeloo, Nat. Park "Dwingelderveld"
AY599246	115911	ericaceous PPO-E8	*Empetrum nigrum*	Dwingeloo, Nat. Park "Dwingelderveld"
AY599247	115912	ericaceous PPO-E9	*C. vulgaris*	Dwingeloo, Nat. Park "Dwingelderveld"

* isolates indicated no longer viable at time of this writing.

Phylogenetic analyses

Fasta searches in the combined EMBL/GenBank/DDBJ database were used to find ITS sequences similar to those we obtained (Table 2). Sequences were aligned in BioEdit (v.5.0.9, Hall 1999) and adjusted manually after the use of the automatic ClustalW option. We used a distance matrix method and prepared a neighbour-joining tree in ClustalX. Positions with gaps were excluded. Stability of clades was tested with 1000 bootstrap iterations. GenBank accession numbers of the isolates studied are given in Table 3. The *Helotiales* ingroup data set was compiled with sequences of *Helotiaceae*, *Dermateaceae* and some related root-derived fungal endophytes, such as *Cryptosporiopsis rhizophila* Verkley & Zijlstra (Verkley *et al*. 2003). The onygenalean taxa *Ajellomyces capsulatus* (Kwon-Chung) McGinnis & Katz and *Coccidioides posadasii* N. Fisher, Koenig, White & Taylor were used as outgroup.

Quantification of fungal colonisation in roots of D. flexuosa

Deschampsia flexuosa roots were cleaned and then stained to determine the amount of AMF and DSE-like colonization. Roots were cleared by heating to 90 ºC in 10 % KOH (wt/vol) for three min and then rinsed with tap water. Cleared roots were boiled for two min in a 0.5 % ink-vinegar solution with pure white household vinegar (Vierheilig *et al*. 1998) and then destained in tap water with a few drops of vinegar to remove coloration from empty cells. For quantification of colonization, 60 1.0-cm root sections of each treatment were mounted on slides (four per slide); the degree of root tissue colonization was estimated by measuring the percentage of total root length colonized by a given fungal type for five plants per treatment. Root sections were examined at 400× under the compound microscope. Structures characteristic of *P. fortinii*-like fungi were noted. AMF colonization was recognized by the presence of vesicles and arbuscules connected to broad, aseptate hyphae. Both arbuscules and vesicles were typically found in the same root specimens.

Synthesis trials

In order to test the effect of the isolated grass endophytes on the nitrogen uptake of both *D. flexuosa* and *C. vulgaris* axenic seedlings, we performed synthesis trials with three grass endophyte isolates strongly differing in genotype and selected as representatives of three different major morphotypes obtained from *D. flexuosa*. The isolate designated “grass isolate PPO-G1” (CBS 115904) represented morphotype 1, while isolates “grass isolate PPO-G2”

Table 4. Fungi used to inoculate roots of *Deschampsia flexuosa* and *Calluna vulgaris* in the *in vitro* synthesis trials, and plant species and location from which they were isolated.

Endophytic species	Host plant	Geographic origin
grass PPO-G1 (Morphotype 1)	*Deschampsia flexuosa* (heathland)	Otterlo, Nat. Park De Hoge Veluwe
grass PPO-G2 (Morphotype 2)	*D. flexuosa* (grass monoculture)	Otterlo, Nat. Park De Hoge Veluwe
grass PPO-G3 (Morphotype 3)	*D. flexuosa* (forest)	Otterlo, Nat. Park De Hoge Veluwe
ericaceous isolate PPO-E6	*Erica tetralix*	Otterlo, Nat. Park De Hoge Veluwe
Cryptosporiopsis rhizophila CBS 109839	*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
Phialocephala fortinii CBS 110241	*Vaccinium vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld

(CBS 115905) and "grass isolate PPO-G3" (CBS 116049) represented morphotypes 2 and 3, respectively ["PPO" designates Praktijkonderzoek Plant & Omgeving (Applied Plant Research), Wageningen University]. To compare the nutrient effect of the endophytic fungi from *D. flexuosa* with that previously described for ERM fungi, we also included three endophytic isolates from ericaceous plants, representing the most abundantly present ericaceous endophytes. These isolates included PPO-E6 (CBS 115910), a nonsporulating mycelium belonging to a species that is unnamed or not identifiable in culture, as well as the *P. fortinii* and *C. rhizophila* isolates listed in Table 4.

Axenic *D. flexuosa* and *C. vulgaris* seedlings were obtained from surface sterilized seeds on water agar. Seeds were sterilized using a 5 min exposure to 1 % hypochlorite followed by three rinses in sterile water. Peat (Fixet Retailgroup, Apeldoorn, The Netherlands) with pH 4, was sieved (1.0 mm mesh size) and autoclaved twice (20 min at 120 °C). Water agar [WA; (Oxoid, Agar technical no. 3, Hampshire, UK) agar, 15 g; distilled water, 1000 mL] was mixed with the sterile peat at 0.5 g peat per 9 cm Petri dish. The next day, the agar in each of these plates was cut in half and one half was removed. At the midpoint of the cut edge of the remaining half, a sterile 2-wk-old *D. flexuosa* or a 4-wk-old *C. vulgaris* seedling was placed.

Each plate was inoculated with one agar plug from an actively growing margin of a fungal colony on Modified Melin Norkrans medium [MMN; malt extract (Oxoid, Hampshire, U.K.), 3 g; agar (Oxoid, Agar technical no.3, Hampshire, U.K.), 15 g; D-glucose, 10 g; $(NH_4)_2HPO_4$, 0.25 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.15 g; $CaCl_2 \cdot 2H_2O$, 67 mg; $FeCl_3$ (1

% solution), 1.2 mL; NaCl, 25 mg; thiamine·HCl, 0.1 mg; distilled water, 1000 mL] (Marx 1969). Five replicates were used for each tested isolate. In the plates with the control plants, an agar plug with sterilized MMN agar was added. The plates with the inoculated and control seedlings were taped with Parafilm (Pechinay Plastic Packaging, Neenah, WI, U.S.A.) to prevent drying out and to avoid contamination. Plates were placed in vertical position, according to a randomized block design in an illuminated growth chamber at 25 °C day temperature and 15 °C night temperature, in a 16 h / 8 h day/night cycle. They were harvested after 5 wk, when the tallest seedlings were grown up to 6 cm length.

Seedling roots were stained in a 0.2 % solution of trypan blue in lactic acid:glycerol:water (3¼ : 3 : 4 by vol.) and then transferred to a storage solution of lactic acid:glycerol:water (1 : 2 : 1 by vol.). They were mounted on a microscopic slide and examined at 400× under the compound microscope for the presence of associated hyphae and intracellular colonisation. The shoots were dried at 70 °C for 24 h and their weight was measured with a microbalance. Dried shoots were pulverised and their N concentrations were measured using an elemental analyser (EA 1108, Fisons Instruments, Nottingham, U.K.). To obtain a value for the absolute amount of nitrogen in the shoots we multiplied the nitrogen concentration in shoots of *D. flexuosa* or *C. vulgaris* by the dry weight of the corresponding shoots at the end of the experimental period.

Statistical analysis

Chi square testing was used to evaluate the significance of differences in isolation proportions of *D. flexuosa* endophyte morphotypes in different habitats. It was also used to evaluate differences in morphotype and species isolation frequencies among ericaceous plant species and the locations at which they were sampled. Data for fungal colonization of *D. flexuosa* roots were arcsine-transformed to obtain a normal distribution and then tested with one-way ANOVA. Average amounts of shoot nitrogen of *D. flexuosa* and *C. vulgaris* seedlings in the fungal treatments were compared with the control treatment by one-way ANOVA. The accepted significance level for all statistical tests was $P < 0.05$.

Table 5. Number of isolates of three different endophyte morphotypes isolated from roots of *Deschampsia flexuosa* sampled in heathlands, forests and grass monocultures. Different letters in vertical direction, indicate significant differences in isolation proportions among habitats (Chi-square test, $P < 0.05$). Different hyphenated numbers in horizontal direction indicate significant differences in isolation proportions among fungal groups within one habitat (Chi-square test, $P < 0.05$).

Number of isolates	Morphotype 1	Morphotype 2	Morphotype 3	Total
Heathland	28^{b2}	3^{a1}	27^{2}	58
Forest	12^{a1}	15^{b1}	35^{2}	62
Grass monoculture	6^{a1}	40^{c3}	22^{2}	68
Total	46	58	84	188

Results

Abundance and occurrence of morphological fungal groups

Culturing of 450 root tips of *Deschampsia flexuosa*, (30 plants, 15 root tips / plant) yielded 257 fungal isolates. Most were sterile mycelia. Most of these mycelia could be placed into one of three major groups based on morphological characteristics. Morphotype 1 was mostly isolated from roots of *D. flexuosa* growing in heathlands and less frequently from plants growing in grasslands or in forest devoid of ericaceous plants (Table 5). In contrast, morphotype 2 was most frequently isolated from *D. flexuosa* plants growing in grasslands. Compared to morphotype 1 and 2 isolates, morphotype 3 isolates were relatively evenly distributed across all three ecosystems.

Culturing of 414 root tips from 115 plants, from five different ericaceous species, yielded 227 isolates. Results for cultures that sporulated and could be characterized morphologically are shown in Tables 6, 7. The largest group consisted of *P. fortinii*-like cultures (22 %). Some cultures sporulated after being cultured for nine months at 4 ºC. Three isolates could be identified based on morphological characteristics as *P. fortinii*. The *P. fortinii*-like isolates occurred in association with most ericaceous species except *V. myrtillus*. They were isolated from all locations, but were most abundant from the Dwingeloo site. Two *Cryptosporiopsis* species made up the second most abundant group, accounting for 8 % of isolates. *C. rhizophila* occurred in most ericaceous plants except *E. nigrum*, and was only found in plants

from the Hoog Buurlo and Dwingeloo sites. *Cryptosporiopsis* sp. 2 was isolated from most locations, but was not obtained from *C. vulgaris* roots. Three isolates belonging to unidentified members of the *Cryptomycetales* were obtained from two different sites at Dwingeloo. Only three isolates of *O. maius* were obtained from roots of *Vaccinium vitis-idaea*.

Phylogenetic analyses

Internal transcribed spacer (ITS) sequences of five fungal isolates from grass roots were compared to sequences in GenBank. One sequence was excluded from further analysis, because it was identified as pertaining to a non-helotialean ascomycete, *Chaetosphaeria vermicularioides* (Saccardo & Roumeguère) W. Gams & Holubová-Jechová. Sequences that appeared to be helotialean and sequences of uncertain affinity were retained. Figure 1 shows the neighbour-joining tree containing the included isolates from grass roots and *Ericaceae*. Four grass isolates clustered well within the *Helotiales*. Grass isolate PPO-G1 (CBS 115904), which was isolated from a heathland, was closely related to *Cadophora hiberna* Bills. It was, however, unrelated to the other grass root isolates and to *Hymenoscyphus* species. The other three grass isolates formed a cluster separate from PPO-G1. Grass isolates PPO-G3 (CBS 116049) and PPO-G4 (CBS 115906) from monocultures clustered with the sequence of *Pyrenopeziza revincta* (P. Karst.) Gremmen as well as sequences of isolates from axenic roots of the grass *Festuca ovina* L. (Vrålstad *et al*. 2002a). Remarkably, the ericaceous isolate PPO-E9 (CBS 115912) clustered also in this group, with a bootstrap support of 90 %. Grass root isolate PPO-G2 (CBS 115905) from forest obtained only 59 % bootstrap support with PPO-G3 and PPO-G4. From the 40 slow-growing ericaceous isolates selected for analysis, nine groups evidenced different RFLP patterns. Within these ericaceous isolates, isolate PPO-E2 (no longer alive) was distinct in being most similar (83 % similarity) to root-associated isolates UBCTRA1436C and UBCTRA1439C, identified as *Hymenoscyphus* sp. and isolated from the ericaceous shrub salal, *Gaultheria shallon* Pursh (Allen *et al*. 2003). A small cluster was formed by ericaceous isolates PPO-E3 (CBS 115908), PPO-E4 (CBS 115909) and PPO-E5 (no longer alive), which showed 100 % similarity with the sequences from the ericoid endophyte isolates GU36 and GU37 from *C. vulgaris* (Sharples *et al*. 2000). There was no bootstrap support for an association of this cluster with the ericoid endophyte isolate GU44, known to produce mycorrhizal coils in *Calluna* roots (Sharples *et al*. 2000).

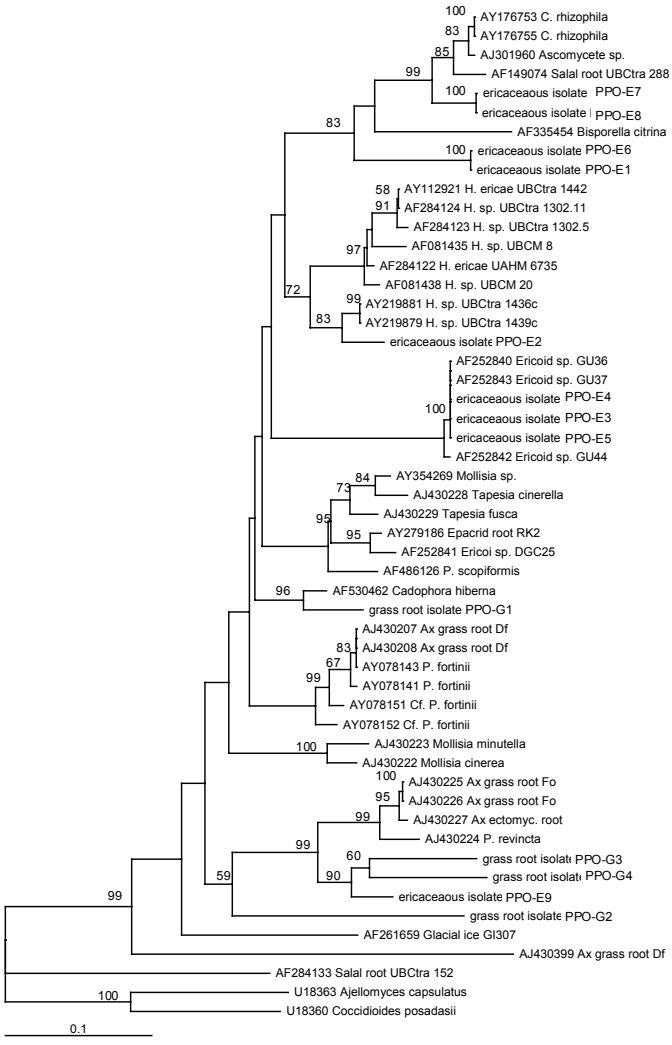

Figure 1. Neighbour-joining tree derived from nuclear ribosomal internal transcribed spacer (ITS) sequences of endophytic root isolates from ericaceous plants (*Calluna vulgaris*, *Erica tetralix*, *Empetrum nigrum*, *Vaccinium myrtillus*, *V. vitis-idaea*) and from the grass *Deschampsia flexuosa* sampled at different locations in the Netherlands. Two species from the order *Onygenales*, *Ajellomyces capsulatus* and *Coccidioides posadasii*, were chosen as outgroup. Bootstrap values of ≥ 50% are indicated above the branches.

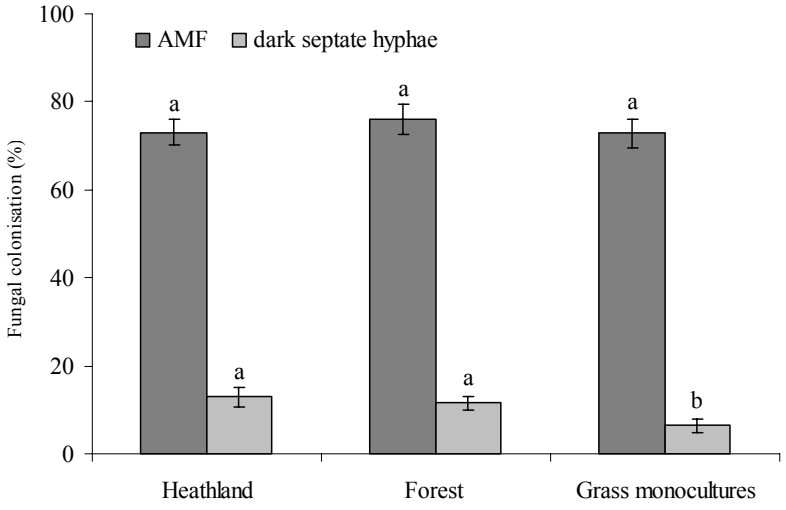

Figure 2. Colonization levels of arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE) in roots of *Deschampsia flexuosa* sampled in heathlands, forests and grass monocultures. Different letters indicate significant differences between habitats (one-way ANOVA, $P< 0.05$).

Isolates PPO-E1 (CBS 115907) and PPO-E6 (CBS 115910) formed a distinct cluster close to the *Cryptosporiopsis* species. This cluster was not closely related to the above-mentioned group of *Hymenoscyphus* isolates. Isolates PPO-E7 (no longer alive) and PPO-E8 (CBS 115911) showed 100 % mutual similarity and were supported in a clade with *C. rhizophila* and two unidentified GenBank sequences, one from *Erica* sp. and one from *Gaultheria shallon*.

Quantification of fungal colonisation in roots of D. flexuosa

Deschampsia flexuosa roots collected in the field contained both AMF and *P. fortinii*-like structures (Fig. 2). At all sites, AMF colonization was found at higher levels than *P. fortinii*-like colonization ($P < 0.001$). Colonization with *P. fortinii*-like fungi were highest in roots from heathlands and forests; the levels in plants from monocultures were significantly lower ($P < 0.05$).

Table 6. List of sporulating endophytes isolated from ericaceous hosts. All strains were from roots collected in the Netherlands. Shown are CBS and GenBank accession numbers, host plant and geographic origin.

Fungal isolate CBS accession no. (GenBank accession no.)	Nr. isol.	Host plant	Geographic origin
*Cryptosporiopsis rhizophila**	9		
CBS 109839 (AY176753)		*Erica tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110602 (AY176754)		*Calluna vulgaris*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110603 (AY176755)		*C. vulgaris*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110604 (AY176756)		*C. vulgaris*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110606 (AY176757)		*E. tetralix*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110609 (AY176758)		*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110612 (AY176759)		*Vaccinium vitis-idaea*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110616 (AY176760)		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110617 (AY176761)		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
Cryptosporiopsis sp. 2	9		
CBS 110611		*Empetrum nigrum*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110613		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110615		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110614		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110605		*E. tetralix*	Otterlo, Nat. Park De Hoge Veluwe
CBS 110608		*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110652		*V. vitis-idaea*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110610		*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110618		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
Cryptomycetales unident.	3		
CBS 110651		*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110654		*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110653		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld
Oidiodendron maius	3		
CBS 110450		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110451		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld

CBS 110452		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld
Phialocephala fortinii	3		
CBS 110240		*E. nigrum*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110241		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110242		*C. vulgaris*	Dwingeloo, Nat. Park Dwingelderveld
P. fortinii-like	46		
JA- 7, 12, 16, 45, 106, 110, 135, 138, 144, 161, 162, 164, 165, 175, 569, 350, 358, 359, 366, 367, 370, 372, 375, 507, 514, 547, 548, 550, 244, 288, 306, 331, 528, 529, 561, 400, 403, 406, 409,413, 414, 426, 430, 436, 541		*C. vulgaris* (4)	Otterlo, Nat. Park De Hoge Veluwe
		C. vulgaris (11)	Dwingeloo, Nat. Park Dwingelderveld
		E. tetralix (1)	Otterlo, Nat. Park De Hoge Veluwe
		E. tetralix (6)	Dwingeloo, Nat. Park Dwingelderveld
		E. nigrum (13)	Dwingeloo, Nat. Park Dwingelderveld
		V. vitis-idaea (1)	Hoog Buurlo, Hoog Buurlosche heide
		V. vitis-idaea (10)	Dwingeloo, Nat. Park Dwingelderveld

*See also Verkley *et al*. (2003).

Synthesis trials

In *D. flexuosa* seedlings inoculated with Helotialean fungi, the amount of nitrogen in the shoots was enhanced over levels seen in controls. This was true not just when endophytic fungi from the same host were tested, but also when ericaceous isolates were used (Fig. 3). The highest amounts of nitrogen were found in seedlings colonized by grass isolate PPO-G1 and the ericoid isolate PPO-E6. In all treatments, staining of inoculated grass roots showed high fungal colonization of the epidermal cells, with 80 to 93 % of cells affected. The nitrogen amount in *C. vulgaris* seedlings was increased when seedlings were inoculated with ericoid endophytic isolates from the same host. In addition, the grass isolates PPO-G1 and PPO-G4 had a positive effect on nitrogen amounts (Fig. 4). The dry weight of the seedlings showed results similar to those obtained for total shoot nitrogen levels (data not shown); however, the nitrogen concentrations of shoots were not significantly different between treatments.

Table 7. Number of isolates of five most abundant fungal morphotypes isolated from ericaceous roots sampled in three different heathland locations. Different letters within columns indicate significant differences among sites (Chi-square test, $P < 0.05$). Different superscript numbers within rows indicate significant differences in isolation proportions among fungal groups within one location or plant (Chi-square test, $P < 0.05$). Chi-square tests were only performed when numbers were sufficient (frequencies of expected values in contingency tables > 5).

Number of isolates	*Cryptosporiopsis rhizophila*	*Cryptosporiopsis* sp. 2	Dark sterile endophyte (DSE)	Identical to ericaceous isolate PPO-3	Identical to ericaceous isolate PPO-6	Total
Origin:						
Total (n=110)	9^{1}	9^{1}	49^{3}	23^{2}	6^{1}	96
Otterlo, Nat. Park De Hoge Veluwe (n=35)	0	1	5^{a}	9^{b}	1	16^{a}
Hoog Buurlo, Hoog Buurlosche heide (n=25)	6	5	1^{a}	1^{a}	2	15^{a}
Dwingeloo, Nat. Park Dwingelderveld (n=50)	3^{1}	3^{1}	43^{b3}	13^{b2}	3^{1}	65^{b}
Otterlo, Nat. Park De Hoge Veluwe (n=35):						
Calluna vulgaris (n=20)	0	0	4	6	0	10
Erica tetralix (n=15)	0	1	1	3	1	6
Hoog Buurlo, Hoog Buurlosche heide (n=25):						
C. vulgaris (n=5)	2	0	0	0	1	3
E. tetralix (n=5)	1	0	0	0	0	1
Vaccinium myrtillus (n=10)	2	4	0	0	1	7
V. vitis-idaea (n=5)	1	1	1	1	0	4
Dwingeloo, Nat. Park Dwingelderveld (n=50):						
C. vulgaris (n=25)	1	0	11^{a}	4	1	17^{a}
E. tetralix (n=15)	2	2	6^{ab}	4	0	14^{ab}
Empetrum nigrum (n=10)	0	1	13^{c}	5	0	19^{c}
V. vitis-idaea (n=10)	0	0	10^{bc}	0	2	12^{abc}

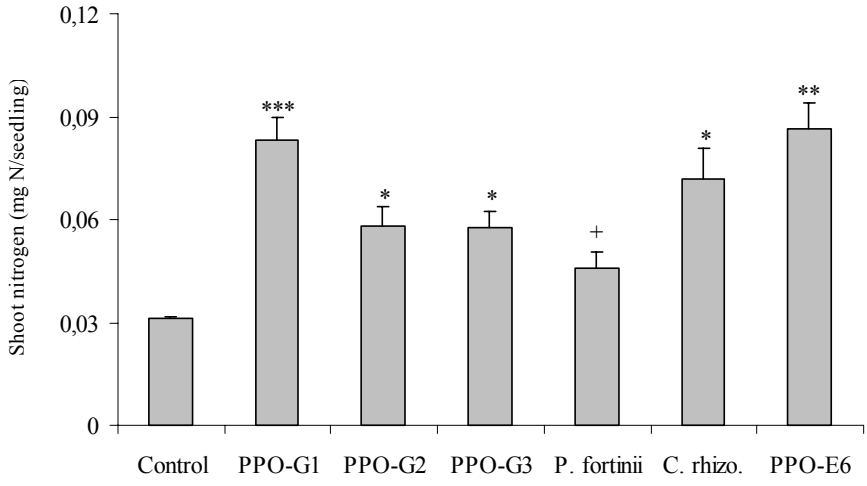

Figure 3. Shoot nitrogen uptake in mg N of *Deschampsia flexuosa* seedlings inoculated with endophytic fungi isolated from *D. flexuosa*: PPO-G1, PPO-G2, PPO-G3 and ericoid endophytic fungal species (*Phialocephala fortinii, Cryptosporiopsis rhizophila* and ericaceous isolate type PPO-E6). Harvest was after 5 weeks. n=5. ANOVA: $P<0.10^{+}$, $P<0.05$*, $P<0.01$**, $P<0.001$***. Values presented are means ± 1 SE.

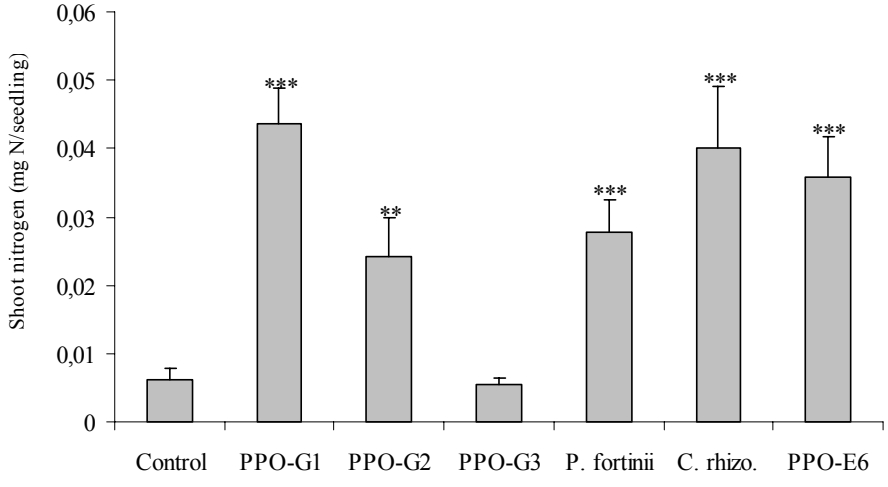

Figure 4. Shoot nitrogen uptake in mg N of *Calluna vulgaris* seedlings inoculated with endophytic fungi isolated from *Deschampsia flexuosa*: PPO-G1, PPO-G2, PPO-G3 and ericoid endophytic fungal species (*Phialocephala fortinii, Cryptosporiopsis rhizophila* and ericaceous isolate type PPO-E6). Harvest was after 5 weeks. n=5. ANOVA: $P<0.01$**, $P<0.001$***. Values presented are means ± 1 SE.

Discussion

We obtained a high diversity of fungal endophytes from ericaceous plants and the grass *D. flexuosa* (Fig. 1). We obtained several species from *D. flexuosa* roots that belonged to the *Helotiales*, the taxonomic order to which the well-known mycorrhiza-formers in the genus *Hymenoscyphus* (now in part transferred to *Rhizoscyphus*) belong. Our finding that three grass root endophyte types and some ericoid endophytic fungi could enhance nitrogen uptake in *D. flexuosa* is unprecedented (Fig. 3). Still more remarkable was the finding that two grass endophyte types could increase nitrogen uptake in *C. vulgaris in vitro* (Fig. 4). To our knowledge, this is the first report that *D. flexuosa* is able to benefit when it is grown with various fungi from the *Helotiales*.

The *Helotiales*, to which four of the grass endophyte types belong, is a diverse order that includes many ericaceous endophytes (Monreal *et al*. 1999, Berch *et al*. 2002, Vrålstad *et al*. 2002a). We found no support for a close relationship of the grass root isolates with *R. ericae* or with the salal isolates identified as *Hymenoscyphus* sp. Even for grass isolate PPO-G1, representing the morphotype (morphotype 1) that was most abundantly isolated from heathland grass roots, there was no bootstrap support for a relationship with these *Hymenoscyphus* species (Fig. 1). Due to the diversity of the sequences, the tree shows long branch attraction. Nevertheless, two of our grass root isolates, PPO-G3 and PPO-G4, clearly show a close relationship with the sequences of *F. ovina* endophytes clustering in the *Dermateaceae*. Moreover, the ericaceous isolate PPO-E9 falls into the same cluster, suggesting that there may be closer relationships between some grass root and ericoid endophytes than has previously been suspected.

Our work confirms that there is a large diversity of ericoid endophytes. Only a few ericaceous isolates clustered within the *H. ericae* aggregate; most of them were not closely related with each other. Monreal *et al*. (1999) were also surprised by the high diversity of their ericaceous isolates, which did not seem closely related to fungi collected from ericoid roots by Xiao & Berch (1996). Isolates related to *Cryptosporiopsis* spp., making up almost 10 % of our isolates, were unusually prominent in our collection (Table 7). The ericaceous isolate types PPO-E7 and PPO-E8, though they could not be induced to sporulate, appeared to be members of this clade. *C. rhizophila* was isolated from various types of ericoid roots; as detailed above,

it conferred nutrient benefits on *D. flexuosa* and *C. vulgaris* seedlings. This species, however, was not isolated from grass. Whether it is truly confined to ericaceous hosts needs to be further investigated.

With our isolation methods, we restricted ourselves primarily to ascomycetes. Recently, however, Allen *et al*. (2003) have shown that basidiomycetes such as *Sebacina* spp. are also abundantly present in salal roots. The difficulty of isolating and propagation of these fungi hampers mycorrhizal experimentation. Some sterile ascomycetous fungi are also difficult to maintain in viable condition in pure culture for several years. In the present study, representative isolates PPO-E2, PPO-E5 and PPO-E7 in our ericaceous fungal collection were lost within a short period after the experiments were finished in both our participating molecular phylogeny centre and our ecological centre. The losses in our collection occurred due to bacterial infection. Sterile fungal isolates may be notably difficult to preserve in pure culture because no conidia or spores are formed, and resistant chlamydospores are formed only in some species (S. Tan, pers. comm.).

In this study we focused on the ecological role of well-known mycorrhizal root endophytes. The occurrence in roots of any fungus does not mean it also is mycorrhizal. Read (1991) emphasized the need for working according to Koch's postulates to test root-inhabiting fungi and to evaluate plant responses as seen in growth parameters or nutrient balance. Tests with ericoid endophytes are, however, mostly restricted to microscopic structural examination, generally only performed with roots obtained from synthesis trials (Monreal *et al*. 1999, Bergero *et al*. 2000, 2003, Sharples *et al*. 2000, Berch *et al*. 2002, Allen *et al*. 2003). With mycorrhiza being defined as mutualistic symbiotic associations, bidirectional transfer of carbon and nutrients should preferably be shown to substantiate that both partners benefit in associations referred to as mycorrhizal (Smith & Read 1997, Lindahl *et al*. 2001). Such testing is planned in our research in the immediate future.

We used only a small selection of ericoid and grass root endophytes in our synthesis trials and did not test a range of nutritional conditions in the growth medium. Our results, however, are compatible with existing suggestions that there is a continuum ranging from loose, nonmycorrhizal root associations to fully mutualistic mycorrhizal associations, and that some fungal species may vary widely in status along this continuum, depending on environmental conditions and host species present (Johnson *et al*. 1997, Perotto *et al*. 2002).

In our syntheses trials, we found significantly increased nitrogen quantities in plants in the fungal treatments (Figs 3, 4). We expect that this was due to the formation of an active mutualistic symbiosis, but we cannot exclude other explanations. For example, a diffusible growth-promoting compound could have been produced by the fungal species. Rudawska & Kieliszewska-Rokicka (1997) showed that ectomycorrhizal fungal strains with high auxin synthesizing capacity induced higher numbers of mycorrhizas than strains with lower capacity for auxin synthesis. This increased mycorrhiza formation, however, was not always accompanied by increased seedling growth. In general, little attention has been paid to the production of plant growth factors by ERM and endophytic fungi (Smith & Read 1997). Perhaps more important in the interpretation of our experiments is that we cannot exclude that the production by endophytes of certain enzymes, e.g. polyphenol oxidases, played a role in mineralizing the organic nitrogen in our test systems, consistent with effects noted by Cairney & Burke (1998). In other *in vitro* experiments we performed, involving the degradation of soluble tannins in the so-called Bavendamm test, we found that some isolates, including *C. rhizophila* (CBS 109839) and ericaceous isolate PPO-E6, gave a positive response. Clearly, more research is needed to elucidate the mechanisms underlying the positive growth effect exerted by these endophytic fungi on host plants.

Though potentially mutualistic *P. fortinii*-like species have a very wide host range (Jumpponen & Trappe 1998, Ahlich *et al*. 1998, Addy *et al*. 2000, Grünig *et al*. 2002a), we did not find these fungi in grass roots. No molecular evidence was found for a close relationship of any of the grass root fungi with species presently or historically known as *Phialophora* or *Phialocephala* (Fig. 1). Dark, septate hyphae were common in *D. flexuosa* roots and, correspondingly, 33 % of our cultures possessed dark hyphae (Table 5). These cultures failed to produce reproductive structures even after nine months, and their appearance differed from that of the *P. fortinii*-like cultures from ericaceous roots. The affinities of these fungal types, e.g. morphotype 1, are still unclear.

In our ericaceous fungal collection, *P. fortinii* and related *P. fortinii*-like species made up a substantial 22 % of isolates (Table 7). Jumpponen and Trappe (1998) reported that *P. fortinii* had been isolated repeatedly from ericaceous plants such as *V. myrtillus*, *V. vitis-idaea*, *C. vulgaris* and *E. nigrum*. *Phialocephala fortinii*-like species have not previously been reported from *E. tetralix*. Also, the positive growth effect of these fungi on both *D. flexuosa* and *C. vulgaris* seedlings has not previously been reported (compare Jumpponen 2001, Stoyke &

Currah 1993). In the present study, we obtained numerous DSE cultures from ericoid sources but identified only the few that sporulated. It would be interesting in future to include additional isolates in molecular analyses to determine their relatedness to various known endophyte groups, including the *H. ericae* aggregate as delineated by Vrålstad (2002a).

The effects of simultaneous colonisation by two different mycorrhizal types are not clear. In *D. flexuosa* roots, we saw that AMF structures were more abundant than dark hyphae (Figure 2). Dual colonisation has been found in a wide range of host plants, most of which conventionally were considered to host only AMF (Read 1991). A negative effect of dual colonisation can be suppression of the original mycorrhizal type. A field survey by Genney *et al*. (2001) revealed that *Nardus stricta* transplants within *C. vulgaris* swards developed little or no AMF colonisation. In our grass roots, however, we found no differences in AMF colonisation percentages among locations. The relatively dense colonization of grass roots by dark fungi at heathland and forest sites, contrasting with lower levels seen in grass monocultures, can be tentatively attributed to high inoculum densities produced by neighbouring non-grass host plants in heaths and forests. In our synthesis trials, where there was no competition from AMF, we found that grass roots were strongly colonised by the grass endophyte isolate PPO-G2, our chosen representative of the relatively pale-coloured morphotype 2.

Whether AMF occur in ericoid plants is currently under debate. Davies *et al*. (2003) found that hair roots of epacrids were colonised by hyphae and vesicles typical of AMF. Earlier, Read (1991) pointed out that in the presence of a true AMF host the inoculum potential may be high enough to cause contaminating ingress into nearby ericaceous roots. In the present study, we found no AMF structures in our ericoid samples, even though grass AMF hosts were present at all collection sites.

In our ericaceous plants, the abundance of *O. maius* was low (Table 6). Johansson (2001) and Sharples *et al*. (2000), who also isolated ericoid endophytes from root pieces, obtained very low *O. maius* numbers, whereas they obtained *R. ericae* abundantly. A high abundance of *O. maius* in ericaceous roots was shown, however, in sites in Alberta and on Vancouver Island, Canada, and in northern Italy (Perotto *et al*. 1996, Hambleton & Currah 1997, Monreal *et al*. 1999). It appears that the abundance of *O. maius* isolates is related to particular site conditions (Perotto *et al*. 2002).

Our demonstration of a positive effect of ericoid endophytes on nutrient uptake in *D. flexuosa* can be considered preliminary. We realize that the role of Helotialean endophytes in the uptake of organic nitrogen by *D. flexuosa* should be clarified with isotopic labelling experiments. Also, as mentioned above, a true mycorrhizal association implies bidirectional transfer of nutrients and carbon (Smith & Read 1997, Read 2002). Nevertheless, the isolation of beneficial Helotialean endophytes from *D. flexuosa* encourages us to re-examine our ideas on organic nitrogen uptake by grasses in nutrient-poor ecosystems, and to further investigate the nitrogen-related effects this fungal association might have on competition between *D. flexuosa* and ericaceous plants.

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Chapter 5

Phylogeny and taxonomy of root-inhabiting *Cryptosporiopsis* species, and *C. rhizophila* sp. nov., a fungus inhabiting roots of several *Ericaceae*

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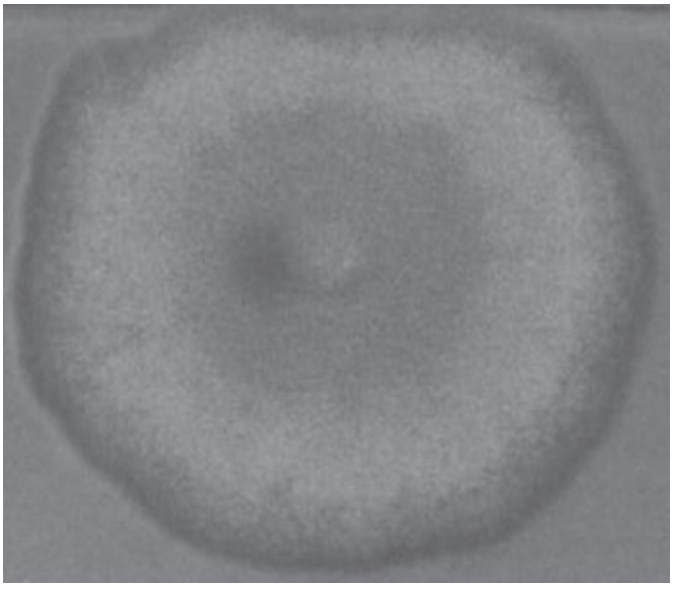


Abstract

Three *Cryptosporiopsis* species have thus far been isolated from roots of woody plants. A fourth species, which was recently isolated from roots of *Calluna vulgaris*, *Erica tetralix*, *Vaccinium vitis-idaea*, and *V. myrtillus* in the Netherlands, is described here as a new species. Sporulation on the natural substratum has not been observed and the morphological description of this fungus is therefore based on characters expressed on oatmeal and malt extract agars. The phenotypic characters indicated a close relationship with the other root-inhabiting species of *Cryptosporiopsis* and species of the associated teleomorph genus *Pezicula*. This relationship was confirmed by phylogenetic analyses using sequence data of the 5.8 S nuclear ribosomal DNA and flanking internal transcribed spacers. In order to facilitate recognition of this possibly under-recognized category of root inhabitants, a key to the root-inhabiting *Cryptosporiopsis* species based on characters *in vitro* is given.

Key words: Cryptosporiopsis, *Ericaceae*, *Pezicula,* phylogenetic analyses, taxonomy

Introduction

Root inhabiting ascomycetes have been implicated to play an important role in functioning of ecosystems. For example, as a result of their symbiosis with ericoid mycorrhizal fungi, ericaceous plants are capable of growing in nutrient-stressed and even in highly polluted environments (Smith & Read 1997). Inoculation with specific endophytic or mycorrhizal ascomycetes can increase resistance against certain root-pathogenic fungi (Sylvia & Chemelli 2001). The biodiversity of root-inhabiting fungi is becoming a major topic in soil ecology, rhizosphere and mycorrhizal research (Sylvia & Chemelli 2001, Perotto, Girlanda & Martino 2002, Vandenkoornhuyse *et al.* 2002). Molecular approaches are revealing an unexpected taxonomic and genetic diversity among the ascomycetes that are isolated from healthy roots, particularly of members of the *Ericaceae*. Ever more sterile morphotypes are tentatively identified as members of the discomycete order *Helotiales* by comparisons of ITS sequences with those in GenBank and EMBL databases. Some have already been recognized as ericoid mycorrhizal fungi (Berch, Allen & Berbee 2002, Monreal, Berch & Berbee 1999, Perotto *et al.* 2002).

Three species of *Cryptosporiopsis* have thus far been isolated from roots of woody plants. *Cryptosporiopsis* species are the anamorphs of *Pezicula* and *Neofabraea*, two genera of the *Helotiales* which are mainly known as endophytes or pathogens of above ground parts of woody plants (Verkley 1999, Abeln, De Pagter & Verkley 2000, De Jong *et al.* 2001). Some species are producers of secondary metabolites with antibacterial, fungicidal and herbicidal activity (Noble *et al.* 1991, Schulz *et al.* 1995, 2002). According to Kowalski (1983), the wood and bark endophyte *Pezicula cinnamomea*, with anamorph *Cryptosporiopsis grisea*, can also spread into the roots of dying trees. Thus far, only two *Cryptosporiopsis* species that were isolated exclusively from roots have been formally described based on morphological characters *in vitro*, viz., *C. radicicola*, from roots of *Quercus robur* (Kowalski & Bartnik 1995), and *C. melanogena*, from roots of *Q. robur* and *Q. petraea* (Kowalski, Halmschlager & Schrader 1998). The teleomorphs of these species are unknown, but on the basis of partial 18S rDNA and ITS sequence analyses, Abeln *et al.* (2000) concluded that they belong to the monophyletic genus *Pezicula* sensu Verkley (1999), a concept including the former genus *Ocellaria*.

As part of an ecophysiological study of ericaceous plant communities in the Netherlands, we repeatedly isolated a fungus from healthy, surface-sterilized roots of several Ericaceae. No sporulation was observed in nature, but the morphological features expressed by some isolates on oatmeal agar indicated that it was a species of *Cryptosporiopsis* resembling *C. radicicola* and *C. melanogena* of oak roots. To test our hypothesis that the fungus isolated from the roots of *Ericaceae* is a genetically distinct entity within the genus *Pezicula*, we performed ITS sequence analyses comparing data derived from earlier work (Abeln *et al*. 2000) and additional data from GenBank. Because the teleomorph is as yet unknown and only an anamorph name can be applied, we describe this fungus as a new species of *Cryptosporiopsis* based on morphological characters *in vitro*.

Materials & Methods

Isolation and phenotypic characterization of root-inhabiting fungi

New strains used in this study are listed in Table 1. Whole plants of *Calluna vulgaris*, *Erica tetralix*, *Vaccinium vitis-idaea*, and *V. myrtillus* were collected in heather and vicinal forest vegetations in the Netherlands, and placed with intact root system and surrounding soil in plastic bags. Plants were regularly moistened and within 14 d treated in the laboratory as follows. Soil and superficial debris were removed from the roots by rinsing in tap water. Root tips were cut off 1 cm behind the apex and attached soil particles were removed with forceps under a stereo microscope. Tips were surface-sterilized in 4 times diluted domestic bleach water (4% chlorine, final concentration 1%) for 3 min, followed by three rinses in sterile water. Three tips were placed in each petri dish on 2% malt extract agar (MEA) or potato dextrose agar (PDA) with 20 mg/l streptomycin to inhibit bacterial growth. Mycelia growing out of the root tips were transferred after about 7 days to 2% MEA and PDA. Pure cultures were regularly checked for sporulation. For morphological description, strains were incubated on oatmeal agar (OA) and 3% MEA, prepared according to CBS List of Cultures, Fungi and Yeasts, 35th ed., 2001. Petri dishes were placed in an incubator at 15 ºC in the dark, and at the same temperature with n-UV (12 h rhythm). The colours were described according to Rayner (1970).

Table 1. Isolates of *Cryptosporiopsis rhizophila* used in this study. All strains were isolated from roots of plants collected in the Netherlands.

GenBank	CBS accession nr	Host	Geographic origin
AY176753	109839	*Erica tetralix*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld
AY176754	110602	*Calluna vulgaris*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176755	110603	*Calluna vulgaris*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176756	110604	*Calluna vulgaris*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld
AY176757	110606	*Erica tetralix*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176758	110609	*Erica tetralix*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld
AY176759	110612	*Vaccinium vitis-idaea*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176760	110616	*Vaccinium myrtillus*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176761	110617	*Vaccinium myrtillus*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide

DNA extraction and sequencing

Strains were transferred from agar cultures to 2 mL liquid medium (2% malt extract) and incubated on a rotary shaker (300 rpm) for 2–3 wk at room temperature. Liquid cultures were transferred to 2-mL tubes, centrifuged and washed twice with sterile water. DNA was extracted using the FastDNA kit (Omnilabo 6050073, BIO 101, CA) according to the manufacturer's instructions. For ITS sequence analysis part of the ribosomal RNA gene cluster was amplified by PCR using primer pairs V9G (De Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990). PCR was performed in 50 µL reaction volumes and each reaction contained 10-100 ng of genomic DNA, 25 pM of each primer, 40 µM dNTP, 1.0 unit Supertaq DNA polymerase and 5 µL 10x PCR buffer (SphaeroQ, Leiden, the Netherlands). The amplification was performed in an Applied Biosystems (Foster City, CA) thermocycler with the following program: 1 min 95ºC, 30x {1 min 95ºC, 1 min 55ºC, 2 min 72ºC} followed by a final extension of 5 min at 72ºC. PCR products were cleaned with GFX columns (Amersham Pharmacia, NJ, 27-9602-01) and analyzed on a 2% agarose gel to estimate the concentration. The PCR products were sequenced using internal primers ITS1 and ITS4 (White *et al.* 1990). Sequencing was performed with the BigDye terminator

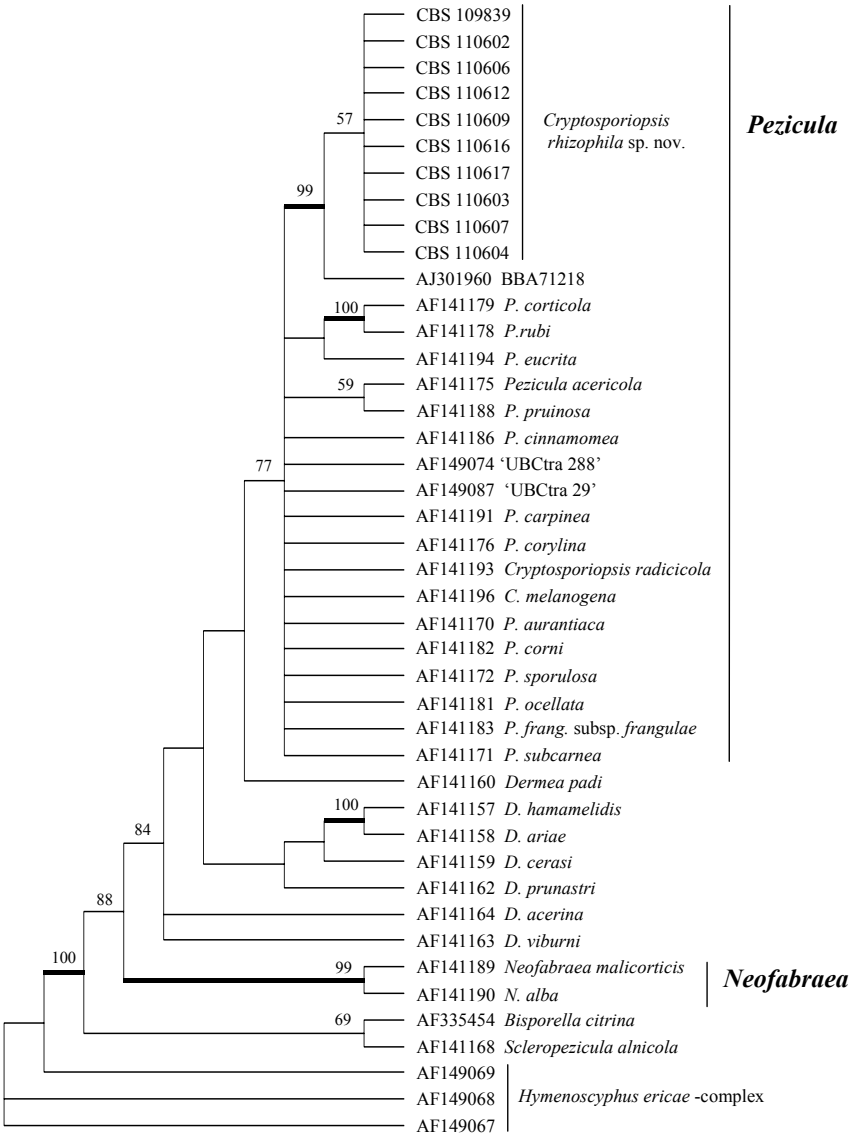


Figure 1. Strict consensus tree of 95 MPT's of 409 steps using 135 parsimony-informative characters of the ITS region. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Branches supported by 90% or higher values are in bold. Accession numbers of sequences taken from GenBank are indicated before the taxon name. GenBank numbers of sequences of *C. rhizophila* strains are given in Table 1. Species are presented by teleomorph name, if known. Sequences of the *Hymenoscyphus ericae* complex were used as outgroup to root the tree.

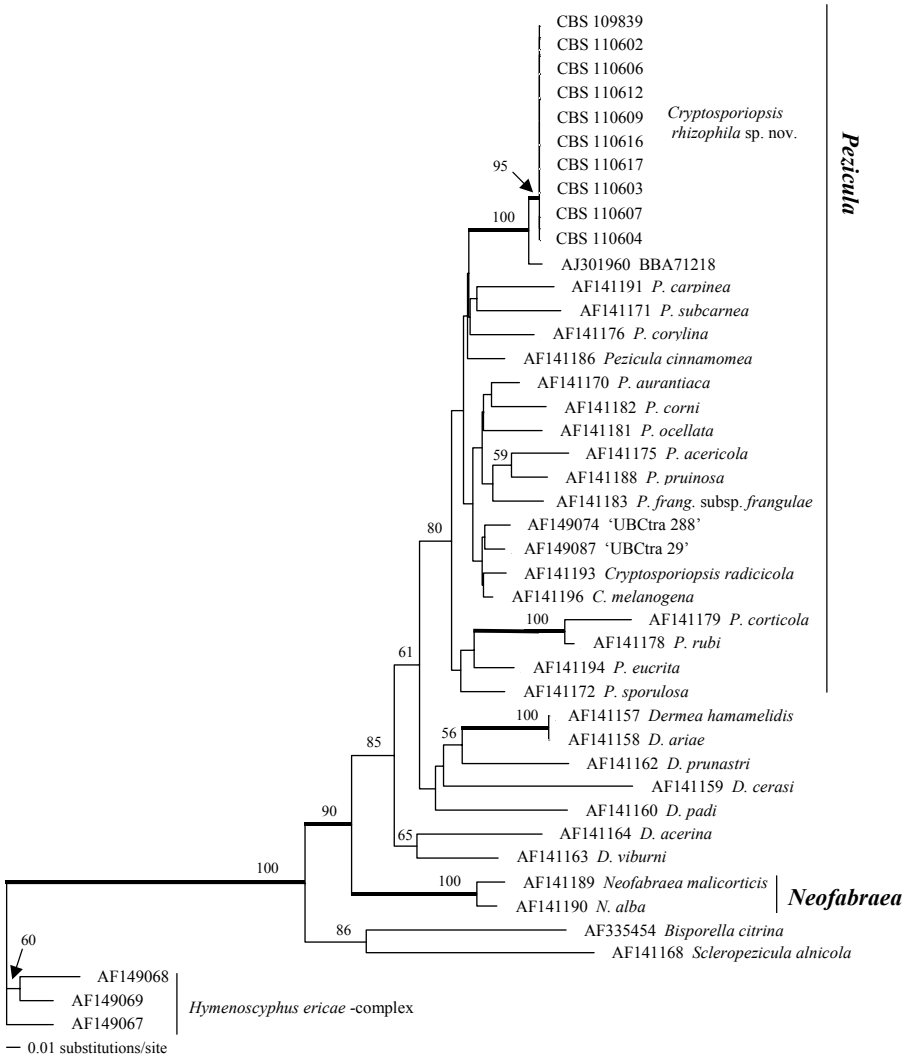


Figure 2. Neighbour-joining tree derived from 135 parsimony-informative and 42 autapomorphic characters of the ITS region, calculated in PAUP without pairwise corrections. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Branches supported by 90% or higher values are in bold. Length of branches is proportional to number of changes. Species are presented by teleomorph name, if known. Sequences of the *Hymenoscyphus ericae* complex were used as outgroup to root the tree.

chemistry (Part number 403049, Applied Biosystems) following the manufacturer's instructions. The sequencing products were cleaned with G50 Superfine Sephadex columns (Amersham Pharmacia 17-0041-01), and separated and analyzed on an automated sequencer (ABI Prism 3700 DNA Analyzer, Applied Biosystems). Forward and reverse sequences were matched using SeqMan (DNAstar Inc., WI).

Phylogenetic analyses

Pairwise and global alignment of consensus sequences were performed in Bionumerics 2.5 (Applied Maths, Kortrijk, Belgium). Manual adjustments were made in the global alignment where necessary. Maximum parsimony methods and neighbour-joining distance methods were used to infer phylogenetic hypotheses. Parsimony analyses were done using PAUP v. 4.0b10 (Swofford 2002). The heuristic searches were performed with the following parameters: characters were unordered with equal weight, and random taxon addition. The tree bisection-reconnection (TBR) algorithm was used in branch swapping, with branches collapsing if the maximum branch length was zero. The maximum number of trees was set at 10.000. Alignment gaps were treated as missing characters. Parsimony bootstrap analyses were performed using the full heuristic search option, random stepwise addition, and 1000 replicates, with maxtrees set at 100.

Neighbour-joining analyses were performed in Bionumerics and PAUP, in both cases without pairwise corrections. Stability of clades was tested with 1000 neighbour-joining bootstrap replications. BLAST searches in GenBank revealed highest similarity to species of *Pezicula*, *Dermea*, and *Cryptosporiopsis*, of which part of the 18S, ITS 1, 5.8S rDNA, and ITS 2 had been sequenced by Abeln *et al.* (2000). In our analyses only the ITS region of these sequences was included. Three additional sequences from GenBank were also included: one highly similar sequence of an unidentified ascomycete (BBA71218), and two of isolates from roots of *Gaultheria shallon*. GenBank accession numbers, taxon names and other information about these sequences are given Table 2. GenBank accession numbers of the strains of *C. rhizophila* are given in Table 1. Two species classified in the family *Helotiaceae* of the same order were also included: *Bisporella citrina*, AF335454, and *Hymenoscyphus ericae*, AF149067, 149068, 149069. The last three sequences were defined as outgroup.

Results

Phylogenetic analyses

The investigated strains of *C. rhizophila* showed 100% identity in ITS 1-5.8 S rDNA-ITS 2. The alignment of all 43 taxa comprised 499 characters, 135 (27%) of which were parsimony-informative. The remaining 364 characters were all uninformative and were excluded from the parsimony analyses. The heuristic search using 5000 random sequence input orders yielded 95 most-parsimonious-trees (MPT) of 409 steps, with consistency index (CI) 0.494, retention index (RI) 0.697, rescaled consistency index (RCI) 0.344, and homoplasy index (HI) 0.506. The strict consensus tree is depicted in Figure 1. Bootstrap supports over 50 % are indicated. *Cryptosporiopsis rhizophila* and the strain BBA71218 formed a highly supported clade (99%), which was nested within the *Pezicula* clade, comprising the oak root endophytes *C. radicicola* and *C. melanogena*, the two strains isolated from the roots of *Gaultheria shallon* (UBCtra 288 and 29), and all included *Pezicula* species. This clade was supported by 77 % of the bootstrap replications. The two species of *Neofabraea* grouped in a well-supported clade, but the species of *Dermea* showed a paraphyletic arrangement. *Scleropezicula alnicola* grouped with *Bisporella citrina* (69 % bootstrap support). In addition to the 135 informative characters, 42 autapomorphic characters were also included the neighbour-joining analysis. The results of this analysis were similar to those of the parsimony analysis, showing 80 % bootstrap support for the *Pezicula* cluster, which included the root-inhabiting species as well as *C. rhizophila* (Figure 7). *C. radicicola* and *C. melanogena* clustered with the two strains isolated from the roots of *Gaultheria shallon* (UBCtra 288 and 29), but bootstrap support was low. As in the parsimony analysis, the cluster of *C. rhizophila* strains and BBA71218 obtained very high bootstrap support. The cluster comprising only the *C. rhizophila* strains received much higher support in the neighbour-joining analysis (95%) than in the parsimony analysis (57%).

Key to the species of *Cryptosporiopsis* isolated from roots

The key is based on characters expressed on OA and MEA in the dark at 15 ºC. It is followed by a formal description of the new species from *Ericaceae*. Previously described *Cryptosporiopsis* species from roots were treated by Kowalski & Bartnik (1995), Kowalski *et al.* (1998), and Verkley (1999).

1 Colonies on OA initially colourless, but later becoming greyish, Buff or brownish, or almost black with age; aerial mycelium may be well-developed, but without elevated surface structures; conidiogenous cells borne directly on vegetative hyphae, or in sporodochial conidiomata provided with seta-like brown-walled hyphae; macroconidia up to 37 µm long and 9 µm wide, containing oil droplets up to 3 µm diam 2

1 Colonies on OA soon becoming brownish, Cinnamon or Olivaceous, with distinct globular to columnar surface structures which are composed of entangling hyphae and rise well above the aerial mycelium; sporulating only in simple to complex, intially closed, eustromatic conidiomata; macroconidia often very large, 29–58 × 9.5–16 µm, with oil droplets up to 2.0 µm diam. ..... *C. grisea* (teleomorph: *Pezicula cinnamomea*)

2 Chlamydospores present; basal cell of the seta-like hyphae swollen; macroconidia 22–37 µm long 3

2 Chlamydospores absent, basal cell of seta-like hyphae not swollen, macroconidia 16.2–25 µm long ..... *C. rhizophila*

3 Colonies becoming homogenously grey to black on MEA, forming scattered, very dark brown areas on OA; macroconidia 25–37 × 5.5–9 µm, usually formed within a few weeks ..... *C. melanogena*

3 Colonies beige, pale or dark brown on MEA; macroconidia 22–35 × 6–7.5 µm, usually formed sparsely and later ..... *C. radicicola*

TAXONOMY

Cryptosporiopsis rhizophila Verkley & Zijlstra, **sp. nov.**

Figs 3–7

Etym.: rhizophilus, root-loving

Conidiomata in vitro typice sporodochia et plerumque setis fuscis septatis praedita. *Cellulae macroconidiogenae* plerumque in conidiophoris simplicer vel interdum ramosae, septatae, acrogenae vel acropleurogenae integratae, phialidicae, cylindricae vel clavatae, 7–13(–18) × 3–5 μm. *Macroconidia* ellipsoidea vel breve cylindrica, vulgo curvata, hyalina, continua, interdum 1-septata, guttas numerosas 1.0–2.5(–3.0) μm diametro continentia, 16.2–25.0 × 6.0–7.6 μm. *Microconidia* ellipsoidea, apice rotundato et basi late truncata vel leviter attenuata, hyalina, continua, 4.0–5.5 × 1.2–2.0 μm.
Typus: **The Netherlands**: Prov. Drenthe, Nationaal Park Dwingelderveld, near 'schaapskooi', isol. ex root of *Erica tetralix*, Sep. 2000, *J. D. Zijlstra* 335 (dried culture on OA, CBS, holotypus; CBS 109839, living culture).

Sporulation occurring directly on immersed or superficial vegetative hyphae, or in superficial, hemispherical sporodochia which may become surrounded by tufts of 70–140 μm long, septate seta-like hyphae with somewhat thickened, smooth, pale to dark brown walls, ending in a hyaline blunt tip, up to 4 μm wide at the base, and often rising above the surface in sticky bundles. In addition, erect synnema-like columns are also formed which are composed of entangled hyphae bearing conidiogenous cells in the lower part. *Conidiogenous cells* mostly integrated in simple, rarely branched, septate, acrogenous or acropleurogenous conidiophores, more rarely discrete and borne on rather undifferentiated sterile tissue consisting of hyphal to isodiametric cells, determinate, phialidic, older ones with a well-visible periclinal thickening, cylindrical to clavate, widest just below the apex, 7–13(–18) × 3–5 μm. *Macroconidia* ellipsoid to short-cylindrical, mostly curved, aseptate, hyaline, with age occasionally medianly 1-septate and golden-yellow, containing numerous oil droplets 1–2.5(–3) μm diam. Measurements in water: dark (on average 22.5 × 7 μm; N = 20): (18–)20–24(–25) × (6–)6.5–7(–7.5) μm, n-UV (on average 20 × 7 μm; N = 20): (16–)18–21.5(–23) × (6–)6.5–7(–7.5) μm. Conidial masses whitish, with age yellow or cinnamon. *Microconidiogenous cells* integrated in separate cylindrical, acrogenous or acropleurogenous

conidiophores, phialidic, with a periclinal thickening at the apex and often a minute collarette. *Microconidia* ellipsoid, with a rounded apex and a broadly truncate or slightly attenuated base, hyaline, aseptate, with granular contents, 4.0–5.5 × 1.2–2.0 µm (dark and n-UV).

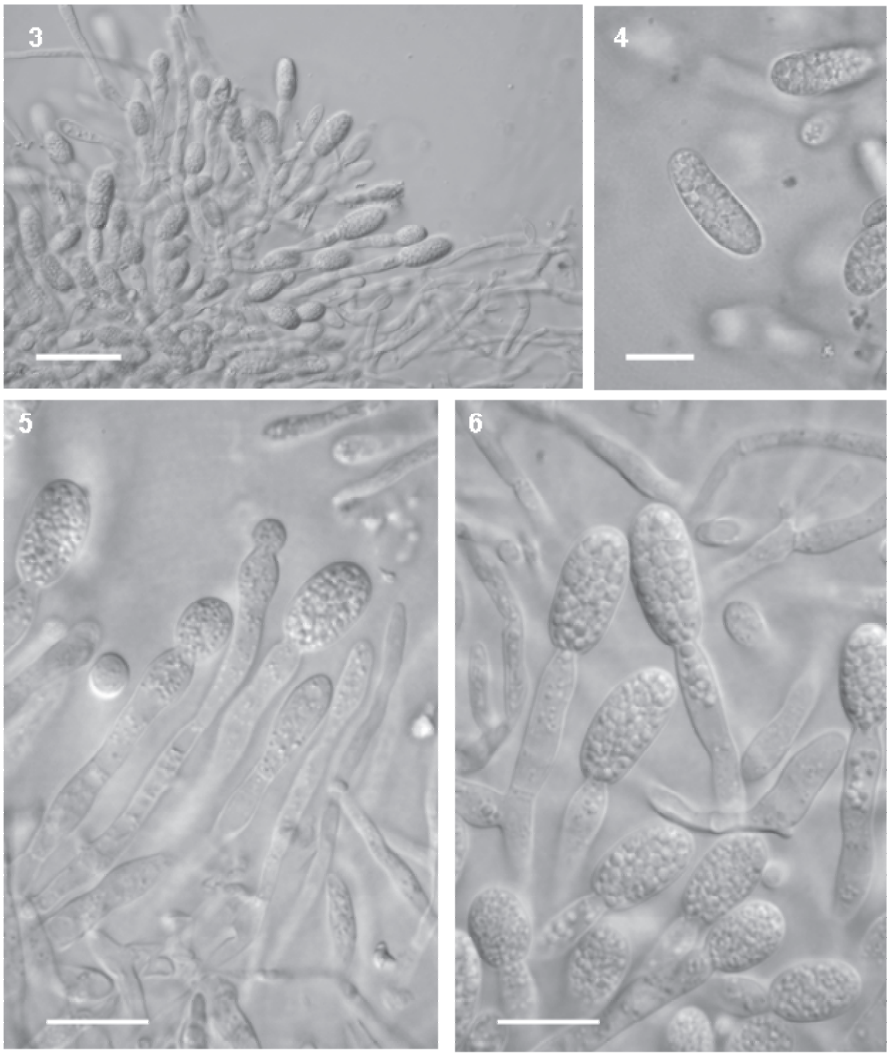
Colony morphology: on OA reaching a diam of 44 mm in 10 d (80 mm in 21 d), with an even or slightly ruffled margin; first glabrous and colourless, but after 3–4 weeks immersed mycelium becoming Beige to Buff, and in the centre and some scattered areas Umber, slightly Olivaceous or Sepia, reverse in these areas becoming dull Hazel; aerial mycelium variable, remaining very scanty or becoming well-developed within a few weeks, whitish to Buff, woolly-floccose, covering most of the colony surface; sporulation starting at about 10–14 d, at first only in the centre from small clusters of macro- and microconidiogenous cells arising from undifferentiated hyphae, but later also in sporodochia which are scattered over the colony surface. On MEA reaching a diam of 10 - 12 mm in 10 d (20 mm in 21 d), with an even to irregular, later often distinctly lobed, glabrous, Saffron to Ochreous margin; surface mostly covered by dense, pure white, woolly aerial mycelium, which near the margin becomes Salmon with some yellow after several weeks; reverse homogeneously Cinnamon or Bay, later Chestnut in the centre. A red diffusible pigment is clearly visible in the medium surrounding 3-week-old colonies. Isolates examined are listed in Table 1. Sporulation was observed on OA in type strain CBS 109839, and also in CBS 110604, 110606 and 110612.

Hosts: isolated from root tips of *Calluna vulgaris*, *Erica tetralix*, *Vaccinium vitis-idaea*, and *V. myrtillus*.

Distribution: Known only from two localities in The Netherlands.

Discussion

In the extended body of literature on fungi isolated from stringently washed or surface-disinfected roots of woody plants, reports of *Cryptosporiopsis* species are very rare. Previously, however, we have noted that unidentified *Cryptosporiopsis* species could regularly be obtained from serially washed roots of apparently healthy *Cornus canadensis* (*Cornaceae*) plants in Ontario, Canada (Summerbell 1989). Since *Cryptosporiopsis* species are often slow to sporulate in culture and also may be difficult to recognize as coelomycetes –



Figures 3–6. Cryptosporiopsis rhizophila, CBS 109839 on OA. 3. Sporodochium (bar = 25 μm). 4. Conidia (bar = 10 μm). 5, 6. Conidiogenous cells (bars = 10 μm).

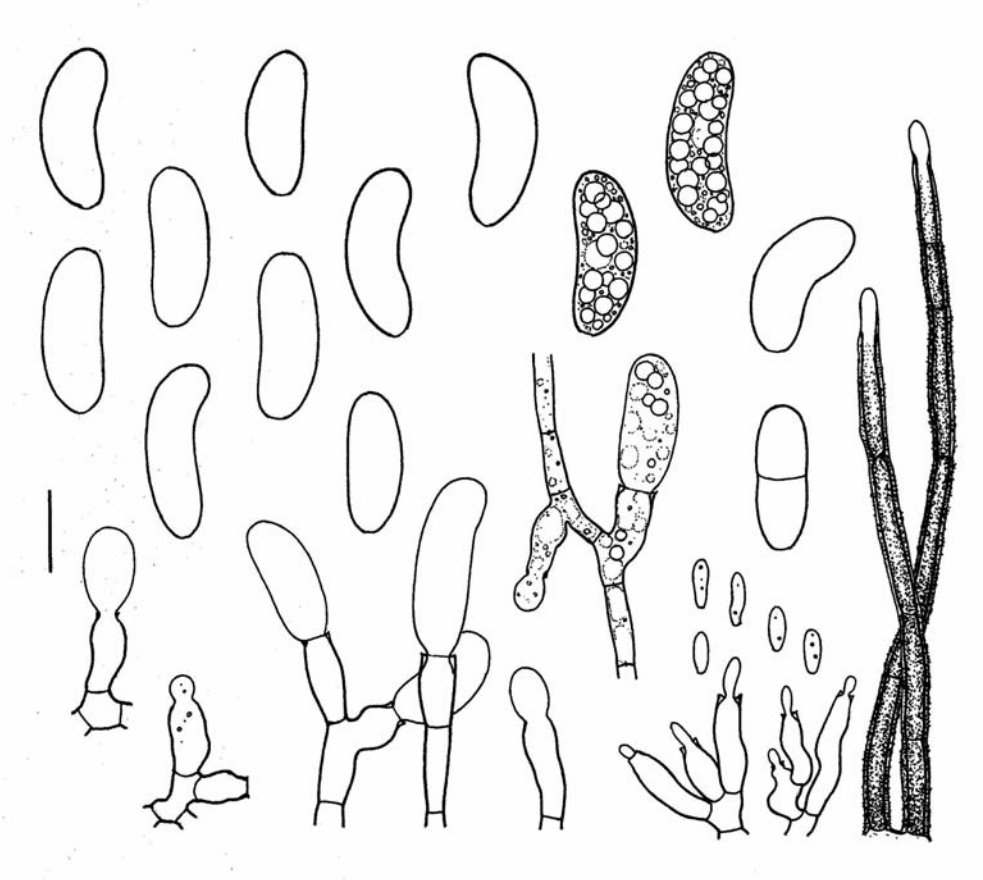


Figure 7. Cryptosporiopsis rhizophila, CBS 109839 on OA (bar = 10 μm). A. Macroconidia and macroconidiogenous cells. B. Microconidia and microconidiogenous cells. C. Setae.

a step that is necessary in order to access useful morphological identification literature – it is possible that such isolates have been seen in other studies but not identified.

Cryptosporiopsis rhizophila can be placed in *Cryptosporiopsis* on the grounds of the macro- and microconidiogenous cells which are integrated, phialidic, determinate, and hyaline. The presence of ellipsoid, pluriguttulate, 0-septate macroconidia and much smaller microconidia are also characteristic of this anamorphic genus (Verkley 1999). The oak-root-inhabiting *C. radicicola* and *C. melanogena* have several features in common with *C. rhizophila*. The macroconidia of these fungi are more or less distinctly curved, and contain oil droplets that are larger than those found in other *Cryptosporiopsis* species. The phialides are frequently directly borne on vegetative hyphae. Conidiomata are sporodochial and often provided with

seta-like hyphae that are not found in other *Cryptosporiopsis* species. There are, however, also differences between the three root fungi. The two species from oak roots form chlamydospores, but these are not found in *C. rhizophila*. The basal cells of the setae of *C. radicicola* and *C. melanogena* are swollen, whilst those of *C. rhizophila* are not. In *C. melanogena* macroconidia are 25–37 × 5.5–9.0 µm (Kowalski *et al*. 1998), and in *C. radicicola* they are 22–35 × 6.2–7.5 µm, while in *C. rhizophila* they are on average shorter, and never over 25 µm long. Some isolates of *C. rhizophila* produced a red diffusible pigment on MEA, and this has not been observed in the other two species.

In congruence with morphological data, the ITS sequence analyses also indicate that *C. rhizophila* is a member of the genus *Pezicula*, and that it is congeneric with *C. radicicola* and *C. melanogena*. The internal topology of the *Pezicula* clade is, however, largely unresolved. Thus far, ITS sequences show no variation within species of *Pezicula*, and also the strains of *C. rhizophila* all had identical ITS sequences. The ITS sequence of isolate BBA 71218 differs by three base positions from that of *C. rhizophila*, indicating that it is most likely specifically distinct. Unfortunately, we have so far been unable to obtain any information about the phenotype of this fungus which was isolated from roots of *Erica* sp. (pers. comm., dr H. I. Nirenberg).

The neighbour-joining analysis indicates that *C. rhizophila* could also be closely related to *Pezicula carpinea*, *P. subcarnea*, *P. cinnamomea* and *P. corylina*, rather than to the other root isolates including *C. radicicola*, *C. melanogena*, and the unidentified strains from *Gaultheria*. However, sequencing of more loci will be necessary to clarify whether the root-inhabiting species indeed represent multiple lineages within *Pezicula*, or a single lineage. The conidiomata of the anamorph of *P. carpinea* (*C. fasciculata*), are relatively similar to those of *C. rhizophila*, because they are also sporodochial or ‘acervuloid’ in culture (Verkley 1999), while *in planta* they develop as true acervuli. *Pezicula carpinea*, which is the type species of the genus *Pezicula*, occurs mainly on *Carpinus betulus* in Eurasia and on *Ca. caroliniana* in North America, but also on *Fagus sylvatica*. It has also, however, been isolated from living bark of other trees in a study by Kowalski & Kehr (1992) on endophytes in forest tree species. As far as is known, it has not been isolated from roots. *Pezicula corylina* is so far only known from North America, where it is confined to *Corylus* spp. In culture, this species forms

eustromatic, initially closed conidiomata resembling those formed *in planta* (Groves 1941, Verkley 1999). Such conidiomata are also formed by the ubiquitous *P. cinnamomea*.

Cryptosporiopsis rhizophila is to our knowledge the first morphologically described species of *Cryptosporiopsis* from roots of *Ericaceae*. Because it has been repeatedly isolated from surface-sterilized, healthy roots of several *Ericaceae*, it can be regarded as an endophytic fungus. The association of *C. rhizophila* with the plants has been confirmed by microscopical observations of hyphae in living, healthy rootlets of sterile *Calluna* seedlings grown in vitro, that were successfully infected after inoculation with this fungus (Zijlstra *et al.*, unpublished results). Some *Helotiales* are experimentally confirmed ericoid mycorrhizal symbionts, viz., *Hymenoscyphus ericae*, and several probably closely related, unnamed *mycelia sterilia* (Read 1974, Monreal *et al.* 1999, Vrålstad, Schumacher & Taylor 2002, Vrålstad, Myre & Schumacher 2002). Berch *et al.* (2002) reported that resynthesis experiments conducted with the salal root isolates UBCtra 288 and 29 had been unsuccessful. Little is known about the role of these apparently root-associated members of *Pezicula*. The resynthesis experiments recently initiated in Wageningen are expected to shed more light on the possible role of *C. rhizophila* as a mycorrhizal partner.

Endophytic fungi that were reported as isolated from twigs and branches of ericaceous plants and identified as *Cryptosporiopsis* sp. (Fisher *et al*. 1984), may have been *P. myrtillina* or *P. acericola*, both of which have been found on above-ground parts of ericaceous hosts. *Pezicula myrtillina* occurs in Europe and North America on recently dead twigs and branches of several *Ericaceae*, viz., *Calluna vulgaris*, *Vaccinium myrtillus*, and *V. uliginosum*, and also on *Rhododendron ferrugineum* and *R. maximum*. No cultures are available of this species, and the anamorph is unknown (Verkley 1999). Morphologically, the apothecia of *P. myrtillina* resemble those of *P. rubi* and *P. eucrita*, species which in our sequence analyses are more distantly related to *C. rhizophila* than are the oak fungi (31 and 20 base positions difference in ITS1 and ITS2, respectively, with *C. rhizophila*). *Pezicula acericola* normally occurs on *Acer*, *Cornus* and *Quercus* spp., but also on *Rhododendron ferrugineum*. This species is different from *C. rhizophila* in morphology and ITS sequence.

Acknowledgements

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Chapter 6

The ecological role of *Cryptosporiopsis rhizophila* in Dutch ericoid plants

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Abstract

Cryptosporiopsis rhizophila was isolated from the roots of several Dutch ericoid species. The ecological role of this fungal species with respect to the ericoid host plant was unclear. We tested the abilities of this species to enhance the nutrient status of *Calluna vulgaris* seedlings by means of synthesis trials *in vitro*. Additionally, Bavendamm tests were performed, to test the saprotrophic ability of this species to produce enzymes that degrade phenolics. Dutch isolates of *Rhizoscyphus ericae* and *Oidiodendron maius* were included in the tests. Results of the synthesis trials showed that three isolates of *C. rhizophila* colonized the *C. vulgaris* roots successfully and could be seen to significantly enhance nitrogen uptake of inoculated *C. vulgaris* seedlings when these were compared to the controls. The fungal structures produced by *C. rhizophila* in the epidermal cells were different from those produced by *R. ericae* and *O. maius*. All tested isolates of *C. rhizophila* were able to produce phenol-oxidizing enzymes in the Bavendamm tests. We conclude that *C. rhizophila* isolates are able to associate with roots of *C. vulgaris* and have the potential to fulfill the same ecological function as well-known mycorrhiza formers towards their ericaceous host.

Key words: Bavendamm test, *Calluna vulgaris,* ericoid mycorrhizal fungi, nitrogen uptake, soluble phenolics, synthesis trials.

Introduction

In our previous work *Cryptosporiopsis rhizophila* Verkley & Zijlstra was isolated from young roots of *Calluna vulgaris* (L.) Hull, *Erica tetralix* L.*, Vaccinium vitis-idaea* L. and *V. myrtillus* L. in Dutch heathlands (Verkley *et al.* 2003, Zijlstra *et al.* 2005). *Cryptosporiopsis* species are anamorphs of ascomycetes in the genera *Pezicula* and *Neofabraea* (*Dermataceae*). Their occurrence in the Dutch heathland plants is noteworthy, because this fungal species constituted about 4% of the total slow-growing fungal isolate collection, and with another unknown *Cryptosporiopsis* species almost 8% (Zijlstra *et al.* 2005). As we were able to maintain several viable cultures of this new species, we had the opportunity to examine in more detail the ecological role of *Cryptosporiopsis* species in ericaceous hosts.

The role of these newly isolated endophytic *Cryptosporiopsis* species with respect to the ericoid host plant is still unclear. From non-ericaecous hosts, both pathogenic and non-pathogenic strains were isolated as endophytes (Fisher *et al.* 1984). Two *Cryptosporiopsis* species are known as root-invaders, e.g. *C. radicicola* Kowalski & Bartnik in roots of *Quercus robur* L. (Kowalski & Bartnik 1995) and *C. melanogena* Kowalski & Halmschlager in *Q. robur* and *Q. petraea* (Mattuschka) Lieblein (Kowalski *et al*. 1998). Only recently, Sigler *et al.* (2005) described two new *Cryptosporiopsis* species from roots of ericaceous hosts in western North America. *Cryptosporiopis ericae* Sigler was isolated from roots of *Vaccinium membranaceum*, *V. ovalifolium* and *Gaultheria shallon* (salal). *Cryptosporiopis brunnea* Sigler was isolated from roots of *G. shallon*. These species appear to be uncommon in roots of western North American ericoid plants as there was an infrequent recovery of the isolates in culture and an inability to detect them in direct PCR amplification from roots (Berch *et al.* 2002, Allen *et al.* 2003). At the moment, however, little is known about their role in roots. In synthesis trials *in vitro*, no formation of mycorrhizal structures was seen in experiments done with salal and with *C. brunnea* (Allen *et al.* 2003) or *C. ericae* (Berch *et al.* 2002).

In heathlands, most ericaceous plants roots are occupied by a variety of endophytic, mutualistic fungi (Monreal *et al*. 1999, Perotto *et al*. 2002, Berch *et al*. 2002). The role of these fungi is diverse. Some of them have already been described as ericoid mycorrhiza. Ericoid mycorrhizae are defined as associations between mainly ascomycetous fungi and plant species belonging to the families of the *Ericaceae* and *Empetraceae*. Ericoid

mycorrhizal fungi produce distinct hyphal coils in cells of the hair roots of plants (Smith & Read 1997). To date, only a few fungal species are described as forming ericoid mycorrhiza. The most important among them are ascribed to genera in the *Helotiales*, for example *Rhizoscyphus ericae* (Read) Korf & Kernan (Read 1974) or *Myxotrichaceae, Oidiodendron maius* G.L. Barron (Dalpé 1986, Hambleton *et al.* 1998). Only the referred species are confirmed to be mycorrhizal in *in vitro* synthesis experiments.

Molecular characterization of ericoid roots has shown that there is a wider variety of ericoid mycorrhizal fungal species than once thought, which includes several species probably closely related to the *H. ericae* aggregate, listed as unnamed *mycelia sterilia* (Monreal *et al*. 1999, Perotto *et al*. 2002, Berch *et al*. 2002, Vrålstad *et al.* 2002 a, b). A group of these root-associated fungal isolates can now be accomodated in the new genus *Meliniomyces*, which is a new anamorph genus for root-associated fungi with phylogenetic affinities to *Rhizoscyphus ericae* (≡ *Hymenoscyphus ericae*) (Hambleton & Sigler 2005). Previously, Hambleton & Currah (1997) referred to this group as the 'Variable White Taxon' (VWT). Although some synthesis trials in vitro were performed, no clear mycorrhizal structures were produced in comparison to an isolate of *R. ericae* or *O. maius* (Hambleton & Currah 1997). So, the function of *Meliniomyces variabilis* in ericaceous host is not yet clear (Hambleton & Sigler 2005). Nevertheless, many endophytic fungi isolated from ericoid roots were found to take part in the formation of mycorrhiza. For example, Bergero *et al.* (2000) isolated several sterile mycorrhizal endophytes from *Erica arborea,* which were able to form ericoid mycorrhiza in synthesis trials. In addition, it is reported that ericoid mycorrhizal fungi may coexist in ericaceous and ectomycorrhizal plants (Bergero *et al*. 2003, Vrålstad *et al*. 2002b).

The association of ericoid mycorrhiza is adapted to benefit plant growth in nutrient-poor soils (Read 1996, Cornelissen *et al.* 2001). In heathland ecosystems, polyphenolics released from degrading litter, characterized by low nitrogen and phosphorus concentrations, can cause complexing of proteins and inhibit nitrogen cycling (Hättenschwiler & Vitousek 2000). Studies with isolates of *Rhizoscyphus* and *Oidiodendron* revealed that by production of extracellular enzymes these fungi are able to degrade complex organic residues that prevail in surface layers of heathland soil (Leake and Read 1997, Read and Perez-Moreno 2003). These enzyme activities might facilitate the degradation of polymeric carbon sources and exposure of nutrients to decomposition processes, and additionally they could be involved in providing direct access to the nutrients themselves (Read *et al.* 2004). Ericoid mycorrhizal fungi, e.g.

R. ericae, have been found to excrete polyphenol oxidase, laccase and catechol oxidase enzymes which are responsible for oxidation of phenolics acids and tannins (see references in Read *et al.* 2004). Therefore these mycosymbionts are able to compete with saprotrophic fungi, which is a prerequisite for efficient nutrient scavenging by the mycosymbionts (Bending & Read 1997, Burke & Cairney 2002).

The aims of this investigation were to compare the nutritional effects of *Cryptosporiopsis rhizophila* with well-known mycorrhiza formers and to test for the presence of polyphenol degrading abilities in this species. We performed synthesis trials of *C. rhizophila* with *Calluna vulgaris* seedlings *in vitro* to determine whether coiled structures were produced in the epidermal cells and to test both the growth stimulus and the nitrogen uptake of *C. vulgaris* seedlings. The polyphenol-degrading abilities of *C. rhizophila* were tested with the Bavendamm reaction. This is an established diagnostic test, which detects secretion of phenol oxidases (Burke & Cairney 2002). In both the synthesis trials and the tests for polyphenolic degradation, *C. rhizophila* was compared with Dutch isolates of *Rhizoscyphus ericae* and *Oidiodendron maius*.

Materials & Methods

Fungal isolates

The fungal isolates were collected from roots of *Ericaceous* plants from several habitats in the Netherlands (Table 1). Root tips from individual plants were cleaned from organic material, surface-sterilized for 3 min with 1% household bleach and rinsed three times with sterile water. Root tips were placed on 2% malt extract agar with 20 mg/l streptomycin sulfate to inhibit bacterial growth. To obtain sterile cultures, mycelia growing out of the root tips were transferred on fresh plates with 2% malt extract, potato dextrose agar and Modified Melin Norkrans medium (Marx 1969). Cultures were regularly checked for sporulation. Identification was based on morphological and molecular characteristics of the strains, see also Verkley *et al*. (2003).

Mycorrhizal synthesis trials

Cryptosporiopsis rhizophila, *R. ericae* and *O. maius* were tested for their ability to produce ericoid mycorrhizas on axenic *C. vulgaris* seedlings obtained from surface-sterilized seeds on

Table 1. Overview of isolates collected in september 2000 from Ericaceous roots in the Netherlands and results of the presumptive assays for soluble phenolic degradation by isolates of *C. rhizophila* and ericoid mycorrhizal fungi (*Rhizoscyphus ericae* and *Oidiodendron maius*).

Species	Accession number	Host plant	Geographic origin	Bavendamm reaction[1]
Cryptosporiopsis rhizophila	CBS 109839	*Erica tetralix*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld	+
Cryptosporiopsis rhizophila	CBS 110603	*Calluna vulgaris*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide	+++
Cryptosporiopsis rhizophila	CBS 110604	*Calluna vulgaris*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld	+++
Cryptosporiopsis rhizophila	CBS 110606	*Erica tetralix*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide	+++
Cryptosporiopsis rhizophila	CBS 110616	*Vaccinium myrtillus*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide	+++
Rhizoscyphus ericae	PPO 100	*Calluna vulgaris*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide	++
Rhizoscyphus ericae	PPO 246	*Erica tetralix*	Prov. Gelderland, Otterlo, Nat. Park De Hoge Veluwe	+++
Oidiodendron maius	CBS 110450	*Vaccinium vitis-idaea*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld	++
Oidiodendron maius	CBS 110452	*Vaccinium vitis-idaea*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld	++

[1] - no colour change, +/- colour change disappeared within an hour, + weak colour change, ++ moderate colour change, +++ strong colour change.

water agar (3 min 1% hypochlorite, rinsed three times in sterile water). The method used was according to Pearson and Read (1973). Peat (Fixet Gardenpeat, pH 4) was dried, sieved (2.0 mm mesh size) and autoclaved twice (20 min at 120ºC). Distilled water agar (1%) was poured over small clumps of the sterilized peat (0.5 g) in Petri dishes. The next day, one half of the water agar discs with peat was removed from the Petri dish and one four-weeks-old *C. vulgaris* seedling was placed at the centre on the cut edge. Each Petri dish was inoculated with two agar plugs from actively growing colony margins of fungal isolates maintained on Modified Melin Norkrans medium (Figure 1). Six replicates were used for each tested isolate.

The Petri dishes with inoculated seedlings were taped with parafilm to prevent drying out and avoid contamination. Petri dishes were placed in vertical position in an illuminated growth chamber at 20ºC day temperature and 15ºC night temperature, 16h/8h day/night cycle for 10 weeks. The Petri dishes were placed in a randomized block design.

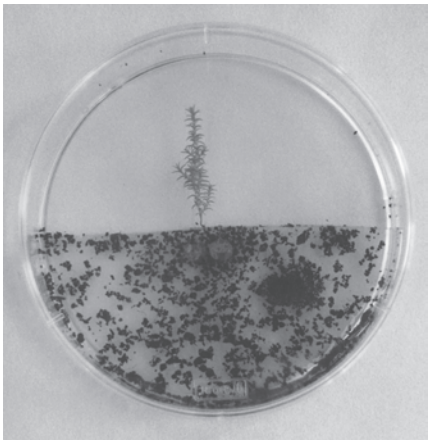


Figure 1. Example of an experimental unit of the synthesis trial *in vitro* with *Calluna vulgaris* seedlings, inoculated with an isolate of *Cryptosporiopsis rhizophila*. Plate incubated for 10 weeks.

Root colonization

Roots of *C. vulgaris* seedlings were stained in a 0.2% solution of trypan blue in lactic acid: glycerol: water (3.25:3:4 by vol.) and then transferred to a storage solution of lactic acid: glycerol: water (1:2:1 by vol.). Roots were mounted on a microscopic slide and the colonization of epidermal cells was observed under a microscope.

Growth response and nitrogen uptake of *Calluna vulgaris*

From plants grown in the synthesis trials, the weight of the above-ground part was measured with a microbalance after drying them at 70ºC during 24 hours. The dried leaves (70 ^{o}C) were pulverised and the N concentrations were measured using an elemental analyser (Fisions Instruments, EA 1108). Nitrogen uptake was calculated by multiplying the percentage nitrogen with the dry weight.

Statistical analysis

Average dry leaf weights and nitrogen uptake of the *C. vulgaris* seedlings in the fungal treatments were log-transformed prior to analysis to obtain homogeneity of variances between the treatments. Means between the different fungal treatments were compared in a one-way analysis of variance by a post-hoc Tukey test ($P < 0.05$).

Assay for degradation of soluble phenolics (the Bavendamm reaction)

The Bavendamm reaction was used according to the method of Giltrap (1982) to determine the ability of fungal isolates to degrade soluble phenolics. A stock solution was prepared by dissolving 0.5 g tannic acid (Aldrich Chemical Co.) in 10 ml of distilled H_2O, and adjusting its pH to 4.7 with 1 M NaOH. The solution was sterilized through a 0.2 µm pore Millipore filter, and added to cool autoclave-sterilized MMN agar (minus malt extract and containing 5 g/l glucose). Agar plugs of 6 mm diam, taken from the margin of actively growing cultures, were used to inoculate the plates. Three replicates were set up for each isolate. Plates were incubated in the dark at 20º C. Fungal secretion of phenol oxidases results in oxidative polymerisation of phenolic acids to brown reaction products. So fungi with the capacity to degrade tannic acid turn the colour of the agar from purple-grey to brown. The intensity of the coloration gives an indication of relative degradative ability. Cultures were examined periodically over seven weeks, and the extent of the reaction was recorded (Bending & Read 1997).

Results

Mycorrhizal synthesis trials

C. rhizophila isolates colonized the epidermal cells of *C. vulgaris* roots successfully and produced hyphal structures in individual epidermal cells (Figure 2). Figure 2c shows clearly that some of the structures, which are seen in the epidermal cell have connections with fungal hyphae outside the cell. The form of the structures seemed to be different from those seen in de *C. vulgaris* roots which were colonized by *R. ericae* or *O. maius* (Figure 3).

Three isolates of *C. rhizophila* increased the dry weight and nitrogen uptake of *C. vulgaris* seedlings compared to the uninoculated seedlings in the control treatment. These *C. rhizophila* isolates were equally effective as *O. maius* and *R. ericae* isolates (Figure 4). In this experiment, the isolates *C. rhizophila* 110603 and 110616 were exceptions, because these fungi colonized the roots without stimulating growth of *C. vulgaris* seedlings. Isolate *C. rhizophila* 110616 even had a slightly detrimental effect on the *C. vulgaris* seedlings compared to the uninoculated seedlings in the control treatment. However, also within the *R. ericae* isolates we noticed a natural variation in the total N uptake.

Figure 2. Patterns of colonisation produced by *Cryptosporiopsis rhizophila* in *Calluna vulgaris* hair roots, visible after staining with tryphan blue (a-c). Hyphal structures of *C. rhizophila* 109839 in some epidermal cells, × 400; (d-f) hyphal structures of *C. rhizophila* 110604 in some epidermal cells, × 400 epidermal cells.

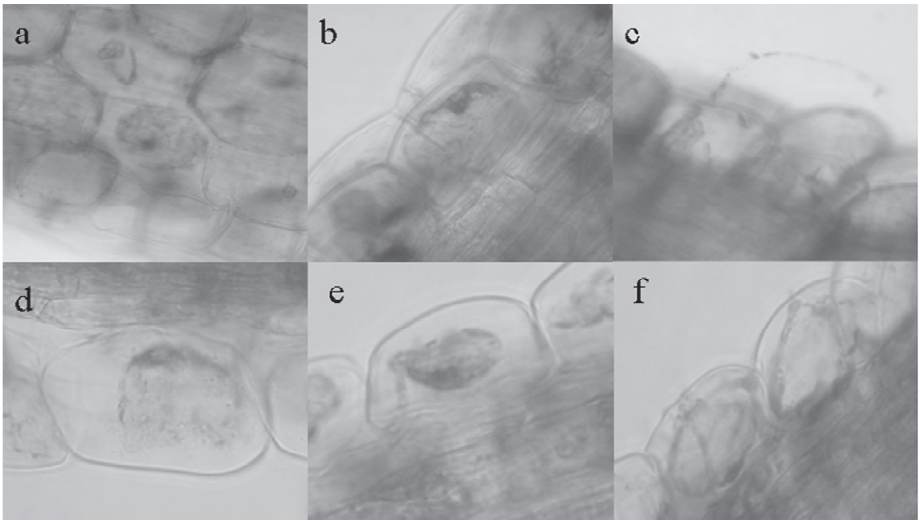
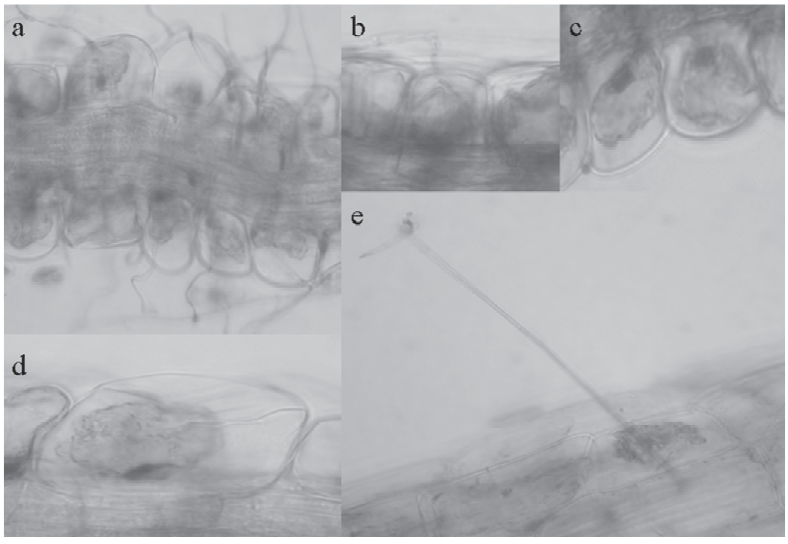


Figure 3. Mycorrhizal colonisation by *Rhizoscyphus ericae* and *Oidiodendron maius* in *Calluna vulgaris* hair roots, stained with a tryphan blue (a-c) hyphal coils produced by *R. ericae* isolate 100, × 400; (d, e) hyphal coils and conidiophore produced by a *O. maius* isolate 405, × 400.



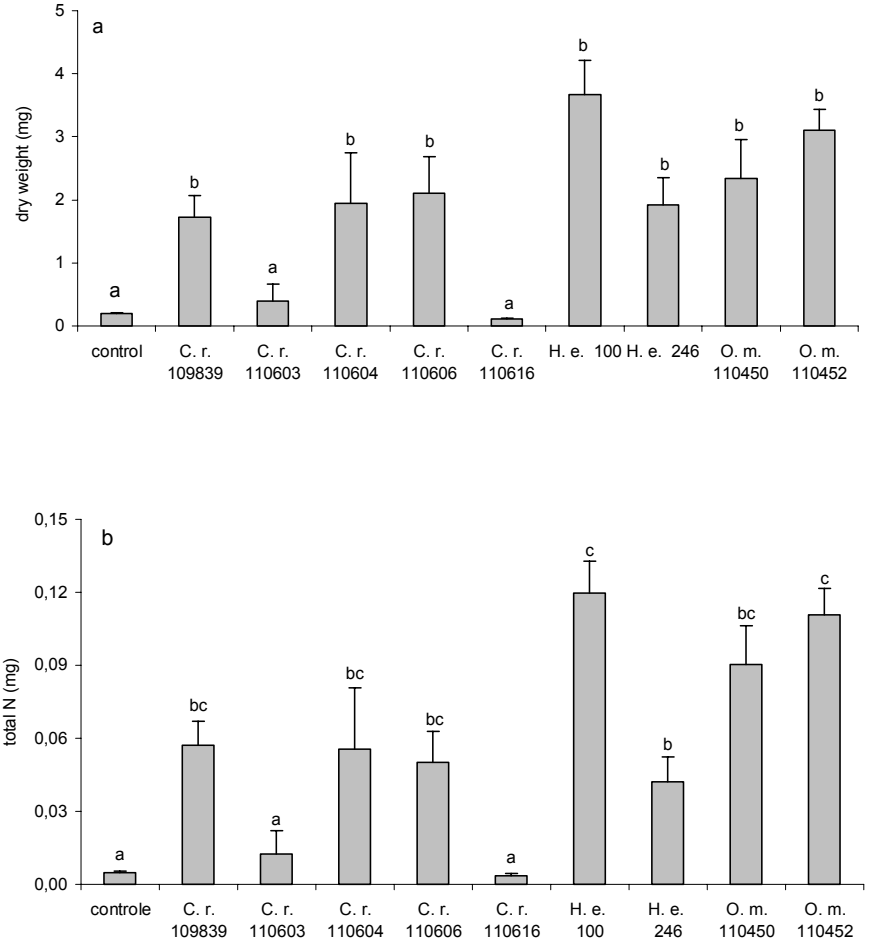


Figure 4. Dry weight in mg (a) and nitrogen uptake in mg N (b) of *Calluna* seedlings inoculated with isolates of *C. rhizophila* and ericoid mycorrhizal fungi (*Rhizoscyphus ericae* and *Oidiodendron maius*), harvested after 10 weeks. n=6. Different letters indicate statistically different values using ANOVA ($P < 0.05$). Columns show means ± SE.

Degradation of soluble phenolics (the Bavendamm reaction)

All *Cryptosporiopis* isolates produced intense brown coloration of the agar when grown with tannic acid (Table 1), and were able to degrade the soluble phenolics. Isolate CBS 109839 was the exception, with a weak darkening. *O. maius* isolates developed the colour later than *Cryptosporiopsis* isolates did, and the colour was less intense. From the isolates of *R. ericae*,

PPO 246 showed a very dark brown colour comparable to that formed by the *Cryptosporiopsis* isolates.

Discussion

Isolates of *Cryptosporiopsis rhizophila* are able to form hyphal complexes in epidermal cells of hair roots and have a beneficial nutrient effect on *C. vulgaris* seedlings. We are aware of the fact that this *in vitro* experiment could show another type of colonisation in the epidermal cells, compared to plants, which are infected in the field by this fungal species. Coils of ericoid mycorrhiza are easily identified in plant roots collected from the field. In synthesis trials, however, formation of coils can be hampered. For example, *O. maius* is widely recognized as an ericoid mycorrhizal fungus, but it is also known that this species sometimes fails to form the typical coil structures in synthesis trials or only after a period of 6-9 months (Bergero *et al.* 2000). In some cases collapsed cytoplasm can be mistaken for hyphal structures. Nevertheless, Figure 2 shows clearly that some of the structures which are seen in the epidermal cells have connections with fungal hyphae outside the cell.

In our experiment three isolates of *C. rhizophila* and both isolates of *O. maius* were as effective as the *R. ericae* isolates in enhancing nutrient uptake, and most roots were densely colonized. The nutritional benefit is expected to be closely related to the fungal structures, which are shown in the seedling roots colonized by *C. rhizophila.* Piercey *et al.* (2002) showed that lack of coil structures of *O. maius* in hair roots of *Picea mariana* (*Pinaceae*), also caused a retarded root growth. So, it seems that the presence of coils is indicative of positive growth and nutrient effects for the host plant.

We show that the nitrogen uptake by *C. vulgaris* seedlings in this experiment is not a side effect of the peat, but a direct effect of the fungal treatment. Sterilisation of the peat could have resulted in detoxifying negative effects of peat, which could also be responsible for the positive growth effect. However, some fungi colonized the roots without stimulating growth of *Calluna* seedlings. In addition, the Bavendamm tests showed clearly that *C. rhizophila* on its own is able to degrade soluble phenolics.

Cryptosporiopsis rhizophila was not previously described as an ericoid mycorrhiza, but this species is now shown to have important abilities in common with other ericoid mycorrhizal fungal species. It also became clear that not all the isolates of *C. rhizophila* were equally effective. We noticed a natural variation within the species in its stimulatory ability. An isolate with a detrimental effect on seedling growth even shows that isolates from the same endophytic fungal species can vary from parasitism to mutualism (Jumpponen 2001). This also seems to be the case in the group of dark septate endophytes (DSE), which is a miscellaneous group of endophytic fungal species that colonize root tissues intracellularly and intercellularly (Jumpponen 2001). While positive host responses have been reported in *Pinus contorta* inoculated by *Phialocephala fortinii* (Jumpponen *et al.* 1998), Stoyke & Currah (1993) their isolate of *P. fortinii* from overgrew the ericaceous host plant, *Menziesia ferruginea*.

Our results demonstrate that all tested *C. rhizophila* isolates were able to degrade soluble phenolics. The Bavendamm reaction was as strong as, or sometimes even stronger than that seen with known ericoid mycorrhizal fungi. This can be ascribed to the phenol-oxidizing enzymes, but whether the *C. rhizophila* isolates also produce peroxidase enzymes remains not yet known. The production of peroxidase enzymes was tested in preliminary tests with positive results by means of the browning reaction of pyrogallol in absence of H_2O_2. Detailed data are not shown, because the testing method is under debate. The added $FeCl_3$ contained in the MMN agar contains sufficient Fe^{3+} to form hydroxyl radicals when H_2O_2 is added to fresh medium (Cairney & Burke 1998). The saprotrophic ability to degrade phenolics enables *C. rhizophila* to release nutrients from organic polymers in the soil and make them available to the root of the host plant. Furthermore, isolates of *C. rhizophila* could assist in enabling ericaceous plants to compete successfully with other plant species, which are not able to access organic N and P sources (Perotto *et al.* 2002).

Molecular techniques have revealed a large diversity of fungal species in ericaceous plants in addition to the known ericoid mycorrhizal fungal species (Straker 1996, Hambleton & Currah 1997, McLean *et al*. 1999). Remarkably enough, *Cryptosporiopsis* species were not identified as such before. Problably, this is related to the problems existing with the current identification techniques. *Cryptosporiopsis* isolates are often slow to sporulate in culture and also may be difficult to recognize as *Coelomycetes*. To our benefit, *C. rhizophila* isolates were able to produce spores and therefore could be identified as such on both morphological and

molecular characteristics (Verkley *et al.* 2003). Only recently, Sigler *et al.* (2005) described two new *Cryptosporiopsis* species, *C. ericae* and *C. brunnea* from roots of *Vaccinium membranaceum*, *V. ovalifolium* and *Gaultheria shallon.* Nevertheless, the ecological role of these *Cryptosporiopis* species in ericaceous hosts remains unclear as in the synthesis trials *in vitro* no formation of mycorrhizal structures was seen in experiments done with salal and with *C. brunnea* (Allen *et al.* 2003) or *C. ericae* (Berch *et al.* 2002).

At present, it is unknown whether the host range of *C. rhizophila* could extend to other plant species outside the family of *Ericaceae*. The other two related root-invader species, *C. radicicola* and *C. melanogena* seem to be rather host specific, and occur only in roots of *Q. robur* or *Q. melanogena* (Verkley 1999). Consequently, *C. rhizophila* could also be host-specific towards ericaceous plants. In contrast, Bergero *et al.* (2000) showed that ericoid mycorrhizal fungi are less host-specific than previously thought. They found that *Neofabraea alba*-like species were able to persist in a Mediterranean *Quercus ilex* climax forest where the host plant *Erica arborea* was absent, and the fungal species were found as common root associates of *Quercus ilex*. Clearly, the host specificity of *C. rhizophila* needs to be further investigated and we hope that our work stimulates more researchers to identify *C. rhizophila* among their unidentified ericoid endophytic isolates, and unravel their function in ecological processes.

In summary, *C. rhizophila* was isolated from several *Ericaceous* species at different locations in the Netherlands. It seems that this fungal species occurs frequently at Dutch heathland sites and is able to colonize roots of several ericoid plant species. A beneficial role towards *C. vulgaris* seedlings *in vitro* has been shown, possibly due to its ability to degrade soluble phenolics. Further research is needed to elucidate the relative importance of these new ericoid mycorrhizal fungi species in the field, compared to *Rhizoscyphus ericae* and *Oidiodendron maius*.

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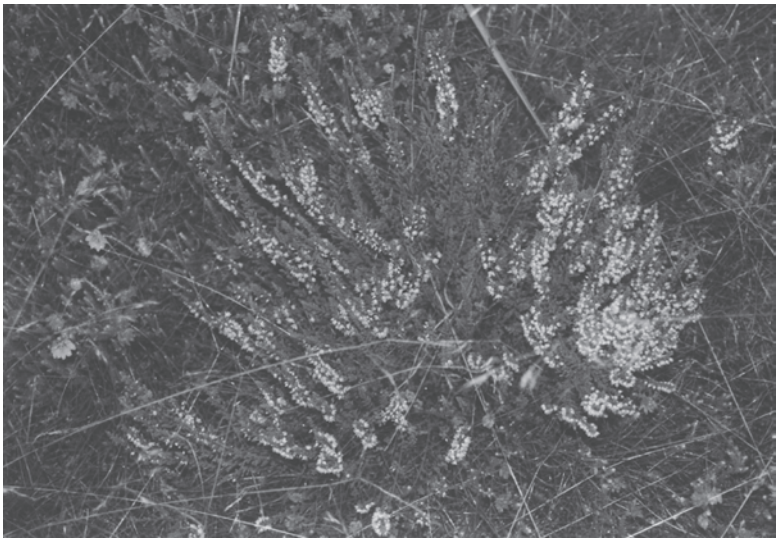
Chapter 7

Effects of tannins in litter on the competition between *Calluna vulgaris* and *Deschampsia flexuosa*

Jantineke D. Zijlstra & Frank Berendse



Abstract

It has been suggested that the competition between ericaceous plants and grasses is strongly affected by the high tannins content in litter of the two species. To test the effect of tannin-rich litter on soil nitrogen and the outcome of the competition between the species, we conducted a greenhouse experiment with *Calluna vulgaris* and *Deschampsia flexuosa* and their leaf litters. Two litters of *C. vulgaris* were used, with the same nitrogen concentration but different (high and low) tannin concentrations. The *D. flexuosa* leaf litter contained lower concentrations of tannins than the *C. vulgaris* litters. The plants were grown in monocultures and in mixed cultures. Inorganic and dissolved organic nitrogen were measured monthly during the experiment. After five months, we measured above- and below-ground biomass and the nutrient concentrations in leaves and roots.

In monocultures, *C. vulgaris* produced most biomass when grown on its own litter. In mixes cultures with grass plants, *C. vulgaris* plants produced significantly less biomass. If grown on grass litter the shrubs were unable to compete with the grass. *D. flexuosa* plants produced most biomass on their own litter, whether in monocultures or mixed with *C. vulgaris* plants. Addition of shrub litter did not lead to tannin-related inhibition of the growth of *D. flexuosa*, as the grass plants were able to outcompete both on shrub litter and on grass litter. In this short experiment, *C. vulgaris* plants were unable to outcompete *D. flexuosa*. Grass plants benefited more efficiently from the available soil nitrogen released from the added litter. The competitive suppression of shrub plants by the grasses was least in the treatments with low nutrient availability. The two kinds of *C. vulgaris* litters that differed in concentration of tannins did not differ in their effects on the competition between the species or on the production of inorganic and dissolved organic nitrogen. From this we conclude that the nitrogen content of the litter is more important as a mechanism driving competition between shrubs and grasses than the tannin content.

Keywords: Calluna vulgaris, competition, *Deschampsia flexuosa*, dissolved organic nitrogen, tannins.

Introduction

Dutch heathlands used to be dominated by the evergreen dwarf shrubs *Calluna vulgaris* (L.) Hull and *Erica tetralix* L., but during the past few decades these species have been increasingly replaced by the grasses *Deschampsia flexuosa* (L.) Trin. and *Molinia caerulea* (L.) Moench. Heathlands are threatened by direct and indirect effects of increased atmospheric nitrogen deposition (Berendse & Aerts 1984, Aerts 1993, Berendse 1994). Additional supplies of phosphorus can also disturb the vulnerable heathland ecosystem, as phosphorus can be an important limiting factor for grass growth as well (Aerts & Berendse 1988, Diemont 1996). Previous experiments investigating competition between dwarf shrubs and dominant grasses have shown that the dominant grasses benefit faster from an increased nutrient supply than the dwarf shrubs (Berendse & Aerts 1984, Heil & Bruggink 1987, Aerts & Berendse 1988, Aerts *et al.*1991). This was attributed primarily to the faster growth rate of grasses at higher nutrient levels; at lower nutrient levels ericaceous plant species are more competitive, as they are better able to conserve nutrients (Berendse & Aerts 1984, Berendse & Elberse 1990).

Among plant species there appears to be a trade-off between features that reduce nutrient losses and the ability to respond rapidly to enhanced nutrient levels. It has been suggested that because of this capacity to avoid nutrient losses, ericaceous plants can suppress nitrogen mineralisation by producing slowly decomposable litters rich in tannins. Tannins from degrading litter can react with protein sources in the soil to form tannin-protein complexes. These nitrogen-rich complexes release inorganic nitrogen slowly and increase the production of dissolved organic nitrogen relative to that of NH_4^+ and NO_3^- (Northup *et al.* 1995). As ericoid mycorrhizal fungi are able to degrade these tannin-protein sources (Bending & Read 1997), dwarf shrubs colonized by these fungi are able to absorb more organic nitrogen than non-colonized plants (Smith & Read 1997, Sokolovski *et al.* 2001). Grass species, on the other hand, which are predominantly colonized by arbuscular mycorrhizal fungi, are thought to use organic phosphate primarily, rather than organic nitrogen. Despite evidence that grasses are able to use organic nitrogen (Näsholm *et al.* 1998), there is still debate about the extent to which arbuscular mycorrhizal fungi can enhance amino acid absorption in plants (Hodge 2001, Hodge *et al.* 2001). It is therefore unclear whether *C. vulgaris* and *D. flexuosa* compete for the same source of organic nitrogen. Do dwarf shrubs really gain a competitive advantage

over dominant grasses by producing recalcitrant litter with high tannin contents that suppress grass growth?

It has been suggested (Northup *et al.*1995, 1998, Inderjit *et al.*1999, Kraus *et al*. 2003) that secondary plant compounds such as tannins influence the nutrient cycling by affecting the mineralization rates of organic and inorganic nitrogen and the ratio between the availabilities of these types of nitrogen. To date, the effect of tannins on the competition between heather and grasses has not been investigated. Previous experiments on competition between ericaceous plant species and dominant grasses have focused primarily on the direct effect of additional inorganic nitrogen on the outcome of the competition (Berendse & Aerts 1984, Aerts & Berendse 1988, Aerts *et al.* 1990, 1991, Mickel *et al*. 1991, Alonso *et al.* 2001, Britton *et al.* 2003, Barker *et al*. 2004). Our hypothesis was that thanks to ericoid mycorrhizal fungi, ericaceous plants compete more efficiently with *D. flexuosa* on nutrient-poor soils amended with tannin-rich litter than on nutrient-poor soils amended with tannin-poor litter. *D. flexuosa* roots are mainly colonized by arbuscular mycorrhizal fungi, so though the species is able to use organic nitrogen, we expect it to do so less than *C. vulgaris*.

In the study reported here, we examined competitive interactions between *D. flexuosa* and *C. vulgaris* in a greenhouse experiment. Specifically, we studied the effects of litter amendments with high and low concentrations of tannins on this interaction. We measured both above- and belowground plant growth and the production of inorganic and organic soil nitrogen.

Table 1. Overview of litter treatments included in this study. Each treatment consisted of 2 x 6 monocultures, 6 mixtures and 6 control pots without plants of *C. vulgaris* and *D. flexuosa*.

Litter codes (96 pots)	Litter treatments
No litter (24 pots)	No litter
Cv-h (24 pots)	*C. vulgaris* (high tannins)
Cv-l (24 pots)	*C. vulgaris* (low tannins)
Df (24 pots)	*D. flexuosa*

Table 2. Overview of litter characteristics of *C. vulgaris* and *D. flexuosa* used as treatments in the mono and mixed cultures. Concentrations of total phenolics are indicated as tannic acid equivalents per gram dry weight and concentrations of condensed tannins as the absorbance measured at 550 nm wavelength. Different letters indicate significant differences between *C. vulgaris* litter with high and low concentrations of total phenolics (Tukey test, $P<0.05$).

Litter	Total phenolics (mg tae/ g dw)	Condensed tannins (A_{550}/0.19 g dw)	%C	%N
D. flexuosa	1.27	0.00	44.71	2.43
C. vulgaris (high tannins)	42.2^{b}	0.02^{ns}	50.36^{ns}	1.64^{ns}
C. vulgaris (low tannins)	27.05^{a}	0.00^{ns}	51.73^{ns}	1.84^{ns}

Materials & Methods

Litter treatments

We performed a competition experiment between *D. flexuosa* and *C. vulgaris* in a greenhouse. As litter treatments we added grass and shrub litters with high and low concentrations of tannins (Table 1). *C. vulgaris* litter was collected from *C. vulgaris* plants in shaded (low tannins) and non-shaded (high tannins) treatments (50% light reduction) in a field experiment in De Hoge Veluwe National Park that ran from September 2001 to March 2003. Leaf material of *D. flexuosa* was collected in the same area. The leaves were dried at 38 ºC for two days and sieved (1 mm mesh size) before grinding. Total phenolics (measuring both concentrations of hydrolysable and condensed tannins) were analysed with the Folin Ciocalteu method as described in Waterman & Mole (1994). The concentration of condensed tannins was analysed following the method of Porter, Hrstich & Chan (1986). Given the problems and complexities of applying an appropriate standard for the proanthocyanidin method, the data are presented as final absorbance at 550 nm. A subsample of the plant material was dried at 70 ºC and pulverized, in order to measure the C and N concentrations with an elemental analyser (Fisons Instruments, EA 1108). The ericaceous litter collected from the treatments was found to have similar nitrogen concentrations, but significantly different concentrations of total phenolics (Table 2).

Experiment

In the litter treatments, 5 g litter (dw), with particles smaller than 1 mm diameter was mixed with 1.7 kg soil (sand mixed with soil with a high organic matter content, 5:1, v/v) and used to fill plastic pots (14 cm diam). Control pots were filled with soil without added litter. *C. vulgaris* seedlings up to one-year old were collected from De Hoge Veluwe National Park. *D. flexuosa* seeds supplied by De Cruydthoek, the Netherlands, were germinated on soil and were two weeks old at the start of the experiment in May 2003. The plants were grown either in monocultures or mixed with the other species at densities of six plants (six *C. vulgaris* plants, six *D. flexuosa* seedlings or three of each). At the start of the experiment the plants were sufficiently small (average height *C. vulgaris*, 3.8 ± 0.5 cm, *D. flexuosa* 4.6 ± 0.4 cm) to be spaced far enough apart to ensure minimum competition for light. To avoid disturbing the array of pots during the growth period, we included control pots without plants in order to be able to measure the amounts of inorganic and dissolved organic soil nitrogen and pH-KCl during the experimental period of 20 weeks. Each treatment was replicated six times. The treatments were set out randomly in a greenhouse with controlled climatic conditions (light/dark: 14/10 h., light intensity 50 $W.m^{-2}$, temperature 20 ºC, 70% R.H.). The pots were weighed and watered three times a week to keep the soil moisture at 60% of water saturation.

Table 3. Effects of litter treatment and competition mode on the biomass and nutrient uptake in shoots and roots of *C. vulgaris* and *D. flexuosa* plants. Yields in monocultures and mixtures were calculated per plant. Results of two-way ANOVA: *** $P<0.001$, ** $P< 0.01$, * $P < 0.05$, NS= non-significant.

Plant	Litter $F_{3, 15}$	Competition $F_{1, 15}$	Litter x Competition $F_{3, 15}$
C. vulgaris			
green shoot biomass	29.696***	21.559***	4.318**
dead shoot biomass	17.071***	0.107^{ns}	0.955^{ns}
root mass	23.049***	2.932	1.343^{ns}
shoot: root ratio	2.247^{ns}	1.112^{ns}	0.076^{ns}
flower mass	15.334***	28.071***	7.113***
Amount of N in plant	20.954***	15.931***	1.955^{ns}
Amount of C in plant	30.874***	13.108***	3.255*
C:N ratio	35.463***	36.500***	3.636*
D. flexuosa			
green shoots	15.777***	31.124***	3.072*
dead shoots	4.530**	0.044^{ns}	1.233^{ns}
roots	8.689***	8.825**	0.804^{ns}
shoot:root ratio	1.744^{ns}	3.663^{ns}	0.937^{ns}
N uptake	42.347***	48.449***	3.379*
C uptake	12.866***	24.641***	2.644^{ns}
C:N ratio	7.787***	17.781**	0.435^{ns}

Measurements

Plants were harvested on 29 September 2003 and then dried at 70 ºC to measure dry weight. The C and N concentrations were measured with an elemental analyser (Fisons Instruments, EA 1108). At harvest, a soil sample was collected from every pot. Extractable NH_4-N and NO_3-N in the soil were determined in 10 g fresh soil from which roots had been removed, which was extracted in 25 ml 1 M KCl. The extracts were filtered through filter paper (Schleicher & Schüll no. 589[3]). Concentrations of the extractable ions in the soil solution were calculated from the concentrations in the extract on the basis of the soil water content. The pH-KCl of the soil was also measured in the same soil extract. The pH-water was measured in an extract of 10 g fresh soil with 25 ml water. Total dissolved nitrogen (DON + inorganic N) was determined conductimetrically after persulphate oxidation of the extract (Yu *et al*., 1993). DON was calculated by subtracting inorganic nitrogen from the total dissolved nitrogen. To measure the soil water contents a subsample of 5 g soil was dried (105 ºC) overnight. Organic matter was determined by loss on ignition at 550 ºC. The C and N concentrations were measured with an elemental analyser (Fisons Instruments, EA 1108).

Statistical analyses

The effects of litter and competition on plant characteristics and soil nutrients were analysed by two-way ANOVA. The analyses were applied to the shoot and root mass per plant; the fixed factors were plant treatment (no plant, mono or mixture) and litter treatment (no litter, *C. vulgaris* high tannins, *C. vulgaris* low tannins and *D. flexuosa*). Block effects were initially included as a random factor, but removed from further ANOVA analyses because they were found to be not significant. Multiple comparisons between treatments were made using Tukey's test ($P<0.05$). We analysed the differences in shoot biomass production between the two species using the ratio between the biomass of the grass and the biomass of *C. vulgaris* in mixed culture. The same was done for the root biomass and shoot:root ratios. Effects of litter were analysed by one-way ANOVA (Tukey's test, $P<0.05$). We used repeated measurements for soil inorganic nitrogen, dissolved organic nitrogen, ratio organic nitrogen:inorganic nitrogen and pH-KCl. To avoid disturbing plant growth, we measured control pots without plants only; therefore there are no plant treatments in this analysis.

Table 4. Effects of litter treatment on the ratio between the shoot biomass, root biomass and shoot: root ratio of *D. flexuosa* and *C. vulgaris* in mixtures. Results shown as mean ± se. Different letters indicate significant differences between litter treatments (Tukey's test, $P<0.05$).

Litter treatment	Biomass ratio in mixture shoot mass	Biomass ratio in mixture root mass	Biomass ratio in mixture shoot: root
No litter	1.54 ± 0.24^{a}	0.20 ± 0.06^{a}	9.98 ± 1.81^{a}
Calluna (high tannins)	5.14 ± 1.61^{b}	2.43 ± 1.30^{a}	8.76 ± 4.27^{a}
Calluna (low tannins)	3.97 ± 1.21^{ab}	0.86 ± 0.58^{a}	10.95 ± 3.25^{a}
Deschampsia	17.93 ± 2.98^{c}	10.96 ± 4.01^{b}	$2{,}85 \pm 0.70^{a}$

Results

Plant measurements

C. vulgaris plants in the monocultures produced significantly more shoot biomass on their own litter than on the grass litter and in the control treatment (Table 3, Fig. 1). Addition of *D. flexuosa* litter reduced the amount of *C. vulgaris* shoot biomass. In addition, the plants in this treatment contained most dead shoot mass (Table 3, $P< 0.05$). In the mixed cultures too, *C. vulgaris* plants produced the least shoot biomass on *D. flexuosa* litter. A striking finding was that in the mixtures there was no positive effect of the *C. vulgaris* litter relative to the control treatment. The difference in shoot biomass between *C. vulgaris* and *D. flexuosa* plants in the mixed culture was much smaller on the *C. vulgaris* litter types than in the *D. flexuosa* litter treatment. The effects of the treatments on the competitive balance between the two species was further analysed by calculating the ratios between the biomass of the two species in mixture (Table 4). The greatest biomass production by *C. vulgaris* when grown in competition with *D. flexuosa* occurred in the control treatment, which was the treatment with the lowest nitrogen availability. The shoot:root ratio of *C. vulgaris* varied between 1:4 and 2:5 and tended to be highest in the *D. flexuosa* litter treatments (Fig. 1). We did not find any significant difference in plant parameters between the two *C. vulgaris* litter types.

Grass plants in monoculture produced most biomass on their own litter (Fig.1); in the monocultures, no significant differences were found between the grass and shrub litter

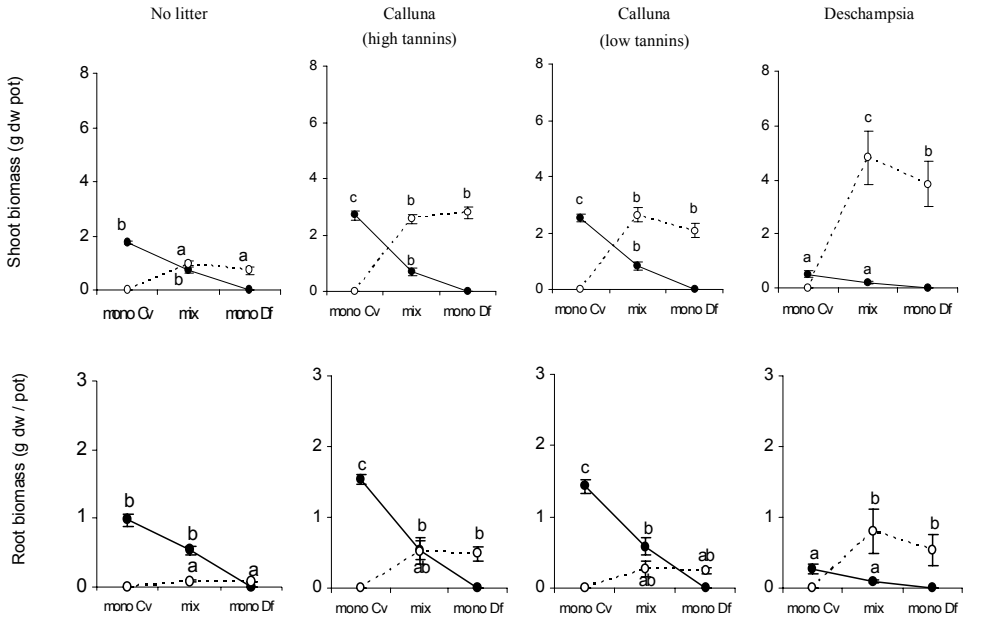


Figure 1. Effects of litter treatments on the competition between plants of *C. vulgaris* and *D. flexuosa*. Figures shown are mean shoot and root biomass (n=6) per pot. Error bars show standard errors of the mean. Different letters indicate significant differences between litter treatments (Tukey test, $P<0.05$). *C. vulgaris* – solid lines, *D. flexuosa* – broken lines.

treatments due to the high biomass variation in the grass treatment. In the mixed cultures, grass plants produced significantly less biomass in the treatments with *C. vulgaris* litter. The differences between shrub litter treatments were not statistically significant. In general, the plant biomass of the grasses was higher in mixtures than in monocultures (Table 3, Fig. 1). In mixtures, the grass benefited greatly from the addition of grass litter, but shrub growth was significantly suppressed (Fig. 3, Table 4). Overall, the shoot:root ratio of *D. flexuosa* was almost triple the shoot:root ratio of the *C. vulgaris* plants and varied between 7.2 and 15.5 (Fig. 1). Furthermore, this ratio tended to be higher in mixed cultures than in monocultures (Table 3).

Nitrogen uptake by plants

The total amounts of nitrogen in *C. vulgaris* plants were highly correlated with the biomass of shoots and roots (linear regression, $r^2= 0.95$, $P<0.001$). *C. vulgaris* plants grown in mono-

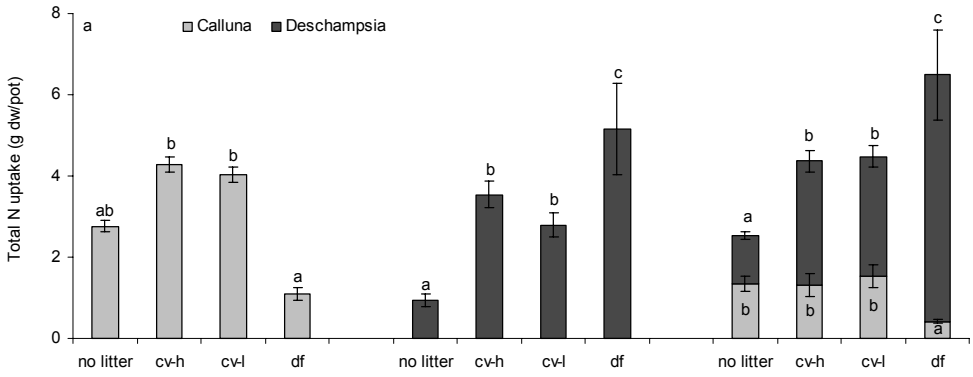


Figure 2. Effects of litter treatments on the total amounts of absorbed nitrogen in the shoots and roots of *C. vulgaris* (light bars) and *D. flexuosa* (dark bars) per plant. Error bars show standard errors of the mean. Different letters indicate significant differences (Tukey test, $P < 0.05$).

cultures showed the highest nitrogen uptake on their own litter, but in the grass litter treatment the nitrogen uptake was strongly reduced (Fig. 2a). In mixtures, the shrub plants were unable to absorb more nitrogen on their own litter compared to the control treatment. In the grass litter treatment they barely absorbed any nitrogen. The combined nitrogen uptake of *C. vulgaris* and *D. flexuosa* was highest in the treatments with *D. flexuosa* litter ($P < 0.05$, Fig. 2). In *D. flexuosa* plants, 6–12% of the total nitrogen was present in the roots. Also in Deschampsia, the total nitrogen uptake correlated strongly with the biomass of shoots and roots (linear regression, *D. flexuosa*: $r^2 = 0.93$, $P<0.001$). In both mono and mixed cultures, *D. flexuosa* plants were able to absorb additional nitrogen released from *C. vulgaris* litter (Fig. 2).

Soil nitrogen measurements

Figure 3 shows the changes in concentrations of inorganic and dissolved organic nitrogen in the treatments without plants. The repeated measures of soil nitrogen parameters show significant differences between litter treatments in the concentration of inorganic and dissolved organic nitrogen (Table 5). Remarkably, in all treatments the concentration of dissolved organic nitrogen is always more than 50% of the total available soil nitrogen (Table 5). The production of inorganic nitrogen and dissolved organic nitrogen in the soil with *D. flexuosa* litter was significantly higher than in the treatments without litter or with *C. vulgaris* litter ($P < 0.05$). After the first two months, *D. flexuosa* litter reduced the ratio DON:inorganic nitrogen, as can be seen from the significant interactions between time and litter (Table 5 and

Table 5. Results of repeated measures of concentrations of inorganic N, DON and pH in the control pots without plants. *** $P<0.001$, ** $P< 0.01$, * $P < 0.05$, NS= non-significant.

Source	Measure	df	*F*
Between-subject effects:			
Litter	Inorganic N	3	44.101***
	DON	3	72.704***
	DON: inorganic N	3	7.738***
	pH (KCl)	3	0.542^{ns}
Within-subject contrasts:			
Time	Inorganic N	4	3.592*
	DON	4	43.818***
	DON: inorganic N	4	15.601***
	pH (KCl)	3	181.560***
Time * Litter	Inorganic N	12	5.355***
	DON	12	2.811**
	DON: inorganic N	12	3.970***
	pH (KCl)	9	2.219*

Fig. 3). In contrast, during the first two months *C. vulgaris* litter greatly increased this ratio. *C. vulgaris* litters with high and low concentrations of tannins showed an immobilization of inorganic nitrogen during the first eight weeks, as evidenced by the inorganic nitrogen being lower than in the control treatment (Fig. 3, $P < 0.05$). Only after 20 weeks did the *C. vulgaris* litters produce significantly more inorganic nitrogen and organic nitrogen than the control treatments ($P < 0.05$).

Discussion

Our results show that *C. vulgaris* plants compete more efficiently with *D. flexuosa* on nutrient-poor soils (no litter amendment or amended with litter relatively rich in tannins) than on soils with grass litter. Only in the treatments without competing grasses could *C. vulgaris* benefit from its own litter. *D. flexuosa* produced most biomass when growing on its own litter, but *D. flexuosa* was able to outcompete *C. vulgaris* both on shrub and grass litter. *Calluna vulgaris* was significantly less able to absorb nitrogen with grass litter than in the other treatments. In contrast, *C. vulgaris* growing in monocultures was able to use all the available nitrogen produced in the treatments with heather litter. The ratio between the biomass of the two species in mixtures showed that the difference in biomass production

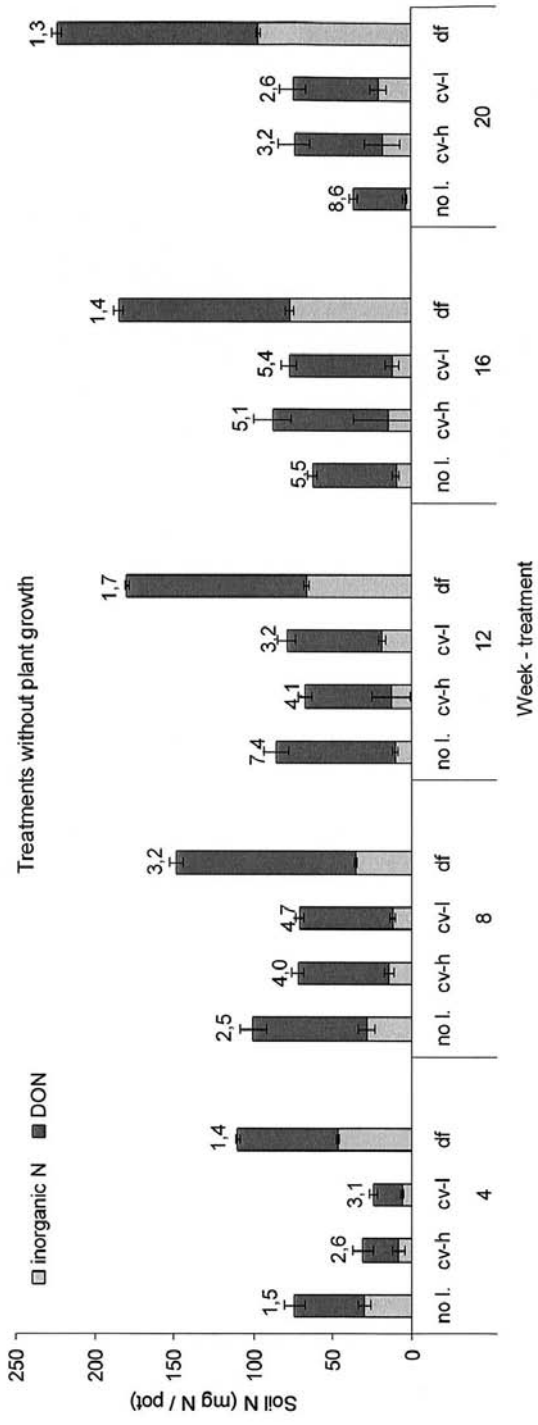


Figure 3. Effect of litter treatments on the concentrations of inorganic and dissolved organic nitrogen in treatments without plants during 20 weeks. Error bars show standard errors of the mean. Numbers above bars indicate ratio DON:inorganic nitrogen.

between the two plant species was largest on the grass litter and was much smaller in the treatments without grass litter.

Shrub plants were strongly inhibited by the presence of grass plants and showed the highest production in monocultures. The plants were not affected by different concentrations of tannins in the two shrub litter types. Northup *et al.* (1995, 1998) argued that plant species with high concentrations of secondary plant metabolites could monopolize the organic nitrogen pool, thereby becoming able to outcompete species unable to absorb organic nitrogen. If this were so, *C. vulgaris* plants should have been able to outgrow *D. flexuosa* plants on their own litter, which is rich in tannins. Our replacement diagrams show, however, that *C. vulgaris* plants were not able to produce more biomass than *D. flexuosa* plants in any of the treatments. Only in monocultures did *C. vulgari*s benefit from its own litter. It seems that the hypothesized monopoly of *C. vulgaris* on the organic nitrogen pool does not increase the competitiveness of *C. vulgaris*.

One explanation of the above behaviour could be that *D. flexuosa* is also able to exploit sources rich in organic nitrogen. In this experiment we found that grass plants were able to use litter sources with high concentrations of tannins equally well as the shrub plants. Previous competition studies between heather and grass, and between *C. vulgaris* and *D. flexuosa* specifically, only considered competition for inorganic nitrogen sources (Berendse & Aerts 1984, Aerts *et al*. 1991, Mickel *et al*. 1991, Berendse 1994, Alonso *et al.* 2001, Britton *et al*. 2003). However, the study by Näsholm *et al*. (1998) with the dual-labelled amino acid glycine in a boreal forest provided the first evidence for significant relevant organic nitrogen uptake by *D. flexuosa*, as 64% of the added glycine was taken up as intact glycine. It was shown that *D. flexuosa* absorbed the same amount (about 30%) of the dual-labelled glycine as the ericaceous shrub *Vaccinium myrtillus*. In addition, Falkengren-Grerup *et al.* (2000) found that *D. flexuosa* plants collected from an acid soil with high nitrogen mineralization in Southern Sweden absorbed even more organic nitrogen (methylamine – with carbon-labelled atoms) than inorganic nitrogen: the ratio was as high as 1.42. It therefore seems unlikely that the mechanism enabling shrubs and grasses to co-exist is niche differentiation determined by organic or inorganic nitrogen, as both plant species seem to be able to compete to an equal extent for the same nitrogen sources.

As shown by the replacement diagrams of the shoot biomass (Fig. 2), *D. flexuosa* was not inhibited by the presence of *C. vulgaris* plants. Moreover, the presence of high concentrations of carbon-based secondary metabolites in *C. vulgaris* litters did not lead to growth inhibition in *D. flexuosa* plants. We had expected that the high concentration of tannins in *C. vulgaris* litter would lead to reduced N mineralization (Kraus *et al.* 2003, Bowman *et al*. 2004), subsequently inhibiting the growth of grass plants on shrub litters compared with the treatments without litter amendment. However, we found no such growth inhibition of the grass plants in the shrub litter treatments, although they produced less biomass by comparison with the grass plants in the grass litter treatments. Other competition studies have shown that dominant grasses are able to benefit more than shrub plants from an increased soil nitrogen supply (Mickel *et al.* 1991, Hartley & Amos 1999, Britton *et al.* 2003). Our results confirm this, as *D. flexuosa* absorbed most nitrogen and produced most biomass on its own litter, which had the highest nitrogen content.

We did not find any difference in plant growth between the two different *C. vulgaris* litter types, even though these varied significantly in the concentration of total phenolics. So, there can be a relation with the presence of low concentrations of condensed tannins in *C. vulgaris* litter, which were similar between the two litter types. The concentration of condensed tannins in the carbon-based secondary compounds in litter is important (Waterman & Mole 1994). Condensed tannins in litter can inhibit mineralization rates significantly due to their e.g. higher protein-binding capacity compared to the hydrolysable tannins (Schimel *et al*. 1996, 1998, Bradley *et al*. 2000, Fierer *et al.* 2001). Bradley *et al*. (2000) reported that adding condensed tannins from *Kalmia* and balsam fir foliage reduced mineral nitrogen availability in black spruce humus. In addition, Schimel *et al*. (1996, 1998) found that high molecular weight fractions of poplar tannins bound N-containing substrates and reduced mineral N pools, whereas the low molecular weight fractions acted as substrates or toxins leading to increased immobilization by the increased microbial growth. In our experiment we also found immobilization of inorganic nitrogen in the shrub litter treatments. The pots with *C. vulgaris* litter produced lower concentrations of total soil nitrogen compared to the control pots and the pots with grass litter during the first 8 weeks (Fig. 3).

It is often assumed that *D. flexuosa* can absorb amino acids without the help of mycorrhiza. Arbuscular mycorrhizal fungi have not been found to be able to degrade protein-phenolic complexes (Hodge *et al*. 2001). Nevertheless, molecular analysis based on the nuclear

ribosomal internal transcribed spacer (ITS) region, revealed that some roots of *D. flexuosa* plants are colonized by beneficial fungal endophytes belonging to the *Helotiales* (Zijlstra *et al*. 2005). The majority (68%) of the ericaceous root isolates analysed were found to belong to the *Helotiales*. Apparently, some grass root endophytes seem to be related to ericaceous root isolates forming ericoid mycorrhiza, e.g. *Rhizoscyphus ericae* complexes of fungal strains, which are able to degrade soluble phenolics (Bending & Read 1997, Zijlstra *et al*. 2005).

Calluna vulgaris could absorb significantly less nitrogen produced in the grass litter treatments. At the start of the experiment, *C. vulgaris* plants established more slowly in this litter treatment – perhaps due to toxic effects of the grass litter on shrub roots. Sánchez-Moreiras *et al*. (2003) reported on allelopathic effects of grasses due to the presence of substances such as phenolic acids, hydroxamic acid, alkaloids and quinones. Unfortunately, there is no literature specifically on growth inhibition of *C. vulgaris* by *D. flexuosa* litter, though Jarvis (1964) showed that *D. flexuosa* humus produces allelopathic effects on plant growth. In Jarvis's litter experiment with *D. flexuosa* and *C. vulgaris* litter, *D. flexuosa* humus inhibited the growth of oak roots drastically (to almost no root growth), whereas in contrast the oak roots developed well on *C. vulgaris* litter, achieving 90% of the growth of the control treatments. Further, not only did the grass humus produce toxic effects on seedlings: the root exudates of *D. flexuosa* were also found to inhibit seedling growth (Jarvis 1964). Before the start of our experiment we measured the concentration of total phenolics in *D. flexuosa* litter and found it was very low compared to the concentrations found in *C. vulgaris* litter. It therefore seems unlikely that the phenolics in grass litter would be responsible for toxic effects. From an analysis of leached DOC from *D. flexuosa* litter, Bowman *et al.* (2004) also concluded that carbohydrates are present in a greater proportion than phenolics. However, we cannot exclude the possibility that components other than phenolics could have been playing an inhibitory role. Clearly, the results of our experiment require further investigation to elucidate the possible allelopathic effects in litter of *D. flexuosa*: not only plant growth may be affected, but also mycorrhizal fungi present in the ericaceous roots could have been inhibited by the release of toxic substances or the activity of other litter-feeding organisms (Jarvis 1964, Rose *et al.* 1983).

Our results show again that *C. vulgaris* plants are much less able to outcompete *D. flexuosa* under nutrient-rich conditions than under less fertile conditions. This is additional evidence to the observation that increased atmospheric nitrogen deposition weakens the competitive

ability of *C. vulgaris* plants in the heathland ecosystem. Lower concentrations of carbon-based secondary compounds of ericaceous litter do not have any effect on the competition between the two species. It is clear that the notion that *C. vulgaris* plants can dominate the organic nitrogen pool because they produce tannin-rich leaves and have an exclusive symbiosis with ericoid mycorrhizal fungi has to be revised. *D. flexuosa* seems to have adjusted to the nutrient-poor system too and is well able to use litter rich in organic nitrogen. In addition, the fungal endophytes thought to be specific to ericaceous plants have turned out to be not that specific (Zijlstra *et al*. 2005). It seems we are only beginning to understand the mechanisms driving the competition between heather and dominant grasses.

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Summary

Summary

Dutch heathland ecosystems form an important part of the West European heathlands with a high conservation value, but after the introduction of artificial fertilizer, this extensive farming system declined rapidly and is now only 7 % of the area present in 1833. The evergreen dwarf shrubs *Calluna vulgaris* and *Erica tetralix* used to dominate the Dutch heathlands, but during the 1970s and 1980s they were increasingly displaced by the grasses *Deschampsia flexuosa* and *Molinia caerulea*. Among the causes of these changes are the direct and indirect effects of high deposition rates of atmospheric nitrogen (40–60 kg N ha/yr), largely derived from agricultural sources. Research in the 1980s and 1990s demonstrated that grasses were able to profit more rapidly from the increased nutrient supply due to their high maximum growth rates. Heather plants also responded positively to nitrogen additions, but with lower growth rates than dominant grasses. Research published since 1995, however, has shown that in the analysis of the nitrogen deposition effects, it is not only the biomass produced that can create positive feedbacks, but that the chemical composition of plant tissue can also be crucial.

The focus of this thesis is on the role of organic nitrogen uptake and endophytic, mutualistic fungi in Dutch heathland ecosystems. In nutrient-poor, acidic soils the amounts of inorganic nitrogen forms (IN) e.g. ammonium (NH_4^+) and nitrate (NO_3^-) are generally small. In contrast, forms of organic nitrogen (DON), e.g. amino acids, are abundantly present. It has long been thought that what restricted the supply of nutrients to plants was the mineralization of organic nitrogen to ammonium and its subsequent oxidation to nitrates. However, evidence is building up that plants are able to bypass the nitrogen cycle by taking up organic nitrogen directly from the DON pool. Endophytic, mutualistic fungal species can facilitate the bypassing, as these organisms are able to degrade protein–tannin complexes to allow their host plant to access N from organic forms like proteins and amino acids. It has been proposed that plant species with high concentrations of litter tannin are able to monopolize the nutrient cycle by enhancing organic forms of nitrogen relative to inorganic forms of nitrogen. Higher DON:IN ratios would favour the species able to absorb organic nitrogen compounds through their associations with ericoid or ectomycorrhizal fungi.

To test this hypothesis, the natural variation of tannins in ericaceous plants and dominant grasses was studied under varying field conditions, with different nutrient supplies and light

intensities. Simultaneously, soil mineralization and mycorrhizal root colonization were measured, to investigate the effects of high tannin concentrations on these processes. In addition, endophytic fungal species were isolated from ericaceous plants and *Deschampsia flexuosa* in order to investigate the presence of fungal species that are adapted to the high nitrogen deposition in the Netherlands and are potentially able to facilitate the process of organic nitrogen uptake. To test the effects of nitrogen and shading on tannins in shrubs under more controlled conditions, a two-factorial field and greenhouse experiment was performed. The effect of tannins in litter on the ratio DON:IN was tested in an incubation experiment. Finally, a greenhouse experiment examined the influence of tannin-rich litter on the competition between *Calluna* and *Deschampsia*.

The field inventory described in Chapter 2 focused on the effects of nutrient supply and light intensity on tannins and mycorrhizal colonization. It tested the hypothesis that additions of nitrogen reduce the concentrations of tannins in ericaceous leaves and retard mycorrhizal colonization in ericaceous plants. Also tested were the negative effects of reduced light intensity on tannins and mycorrhizal colonization in ericaceous plants. A field study was done at three heathland locations in the Netherlands. Leaves of ericaceous plants and grasses were collected and analysed for concentrations of condensed tannins, total phenolics, and nutrients. Similarly, root samples were taken to record mycorrhizal colonization and soil was sampled to measure the soil mineralization. To compare variation in plant chemistry as affected by soil nutrient supply, two dwarf-shrub vegetations on humus-rich and humus-poor sites with *Calluna vulgaris* and *Deschampsia flexuosa* were selected in De Hoge Veluwe National Park. The effects of light intensity were investigated by comparing shaded (50% incident light) and non-shaded sites in Dwingelderveld National Park and in the Hoog Buurlosche Heide forest reserve. The comparison included *Quercus robur* woodlands with a herbaceous layer dominated by the ericaceous dwarf shrubs *Vaccinium vitis-idaea* or *V. myrtillus*. The grass *Deschampsia flexuosa* was also present in these sites. The non-shaded Hoog Buurlosche Heide site was a mixed heathland with *C. vulgaris*, *Erica tetralix, V. myrtillus, V. vitis-idaea, M. caerulea and D. flexuosa.* In addition to the field inventory, two-factorial experiments were conducted with *C. vulgaris* plants, in which fertilizer and shade levels were varied under greenhouse and field conditions.

A surprise finding when studying the tannin concentration in plants in the field was the large natural variation between plants growing in shaded or unshaded conditions or growing on

humus-rich versus humus-poor soils. Both humus-rich conditions and shade could negatively affect the concentration of tannins in *Calluna* plants, thereby possibly reducing the plants' competitiveness with respect to grasses. In the field experiments, shading of plants resulted in significantly less mycorrhizal colonization. Only in the greenhouse experiment did addition of nitrogen reduce mycorrhizal colonization. The results imply that increased atmospheric nitrogen deposition can depress the carbon-based secondary compounds in ericaceous plants and the mycorrhizal colonization in roots, thereby reducing the plants' competitiveness with respect to grasses. Additionally, if ericaceous plants are shaded by grasses that have become dominant due to increased nitrogen supplies, these effects will be intensified and competition-driven displacement will be accelerated.

Chapter 3 describes an incubation experiment to test the hypothesis that large amounts of tannins in the litter decrease the amounts of inorganic nitrogen in incubated soils, while concomitantly increasing the amounts of dissolved organic nitrogen. Also tested were the effects of plant extracts, pure phenolics and pure tannic acid on the amounts of inorganic and dissolved organic nitrogen produced. An incubation experiment was performed with different types of shrub litter (*C. vulgaris, V. vitis-idaea* and *V. myrtillus*), grass litter (*D. flexuosa*), plant extracts, pure phenolics and pure tannic acid. For 16 weeks the inorganic and organic nitrogen was measured every two weeks. The incubation units were small audiothene bags (17.5 x 8 cm) with peat as a soil medium. Most pure phenolics showed no effects on the amount of inorganic nitrogen or the DON:IN ratio. Adding tannic acid clearly inhibited the production of inorganic nitrogen; the amounts of DON also tended to decrease compared to the nitrogen treatments without tannic acid, but more slowly. Plant extracts of the shrub litter had no effect on the amount of inorganic nitrogen compared to the control with water. Litters with C:N ratios above 30 and high tannin concentrations, e.g. *Calluna* and *V. vitis-idaea* decreased the amounts of inorganic nitrogen and concomitantly increased the amounts of dissolved organic nitrogen in soils. A striking result from this experiment was that though the soil amended with *V. myrtillus* litter, which had the highest total phenolics concentration, initially immobilized inorganic nitrogen, as did the other ericaceous litters, after six weeks it released nitrogen at the same rates as *D. flexuosa* litters. At this turning point, IN took over from DON as the main nitrogen form in the substrate.

For several decades, mycologists have known that shrubs have symbiotic relationships with endophytic fungal species and that these organisms promote nutrient uptake, especially of

organic nitrogen. Furthermore, awareness is growing that not every symbiotic fungal species is equally efficient in nutrient transport. This being so, it was decided to investigate the endophytic fungal species present in Dutch ericaceous plants, as no such survey had been done before. As explained in Chapter 4, the aim was to find out which ericoid mycorrhizal fungi species are present in plant roots from Dutch heathland ecosystems. Most of the root samples examined were collected from the plants described in the field study in Chapter 2. This dedicated search for root inhabitants produced a surprising result|: a high diversity of endophytic fungal species was found. Some species occurred more often in ericaceous plants: for example, a variety of fungal species belonging to the *Rhizoscyphys ericae* aggregate, *Oidiodendron maius*, *Phialocephala fortinii* and *P. fortinii*-like species. Several of the fungi species identified had not previously been described as endophytic fungal species from ericaceous plants: *Cryptosporiopsis rhizophila, Cryptosporiopsis sp.* and *Verticillium sp*. It had been assumed that the symbiosis in ericoid roots was highly specific between fungal species and ericaceous hosts. However, when root fungi were isolated from grass roots in this research, *Deschampsia flexuosa* roots were found to contain endophytic fungal species, which belonged to the *Helotiales*, the phylogenetic group to which most of the ericaceous fungal isolates belonged. Although this finding sheds new light on a possible mechanism behind the capacity of *Deschampsia* to absorb nitrogen, it remains unclear whether these fungi in grass roots can fulfil the same role in organic nitrogen breakdown and uptake as they do in ericaceous plant roots.

Finding new fungal species does not seem to be that difficult. Unfortunately, not all fungal species are equally willing to grow and sporulate *in vitro* on artificial substrates, which until recently was a prerequisite for publication. This greatly hampers their description. Nevertheless, the fungus, *Cryptosporiopsis rhizophila* Verkley & Zijlstra could be published based on sporulating structures and phylogenetic analyses using data on the 5.8S nuclear rDNA and flanking internal transcribed spacers (Chapter 5). Subsequently Lynn Sigler published her work on two new *Cryptosporiopsis* species from roots of ericaceous hosts in western North America.

Chapter 6 describes how the ability of *C. rhizophila* to enhance the nutrient status of *Calluna* seedlings and to induce formation of ericoid mycorrhizal coils was tested by means of synthesis trials. Additionally, Bavendamm tests were done to test the saprotrophic ability of this species to produce enzymes that degrade phenolics. For comparison, Dutch isolates of

Hymenoscyphus ericae and *Oidiodendron maius* were included in the tests. It was found that three isolates of *C. rhizophila* were able to enhance the nitrogen uptake compared to the uptake in non-inoculated seedlings, and produced intracellular fungal structures in the epidermal cells. All isolates of *C. rhizophila* tested were able to produce phenol-oxidizing enzymes and to degrade soluble phenolics. Further research needs to be done to reveal whether this ability can also boost the organic nitrogen uptake of the symbiotic host.

The competition experiment in Chapter 7 demonstrates the effects of tannin-rich litter types on soil nitrogen and on the outcome of the competition between grass and shrub species. It is hypothesized that the competition between ericaceous plants and grasses is strongly affected by the difference in tannins in the litter of the two species. To test this hypothesis, a greenhouse experiment was conducted with *C. vulgaris* and *D. flexuosa*. Two litters of *C. vulgaris* were used, with the same nitrogen concentration but different (high and low) tannin concentrations. The *D. flexuosa* leaf litter with the highest nitrogen concentrations contained lower concentrations of tannins than the *C. vulgaris* litters. The plants were grown in monocultures and in mixed cultures. Inorganic and dissolved organic nitrogen were measured monthly. After four months, the above- and below-ground biomass and the nutrient concentrations in leaves and roots were measured. In the experiment, *C. vulgaris* plants were unable to outcompete *D. flexuosa*. In contrast, grass plants were able to benefit more efficiently from the available soil nitrogen released from the added litter. Furthermore, the competitive suppression of shrub plants by the grasses was almost absent in the treatments with low nutrient availability. It is therefore concluded that the nitrogen content of the litter is more important as a mechanism driving competition between shrubs and grasses than the tannin content.

Studying the heathland ecosystem is a tremendous challenge due to the intriguing interactions between plants that influence soil nitrogen processes and to the symbiotic relationships between plants and endophytic fungi. The results of the research described in this thesis show that high tannin concentrations in ericaceous litter positively influence the amounts of organic forms of nitrogen relative to inorganic forms of nitrogen in the soil. However, these tannin concentrations can be negatively influenced by nitrogen additions and shading effects. It is also shown that grasses from the heathland systems seem to be perfectly adapted to the high organic nitrogen conditions, including adaptation to related ericaceous fungal symbionts. Therefore the ability of ericaceous plants to dominate the nutrient pool by producing tannin-

rich litter seems to be limited. The previously undiscovered abundant fungal species in heathland roots immediately grasped my attention, but the beauty and complexity of the heathland plants should raise in anyone an awareness that this ecosystem is worth every effort to sustain and cherish it as our precious inheritance.

Samenvatting

Samenvatting

Nederlandse heide-ecosystemen vormen een belangrijk onderdeel van de West-Europese heidegebieden. Daarom is behoud door verantwoord natuurbeheer belangrijk. Na de introductie van de kunstmest is het snel achteruit gegaan met de extensief beheerde heidegebieden. Het heide areaal is nu ongeveer 42.000 ha. Dit is nog maar 7% van het aanwezige areaal in 1833. De dwergstruiken *Calluna vulgaris* en *Erica tetralix* waren voorheen de dominante plantensoorten. In de jaren zeventig en tachtig worden deze soorten echter steeds meer verdrongen door de grassen *Deschampsia flexuosa* and *Molinia caerulea*. Dit komt mede door de directe en indirecte effecten van een hoge stikstofdepositie via de atmosfeer (40-60 kg N ha^{-1} jr^{-1}). Een merendeel hiervan is van landbouwkundige oorsprong. Onderzoek in de jaren tachtig en negentig laat zien dat grassen in staat zijn om sneller van het toegenomen stikstof aanbod te profiteren. Dit is mede doordat ze hogere maximale groeisnelheden kunnen bereiken. Heideplanten kunnen ook een positieve respons laten zien bij extra stikstofgiften, maar de groeisnelheden zijn lager in vergelijking met grassoorten. In de literatuur na 1995 kwam naast de productie van de hoeveelheid biomassa een alternatieve verklaring voor de analyse van stikstofdepositie effecten naar voren. De verschuiving in dominantie van heide naar gras kan ook gedeeltelijk verklaard worden door de veranderde chemische samenstelling van de planten.

Het doel van dit proefschrift is om de rol van organische stikstof opname en de interactie met symbiotische schimmels in Nederlandse heide-ecosystemen te onderzoeken. In voedselarme, zure gronden zijn minerale stikstofvormen (IN) zoals ammonium (NH_4^+) en nitraat (NO_3^-) meestal in kleine hoeveelheden aanwezig. De belangrijkste stikstofbronnen zijn de organische vormen van stikstof (DON), zoals aminozuren. Traditioneel werd gedacht dat de mineralisatie van organische stikstof naar ammonium en via het oxidatie proces naar nitraat, de beperkende factor was in het nutrienten aanbod voor planten. Tegenwoordig komt er steeds meer wetenschappelijk bewijs dat planten rechtstreeks organische stikstof uit de DON pool kunnen opnemen en daarmee niet meer afhankelijk zijn van de omzettingsprocessen via ammonium en nitraat. Symbiotische schimmels kunnen hierin een belangrijke bevorderende rol spelen, omdat ze in staat zijn om sterk organische gebonden stikstofvormen af te breken. De afbraakproducten, zoals aminozuren zijn vervolgens makkelijk op te nemen door de plant. Halverwege de jaren negentig is een hypothese naar voren gekomen die stelt dat planten die

strooisel produceren met hoge tannine concentraties in staat zijn de stikstofkringloop te domineren. Strooisel met hoge tannine gehaltes kunnen de toename van organische stikstof bevorderen en de aanwezigheid van minerale stikstof remmen. Daarnaast wordt verondersteld dat hogere DON:IN verhoudingen in de bodem die plantensoorten bevorderen die in staat zijn om organische stikstof op te nemen via hun associaties met ericoïde of ectomycorrhiza schimmels.

Om bovenstaande hypothese te toetsen hebben we eerst de natuurlijke variatie van tannine in heideplanten en dominante grassen onderzocht onder verschillende veldomstandigheden van stikstofmineralisatie en lichtintensiteit. Tegelijkertijd is de bodemmineralisatie gemeten en de kolonisatie van de wortels door mycorrhiza vormende schimmels. Als aanvulling hebben we endofytische schimmels uit de wortels van heideplanten en *Deschampsia flexuosa* geïsoleerd. We wilden namelijk weten of deze schimmelsoorten aangepast zijn aan de hoge stikstofdepositie, zoals die in Nederland voorkomt en of ze een rol kunnen vervullen in het bevorderen van de organische stikstofopname. Als vervolgonderzoek op de veldstudie zijn een kasproef en een veldexperiment opgezet om de effecten van stikstof en schaduw op het tannine gehalte in heideplanten te testen onder gecontroleerde omstandigheden. Een incubatie experiment is ingezet om de effecten te toetsen van strooisel met hoge tannine gehaltes op de ratio DON:IN. Het competitie experiment laat de invloed zien van strooisel met hoge tannine gehaltes op de competitie verhouding tussen *Calluna* and *Deschampsia*.

Het veldwerk zoals dat beschreven is in Hoofdstuk 2, richt zich vooral op de effecten van nutrienten en lichtintensiteit op de tannine concentraties in planten en de aanwezigheid van mycorrhiza structuren in wortels. We wilden de hypothese testen dat heideplanten die op bodems groeien met een hogere stikstofmineralisatie een lagere tannine concentratie hebben dan planten die op bodems groeien met een lagere stikstofmineralisatie. Daarnaast wilden we testen of stikstofrijke planten een lagere mycorrhiza kolonisatie hebben. Tevens zijn de negatieve effecten onderzocht van een beperkte lichtintensiteit op de concentratie van tannine in heideplanten en de mycorrhiza kolonisatie. Onze veldstudie is uitgevoerd op drie verschillende lokaties in Nederland. Bladmateriaal van heideplanten en grassen werd verzameld en geanalyseerd op concentraties aan gecondenseerde tannines, totale fenolen en nutrienten. Tegelijkertijd hebben we wortelmonsters verzameld om de mycorrhiza kolonisatie te meten en grondmonsters om de bodemmineralisatie te bepalen. Om de variatie in de chemische samenstelling van de planten te meten onder invloed van bodemnutrienten zijn

heidevegetaties geselecteerd in het Nationaal Park De Hoge Veluwe en het Nationaal Park Dwingelderveld. Eén op een geplagde en één op een niet-geplagde locatie met daarin *Calluna vulgaris* and *Deschampsia flexuosa*. De effecten van lichtintensiteit onderzochten we op beschaduwde (50% lichtreductie) en niet-beschaduwde lokaties in het Nationaal Park Dwingelderveld en de boswachterij Hoog Buurlosche Heide. In de vergelijking hebben we eikenhakhoutbosjes (*Quercus robur*) opgenomen waarbij de kruidachtige laag gedomineerd werd door rode (*Vaccinium vitis-idaea*) en blauwe bosbes (*V. myrtillus*). Daarnaast was het gras, *Deschampsia flexuosa* aanwezig. Op de niet-beschaduwde lokatie op Boswachterij de Hoog Buurlosche Heide groeide een variatie aan heideplanten: *C. vulgaris*, *E. tetralix, V. myrtillus, V. vitis-idaea* en de grassen*, M. caerulea* en *D. flexuosa.* Als vervolgonderzoek op de veldstudie hebben we een kasproef en een veldexperiment opgezet om de effecten van stikstof en schaduw op het tannine gehalte in heideplanten te testen onder gecontroleerde omstandigheden.

Bij het bestuderen van de tannine concentraties van planten uit het veld vonden we een hoge natuurlijke variatie tussen planten die groeiden in beschaduwde of onbeschaduwde omstandigheden of op geplagde versus niet-geplagde gronden. Zowel niet-geplagde omstandigheden als schaduw gaven een negatieve correlatie met de aanwezige tannine concentraties in *Calluna* planten. In de veldexperimenten, resulteerde schaduw duidelijk in een lagere concentratie van mycorrhiza kolonisatie. Terwijl in het kasexperiment de stikstofbehandeling resulteerde in een lagere concentratie van mycorrhiza kolonisatie. Onze resultaten geven aan dat een verhoogde toename van atmospherische depositie kan leiden tot vermindering van tannine concentraties in heideplanten en tot een verminderde mycorrhiza kolonisatie van de wortels. Hierdoor is het mogelijk dat het competitieve vermogen van heideplanten ten opzichte van grassen negatief wordt beïnvloed. Bovendien zal het proces van verdringing bevordert worden als dominante grassen door de extra stikstofopname de heideplanten gaan overschaduwen.

In Hoofdstuk 3 beschrijven we een incubatie experiment. Hierbij testen we de hypothese dat strooisel met hoge tannine gehaltes de hoeveelheid minerale stikstof in de bodem verlaagt, maar tegelijkertijd de hoeveelheid van oplosbare organische stikstof verhoogt. We gebruikten in het experiment strooisel van heideplanten (*C. vulgaris, V. vitis-idaea* en *V. myrtillus*) en van het gras, *D. flexuosa*. Daarnaast hebben we de effecten getest van plant extracten, pure fenolen en puur tanninezuur op de hoeveelheden geproduceerde minerale en organische

stikstof. De opgeloste minerale en organische stikstof werd met tussenpozen van twee weken gemeten, gedurende 16 weken. Als incubatie eenheden gebruikten we kleine, plastic zakken (17.5 x 8 cm) met turf als grondmedium. De meeste pure fenolen en plantextracten gaven geen effect op de geproduceerde hoeveelheid minerale stikstof of op de ratio DON:IN. Toevoeging van tanninezuur gaf echter wel een duidelijke remming van de minerale en organische stikstof productie. Bij strooiseltypes die gekenmerkt werden door C:N ratios hoger dan 30 en met hoge tannine concentraties, zoals *C. vulgaris* en *V. vitis-idaea* zagen we duidelijk dat er minder minerale stikstof werd geproduceerd en meer organische stikstof waardoor de ratio DON:IN sterk toenam ten opzichte van de andere strooiseltypes met lagere C:N ratios. Een opvallend resultaat was dat grond met *V. myrtillus* strooisel (C:N<30), die in het begin het hoogste tannine gehalte bezat, de eerste weken minerale stikstof immobiliseerde net zoals de andere heidestrooisels. Echter na zes weken werd er in totaal net zoveel stikstof geproduceerd als in het grasstrooisel. Daarbij veranderde de ratio DON:IN van groter dan één naar kleiner dan één.

Mycologen weten al enkele tientallen jaren dat heideplanten symbiotische relaties hebben met endofytische schimmelsoorten. Bovendien is bekend dat deze organismen een positieve bijdrage kunnen leveren aan de voedselopname van planten, vooral in de opname van organische stikstof. Er is een toenemend besef dat niet elke symbiotische schimmelsoort even efficiënt is in het voedseltransport. Daarom vonden we het belangrijk om eerst te onderzoeken welke endofytische schimmelsoorten aanwezig zijn in Nederlandse heideplanten. Dit onderzoek wordt weergegeven in Hoofdstuk 4. De wortelmonsters zijn verzameld van dezelfde planten als die beschreven staan in Hoofdstuk 2. In onze zoektocht naar endofytische schimmelsoorten, werden we flink verrast door de hoge diversiteit van schimmels die we aantroffen. Sommige soorten zoals de schimmels die behoren tot het *Rhizoscyhus ericae*-complex, *Oidiodendron maius*, *Phialocephala fortinii* en *P. fortinii*-achtige soorten werden ook door andere onderzoekers in heideplanten gevonden. Daarentegen troffen we ook nieuwe, nog niet eerder beschreven schimmelsoorten aan zoals *Cryptosporiopsis rhizophila, Cryptosporiopsis sp.* en *Verticillium sp.* Voorheen werd veronderstelt dat de symbiose tussen schimmelsoorten en heidewortels zeer specifiek was. Schimmels die we echter isoleerden *D. flexuosa* bleken via fylogenetische analyses gerelateerd te zijn aan de orde van de *Helotiales*. In deze groep clusteren ook veel van onze schimmelsoorten die uit heideplanten zijn geïsoleerd. Dit resultaat werpt een nieuwe licht op het vermogen van *D. flexuosa* om organische stikstof op te nemen. Een vraag die nog niet is beantwoord, is of deze gras-

endofyten in graswortels eenzelfde rol vervullen ten aanzien van bevordering van organische stikstofopname als heide-endofyten in wortels van heideplanten.

Ons onderzoek geeft aan dat het niet zo moeilijk is om nieuwe schimmelsoorten te vinden. Niettemin zijn niet alle schimmelsoorten even makkelijk onder kunstmatige omstandigheden op te kweken en tot de productie van sporen aan te zetten. Dit was tot voor kort een belangrijke en belemmerende voorwaarde om een nieuwe soort te beschrijven. Gelukkig liet de schimmel *Cryptosporiopsis rhizophila* Verkley & Zijlstra wel duidelijke sporulatiestructuren zien. Tevens kon deze schimmel via moleculaire analyses gekarakteriseerd worden aan de hand van genetische stukken die aanwezig zijn op het 5.8S ribosomaal DNA en de flankerende ITS (internal transcribed spacers) regios (Hoofdstuk 5). Lynn Sigler heeft inmiddels haar werk gepresenteerd over twee nieuwe *Cryptosporiopsis* soorten die eveneens werden geïsoleerd uit wortels van heideplanten, afkomstig uit het westen van Noord-Amerika.

In Hoofdstuk 6 hebben we getest of isolaten van *C. rhizophila* in staat waren om de opname van nutrienten van *Calluna* zaailingen te bevorderen door middel van synthese proeven onder steriele omstandigheden (*in vitro*). Met behulp van zg. Bavendamm testen wilden we het saprotrofe vermogen van de schimmelisolaten testen om enzymen te produceren die fenolen kunnen afbreken. De uitkomsten werden vergeleken met die van bekende mycorrhiza vormende schimmels zoals *Rhizoscyphus ericae* en *Oidiodendron maius*. Drie isolaten van *C. rhizophila* verhoogden de stikstof opname van *Calluna* zaailingen ten opzichte van de controle planten en produceerden schimmelstructuren in de wortelcellen. Alle geteste isolaten van *C. rhizophila* produceerden fenol oxiderende enzymen waardoor ze oplosbare fenolen kunnen afbreken. Verder onderzoek moet laten zien of deze eigenschap ook een toename van organische stikstof opname kan bewerkstelligen door de gastheer.

In het competitie experiment in Hoofdstuk 7 testen we de hypothese dat concurrentie tussen heideplanten en grassen sterk beïnvloed wordt door de verschillen in tannine concentraties in het strooisel van beide soorten. We hebben hiervoor een kasproef ingezet met *C. vulgaris* en *D. flexuosa* planten. De behandelingen bestonden uit twee strooiseltypes van *C. vulgaris*, die dezelfde stikstof concentraties hadden, maar van elkaar verschilden door een hoog of een laag gehalte aan tannine. Het grasstrooisel met de hoogste stikstofconcentraties heeft het laagste tannine gehalte. De planten werden geplaatst in monocultures en in mengcultures. Gedurende

vijf maanden werd maandelijks de minerale en organische stikstof in de bodemoplossing gemeten. Aan het eind van het experiment werden de biomassa en de nutrienten concentraties gemeten van de bovengrondse plantendelen en de wortels. In ons experiment waren *C. vulgaris* planten niet in staat om via hun strooisel *D. flexuosa* te domineren. Daarentegen waren de grasplanten in staat om efficiënt de beschikbare bodemstikstof te benutten die beschikbaar kwam uit de toegevoegde strooiseltypes. Opvallend was dat de onderdrukking van heideplanten door grasplanten nihil was in de strooiselbehandelingen met lage stikstofgehaltes. Wij concluderen hieruit dat het stikstofgehalte van het strooisel belangrijker is om de concurrentie verhoudingen tussen heideplanten en grassen te bepalen dan het tannine gehalte.

Het is een geweldige uitdaging om onderzoek te doen naar het functioneren van het heide-ecosysteem. Vooral door de boeiende interacties tussen planten, die bodemprocessen beïnvloeden via hun strooisel, en hun symbiotische schimmelpartners. De resultaten van dit onderzoek laten zien dat heideplanten met hoge tannine gehaltes in hun strooisel de DON:IN ratio positief kunnen beïnvloeden. Effecten van schaduw en stikstofverrijking kunnen daarentegen tannine gehaltes in heideplanten reduceren en de concurrentiepositie verzwakken. Bovendien laat ons onderzoek zien dat een dominant gras, zoals *D. flexuosa* in staat is om zich goed aan te passen aan bodemomstandigheden met hoge organische stikstofgehaltes. Misschien mede doordat deze plant overeenkomstige symbiotische schimmels bezit als heideplanten. Het vermogen van heideplanten om de nutrientenkringloop te domineren door de productie van strooisel met hoge tannine gehaltes lijkt daardoor beperkt. De nog onontdekte en uitbundige rijkdom van schimmels in heidewortels trok direct mijn aandacht. Ik hoop dat de schoonheid en diepere achtergronden van heideplanten in iedereen een besef wakker maakt dat dit ecosysteem de moeite waard is om te behouden en te koesteren als een bijzonder erfgoed.

Dankwoord

Curriculum vitae

List of publications

Affiliations of co-authors

Dankwoord

Amicus certus in re incerta cernitur.
Een echte vriend wordt in onzekere tijden opgemerkt.

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Curriculum vitae

Jantineke Zijlstra was born on 8 August 1972 in Uithuizermeeden (Netherlands). After attending the HAVO in Leeuwarden for her secondary education she studied Tropical Agriculture and Rural Development at Larenstein in Deventer from 1990 to 1995. For her theological education she spent one year at the De Wittenberg Bible School in Zeist. From September 1996 to November 1999 she studied Science in Plant Breeding and Crop Protection at Wageningen University. Her MSc projects were in Entomology and Ecological Phytopathology. For her project in Entomology, she spent six months in the southern part of Hungary, Hódmezővásárhely, studying the potential of entomopathogenic nematodes and fungi for biological crop protection of the western corn root worm (*Diabrotica virgifera virgifera*). This project was supervised by Prof. Joop van Lenteren. Her second MSc thesis was an inventory research to find new hyperparasites of the microsclerotia of *Verticillium dahliae*. This project was supervised by Dr. Aad Termorshuizen and Prof. Walter Gams. It resulted in a new fungal species, *Spermatoloncha* sp., which was derived from a sandy soil from the garden of Dr. Frans Tjallingii in Wageningen Hoog.

Before completing her degree she took up employment at the Utrecht University "Biology Science Shop", where she remained from May to October 1999. Supervised by Dr. Peter Bakker of Utrecht University and Manon Vaal of the Biology Science Shop she did a literature study on the biological control of fungal diseases in lettuce and strawberry. From November 1999 to March 2000 she worked as an analytical chemist with Dr. Wim Blok on a project about the general disease resistance of compost at the Biological Farming Systems Group of Wageningen University. In April 2000 she began her PhD project at the Nature Conservation and Plant Ecology Group, Wageningen University under the supervision of Prof. Frank Berendse. From December 2004 to March 2005 she was stationed in Wageningen as a Database Content Developer at the Plant Protection Service, Ministry of Agriculture, Nature and Food Quality. In January 2005 she took up her current post as RISCover® Project manager / Phytopathologist at the Blgg Laboratory in Naaldwijk.

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