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FACTORS INFLUENCING PLANT RESPONSE DURING MYCORRHIZAL ESTABLISHMENT AND FORMATION: THE COST-BENEFITS IN A SYMBIOTIC CONTINUUM

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Declaração

Na elaboração desta dissertação foi feito o aproveitamento total de resultados de trabalhos já publicados ou submetidos para publicação, que constituem alguns capítulos da presente tese. Uma vez que estes trabalhos foram realizados em colaboração, e de acordo com o disposto no nº 1 do Artigo 15º do Regulamento de Doutoramento da Universidade de Lisboa, publicado no Diário da República – II Série Nº 194, de 19-08-1993, o candidato esclarece que participou na obtenção, análise e discussão dos resultados, bem como na redacção dos manuscritos.

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Summary

The goal of this thesis was to understand: i) What factors determine the variance in plant response to mycorrhization, and how; ii) How does this variance relate to the balance between mycorrhizal cost and benefit.

Two main and different proxies were used: i) The effects of mycorrhization on plant productivity and vitality; ii) C and N metabolism readjustments in response to mycorrhizal formation.

The mycorrhizal partners chosen were *Pinus pinaster* L. and *Pisolithus tinctorius* (Pers.) Coker & Couch.

The effects of mycorrhization over the plants productivity were tested in conditions that went from severely N limited to near optimum N supply. In these conditions the plants response to mycorrhization was consistently found to be determined by its effect on the plants' N uptake. It is concluded that the cost efficiency of N acquisition is not important in the outcome of the symbiosis, but only the balance between the N supply by the fungus and the N demand by the plant. A new index is proposed, the mycorrhizal N demand/supply ratio (MDS_N), which was found to accurately explain how this balance changes.

The plant's photosynthetic performance (PI_{abs}) was used as an indicator of plant vitality/stress, and was not reliably correlated with mycorrhizal effects on growth or N status, confirming that these two parameters are not good indicators of stress or plant fitness.

It was also demonstrated that investing more in fungal growth at one particular moment is not necessarily a disadvantage in the long term.

The validity of the proposal of parasitism in ECM interactions is discussed. It is concluded that the hypothesis that the benefits exchanged by the partners are by-products instead of costly benefits, explains the reported results.

Key words: ECM, N, C, cost:benefit, mycorrhizal response, resource allocation.

Resumo

A micorrização é tradicionalmente considerada benéfica para a planta hospedeira, uma vez que, de modo geral, aumenta a tomada de nutrientes, e subsequente aumento do crescimento da planta. Nomeadamente as interacções ectomicorrízicas (ECM) são particularmente importantes em ecossistemas limitados pelo N. O melhoramento da tomada e concentrações na planta deste nutriente como resultado da formação de ECM têm sido frequentemente observados.

No entanto, o número de publicações relatando respostas negativas ou nulas à micorrização, em termos de produtividade, tem vindo a aumentar. Estas observações têm sustentado a hipótese de que esta associação possa apresentar um contínuo de respostas, variando de mutualista a antagonista. Pensa-se ainda que em qualquer combinação planta-fungo, a resposta à micorrização possa deslocar-se ao longo deste contínuo.

É ainda incerto quais os factores que determinam o tipo de resposta. Presume-se no entanto que o resultado de uma relação micorrízica dependa do balanço entre a necessidade de energia pelo fungo e a de nutrientes pela planta.

O objectivo da presente tese foi compreender: i) Quais os factores que determinam a variação da resposta das plantas à formação de ECM; ii) Como se relaciona esta variação com o balanço custo:benefício associado à interacção micorrízica.

Foram realizadas duas abordagens distintas para dar resposta às questões colocadas: a) estudo dos efeitos da micorrização na produtividade e vitalidade da planta; b) avaliação das alterações dos metabolismos de C e N em resposta à micorrização. Os parceiros micorrízicos escolhidos foram *Pinus pinaster* L. e *Pisolithus tinctorius* (Pers.) Coker & Couch.

Numa primeira fase do trabalho, foi avaliada a resposta da planta durante o processo de estabelecimento da ectomicorriza, e procurou analisar-se se esta resposta é dependente do "status" nutricional ou da idade da planta, ou da quantidade de N fornecida à planta.

Grupos de *P. pinaster* de diferentes idades e "status" nutricionais foram inoculados com micélio de *P. tinctorius* vivo (plantas micorrizadas; M), ou morto (controlos não micorrizados; NM). As plantas receberam 1.9 mM ou 3.8 mM amónio (NH_4^+) como fonte de N. O estabelecimento de ECM foi monitorizado até um mês depois da data de inoculação através de medições diárias de fluorescência da clorofila *a* e da análise do transiente de fluorescência O-J-I-P (teste JIP). Determinaram-se ainda biomassas e taxas fotossintéticas.

Observou-se que as plantas reagiram de modo distinto à ectomicorrização consoante a sua idade (estádio de desenvolvimento, área foliar), "status" nutricional inicial e quantidade de N fornecido. Dependendo da idade da planta, a formação de ECM constituiu um stress. Maior disponibilidade de N suavizou ou eliminou o impacto negativo da formação de ECM. As plantas mais novas no momento da inoculação foram as únicas a apresentarem taxas fotossintéticas mais elevadas quando micorrizadas. Observou-se ainda que a micorrização teve um efeito tampão, minorando os efeitos de stresses nutricionais e mecânicos, independentemente dos seus efeitos sobre o crescimento ou nutrição.

Concluiu-se que a formação de ECM pode ter um efeito negativo na produtividade da planta hospedeira durante o seu estabelecimento e fases iniciais de desenvolvimento, dependendo da quantidade de N disponível à planta, do seu "status" nutricional, e idade. A fluorescência da clorofila *a* demonstrou ser uma ferramenta não destrutiva, não invasiva e fiável, capaz de identificar os primeiros sinais da interacção planta-fungo micorrízico.

As respostas negativas à formação de ECM podem resultar de perda excessiva de C na direcção do fungo, ou de imobilização de N pelo parceiro fúngico. Em seguida, procurou-se compreender qual destes efeitos foi o responsável pelas respostas negativas do crescimento e eficiência fotossintética previamente observadas.

Utilizando o mesmo desenho experimental, monitorizou-se o estabelecimento das ECM através de medições de ergosterol. As plantas foram colhidas 29 dias após a inoculação, determinaram-se pesos secos, concentrações de C e N, e calcularam-se vários parâmetros de crescimento e produtividade: taxas relativas de crescimento (RGR) e de tomada de N (RN), e produtividade de N (NP).

As plantas M tomaram menos N (RN). Isto resultou em menor produtividade de N (NP) e crescimento (RGR) para as plantas mais velhas (4 semanas), mas não para as mais novas. As plantas M mais velhas tiveram NP mais baixo do que plantas NM com valores semelhantes de RN.

Concluiu-se com este trabalho que os efeitos negativos observados sobre o crescimento e vitalidade da planta hospedeira foram maioritariamente uma consequência de menor tomada de N, e consequentemente de imobilização de N pelo fungo. A extensão da limitação de N experimentada pelas plantas foi o factor mais importante no modo como a planta respondeu à micorrização. Em plantas M mais velhas foram ainda observadas evidências de um efeito negativo sobre o crescimento, resultante de uma limitação que não a de N, mas dependente da limitação de N. O efeito

da micorrização foi mais pronunciado em plantas que receberam menos N e/ou com menor "status" de N, o que se deveu provavelmente aos maiores graus de micorrização nessas condições.

Os efeitos da micorrização foram de seguida testados em plantas com uma maior e ampla variedade de combinações entre disponibilidade de N (RAR_N), concentração de N em tecidos da planta e grau de micorrização. De modo a obter esta variedade eliminando simultaneamente o efeito da idade ou estádio de desenvolvimento da planta, foi seguido um desenho experimental semelhante aos anteriores, com amostragens periódicas a cada 4 semanas, mantendo o fornecimento de N constante. A disponibilidade de N, o "status" de N da planta e o grau de micorrização foram avaliados como possíveis determinantes da variação dos efeitos da micorrização. Calcularam-se vários parâmetros de crescimento e produtividade (RGR, RN, NP, U), determinaram-se as concentrações de N, taxas fotossintéticas e graus de colonização. A fluorescência da clorofila *a* (JIP test) foi usada como indicador de stress/vitalidade da planta.

Verificou-se que o N foi limitante em todas as condições testadas. Os efeitos da micorrização sobre o crescimento da planta (RGR) e tomada de N (RN) correlacionaram-se forte e positivamente. Estes efeitos variaram de positivos a negativos. Foi sugerido um novo índice, a razão micorrízica de procura/oferta de N (MDS_N), que considera que este balanço varia com o grau de micorrização e a disponibilidade de N (RAR_N), e que permitiu explicar esta variação. O efeito da micorrização no crescimento da planta hospedeira não esteve correlacionado com o efeito na eficiência fotossintética da planta (PI_{abs}), e portanto na sua vitalidade.

Concluiu-se que o efeito da micorrização sobre a tomada de N determina o crescimento da planta hospedeira em condições de limitação de N, e que varia de acordo com MDS_N. As diferenças no crescimento reflectiram alterações na distribuição de recursos entre a planta hospedeira e o fungo micorrízico, e não na vitalidade da planta. Os resultados foram interpretados à luz de vários modelos e hipóteses propostos.

A avaliação sobre a vantagem ou desvantagem da micorrização foi também feita através do estudo das alterações dos metabolismos de C e N, e consequentes alterações do balanço energético das plantas. Indicações de que as ECM levam a alterações e reajustes nos metabolismos de N e C, e de que poderão levar a uma desaceleração das vias metabólicas de regulação da planta, com pelo menos uma parte das despesas metabólicas passando para o fungo, têm sido obtidas em vários estudos. Foram, assim, investigadas as alterações nos metabolismos de C e N desde o momento da inoculação

até ao estabelecimento pleno da interacção ectomicorrízica, e a sua dependência da quantidade de N fornecido à planta. O grupo de plantas inoculadas no primeiro mês após a germinação foi escolhido para investigar esta hipótese por ter sido o único grupo em que se verificou uma perda de eficiência fotossintética, e portanto de vitalidade, em resultado da micorrização, em plantas que receberam menos N.

Plantas de *P. pinaster* foram inoculadas com *P. tinctorius* vivo (M) ou morto (NM), foi-lhes fornecido 1.9 ou 3.8 mM amónio como fonte de N, e o seu desenvolvimento foi seguido durante 29 dias. Concentrações de ergosterol e actividades de enzimas sucrolíticas na raiz, da fosfoenolpiruvato (PEP) carboxilase em raízes, folhas e cotilédones, tal como concentrações de hidratos de carbono solúveis, amido, frutose-2-6-bifosfato (F26BP), N e amino ácidos livres foram determinados. Taxas fotossintéticas, fluorescência da clorofila *a* e pesos secos foram ainda registados periodicamente, e taxas relativas de crescimento (RGR) e tomada de N (RN) foram calculadas. As actividades enzimáticas medidas foram seleccionadas pela sua relevância nas trocas de C e N entre simbiontes, mas também por serem específicas das plantas, permitindo o isolamento da resposta da planta ao nível da raiz.

Não se verificaram alterações qualitativas, mas apenas quantitativas, no metabolismo de N ou C nas plantas micorrizadas. Foram observados períodos alternados de activação e desactivação metabólicas em plantas micorrizadas, que se relacionaram, respectivamente, com períodos de maior ou menor investimento subterrâneo.

Os resultados indicam que o crescimento do fungo e a taxa de colonização da raiz pode não ser constante no tempo, mas variar, resultando em períodos de maior ou menor demanda de C e N pelo fungo. Isto reflecte-se em alterações temporárias do metabolismo da planta hospedeira. Estas flutuações são provavelmente resultado de uma alternância entre períodos em que os recursos são suficientes e períodos em que é necessário um maior investimento em crescimento fúngico de modo a alcançar novas fontes de nutrientes. Os resultados para a planta em termos de produtividade ou vitalidade não foram, no entanto, influenciados por estes desvios, mas apenas pela quantidade de N disponível e pelo efeito da micorrização sobre a tomada de N.

A micorrização só resultou em vantagem para a planta hospedeira com o fornecimento de maior concentração de N levando a uma menor distribuição de N para a parte subterrânea e maior ganho de N. Por outro lado, com menos N a maior distribuição de C para a parte subterrânea foi constante e a tomada de N menor, resultando num efeito negativo na eficiência fotossintética.

Em conclusão, na presente tese, os efeitos da micorrização sobre a produtividade da planta hospedeira foram testados em condições que variaram desde limitação extrema de N até próximo do fornecimento óptimo de N. Nestas condições a resposta das plantas à micorrização foi sempre determinada pelo seu efeito sobre a tomada de N pela planta. Concluiu-se que o custo da aquisição de N não é importante para o resultado da simbiose nestas condições, mas apenas o balanço entre fornecimento de N pelo fungo e demanda de N pela planta. Isto é contrário ao que tem sido proposto até agora. O novo índice proposto, a razão micorrízica de procura/oferta de N (MDS_N) explica adequadamente a sua variação.

A validade da hipótese de existência de parasitismos na simbiose micorrízica é discutida. Conclui-se que a hipótese de os benefícios trocados pelos simbiontes serem excedentes e não representarem um custo para os simbiontes explica os resultados obtidos.

A eficiência fotossintética da planta foi usada como indicador de vitalidade da planta, e não se verificou nunca estar correlacionada com efeitos no crescimento ou "status" nutricional, confirmando que nenhum destes parâmetros são bons indicadores de stress ou "fitness", apesar de serem usualmente utilizados na avaliação dos efeitos da micorrização.

Demonstrou-se ainda que o maior investimento no crescimento do fungo em determinado momento não constitui necessariamente uma desvantagem a longo prazo.

O tipo de análise do crescimento da planta utilizado foi essencial na caracterização e análise e compreensão das situações estudadas. Nomeadamente a utilização da taxa relativa de adição de N (RAR_N), que permitiu a avaliação da disponibilidade de N em relação às necessidades da planta, foi de extrema importância. Também a análise da fluorescência da clorofila *a*, e em particular a utilização do teste JIP e do índice de desempenho fotossintético PI_{abs}, não usadas frequentemente em pesquisa de micorrizas, provaram ser extremamente valiosos na avaliação de situações de stress e do efeito da micorriza na vitalidade da planta.

Key words: ectomicorrizas, N, C, custo:benefício, resposta à micorrização, distribuição de recursos.

CHAPTER 1

Introduction

CHAPTER 1. Introduction

1. The ECM

The great majority of higher plants form symbiotic associations with fungi at the root level. Mycorrhizae are the structures that result from these associations and have been considered to be the chief organs involved in nutrient uptake of most land plants (Smith and Read, 1997).

The most widely known classification of mycorrhizae divides them into ectomycorrhizae (ECM), vesicular-arbuscular mycorrhizae (AM), ectendo, arbutoid, monotropoid, ericoid and orchid mycorrhizae (Smith and Read, 1997), of which the first two are the most common. This classification takes into consideration the fungal and plant associates, whether the colonization is intra or intercellular, and the type of structures formed.

ECM are found almost entirely on woody perennials. They are established with the fine roots of trees and shrubs, especially of the families *Betulaceae, Pinaceae, Fagaceae, Salicaceae and Dipterocarpaceae*, but were also found in some monocots and ferns. The fungal partners belong to the *Basidiomycota* and *Ascomycota* (Smith and Read, 1997; Brundrett, 1999).

Communities of ECM trees are dominant in coniferous forests, especially in boreal and or alpine regions, trees and bushes are common in temperate and mediterranean ecosystems, and also occur in certain tropical or sub-tropical rain forest habitats (Brundrett, 1999).

In ECM the fungal tissue is located in intercellular spaces and never penetrates the root's cells. A mantle of hyphae forms around the fine roots, enclosing them. From there, hyphae penetrate into the apoplast of the rhizodermis, or, in the case of the gymnosperms, also of the root cortex, where they ramify between the cells forming highly branched networks, in a structure called the Hartig net. The fungal mantel and Hartig net are characteristic of all ECM.

At the very core of the mycorrhizal interaction lies the exchange of water and nutrients, supplied by the fungal partner, for photoassimilates, supplied by the host plant, and the Hartig net constitutes the contact surface between the fungus and the root through which they can be exchanged. This surface's size is large but variable, going from a shallow Hartig net present, for instance, in superficial coating ECM of some Eucalyptus species, to an extensive and deep Hartig net like the one that develops in

most of the pine ECM. The contact zone of hyphae and rot cells also differ whether the association is established with a basididio- or a ascomycete (Gonçalves, 2000). Furthermore, there is great variation in degree of development, amount of extra-matrical mycelium, mantle thickness, and percentage of colonized root area (Brundrett, 2004).

From the mantle, hyphae radiate into the substrate and exploit it for nutrients and water. Some ECM also posses mycelial cords that transport water and nutrients along great distances, and in some cases through unspecialized non-living hyphae (Smith and Read, 1997).

The importance of ECM fungi in the improvement of the host plants mineral nutrition and growth has long been recognized. In general, mycorrhizal infection has been found to improve the nutrient uptake leading to the host plant's increased growth (Burgess *et al*., 1992; Brunner and Scheidegger, 1994). Mycorrhizal stimulation of nutrient uptake is frequently attributed to an improved exploitation of the substrate that eliminates spatial limitations to the uptake of nutrients. The fungal hyphae constitute an extension of the nutrient absorption surface since they penetrate the soil more extensively than the root. The volume of exploited soil is greatly increased in this way, particularly in the cases where the fungal mantle is linked to an extensive mycelial system. The nutrients can be translocated through the depletion zones surrounding the roots, and inaccessible sources of nutrients can be mobilized. Due to their high surface-to-volume ratio and capacity for rapid exponential growth, fungal hyphae are superior to roots in the acquisition of immobile nutrient ions from the low and both spatially and temporally variable nutrient concentrations in natural soils. Fungi may also have access to other nutrient sources than plant roots, namely through production of degradative extracellular enzymes or organic acids (Leake and Read, 1990; Zhu *et al*., 1994; Smith and Read, 1997; Jones and Smith, 2004).

The effects of ECM are, however, not restricted to physical changes in nutrient absorption area and distribution, as it can also result in higher nutrient uptake rates (Vézina *et al*, 1989; Javelle *et al.*, 1999; Selle *et al.*, 2005).

At the other end of the mycorrhizal partnership, the fungus is also dependent on the C supply by the plant. A substantial part of the carbon fixed through photosynthesis is allocated to the fungus, for formation, maintenance and functioning of mycorrhizal structures (Jones *et al*., 1991; Marschner and Dell, 1994; Smith and Read, 1997; Wu *et al*., 2002). Host-derived carbohydrates are necessary for the mycorrhizal fungus as precursors for the synthesis of fungus-specific carbohydrates and sugar-alcohols like trehalose, glycogen or mannitol (Hampp *et al*., 1995; Schaeffer *et al*., 1995; Martin *et*

al., 1998; López *et al*., 2007). ECM mycelium (Harris *et al*., 1985) and fruit-body production (Lamhamedi *et al*., 1994) have been shown to be dependent on the current photoassimilates provided by the host plant.

2. C, N and C/N interactions in ECM

ECM fungi have evolved in N limiting ecosystems, and ECM interactions predominate in such ecosystems (Martin and Botton, 1993; Smith and Read, 1997; Read and Pérez-Moreno, 2003). The improvement in nitrogen uptake as a result of mycorrhizae formation is therefore of central importance for the development and competitivity of host. Additionally to the improved spatial exploitation of the substrate, mycorrhizal roots and external hyphae have been found to have more transporters (Selle *et al.*, 2005) and higher N uptake rates (Vézina *et al*, 1989; Cambraia *et al*., 1992; Javelle *et al.*, 1999) than non-mycorrhizal roots. ECM mycelium also promotes the conversion of N into forms more readily used by the root (Finlay *et al*., 1992; Read and Pérez-Moreno, 2003). Furthermore, inorganic N may be available for plant uptake only as brief pulses, arising when conditions for mineralization are favourable, and an extensive uptake surface may be of extreme value in taking advantage of these brief pulses (Warren *et al*., 2003).

The ability of ECM fungi to take up inorganic nitrogen and transport nitrogen-containing solutes to their host plant is well established (France and Reid, 1983; Chalot and Brun, 1998; Chalot *et al*., 2002). More specifically, ECM fungi can help increase ammonium (NH_4^+) uptake, the form of mineral N predominating in most forest ecosystems characterized by profuse ECM development (Smith and Read, 1997). Because it is usually present in low concentrations and its diffusion is severely restricted in dry soils, the competition for this nutrient is intense. Under these conditions its assimilation by ECM likely plays a key role in N nutrition of forest trees.

ECM plant species of different communities have been found to assimilate predominantly ammonium, amino acids or more complex forms of organic N (Turnbull *et al*., 1996b), and ammonium uptake rates in mycorrhizal forest trees are usually higher than the ones measured for nitrate and in non mycorrhizal plants (Javelle *et al*., 1999; Plassard *et al*., 2000). In addition, most of the tree species present in ECM dominated ecosystems prefer ammonium to nitrate as N source (Martins-Loução and Cruz, 1999; Martins-Loução and Lips, 2000; Kronzucker et al., 2003). Most ECM fungi, similarly to their host plants, prefer ammonium to nitrate as N source, presenting higher ammonium uptake rates (Finlay *et al*., 1992; Marschner and Dell, 1994; Eltrop and Marschner,

1996a; Anderson *et al*., 1999; Prima-Putra *et al*., 1999). The effect of mycorrhization on the host plants' N uptake is also dependent on the form of nitrogen available, and while the uptake of ammonium is generally improved the same has less frequently been observed for nitrate (Scheromm *et al*., 1990a; Plassard *et al*., 1994; Eltrop and Marschner, 1996a; Plassard *et al*., 2000; Gobert and Plassard, 2002). The kinetics and energetics of NH_4^+/methylammonium transport in ECM fungi have been characterized (Jongbloed *et al*., 1991; Javelle *et al*., 1999).

The importance of the external hyphae as ammonium absorbing structures in ECM roots has been demonstrated. Hyphal ammonium acquisition was observed to contribute with 45% to 73% of total plant N uptake under N deficiency (Brandes *et al*., 1998; Jentschke *et al*., 2001), and the ^{15}N ammonium uptake by birch seedlings was greatly decreased when the external mycelium was disrupted (Javelle et al., 1999).

N assimilation in ECM fungi, similarly to plants, can be made through the same two main and alternative pathways: the NADP-glutamate-dehidrogenase (GDH, E.C. 1.4.1.4) pathway and the glutamine synthetase (GS, E.C. 6.3.1.2) /glutamate synthase (GOGAT, E.C. 1.4.1.13) pathway. This last involves the sequential action of GS, catalysing the amination of glutamic acid in order to form glutamine, and GOGAT, catalysing glutamate synthesis. Both pathways can be present in ECM fungi, the relative importance of each differing according to fungal species, the physiological state of the mycelium and the mycorrhizal partner (Martin and Botton, 1993; Rudawska *et al*., 1994). The GS-GOGAT, however, seems to be the main assimilation pathway, similarly to what happens in plants (Marschner and Dell, 1994; Turnbull *et al*., 1996a; Prima-Putra *et al*., 1999; Wright *et al*., 2005; Morel *et al*., 2006). When NH_4^+ is assimilated in the fungal mycelium by either GS/GOGAT or GDH, 2-oxoglutarate derived from the tricarboxylic cycle has to be provided as a carbon skeleton for glutamate or glutamine. In fungi, the tricarboxylic acid cycle can be replenished by the anaplerotic enzymes pyruvate carboxylase and phosphoenolpyruvate carboxykinase (Scheromm *et al*., 1990b; Wingler *et al*., 1996), whilst the plant enzyme phosphoenolpyruvate carboxylase (PEPc) is generally not active (Wingler *et al*., 1996).

NH_4^+ is not accumulated as a free ion in the mycelial tissue but quickly assimilated, at considerable distances of the fungal mantle. Several studies suggest amino acids to be the main sink for the assimilated N (Finlay *et al*., 1988; Ek *et al*., 1994; Marschner and Dell, 1994). It seems to be then translocated under the form of amino acids precursors inside the mycelium and to the mantle, where the absorption by the root system takes place (Finlay *et al*., 1988; Ek *et al*., 1994). Indirect evidence from ^{15}N labelling

experiments supports the transfer of amino acids from the fungus to the host (Smith and Read, 1997). The main form of N transferred to the host plant is glutamine (Finlay *et al*., 1988; Marschner and Dell, 1994; Blaudez *et al*., 1998, 2001), but glutamate, alanine and aspartate are also important sinks for newly absorbed ammonium (Finlay *et al*., 1988). The extension of the transference of other N compounds through the mantle is not clear, but the direct transfer of NH_4^+, as hypothesized for AM symbiosis (Govindarajalu *et al*., 2005) has also been suggested for ECM (Selle *et al*., 2005; Chalot *et al*., 2006). Both organic and inorganic N transfer may co-exist. It has been suggested that the transfer of organic N may be larger in conditions of C sufficiency, when larger incorporation of NH_4^+ into C skeletons may be expected, and that transfer of inorganic acid may be increased under C deficiency (Chalot *et al*., 2006).

The physiological processes involved in the transfer of N from fungus to plant within the symbiotic tissue are still poorly understood. N transporters from the plant and fungal partners have been isolated but their role and regulation is still under investigation, as is their localization in mycorrhizal tissues (Müller *et al*., 2007).

On the other hand, little is known about the metabolic zonation of N assimilation in ECM. The GS-GOGAT cycle may take place entirely in the fungus, and the newly formed glutamine be exported into the plant where it is converted into the remaining amino acids. It may, however, also be separated, and GS could be active in the fungus and GOGAT in the root, in which case there could be a glutamine-glutamate shuttle across the interface (Martin and Botton, 1993).

ECM associations have a deep effect in plant C metabolism, generally increasing the C allocation to the roots (e.g.: Jones *et al*., 1991; Marschner and Dell, 1994; Rygiewicz and Andersen, 1994; Wu *et al*., 2002), although this is not always the case (Durall *et al*., 1994). An important percentage of this C is allocated to mycorrhizae and fungal mycelia, indicating that the fungal partner is an important sink for the hosts' carbohydrates (Cairney and Alexander, 1992; Wu *et al*., 2002). In fact, mycorrhizae attract carbohydrates much more efficiently than non-mycorrhizal fine roots (López *et al*., 2007).

As a result mycorrhization can up-regulate the host plant's rate of net photosynthesis (Dosskey *et al*., 1990, 1991; Colpaert *et al*., 1996; Loewe *et al*., 2000) and sucrose synthesis (Loewe *et al*., 2000). Photosynthesis can also be stimulated by higher shoot N concentrations resulting from an improved N status, but this is not always the case. Mycorrhizal effects on photosynthesis are very varied, and in some cases an explanation for them has not been found (Dosskey *et al*., 1990, 1991).

Sucrose is the main carbohydrate transported by the host plant, but ECM fungi are unable to use it as C source. Its conversion into glucose and fructose seems to be a pre-condition for its use by the fungus since they are able to take up and use both, although they show a preference for glucose (Salzer and Hager, 1991; Chen and Hampp, 1993; Hampp *et al*., 1995; Wiese *et al*., 2000). Several observations indicate that hexoses are the main carbon compounds taken and used by ECM fungi, while the significance of other C sources is unclear (Buscot *et al*., 2000). Since so far no clear evidence of the presence of sucrolitic enzymes in ECM fungi has been found, it is likely that sucrolytic enzymes of the host plant are responsible for sucrose hydrolysis. In fact, the inhibition of mycelial growth observed in *Amanita muscaria*, *Hebeloma crustuliniforme* and *Pisolithus tinctorius* supplemented with sucrose was immediately annulled by the addition of invertase (Salzer and Hager, 1991).

Plants possess two major types of sucrolytic enzymes: invertases and sucrose synthase (SS). Three types of invertase isoenzymes can be distinguished: soluble (AI_s) and insoluble (AI_{ins}) acid invertases, and neutral invertase (NI). SS and NI are located in the cytosol. AI_s occurs primarily in vacuoles, but it is also reported to occur in the apoplastic cell wall space, either as soluble enzyme or bound to the cell wall fraction (Zhu *et al*., 1997). AI_{ins} is located in the apoplastic cell wall space, bound to the cell wall fraction. Because in ECM the plasma membranes of the plant and fungus do not come into direct contact, and mutual interchange has to occur by diffusion across the apoplastic space, the activity of cell wall-bound invertase has been considered to have a potentially major role in this (Salzer and Hager, 1991; Schaeffer *et al.*, 1995; Hampp *et al*., 1999). The kinetic properties of host plants' invertases have also been hypothesized to contribute to the regulation of carbohydrate transfer to the fungus, either through changes in apoplast pH that result from hexose transport, or through the selective uptake of hexoses (Schaeffer *et al*., 1995; Smith and Read, 1997; Hampp *et al*., 1999).

The hexoses taken by the fungus are quickly converted to fungus-specific compounds, therefore maintaining an hexose concentration gradient between plant and fungal tissues, which is thought to maintain the sugar flow towards the fungus (López *et al*., 2007). Sucrose levels were found to be lowest in colonized roots (Martin *et al*., 1998), while fungus-specific compounds, like trehalose and ergosterol were increased (Hampp *et al*., 1995; Martin *et al*., 1998; López *et al*., 2007).

The supply of nutrients by the fungus to the plant is deeply connected to the supply of C to the fungus. The assimilation of inorganic N into amino acids is an important sink for carbohydrates in the fungal mycelium (Martin *et al*., 1988; Wallenda and

Kottke, 1998), which are ultimately supplied from host carbohydrates. The supply of C to the fungus is higher at low N supply and plant productivity (Högberg *et al*., 2003).

The hypothesis that N delivery to the host is dependent on C flow from plant to the fungus has found support in the observation that the transference of ^{14}C from the plant to the fungus was significantly correlated with the transference of ^{15}N from the fungus to the plant, at various N availabilities (Kitöviita, 2005). This supply seems to be controlled by the plant and to depend on the N availability and the plant's needs. Both the potential capacity of ECM fungi to colonize the roots and the growth of extramatrical mycelium are reduced due to increased N availability (Wallander and Nylund, 1991, 1992; Arnebrant, 1994; Brunner and Scheidegger, 1994; Ekblad *et al*., 1995; Wallenda and Kotke, 1998; Nilsson and Wallander, 2003; Treseder, 2004; Nilsson *et al.*, 2005). Evidence has been found that increased N supply causes a switch from gluconeogenesis, i.e. sucrose and starch formation, to glycolisis (Wingler *et al*., 1994; Wallenda *et al*., 1996), and that the decreased sucrose production negatively affected C delivery to the fungus, resulting in decreased fungal biomass (Wallenda *et al*., 1996). It has been proposed that the transport of N into the plant-fungus interface decreases the competition between the fungus and the plant for apoplastic hexoses (Nehls *et al*., 2007), and that the plant reduces the C supply to the fungus if it fails to supply adequate amounts of nutrients.

However, expression studies of fungal and plant monosaccharide transporters at the interface indicate that it is the fungal activity that determines the sink strength in mycorrhizae. The *A. muscaria* monosaccharide transporter AmMST1 expression in mycorrhizae was found to be sugar-regulated, being activated by increased *in vivo* concentration of monosacharides at the plant-fungus interface (Nehls *et al*., 2000) while the expression of monosaccharide transporters in spruce (Nehls *et al*., 1998, 2000) and birch (Wright *et al*., 2000) root cells was decreased in symbiosis.

The effects of mycorrhization on C and N metabolisms are not limited to changes in demand and supply of N and C. Changes in the free amino acids profile and expression and activities of N metabolism enzymes (Vézina *et al*., 1989; Chalot *et al*., 1991; Botton and Dell, 1994; Martin *et al*., 1998) indicate that it is altered and readjusted in mycorrhizal roots. This reorganization of metabolic pathways has been repeatedly observed to occur at very early stages following contact (Blaudez *et al*., 1998; Boiffin *et al*., 1998; Duplessis *et al*., 2005; Frettinger *et al*., 2007), but initial changes may be transient, and later replaced by different adjustments (Boiffin *et al*., 1998; Blaudez *et al*., 1998, 2001).

Several previous studies have revealed molecular and biochemical evidences of a deactivation of the root metabolism, and an activation of the fungal metabolism, in response to mycorrhizae formation (Schaffer *et al*., 1996; Blaudez *et al*., 1998; Johansson *et al*., 2004; Duplessis *et al*., 2005; Frettinger *et al*., 2007; Herrmann and Buscot, 2007). In some cases, these changes have been considered a consequence of a metabolic shift, with some metabolic functions of the root being taken over by the fungus (Vézina *et al*., 1989; Johansson *et al*., 2004), namely N assimilation (Wingler *et al*., 1996). Those results have led to the idea that ECM symbioses allow plants to slow-down regulative pathways, and that this may compensate the cost of photoassimilate transfer to the fungal partner.

3. Cost/benefit and the symbiotic continuum

Mycorrhizae are traditionally accepted as being mutualistic symbioses, therefore resulting in a net beneficial outcome for both plant and fungal partners. However, in an increasing number of reported cases mycorrhization was found to decrease plant productivity (e.g.: Dosskey *et al.*, 1990; Colpaert *et al.*, 1992, 1996; Durall *et al*., 1994; Eltrop and Marschner, 1996a; Plassard *et al*., 2000). This has led to the growing belief that its effects on the host plant can vary from the traditionally accepted mutualistic through to antagonistic in a continuum of responses (Jonhson *et al.*, 1997; Jones and Smith, 2004; Neuhauser and Fargione, 2004; Mazancourt *et al.*, 2005). It has been proposed that in any particular host-plant / fungus combination the response can move along this continuum (Jonhson *et al*., 1997; Gange and Ayres, 1999).

This has led some authors to question the nature of the interaction, since traditional mutualism models do not include the possibility of negative effects between species (Neuhauser and Fargione, 2004). Mycorrhizal fungi have been proposed to shift between mutualism and parasitism (Jonhson *et al*., 1997), to be a conditional mutualism, i.e., mutualistic only in a given environment (de Mazancourt *et al*., 2005), or to be reciprocal exploitations, or reciprocal parasitisms, where conflicts of interest arise that destabilize the partnership (Egger and Hibbet, 2004; Neuhauser and Fargione, 2004). The hypothesis that mutualistic interactions are essentially exploitative with each species exploiting the other to gain a benefit, was also raised (Herre *et al*.,. 1999). However, it has also been considered that the exchange processes of the majority of mycorrhizae are in dynamic equilibrium, meaning that the cost/benefit balance may shift to favour one partner over the other at times, but must later shift back to a more

equitable arrangement, or both partners will be in disadvantage in the long term (Brundrett, 2004).

Mycorrhizae, similarly to other mutualisms, pose a serious challenge for researchers both in terms of the understanding of the conditions favouring their evolution, and of the persistence and stability of the symbiosis (Herre et al., 1999; Hoeksema and Schwartz, 2002). There is, as of yet, no general theory of mutualism. A diversity of modelling approaches has been applied to interspecific mutualisms, including game-theoretical models such as the Iterated Prisoner's Dilemma (IPD; Doebeli and Knowlton, 1998; Hoeksema and Bruna, 2000), population dynamics models (Neuhauser and Fargione, 2004), virulence models (Hoeksema and Bruna, 2000) and biological market models (Schwartz and Hoeksema, 1998; Hoeksema and Schwartz, 2002). The IPD and biological market models are two approaches that focus on the balance of trade in mutualisms and which are of special interest in the mycorrhizal case, namely in answering the question of what factors determine if a species evolves to or behaves as a mutualist or a parasite.

The Iterated Prisoner's Dilemma: An organism receives a certain amount of "payoff" for cooperating with a cooperator, a certain amount for cheating a cooperator, and so on. The decision to cheat or not to cheat is based only on these "payoffs", according to predetermined behavioural strategies. In order to allow for the analysis of how interactions can slide along the gradient between mutualism and parasitism, some IPD models have been proposed that allow continuous variation in the "payoffs" over the course of the game. The player's decision about how much to invest in a partner is based in some quantitative way on a perception of the quality of their partner (Hoeksema and Bruna, 2000). In the IPD model proposed by Doebeli and Knowlton (1998), where the IPD was adapted for the analysis of interspecific mutualisms, the amount invested by an individual varies with the payoff received in the previous iteration. In this way, the long term persistence of mutualism is possible, though it is characterized by large fluctuations in the costs and benefits of the interaction. IPD models do not consider by-product benefits, i.e., resources that are not needed by the giving partner, or that the giving partner is unable to use for its own benefit, but only costly benefits. It also does not allow for multiple players, and therefore it cannot incorporate choice between multiple partners (Schwartz and Hoeksema, 1998).

Biological Market Models: The decision to cooperate, and with whom to cooperate, are based on a comparison of the potential benefits offered by a number of different potential partners attempting to outbid each other for the right to participate in the interaction (Hoeksema and Bruna, 2000). A price of trade is determined by the balance between supply of and demand for the benefits being exchanged, and the price locates the interaction along the mutualism-parasitism continuum. As long as a commodity controlled by an individual on one side of the interaction is in high demand by individuals on the other side, the individuals in control of that commodity can "bargain" for the best offering "price" among the individuals needing it. Thus, an asymmetrical relationship, in which one species benefits much more than the other from the interaction, can develop when one species controls a commodity in high demand by the other (Hoeksema and Bruna, 2000). This theory has been applied to the mycorrhizal interaction (Schwartz and Hoeksema, 1998; Hoeksema and Schwartz, 2002). In this model, whenever two species need each of two resources, and differ in their abilities to obtain at least one of those resources, they will always benefit by specializing in the acquisition of the resource they obtain more efficiently and trading with the other species for the other resource. However, in some situations, two species can each benefit from specialization and trade even if they do not differ in their relative abilities to acquire the two resources. As long as they differ in their relative preferences or needs for the two resources, they can still profit from specialization and trade. Each species should then specialize in acquisition of the resource that they do not prefer, and trade for the one that they do. This mechanism allows a benefit from specialization and trade even if two species have identical abilities to acquire two resources. This mechanism implies that, if two species differ in both their relative preferences for two resources and their relative abilities to acquire those resources, in some cases they may each benefit from specializing in the acquisition and trade of the resource for which they perceive a relative acquisition disadvantage.

A major problem underlying mutualism theory is the evaluation of what is cost and what is benefit in the symbiosis (Herre et al., 1999; Hoeksema and Schwartz, 2002), and the degree to which the benefits exchanged are by-products or costly to the symbionts (Hoeksema and Kummel, 2003; de Mazancourt *et al*., 2005).

It is generally considered that the main benefit to the plant is an improved nutrition, while the cost is the C expended in growth and maintenance of the fungus. The outcome of a mycorrhizal relationship is presumed to depend on the balance between the two, and negative effects of mycorrhizal colonization are expected to occur when the net C

costs for fungal maintenance and growth exceed the net benefits obtained from improved nutrient uptake (Jonhson *et al.*, 1997; Tuomi *et al.*, 2001; Jones and Smith, 2004).

Higher root respiration rates have been repeatedly observed in mycorrhizal plants when compared to non-mycorrhizal plants (Reid *et al*., 1983; Rygiewicz and Andersen, 1994; Colpaert *et al*., 1996; Conjeaud *et al*., 1996; Eltrop and Marschner, 1996b; Heinemeyer *et al*., 2006), although cases have also been observed where no difference was found (Durall *et al*., 1994). Furthermore, some studies suggest that mycorrhizae impose a higher C cost on their hosts under field conditions than under experimental conditions (Rygiewicz and Andersen, 1994). This is not always compensated for by the plant, and the increased belowground carbon allocation may determine decreased growth of mycorrhizal plants (Colpaert *et al.*, 1996; Plassard *et al*., 2000). This also explains increased rates of photosynthesis that were not associated to increased growth relative to non-mycorrhizal plants (Colpaert *et al*., 1996; Conjeaud *et al*., 1996).

High nutrient retention by the mycobiont (Colpaert *et al.*, 1992, 1996) is the other factor that has been considered to determine decreased growth of mycorrhizal plants. ECM fungi have a high potential to take up, accumulate (Wallenda and Kottke, 1998; Plassard *et al*., 2000) and immobilize N (Colpaert *et al.*, 1992, 1996; Plassard *et al*., 2000).

The balance between the fungal demand for energy and the plant's needs for nutrients seems to depend on the amount of nutrients available to the plant. Mycorrhizae have been widely found to improve N and P nutrition of the host plant when these nutrients were in limited supply, but not when they were abundant (Douds *et al*., 1988; Dickson *et al*., 1999; Bücking and Heyser, 2000). It has been considered that the C cost may not always be compensated for by improved nutrition when the nutrients are readily available.

Cost efficiency, i.e., the amount of nutrients acquired per C expended, decreases with increasing mineral nutrient availability, and this decline has been proposed to be steeper for mycorrhizal plants. Mycorrhizal plants have been proposed to perform better (increased nutrient concentrations, nutrient uptake capacity, increased growth) under nutrient poor conditions due to their superior cost efficiency, whereas non-mycorrhizal plants should be more cost efficient and grow better at high nutrient levels (Shwartz and Hoeksema, 1998; Tuomi *et al.*, 2001). Some authors have found the construction and maintenance costs lower for fungal hyphae and mycorrhizal roots than non-mycorrhizal roots (Jones *et al*., 1998, 1991; Jones and Smith, 2004), and this has been considered the

reason for the greater cost efficiency of mycorrhizal plants (Jones *et al*., 1998). However, others have found them higher (Colpaert *et al*., 1996; Smith and Read, 1997).

In addition, although the efficiency in terms of the amount of nutrients acquired per unit weight of tissue is, without a doubt, higher in fungal hyphae than in roots, its higher cost efficiency is still a question. Although less C would be expected to be required to support a unit length of absorbing structure (Jones *et al*., 1991, 1998), the fungus could have a relatively high regeneration rate, or the duration of active uptake by hyphae could be very short, determining that mycorrhizal roots have a very high C demand (Jones *et al*., 1991).

Cost efficiency calculations are complicated and the few reported experimental data indicate that under laboratory conditions it may be higher (Jones *et al*., 1991, 1998), equal (Tuomi *et al*., 2001) or lower (Jones *et al*., 1991) in mycorrhizal compared to non-mycorrhizal roots.

Some models have been proposed to explain the variation of mycorrhizal effects on plant productivity that considered it to depend on the nutrient availability (Morgan *et al*., 2005; Janos, 2007) or on the balance between C cost and nutrient gain (Schwartz and Hoeksema, 1998; Tuomi *et al.*, 2001; Neuhauser and Fargione, 2004). The degree of mycorrhization has also been considered responsible for such variations, and been used to model them, either alone or together with nutrient availability (Gange and Ayres, 1999; Tuomi *et al.*, 2001).

All the models proposed so far are effective at predicting the negative effects on the host plant at high nutrient availabilities. However they do not predict the situations of decreased growth when the nutrients are limiting, which, although they are much less frequent, have also been observed (Ingestad *et al.*, 1986; Colpaert *et al.*, 1999; Hobie, 2006).

Of great importance in the evaluation of benefit is the choice of parameters used in that evaluation. Survival and fecundity and reproductive success are acknowledged as the best parameters when evaluating plant fitness, and therefore the plants' response to mycorrhization, but are, at best, more difficult, and in many cases impossible, to measure, as in the case of plant species with long life cycles. The response to mycorrhization has most often been evaluated through its effect on the growth or nutrient status of the host plant, as these are easily and always measurable, but their meaningfulness as benefit indicators has been questioned (Johnson *et al.*, 1997; Jones and Smith, 2004).

Increased growth rates in mycorrhizal plants have most often been attributed to increased nutrient uptake (Jones *et al*., 1990, 1991). However, nutritional effects are not always reflected in growth, and growth responses are not always correlated with a response in fitness (Jones and Smith, 2004). Fecundity can also be affected differently from growth or nutritional status. Mycorrhizal *Avena fatua* plants produced more biomass and accumulated more N than NM plants, but flowered for a shorter time and tended to produce seeds with a reduced individual mean mass (Koide *et al*., 1988). On the other hand, mycorrhizal plants that grew less have been observed to produce more reproductive structures (Miller *et al*., 2002) or more successful progeny (Nuortila *et al*., 2004) than non-mycorrhizal plants.

Furthermore, for plant species that take many years to reach sexual maturity, lifetime fitness may be determined more by the ability to survive through occasional environmental extremes, such as nutrient or water limitation, than by rapid growth rates during more common, moderate conditions (Jones and Smith, 2004). Mycorrhization has been observed to increase survival in conditions of high stress (Guerin-Laguette *et al.*, 2003; Jones and Smith, 2004), namely of micropropagated plants during the acclimation phase (Romano and Martins-Loução, 1994; Normand *et al*., 1996; Martins *et al*., 1997). Mycorrhization has been considered to have a stress buffering effect, facilitating the plants adaptation to environmental changes (Romano *et al*., 1996; Rivera-Becerril *et al*., 2002; Tsimilli-Michael and Strasser, 2002).

Mycorrhization also have non-nutritional effects, which will not be reflected on nutritional status or growth, but that may determine increased survival and fitness. It provides protection against root and shoot pathogens, namely through the production of phenols or antibiotics, induced resistance, competition for root infection sites, or by providing a physical barrier to infection (Marschner and Dell, 1994; Hampp *et al*., 1999; Whipps, 2004; Akema and Futai, 2005). Mycorrhizae can also be responsible for the excretion of chelating compounds or ectoenzymes (Marschner and Dell, 1994; Hampp *et al*., 1999), confer protection from toxic minerals (Rivera-Becerril *et al*., 2002; Hildebrandt *et al*., 2007) and resistance to drought (Bogeat-Triboulot *et al*., 2004). Furthermore, ECM roots survive longer than non mycorrhizal ones (King *et al*., 2002).

Finally, the time window chosen for observation may also determine that mycorrhization be considered beneficial, or not. The C cost and N gain deriving from the symbiosis fluctuate in time, in a non-synchronized way, as they vary with environmental changes, as well as with developmental stages of the plant, the fungus and the mycorrhizal structures, and the cost:benefit ratio may vary substantially over the

course of time (Jones *et al*., 1991). The usefulness of short term experiments in determining mutual benefit to mycorrhizal symbionts is therefore questionable. Furthermore, the conditions under which mycorrhizal fungi benefit the host plant may be quite specific and may not be encountered during the duration of a given experiment (Jones and Smith, 2004).

4. Outline of the thesis

As mentioned above, the cost/benefit dynamics in mycorrhizal symbiosis is still largely unknown. Namely, there is still a great lack of understanding of what factors determine the effects of mycorrhization, and how these effects are related with the cost/benefit balance in the symbiosis. What represents a cost and a benefit in the symbiosis, is also not yet understood.

The goal of this thesis was to understand:

I. What factors determine the variance in plant response to mycorrhization.

II. How does this variance relate to the balance between mycorrhizal cost and benefit.

Two main and different proxies were used:

1) The effects of mycorrhization on plant productivity and vitality.

The nitrogen (N) availability, the plants' N status, the age of the plant at the time of inoculation and the level of mycorrhizal colonization, were investigated as possible determinants of the variance in mycorrhizal effects.

For the evaluation of these effects, a classical plant growth analysis approach was chosen. Plant growth analysis is an explanatory and integrative approach to interpreting plant form and function. It uses simple primary data in the form of weights, areas, volumes and contents of plant components to investigate processes within and involving the whole plant (Hunt, 1982, 2003; Lambers et al., 1990; van den Driessche and van den Driessche, 1991). It comprises powerful comparative tools that aim to negate, as far as possible, the inherent differences in scale that can exist between different organisms or phases of growth.

The collected primary data (e.g., weights, nutrient contents) can be used to calculate four distinct types of derivate (Hunt, 2003):

i) **Absolute Growth Rates:** Simple rates of change, involving only one plant variable and time. E.g., whole plant rate of dry weight increase.

ii) **Relative Growth Rates:** More complex rates of change, but still only involving one plant variable and time. Examples are the relative growth rate (RGR; whole plants rate of dry weight increase per unit of dry weight) and the N relative uptake rate (RN; whole plants rate of N increase per unit of plant N).

iii) **Simple Ratios:** These involve ratios between two quantities, and may either be ratios between two like quantities, such as total leaf dry weight and whole plant dry weight, or unlike quantities, such as total leaf area and whole plant dry weight.

iv) **Compounded Growth Rates:** Rates of change involving more than one plant variable. Examples are the nitrogen productivity (NP; whole plant rate of dry weight increase per unit of plant N), and the N net uptake rate (U; whole plant rate of N increase per root weight or area).

The use of simple ratios was used in the present thesis as a way to isolate the effect of mycorrhization on several plant variables, by calculating the ratio between a given parameter value for an individual mycorrhizal plant (m) and the mean value for non mycorrhizal plants (NM) grown in the same experimental conditions: m/NM. Similar approaches had been previously suggested (Koide, 1991; Gange and Ayres, 1999).

The concept of nutrient relative addition rate (RAR), developed by Ingestad and collaborators (e.g., Ingestad and Lund, 1979; Ingestad, 1982; Ingestad and Kähr, 1985), was also used in the present thesis. This concept was developed following the realization that external nutrient concentration alone is not sufficient to characterize nutrient supply. The decisive property of the nutrient solutions for the rate of nutrient uptake and growth is not the concentration, but the nutrient amount supplied per unit time in relation to the growth rate. In other words, the same nutrient concentration in the medium will mean different nutrient supplies to plants of different sizes and nutrient contents because their nutrient requirements will differ. The RAR growth technique was therefore developed, in which additions of nutrients increase with time, according to the plants' needs, resulting in a stable growth rate (steady-state growth), after a lag phase during which the plants adapt to the nutrient level characteristic of the treatment. In the present thesis, an alternative use was given to the RAR calculations. The formula used to calculate the adequate nutrient addition to ensure a steady-state growth (constant RAR) was used to characterize the nutrient supply to the plants during a given interval of time.

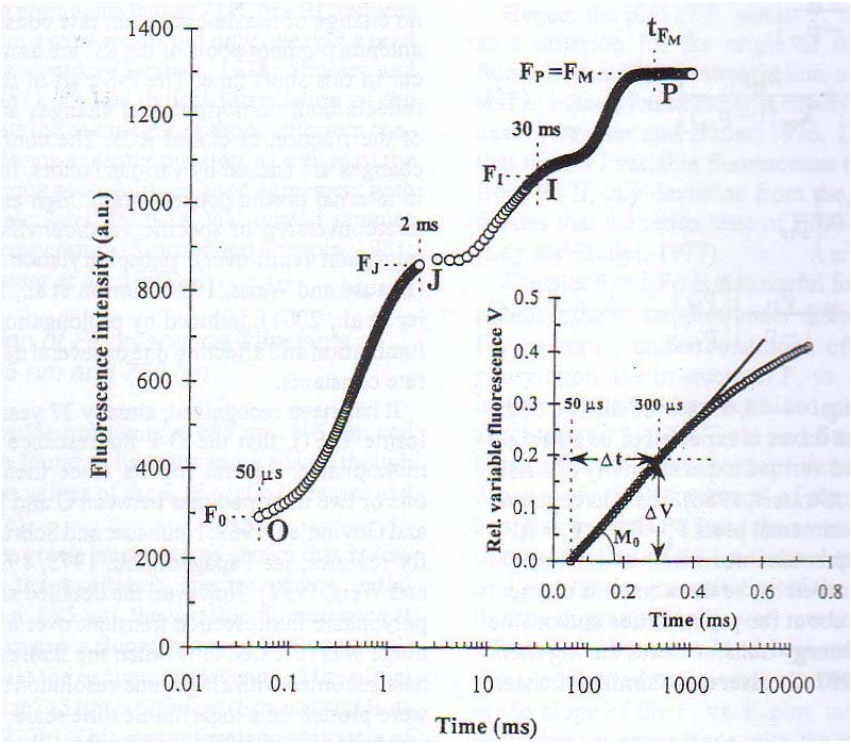

Figure 1. Typical chlorophyll *a* polyphasic fluorescence rise O-J-I-P. The transient is plotted on a logarithmic scale from 50µs to 1s. The marks refer to the selected fluorescence data used by the JIP test for the calculation of structural and functional parameters: the fluorescence intensity F_0 (at 50µs); the fluorescence intensities F_J (at 2ms) and F_I (at 30 ms); the maximal fluorescence intensity, $F_P=F_M$ (at time denoted t_{FM}). The insert shows the transient expressed as the relative variable fluorescence $V= (F-F_0) / (F_M-F_0)$ vs. time, from 50µs to 1 ms on a linear time scale, demonstrating how the initial slope, also used by the JIP test, is calculated: $M_0 = (\Delta_V/\Delta_t)_0 = (V_{300\mu s}) / (0.25 \text{ ms})$. *In* Strasser *et al*. (2000).

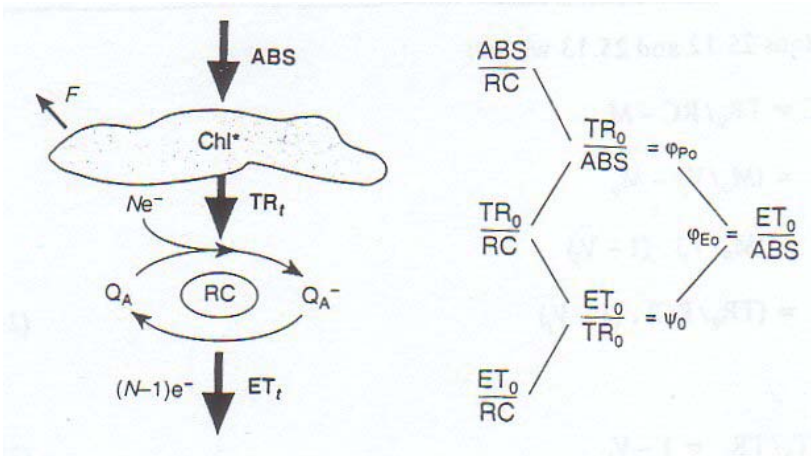

Figure 2. Simplified working model of the energy fluxes in the photosynthetic apparatus. *In* Strasser *et al*. (2000).

Chlorophyll *a* (Chl *a*) fluorescence measurements, and the analysis of fast fluorescence kinetics O-J-I-P according to the JIP test (Strasser *et al.*, 2000) were used as a non-invasive and highly sensitive indicator of stress/plant vitality. All oxygenic photosynthetic materials investigated so far exhibit a polyphasic chlorophyll *a* fluorescence rise upon strong actinic illumination. On a logarithmic time scale, this rise shows a sequence of phases from the initial minimal fluorescence (F_0), passing by the two intermediate steps F_J and F_I, to the maximal fluorescence (F_M) (Fig. 1; Strasser and Govindjee 1992a, b; Strasser *et al.*, 1995). The shape of the O-J-I-P transient changes according to different environmental conditions (Strasser et al., 2004), and its analysis using the JIP test, allows the access to a large amount of information about the behaviour of the photosynthetic apparatus (Strasser *et al.*, 2000, 2004). The JIP test takes into consideration the fluorescence values at 50 μs (F_0), 100 μs (F_{100}), 300 μs (F_{300}), 2 ms (step J), 30 ms (step I) and F_M. It uses these values in the calculation of several flux ratios, specific energy fluxes (per reaction center) and phenomenological energy fluxes (per leaf cross section) of the photossystem II.

Recently, the performance index (PI) has been introduced (Strasser *et al.*, 2000). PI_{abs} combines three parameters favourable to photosynthetic activity: (1) the density of reaction centres (expressed on an absorption basis), (2) the quantum yield of primary photochemistry, and (3) the ability to feed electrons into the electron chain between photosystem II and I (Srivastava *et al.*, 1999). Table 1 summarizes the technical parameters used in calculations as well as the JIP parameters. In Fig. 2 a simplified working model of the energy fluxes in a photosynthetic apparatus considered in the JIP test is shown.

Not so commonly used in mycorrhizal research, the chlorophyll *a* fluorescence analysis is perhaps the best tool available to evaluate stress and plant vitality, and therefore fitness, save from survival and fecundity, since it integrates the multiple effects of mycorrhization, including non-nutritional effects, which can be difficult to evaluate. This method can be carried out quickly, and is non-destructive, facilitating the screening of a large number of samples.

2) C and N metabolism readjustments in response to mycorrhizal formation.

The question of how the modifications in plant C and N metabolism occur from the moment of inoculation and until a fully established mycorrhizal state was investigated, and how they are dependent on N supply.

Technical fluorescence parameters	
Area	Area between fluorescence curve and F_M
F_0	F_{50}, fluorescence intensity at 50μs
F_{100}	Fluorescence intensity at 100μs
F_{300}	Fluorescence intensity at 300μs
F_J	Fluorescence intensity at the J step
F_I	Fluorescence intensity at the I step
F_M	Maximal fluorescence intensity
F_V/F_0	$(F_M/F_0)/ F_0$
$(\Delta V/\Delta t)_0$ or M_0	Slope of the curve at the origin of the fluorescence rise. It is a measure of the rate of primary photochemistry. $M_0 = 4 (F_{300}-F_0) / (F_M-F_0)$
V_J	Variable fluorescence at 2ms. $V_J = (F_J-F_0) / (F_M-F_0)$
V_I	Variable fluorescence at 30ms. $V_I = (F_I-F_0) / (F_M-F_0)$
Quantum efficiency or flux ratios	
φ_{P0} or TR_0/ABS	Trapping probability or quantum yield efficiency. Expresses the probability that an absorbed photon will be trapped by the PSII reaction centre. $\varphi_{P0} = (F_M-F_0) / F_M$
Ψ_0 or ET_0/TR_0	Expresses the probability that a photon absorbed by the PSII reaction centre enters the electron transport chain. $\Psi_0 = 1- V_J$
Specific fluxes or activities	
ABS/RC	$= M_0 (1/ V_J) (1/ \varphi_{P0})$
TR_0/RC	$= M_0 (1/ V_J)$
ET_0/RC	$= M_0 (1/ V_J) \Psi_0$
DI_0/RC	$= (ABS/RC) - (TR_0) (1/ \varphi_{P0})$
Phenomenological fluxes or activities	
ABS/ CS_0	$= F_0$, or other useful expression
TR_0/ CS_0	$= \varphi_{P0} (ABS/ CS_0)$
ET_0/ CS_0	$= \varphi_{P0} \Psi_0 (ABS/ CS_0)$
DI_0/ CS_0	$= (ABS/CS_0) - (TR_0/CS_0)$
RC/CS_0	$= \varphi_{P0} (V_J / M_0) F_0$
Density of reaction centres	
RC/ABS	$(RC/TR_0) (TR_0/ABS) = [V_J/(d_V/d_{t0})] (F_V/F_M)$
Performance index	
PI_{abs}	$(RC/ABS) [\varphi_{P0}/(1- \varphi_{P0})] [\Psi_0/(1- \Psi_0)]$

Table 1. Technical data of the O-J-I-P curves and the JIP test parameters. ABS, energy absorption; TR, energy trapping; ET, electron transport; DI, energy dissipation; RC, reaction center; CS, leaf cross section. Adapted from Strasser *et al*. (2000).

C and N metabolism enzyme activities and metabolite concentrations were determined. The enzymes were chosen for their relevance in the C/N exchange between symbionts, but also because they are plant-specific, thus allowing us to isolate the plant response at the root level.

In addition, the approaches previously described in 1) were also followed.

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CHAPTER 2

Are Mycorrhizae always beneficial?

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CHAPTER 2. Are Mycorrhizae always beneficial?

Summary

In this work we evaluate whether the effect of ectomycorrhiza in the early developmental stages of symbiosis establishment is detrimental or beneficial in plant productivity and whether this effect is dependent on either N nutrition or plant age.

Groups of *Pinus pinaster* L. plants with different ages and nutritional status were inoculated with alive or dead *Pisolithus tinctorius*. The plants were fed either with 1.9 mM or 3.8 mM ammonium as N source. Ectomycorrhiza establishment was monitored until one month after the inoculation through daily chlorophyll *a* fluorescence measurements and the analysis of fast fluorescence kinetics O-J-I-P, biomass increment and photosynthesis.

Our results show that plants react differently to ectomycorrhiza formation depending on their age (stage of development, leaf area), their initial nutritional status, and the amount of nitrogen supplied. Mycorrhiza formation was found to constitute a stress depending on the plants age. Increased availability of N softened or eliminated the negative impact of mycorrhiza formation. Younger plants were the only ones that eventually developed a higher net photosynthesis rate when mycorrhizal.

It is concluded that ectomycorrhiza formation may have a detrimental rather than a beneficial effect in plants productivity during their establishment and early developmental stages, and that this depends on the amount of N available to the plant, on the nutritional status and on the age of the plant. Chlorophyll *a* fluorescence measurements proved to be a non destructive, non invasive and reliable tool able to identify the first signals of plant-mycorrhiza fungi interactions.

Key words: ammonium, chlorophyll *a* fluorescence, mycorrhiza, nitrogen, O-J-I-P test, *Pinus pinaster*, benefit.

Introduction

Although the beneficial role of mycorrhizal symbiosis has been frequently observed, there have been reported cases where mycorrhizal inoculation has led to a decrease in plant productivity, particularly in arbuscular mycorrhizal plants (AM) (e.g.,

Koide, 1985; Baon *et al.*, 1994; Jifon *et al.*, 2002; Schroeder and Janos, 2004). This decrease has often been found to be transient and recovered later, being followed by a positive growth response. No such growth depressions are usually referred to in literature regarding ectomycorrhiza (ECM), which are generally found to enhance plant growth (e.g., Burgess *et al.*, 1994; Lu *et al.*, 1998). However, a number of studies have found no differences in growth between ectomycorrhizal and non-mycorrhizal plants (Thomson *et al.*, 1994; Bâ *et al.*, 1999) and cases of reduced growth have also been found for plants with this type of association (Colpaert *et al.*, 1992; Eltrop and Marschner, 1996a).

Association with ectomycorrhizal fungi can improve nitrogen acquisition by plants, playing a key role in nitrogen nutrition of forest trees. Ammonium is the predominant form of mineral nitrogen in most forest ecosystems that support a profuse ectomycorrhizal development (Wingler et al., 1996; Prima Putra *et al.*, 1999). Ectomycorrhizal fungi are also known to increase ammonium uptake and ammonium assimilation rates in woody plants (Rudawska et al., 1994; Eltrop and Marschner, 1996a; Wingler *et al.*, 1996; Javelle *et al.,* 1999).

Mycorrhiza establishment leads to an increase in the demand of carbohydrates, for fungal maintenance and growth (Reid *et al.*, 1983; Dosskey, *et al.*, 1990; Hampp *et al.*, 1999). Clear evidence exists that *P. tinctorius* acts as a strong C sink at least during the early stages of the symbiosis (Cairney and Chambers, 1997).

Growth depressions following mycorrhizal inoculation are generally attributed to the carbohydrate drain of the mycorrhizal fungus, while positive growth effects of mycorrhiza are thought to occur when the benefits of increased nutrient uptake exceed the carbon cost of the association (Thomson *et al.*, 1994; Schroeder and Janos, 2004).

The photochemical performance of the host plant has also been found to decrease in the first stages of AM establishment, indicating that this can constitute a stress for the host plant. This decrease was transient, and followed by an increase to higher levels than those found prior to mycorrhiza establishment, and in non-mycorrhizal plants (Calantzis, 2002).

In this study we tried to address the following questions:

- Does ectomycorrhizae have a detrimental rather than a beneficial effect in plants productivity during their establishment and early developmental stages?
- Are these responses dependent on i) the amount of N available to the plant, ii) the nutritional status of the plant, iii) or on the age of the plant?

Material and methods

Plant and fungal material

The *P. tinctorius* (Pers.) Coker & Couch isolate PtA from the collection of the University of Lisbon, Plant Biology Department, was grown in pure culture in liquid modified Melin-Norkans (MMN) medium (Marx, 1969).

For inoculation, mycelium of *P. tinctorius* was grown for 2 months in the dark at 24 ºC on a perlite/vermiculite (v/v) mixture moistened with liquid MMN medium.

Pinus pinaster L. seeds were collected in Sines and Santarém, Portugal, and provided by the National Centre of Forest Seeds (CENASEF) of the Portuguese Ministry of Agriculture. The seeds were surface-sterilized with 30 % calcium hypochlorite for 30 minutes, rinsed in several changes of distilled water, and soaked in distilled water at 4 ºC for 48 h. Sowing was carried out in gnotobiotic conditions on a sand/vermiculite (v/v) mixture sterilized at 120 ºC for 1 h. Seedlings were watered with distilled water as needed.

When the second set of leaves appeared, approximately one month after sowing, the seedlings were transferred to 350 mL root trainers (20cm Fleet Roottrainers, Ronaash, Ltd., Roxburghshire, U.K.), with a perlite/vermiculite (v/v) mixture as substrate, sterilized at 120 ºC for 1 h.

Experimental design

At the time of transfer to the root trainers the plants were divided into three groups. Group 1 (G1) was inoculated at the time of transfer from the sowing beds into the root trainers (Fig. 1). Half of the seedlings were inoculated with alive (mycorrhizal - M) and half with dead (non-mycorrhizal control - NM) *P. tinctorius* mycelium. For the inoculation, 100 mL inoculum, previously washed with distilled water, was placed in contact with the roots. For the dead mycelium, the inoculum was sterilized for 1 hour at 120 ºC.

Group 2 (G2) was not inoculated at the time of transfer. The plants were watered twice a week with 25 mL distilled water, for one month after the transfer to the root trainers. They were then divided into two sub-groups, and inoculated with alive or dead *P. tinctorius* mycelium, as already described for G1.

In both G1 and G2, from the moment of inoculation the plants started being fed with MMN medium, from which thiamine was omitted. Each sub-group, M or NM, was

again divided. Half of the plants received 3.8 mM NH_4^+, and the other half 1.9 mM NH_4^+ as N source.

MMN medium has an ammonium concentration of 3.8 mM. In order to obtain a medium with half this concentration (1.9 mM), it was modified, containing 0.95 mM $(NH_4)_2HPO_4$ and 4.6 mM KH_2PO_4.

Group 3 (G3) was also not inoculated at the time of transfer. The plants were divided into 2 sub-groups and started being fed with either 3.8 mM NH_4^+ or 1.9 mM NH_4^+ as N source, as already described. After 1 month each of the 2 sub-groups were again divided and inoculated with either alive or dead inoculum. They continued being fed as before.

In all groups, each plant was watered twice a week with 25 mL of medium. 80 to 100 plants were used in each sub-group.

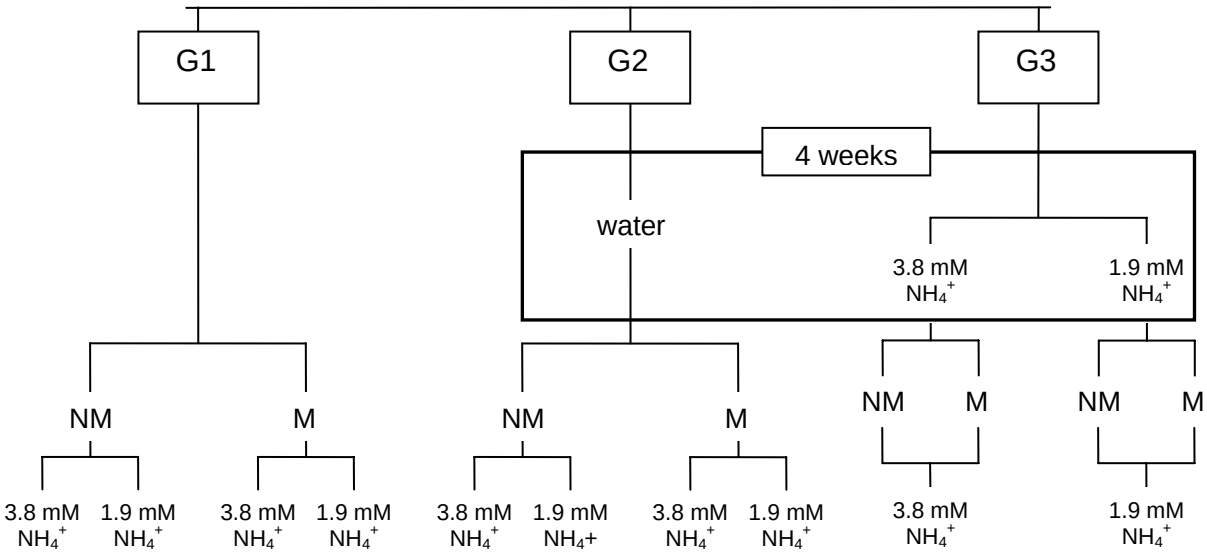

Figure 1. Scheme of the experimental design.

The whole experiment was performed in a growth chamber under a 16 h light / 8 h dark photoperiod at 24 / 18 ºC, approximate 70 % relative humidity and 250 μmol m^{-2} s^{-1} at plant height.

The timing of mycorrhiza formation was established by collecting 6 to 8 plants every 2 days from the day of inoculation until day 14, and every 5 days until day 29.

Visual assessment of the mycorrhizal status and root ergosterol quantification were performed.

The remaining plants were harvested 29 days after the time of inoculation. Shoot and root were collected separately. Fresh and dry weights, after 48 h freeze drying, were recorded.

Chlorophyll a fluorescence measurements

Ectomycorrhiza establishment was monitored through chlorophyll *a* (Chl *a*) fluorescence measurements, and the analysis of fast fluorescence kinetics O-J-I-P according to the JIP test (Strasser *et al.*, 2000). Changes in chl *a* fluorescence were used as a stress/plant vitality indicator during the process of mycorrhiza formation. Full fluorescence transients were recorded in plants before their inoculation (day 0), daily during the first 14 days after inoculation, and every 5 days, for two consecutive days, until day 29. A minimum of 60 measurements was made for each day.

The values were grouped in packages of two consecutive days (e.g. values obtained for day 1 and 2 were pulled together).

Measurements of Chl *a* fluorescence were conducted in fully dark adapted leaves, before the onset of illumination in the culture chamber, with a Plant Efficiency Analyser (PEA, Hansatech Ltd., England). In the dark adapted steady-state the fluorescence rise is considered to be due only to changes in the redox state of PSII reaction centre, and is therefore considered to reflect only the photochemical reactions.

Light was provided by an array of six light-emitting diodes (peak 650 nm) focused on the sample surface. Chl *a* fluorescence signals were detected using a PIN photocell after passing through a long pass filter (50 % transmission at 720 nm). The fluorescence signal was recorded for 1s, starting from 50 μs after the onset of illumination. The data acquisition was every 10 μs for the first 2 ms, and every 1 ms until 1s. On a logarithmic time scale, the rising transient from minimal fluorescence, F_0 (fluorescence at 50 μs) to maximal fluorescence, F_M, had a polyphasic behaviour (Strasser and Govindjee 1992a, b; Strasser *et al.*, 1995). Analysis of the transient took into consideration the fluorescence values at 50 μs (F_0), 100 μs (F_{100}), 300 μs (F_{300}), 2 ms (step J), 30 ms (step I) and F_M. This method is called the JIP test and has been elaborated in terms of both application (Strasser *et al.*, 2000) and theory (Strasser *et al.*, 2004).

Recently, the performance index (PI) has been introduced (Srivastava *et al.*, 1999, Strasser *et al.*, 1999; Strasser *et al.*, 2000; Tsimilli-Michael *et al.*, 2000). PI_{abs} combines three parameters favourable to photosynthetic activity: (1) the density of reaction

centres (expressed on an absorption basis), (2) the quantum yield of primary photochemistry, and (3) the ability to feed electrons into the electron chain between photosystem II and I (Srivastava *et al.*, 1999). Table 1 summarizes the technical parameters used in calculations as well as a selection of the JIP parameters used in this study.

Technical fluorescence parameters	
Area	Area between fluorescence curve and F_M
F_0	F_{50}, fluorescence intensity at 50µs
F_{100}	Fluorescence intensity at 100µs
F_{300}	Fluorescence intensity at 300µs
F_J	Fluorescence intensity at the J step
F_I	Fluorescence intensity at the I step
F_M	Maximal fluorescence intensity
F_V/F_0	$(F_M/F_0)/ F_0$
$(\Delta V/\Delta t)_0$ or M_0	Slope of the curve at the origin of the fluorescence rise. It is a measure of the rate of primary photochemistry. $M_0 = 4 (F_{300}-F_0) / (F_M-F_0)$
V_J	Variable fluorescence at 2ms. $V_J = (F_J-F_0) / (F_M-F_0)$
V_I	Variable fluorescence at 30ms. $V_I = (F_I-F_0) / (F_M-F_0)$
Quantum efficiency or flux ratios	
φ_{P0} or TR_0/ABS	Trapping probability or quantum yield efficiency. Expresses the probability that an absorbed photon will be trapped by the PSII reaction centre. $\varphi_{P0} = (F_M-F_0) / F_M$
Ψ_0 or ET_0/TR_0	Expresses the probability that a photon absorbed by the PSII reaction centre enters the electron transport chain. $\Psi_0 = 1- V_J$
Density of reaction centres	
RC/ABS	$(RC/TR_0)\ (TR_0/ABS) = [V_J/(d_V/d_{t0})]\ (F_V/F_M)$
Performance index	
PI_{abs}	$(RC/ABS)\ [\varphi_{P0}/(1- \varphi_{P0})]\ [\Psi_0/(1- \Psi_0)]$

Table 1. Technical data of the O-J-I-P curves and the selected JIP test parameters used in this study.

Net photosynthesis rate

Gas exchange measurements were made with a compact CO_2 /H_2O porometer CQP-130 coupled with a NBIR gas analyser (Binus 100 Leybold Heraeus, D-6450 Hanau, Germany). Whole shoot net photosynthesis was measured 4, 6, 10, 14, 21 and 29 days after inoculation (day 0) in plants from G1. G2 and G3 were measured 8, 14 and 29 days after inoculation. The light intensity was 250 μmol m^{-2} s^{-1}. 8 to 12 plants were measured for each day and group.

Data analysis

The differences between dry weights were analysed using an ANOVA test, followed by a Tukey test, with $P \leq 0.05$. All differences between control (NM) and mycorrhizal (M) plants in other parameters were analysed using a Student t-test, with $P \leq 0.05$. SPSS software, version 12.02, was used for both tests.

Results

In plants from G1 and G2 the first signs of mycorrhiza establishment, were observed 6 days after inoculation. On G3, this was only observed 12 days after inoculation (data not shown). No mycorrhiza formation was observed in control plants.

Chlorophyll a fluorescence

The photosynthetic Performance Index (PI_{abs}) was found to be the most sensitive of all the calculated parameters.

In plants from G1, that were younger at the time of inoculation, a significant drop in PI_{abs} was observed simultaneously with the first signs of mycorrhiza formation, with both N nutritions (day 6; Fig. 2). With 1.9 mM NH_4^+, M plants kept a lower level of PI_{abs} than NM throughout the rest of the experiment. With 3.8 mM NH_4^+ this drop was recovered to similar levels to the ones found in NM plants (Fig. 2).

The initial drop, simultaneous with the first signs of mycorrhiza formation, was not observed in plants of G2 and G3, older at the time of inoculation (Fig. 3, 4).

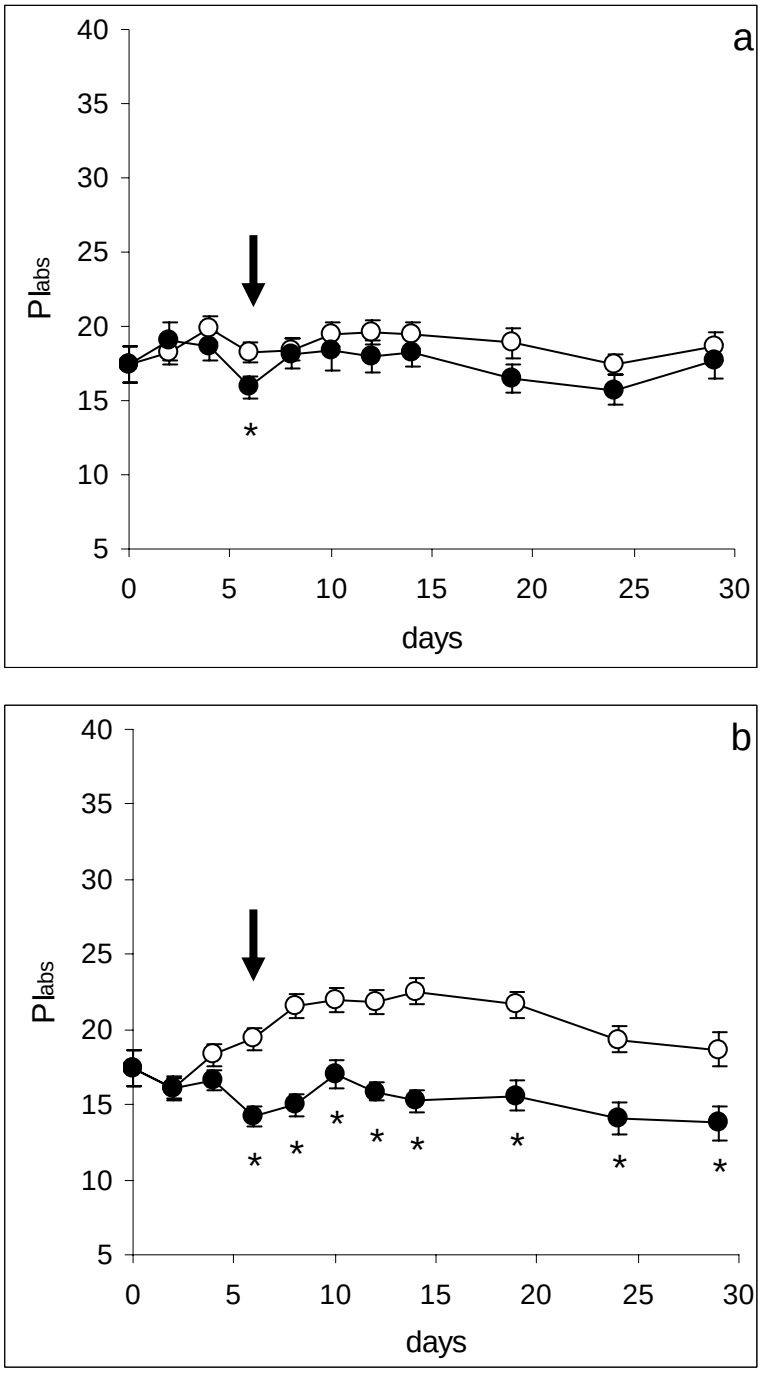

Figure 2. Photosynthetic Performance Index (PI_{abs}) variation during 30 days after inoculation of plants of group 1. At day 0 the plants were inoculated with alive (M; closed circles) or dead (NM; open circles) *P. tinctorius*. The plants were fed with **a)** 1.9 mM or **b)** 3.8 mM NH_4^+. The arrow marks the beginning of mycorrhization. Values are means ± SE. Asterisks indicate significant differences between treatments at $P \leq 0.05$ (Student t-test).

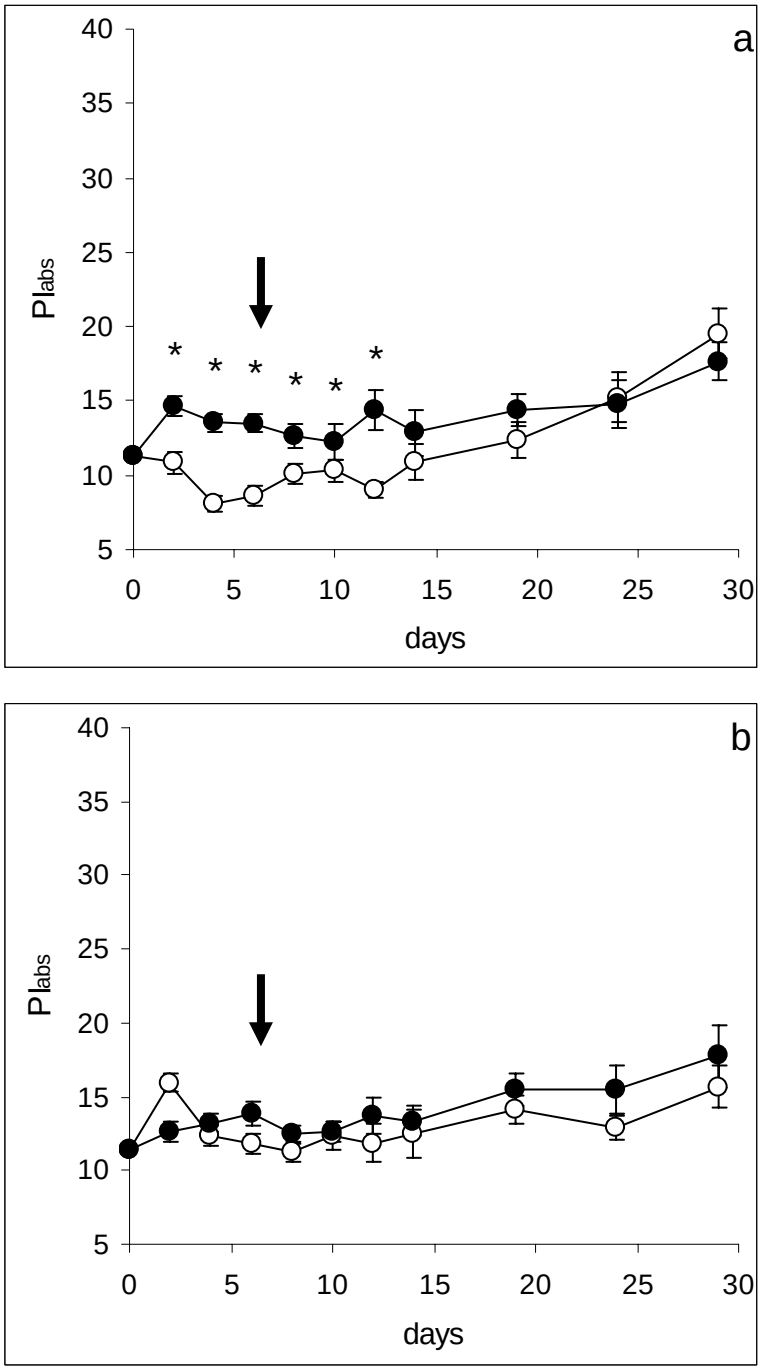

Figure 3. Photosynthetic Performance Index (PI_{abs}) variation during 30 days after inoculation of plants from group 2. At day 0 the plants were inoculated with alive (M; closed circles) or dead (NM; open circles) *P. tinctorius*. The plants were fed with **a)** 1.9 mM or **b)** 3.8 mM NH_4^+. The arrow marks the beginning of mycorrhization. Values are means ± SE. Asterisks indicate significant differences between treatments at $P \leq 0.05$ (Student t-test).

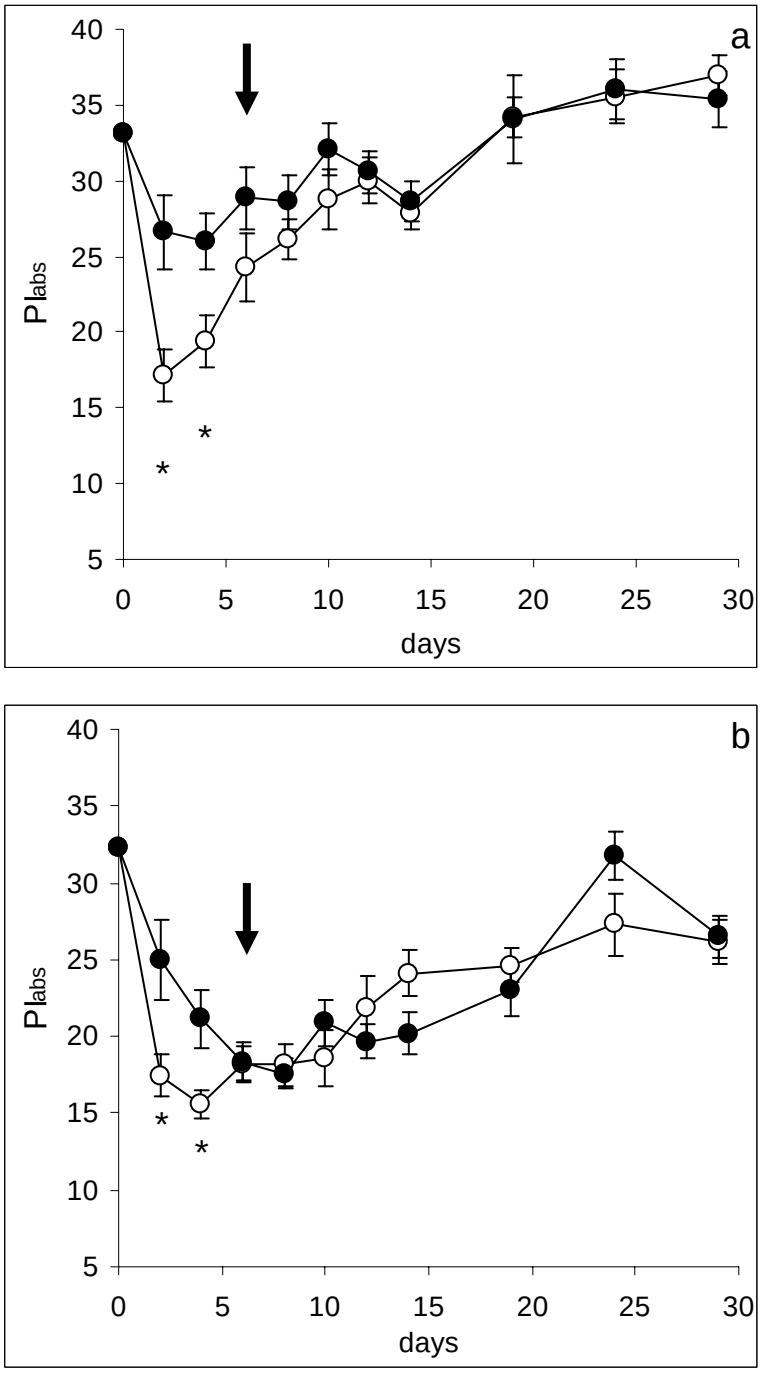

Figure 4. Photosynthetic Performance Index (PI_{abs}) variation during 30 days after inoculation of plants from group 3. At day 0 the plants were inoculated with alive (M; closed circles) or dead (NM; open circles) *P. tinctorius*. The plants were fed with **a)** 1.9 mM or **b)** 3.8 mM NH_4^+. The arrow marks the beginning of mycorrhization. Values are means ± SE. Asterisks indicate significant differences between treatments at $P \leq 0.05$ (Student t-test).

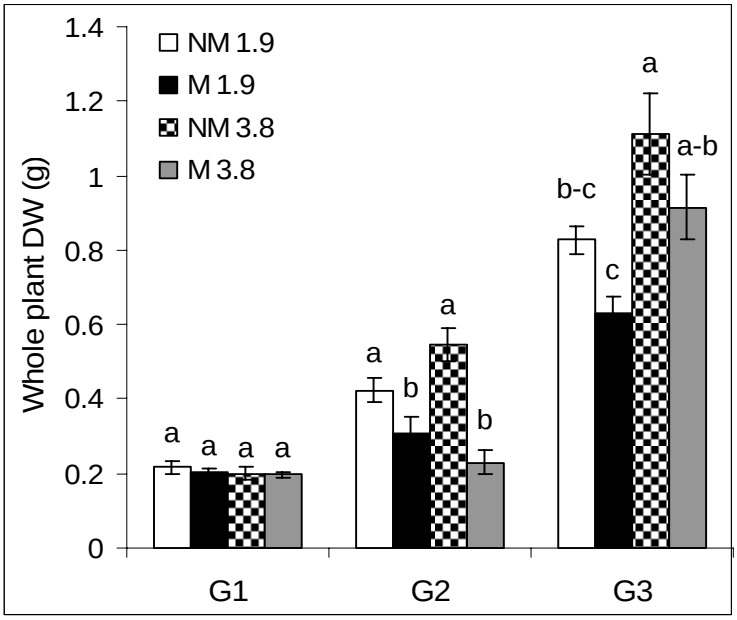

Figure 5. Biomass accumulation in *P. pinaster* plants 29 days after inoculation, in each of the groups of plants studied: group 1 (G1), 2 (G2) and 3 (G3) fed with 1.9 mM (1.9; white and black) or 3.8 mM NH_4^+ (3.8; chequered and grey). The plants were inoculated with dead (NM) or alive (M) *P. tinctorius.* Values are means ± SE. The letters indicate significant differences within groups at $P \leq 0.05$ (one way ANOVA, Tukey).

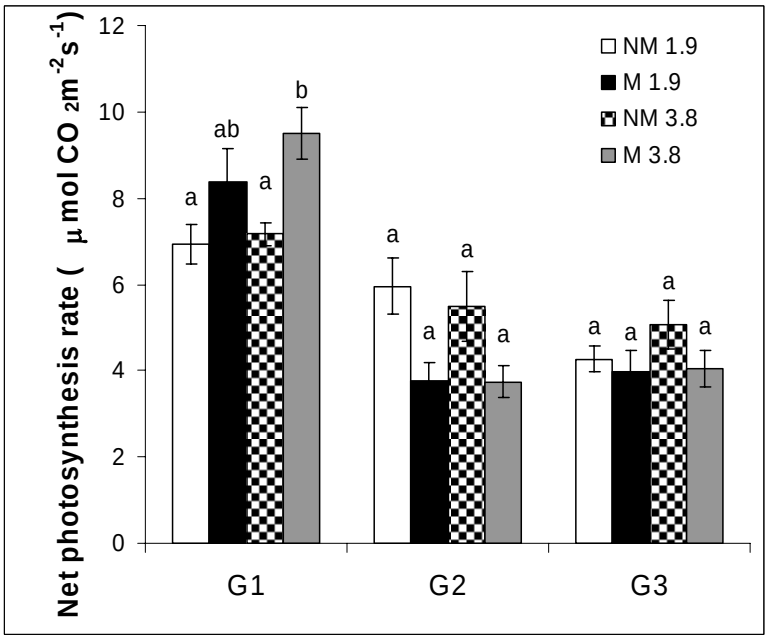

Figure 6. Net photosynthesis rate (µmol CO_2 $m^{-2}s^{-1}$) in *P. pinaster* plants 29 days after inoculation, in each of the groups of plants studied: group 1 (G1), 2 (G2) and 3 (G3) fed with 1.9 mM (1.9; white and black) or 3.8 mM NH_4^+ (3.8; chequered and grey). The plants were inoculated with dead (NM) or alive (M) *P. tinctorius.* Values are means ± SE. The letters indicate significant differences within groups at $P \leq 0.05$ (one way ANOVA, Tukey).

G2 plants presented very low PI_{abs} values at the time of inoculation, indicating that they were severely stressed after a month of starvation (Fig. 3). With 1.9 mM NH_4^+ no significant differences between M and NM plants were observed in this group. With 3.8 mM NH_4^+ a rise of PI_{abs}, indicating a recovery from the starvation stress, was observed in both M and NM plants. This recovery was observed sooner in M plants, which had significantly higher PI_{abs} values during the first 12 days of treatment. After day 14 there were no significant differences between M and NM plants (Fig. 3).

On G3, a pronounced drop in PI_{abs} following inoculation was observed in both M and NM plants (Fig. 4). This drop was clearly less pronounced in M plants, and the difference between M and NM was higher with 3.8 mM NH_4^+. No other differences between M and NM plants were observed throughout the duration of the experiment.

Biomass increment

Mycorrhiza formation never had a positive effect in plant biomass gain (Fig. 5). On G1 there were no significant differences between treatments. On G2, M plants grew less than NM. There were no differences between 1.9 and 3.8 mM NH_4^+ fed plants. On G3 plants fed with 3.8 mM NH_4^+ were bigger than plants fed with 1.9 mM NH_4^+. No differences were found between M and NM plants.

Net photosynthesis rate

On G1, M plants exhibited higher net photosynthesis rate than NM plants after 29 days. However, this difference was only significant for 3.8 mM NH_4^+ fed plants.

M plants of G2 had lower photosynthesis than NM with both N nutritions. However, these differences were not significant. No significant differences were observed in G3 (Fig. 6).

Because the whole shoot was measured, instead of a single isolated needle, there was shading, and the values of the measured CO_2 fixation are, most likely, underestimated. This underestimation is most likely more pronounced in older plants. Although approximately the same amount of plant was measured in plants with similar ages, this makes it hard to compare values between plants of very different ages.

Discussion

In younger plants at the time of inoculation (G1) mycorrhiza had a detrimental effect on the plants photosynthetic performance (PI_{abs}). This was either confined to the very beginning of mycorrhization (3.8 mM NH_4^+) or present throughout the duration of

the experiment (1.9 mM NH_4^+) depending on the amount of N the plants were fed (Fig. 2). A similar detrimental effect of mycorrhiza establishment on the host plants photosynthetic performance has been previously observed in vine plants with AM (Calantzis, 2002). In this previous study, the detrimental effect was transient, and followed by a recovery to a higher level than non-mycorrhizal plants. In our case, a recovery was only observed in plants that received more N, and never to a higher level than non-mycorrhizal plants. ECM formation can therefore have a negative impact on the host plant, similarly to what has been observed in AM interactions. The amount of N provided to the plant, in our case, determined if this negative impact was transient or not, or at least influenced its duration.

In older plants at the time of inoculation (G2 and G3) a negative impact of mycorrhiza on the plants photosynthetic performance was never observed (Fig. 3, 4). This indicates that age is determinant to the impact mycorrhiza will have on the host plant, and on whether this will be a negative one. The smaller total leaf area of younger plants may decrease their capacity to cope with the increased C demand derived from fungus colonization. This has been suggested in previous studies where transient growth depressions in response to AM formation have been observed (Schroeder and Janos, 2004). In addition, faster root colonization was observed in younger plants (data not shown) probably due to their smaller root system, which can be colonized faster and more uniformly by an equal amount of inoculum. This faster colonization may have led to a higher initial impact on the host plant.

The losses in photosynthetic performance (PI_{abs}) observed in younger plants were not reflected in the plants net photosynthesis rate. Younger plants were the only ones that eventually developed a higher photosynthetic activity when mycorrhizal (Fig. 6). This may also be related to their smaller leaf area. Association with ECM fungi has been found to enhance their hosts photosynthetic activity, namely with *P. tinctorius* (Reid *et al.*, 1983; Cairney and Chambers, 1997). No (Cairney and Chambers, 1997; Gavito *et al.*, 2000) or negative (Jifon *et al.*, 2002) effects have also been observed.

In this study, no gain in plant biomass was observed as a result of mycorrhiza formation (Fig. 5). The increased photosynthesis of M plants of G1 was therefore not reflected in a higher biomass production (Fig. 5). It has been reported that increased photosynthetic rates in mycorrhizal plants with *P. tinctorius* can be associated with increases (Reid *et al.*, 1983; Cairney and Chambers, 1997) or decreases (Eltrop and Marschner, 1996b) in host biomass. Higher shoot growth has also been observed with no changes in photosynthesis in AM associations (Gavito *et al.*, 2000).

Mycorrhiza formation led to decreased growth in starved plants (G2) and had no effect in growth of non-starved plants of both ages (G1 and G3) This indicates that the nutritional status of the plant at the time of inoculation was determinant to the way mycorrhization was reflected on plants biomass production. Growth depressions following mycorrhizal inoculation are generally attributed to the carbohydrate drain of the mycorrhizal fungus (Thomson *et al.*, 1994). Clear evidence exists that *P. tinctorius* acts as a strong C sink at least during the early stages of the symbiosis (Cairney and Chambers, 1997). Positive growth effects of mycorrhiza are thought to occur when the benefits of increased nutrient uptake exceed the carbon cost of the association (Schroeder and Janos, 2004). In our study, plants of G2 must not have been able to compensate for this increased C cost, because of their nutritional stress. These plants also had lower photosynthesis, although the differences were not statistically significant, confirming the negative effect of mycorrhiza establishment (Fig. 6).

The age of the host plant also influences the plant growth response, since differences in growth were only observed in older plants. Our study therefore suggests that both age and nutritional status of the host plant are determinant factors in the mycorrhiza effect.

A buffer effect due to the presence of the live fungus was observed in G2 and G3. The pronounced drop in PI_{abs} following inoculation observed in plants of G3 seemed to be due to the mechanical stress of the plants transfer and inoculation process, and independent of mycorrhiza formation (Fig. 4). This was less pronounced in M plants, indicating that the inoculation with living mycelium may help them to cope with this stress (Fig. 4). This effect was present before any mycorrhiza has formed, and seems therefore to be due only to the presence of the fungus. A similar buffer effect was observed in G2 plants with 3.8 mM NH_4^+ (Fig. 3): M plants recover more quickly than NM plants from their starvation, showing an immediate rise in PI_{abs} following the onset of the feeding. Again, this is an effect that is visible prior to any mycorrhiza formation. The presence of live fungus seems therefore to have a stress buffering effect.

In all groups of plants, the higher the N available to the plant, the most positive the impact of mycorrhiza on the host plant photochemical performance / vitality, when compared to NM plants (Fig. 3, 4, 5). N availability seems therefore to be determinant to the impact of ECM formation. This contrasts with what is generally accepted for other inorganic nutrients and AM, since the benefit from AM establishment was found to be diminished as phosphorus availability increased. However, the opposite effect has also been reported (Schroeder and Janos, 2004).

In conclusion, this study shows that plants react differently to ectomycorrhiza formation depending on their age, their initial nutritional status, and the amount of nitrogen supplied. As was reported for AM mycorrhiza, the initial impact of ECM formation can be a negative one. There is a high variation reported in literature for the way productivity parameters, namely growth and photosynthesis, respond to mycorrhiza formation. Our study shows that all factors tested here are important to this response, and determinant to whether mycorrhiza formation will have an initial negative impact on the host plant.

Care should thus be taken when evaluating the effect of mycorrhiza on their host, and when comparing different experiments, since this is clearly an evolving system, influenced not only by external factors, but also by plants developing stage.

The stress buffer effect observed as a consequence of inoculation with live fungus may play an important role in the host plants survival in these young and sensitive stages.

This study also shows the importance of the parameters used for mycorrhiza benefits evaluation. Their selection is determinant to whether mycorrhiza formation will have a negative impact on the host plant or not. Chlorophyll *a* fluorescence measurements were a non destructive, non invasive and reliable tool able to identify the first signals of plant-mycorrhiza fungi interaction.

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CHAPTER 3

Factors influencing the plant's response to ectomycorrhizal establishment

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CHAPTER 3. Factors influencing the plants' response to ectomycorrhizal establishment.

Summary

Plant negative responses to ectomycorrhizae formation may result from excess C drainage or N imobilization by the fungal partner. In this work we evaluated which of these effects was the most responsible for the negative responses in growth and photosynthetic efficiency previously observed in *Pinus pinaster* upon mycorrhization with *Pisolithus tinctorius*.

Groups of *P. pinaster* plants with different ages and nutritional status were inoculated with alive or dead *P. tinctorius*. The plants were fed either with 1.9 mM or 3.8 mM ammonium as N source. Ectomycorrhizal establishment was monitored through ergosterol measurements. The plants were collected 29 days after inoculation. Dry weights, N and C concentrations, and several plant growth and productivity parameters were determined.

Mycorrhizal plants had lower N uptake (RN). This resulted in lower nitrogen productivity (NP) and growth (RGR) in plants that were older (4 weeks) at the time of inoculation, but not in younger plants. Mycorrhizal plants that were older at the time of inoculation had lower NP than non-mycorrhizal plants with similar RN.

The negative effects over the host plants' growth and vitality were found to be mainly a consequence of decreased N uptake, and therefore of N immobilization by the fungus. The extent to which the plants were N limited was found to be most important in the way the plant reacted to mycorrhization. Evidences of a negative effect resulting from a limitation to growth other than N were only observed in older plants, and were dependant on the N limitation. The effect of mycorrhization was more pronounced in plants receiving less N and/or with lower N status, which was likely due to the higher degrees of colonization in those conditions.

Key words: ammonium, ectomycorrhizae, nitrogen, *Pinus pinaster*, cost, benefit.

Introduction

Mycorrhizal symbioses are traditionally considered to be mutualisms, and therefore beneficial to the host plant. Namely, ectomycorrhizae have generally been found to enhance plant growth (e.g., Burgess et al., 1994; Lu et al., 1998). However, the increasing number of reported cases of decreased plant productivity in ectomycorrhizal plants (Colpaert et al., 1992; Eltrop and Marschner, 1996a; Dosskey et al., 1990; Colpaert et al., 1996; Colpaert et al., 1999; Conjeaud et al., 1996) have led to the growing belief that the effects of mycorrhization on the host plant may vary from the traditionally accepted mutualistic through to antagonistic in a continuum of responses (Jonhson et al., 1997; Jones and Smith, 2004; Gange and Ayres, 1999; Mazancourt et al., 2005). Our understanding of the reasons behind this variation in the plants response to mycorrhization is still very limited, but it is essential if we are to understand the nature and dynamics of this interaction.

The outcome of a mycorrhizal relationship has been presumed to depend on the balance between the fungal demand for energy and the plant needs for nutrients (Jonhson et al., 1997; Tuomi et al., 2001; Neuhauser and Fargione, 2004; Schwartz and Hoeksema, 1998), and can therefore be expected to vary with any environmental or developmental factors that affect the C cost and N gain deriving from the symbiosis. Namely, the variation of mycorrhizal effects on plant productivity has been considered to depend on the degree of mycorrhization (Gange and Ayres, 1999; Douds et al., 1988; Tuomi et al., 2001), and on the nutrient availability (Janos, 2007; Tuomi et al., 2001; Neuhauser and Fargione, 2004; Schwartz and Hoeksema, 1998). Reduced seedling growth in response to ectomycorrhizae has been considered to result from increased belowground carbon allocation (Colpaert *et al.*, 1996; Conjeaud *et al.*, 1996) or as a consequence of high nutrient retention by the mycobiont (Colpaert *et al.*, 1992, 1996).

In a previous report (Corrêa et al., 2006) we presented the results of an experiment designed to evaluate the effects of mycorrhizal establishment on growth, photosynthesis and photosynthetic performance. Mycorrhization was found to have a negative effect on the host plant's growth and photosynthetic performance but this effect was dependent on the amount of N available to the plant, and on the nutritional status and age of the plant at the time of inoculation. In this work we tested whether the observed detrimental effects resulted from increased C drainage or from N retention by the fungal partner.

Material and methods

Plant and fungal material

The *P. tinctorius* (Pers.) Coker & Couch isolate PtA from the collection of the University of Lisbon, Plant Biology Department, was grown in pure culture in liquid modified Melin-Norkans (MMN) medium (Marx, 1969). For inoculation, *P. tinctorius* mycelium was grown for 2 months in the dark at 24 ºC on a perlite/vermiculite (v/v) mixture moistened with liquid MMN medium.

P. tinctorius was also grown in Petri dishes, in the dark at 24ºC, with solid modified MMN medium, supplemented with $5gL^{-1}$ glucose, covered with cellophane. The mycelium was collected 3 weeks after inoculation, separated from the cellophane, weighed and stored at -70ºC until freeze-drying. Freeze-dried mycelium was then homogenized with a ball mill, using liquid nitrogen, kept at -70ºC until redrying, and then stored under vacuum at -25ºC.

Pinus pinaster L. seeds were collected in Sines and Santarém, Portugal, and provided by the National Centre of Forest Seeds (CENASEF) of the Portuguese Ministry of Agriculture. They were surface-sterilized with 30 % calcium hypochlorite for 30 minutes, rinsed in several changes of distilled water, and soaked in distilled water at 4 ºC for 48 h. Sowing was carried out in gnotobiotic conditions on a sand/vermiculite (v/v) mixture sterilized at 120 ºC for 1 h. Seedlings were watered with distilled water as needed.

When the second set of leaves appeared, approximately one month after sowing, the seedlings were transferred to 350 mL root trainers (20cm Fleet Roottrainers, Ronaash, Ltd., Roxburghshire, U.K.), with a perlite/vermiculite (v/v) mixture as substrate, sterilized at 120 ºC for 1 h.

Experimental design

At the time of transfer to the root trainers the plants were divided into three groups. Group 1 (G1) was inoculated at the time of transfer from the sowing beds into the root trainers (Fig. 1). Half of the seedlings were inoculated with alive (mycorrhizal - M) and half with dead (non-mycorrhizal control - NM) *P. tinctorius* mycelium. For the inoculation, 100 mL inoculum, previously washed with distilled water, was placed in

contact with the roots. For the dead mycelium, the inoculum was sterilized for 1 hour at 120 ºC.

Group 2 (G2) was not inoculated at the time of transfer. The plants were watered twice a week with 25 mL distilled water, for one month after the transfer to the root trainers. They were then divided into two sub-groups, and inoculated with alive or dead *P. tinctorius* mycelium, as described for G1.

In both G1 and G2, from the moment of inoculation the plants started being fed with MMN medium, from which thiamine was omitted. Half of the plants in each sub-group, M or NM, received 3.8 mM NH_4^+, and the other half 1.9 mM NH_4^+ as N source. Each plant received 25 mL of medium twice a week.

MMN medium has an ammonium concentration of 3.8 mM. In order to obtain a medium with half this concentration (1.9 mM), it was modified, containing 0.95 mM $(NH_4)_2HPO_4$ and 4.6 mM KH_2PO_4.

Group 3 (G3) was also not inoculated at the time of transfer. The plants were divided into 2 sub-groups and started being fed with either 3.8 mM NH_4^+ or 1.9 mM NH_4^+ as N source, as already described. After 1 month each of the 2 sub-groups were again divided and inoculated with either alive or dead inoculum. They continued being fed as before.

The whole experiment was performed in a growth chamber under a 16 h light / 8 h dark photoperiod at 24 / 18 ºC, approximate 70 % relative humidity and 250 μmol m^{-2} s^{-1} at plant height.

Between 8 and 12 plants were harvested prior to inoculation (day 0), and 8, 14 and 29 days following inoculation. Shoots and roots were separated, the roots were washed in running water as quickly as possible, and excess water was removed. Shoots and roots were frozen immediately at -70ºC. The samples were freeze-dried for 72 h, and stored at -20ºC, in vacuum. They were then homogenized in liquid N_2 using a ball mill, and freeze dried again for 24 h. All freeze dried samples were kept under vacuum at -20ºC. The dry weights were recorded after freeze drying.

Extraction and determination of ergosterol

The metabolically active fungal biomass in mycorrhizal roots (mantel + Hartig net) was determined from ergosterol, and used as a measure of mycorrhizal colonization. Free ergosterol of approximately 5mg freeze-dried sample was extracted in 1ml ice cooled absolute ethanol, for 15 minutes, with occasional vortexing. After centrifugation

(10 min, 14000*g*), the supernatant was used for determination of ergosterol, without further purification.

Twenty five µl of the supernatant were separated by HPLC system on a Spherisorb S5 ODS2 (4.6 x 250 mm) column (Phase Separations), using pure methanol as eluant. Ergosterol was detected at 280 nm with a UV detector (UV 2000, Spectra Physics).

The ergosterol content of *P. tinctorius* grown in pure culture in Petri dishes was used to convert the ergosterol in roots to fungal biomass.

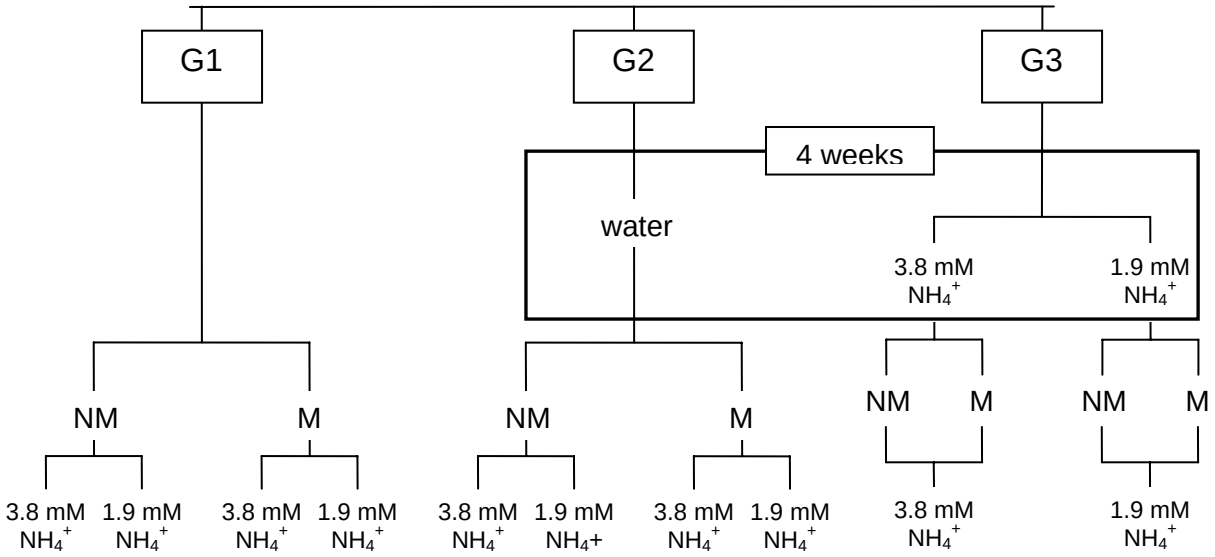

Figure 1. Scheme of the experimental design.

Determination of C and N concentrations

C and N concentrations were determined in freeze-dried shoots and roots with an elemental analyser Euro EA 3000 (EuroVector CHNS-O Elemental Analyser; Callidus Software Interface-Version 4.1). The quantification was made using the method of external pattern, through linear calibration. The pattern used was the reference material Wheat Flour (OAS) calibrated for NIST patterns. The separation was made using a gaseous chromatography column, and the detector was a thermal conductivity detector (TCD). The integration of the chromatographic peaks was made using the Callidus software, version 4.1 (EuroVector).

Parameter	Equation	Units	References
Relative growth rate **RGR**	**(1 / W) × (dW / dt)** = $(\ln W_2 - \ln W_1) / (t_2 - t_1)$	Weight weight^{-1} time^{-1}	Hunt, 1982; Freijsen and Veen, 1990; van den Driessche and van den Driessche, 1991
Nitrogen relative uptake rate **RN**	**(1 / N) × (dN / dt)** = $(\ln N_2 - \ln N_1) / (t_2 - t_1)$	Weight weight^{-1} time^{-1} or, mole mole^{-1} time^{-1}	Hunt, 1982; Freijsen and Veen, 1990; van den Driessche and van den Driessche, 1991
Nitrogen productivity **NP**	**(1 / N) × (dW / dt)** = $[(W_2 - W_1) \times (\ln N_2 - \ln N_1)] / [(N_2 - N_1) \times (t_2 - t_1)]$	Weight weight^{-1} time^{-1}	Freijsen and Veen, 1990; van den Driessche and van den Driessche, 1991; Ingestad, 1979; Lambers *et al*., 1998

Table 1. Equations for the calculation of the plant growth analysis parameters used. W: plant weight; N: plant N content; t: time; R: root weight or area.

Plant Growth and Productivity Parameters

The relative growth rate (RGR), N relative uptake rate (RN) and the N productivity (NP) were calculated (Table 1). The pairing method (Hunt, 1978) was used in the calculations of RGR and RN. RGR and NP were calculated on a dry weight basis.

Data analysis

The effects of the two variables tested, mycorrhization and N supply, were analysed using a two-way ANOVA test. The Tukey test was used to determine significant differences between means, with $P \leq 0.05$.

Exponential equations were fitted to the correlations between RN and RGR, and RN and NP. The validity of assumptions was checked, and the goodness of fit of the model was evaluated using a one-way ANOVA.

In all cases preliminary analyses were performed to ensure no violation of the assumptions regarding each test. SPSS software, version 15.0, was used for all tests.

Results

Mycorrhizal colonization

Mycorrhizal colonization was strongly influenced by the N status and N supply of the host plants. In all 3 groups of plants, mycorrhizal degree was higher in plants receiving less N, and the plants in G2 (starved) were more strongly mycorrhizal than in the other 2 groups, with both N supplies (Fig. 2).

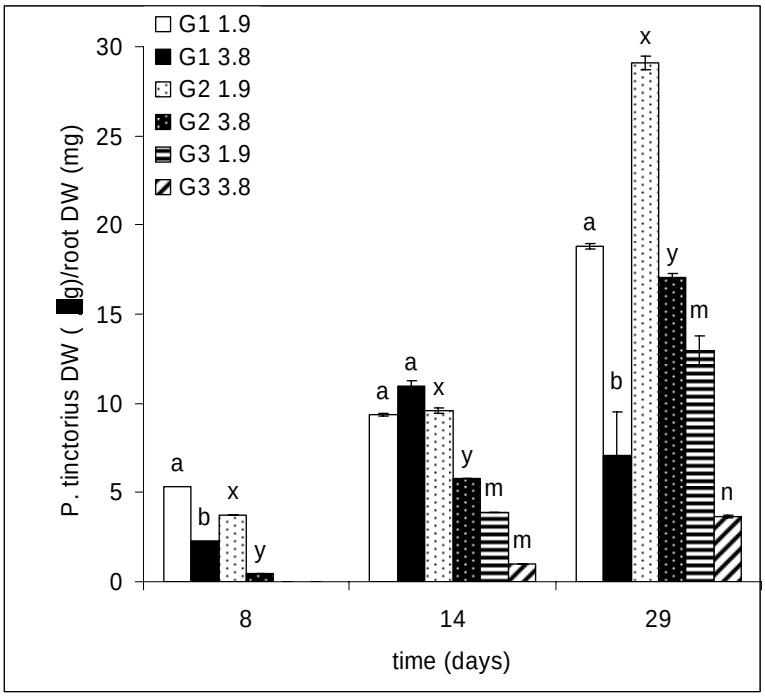

Figure 2. Concentrations of *P. tinctorius* biomass in mycorrhizal whole roots in G1, G2 and G3. Fungal biomass was estimated using the mean value of the ergosterol content of *P. tinctorius* mycelia grown in pure culture as a conversion factor (6.74 μg mg^{-1} DW). The plants were fed a nutrient solution containing 1.9 mM NH_4^+ (1.9) or 3.8 mM NH_4^+ (3.8) as N source. Values are averages ± S.E. (n=4). A one-way ANOVA followed by a Tukey test was used to test for significant differences between means. Different letters represent significant differences between plants receiving different N supplies within the same group at $p \leq 0.05$.

In G3, mycorrhization began later than in the other two groups, and was the lowest of all three groups by the end of the experiment (Fig. 2).

Plant Growth and Productivity Parameters

Due to the low growth rates observed during this work all parameters were calculated for the whole experimental period, since the significance of the small differences found in values calculated for periods of one or two weeks was dubious, and the results were unclear.

Mycorrhization resulted in lower RN in all groups, although this effect was only significant in G1 and G2 (Table 2).

	N supply (mM NH_4^+)	Mycorrhizal status	G1	G2	G3
RN	1.9	NM	1.87 ± 0.13^{a}	0.01 ± 0.00^{a}	1.20 ± 0.09^{ab}
		M	1.29 ± 0.10^{b}	-0.08 ± 0.01^{a}	0.75 ± 0.11^{b}
	3.8	NM	1.91 ± 0.11^{a}	-0.55 ± 0.16^{a}	1.55 ± 0.09^{a}
		M	1.55 ± 0.04^{ab}	-1.13 ± 0.19^{b}	1.53 ± 0.17^{ab}
	ANOVA effect	M	0.004	0.026	0.052
		N	0.163	< 0.001	< 0.001
		M x N	0.298	0.084	0.078
NP	1.9	NM	9.70 ± 0.18^{a}	11.32 ± 0.14^{a}	12.50 ± 0.09^{a}
		M	9.07 ± 0.16^{ab}	3.64 ± 0.05^{c}	6.82 ± 0.12^{c}
	3.8	NM	8.12 ± 0.15^{c}	5.80 ± 0.13^{b}	14.05 ± 0.92^{a}
		M	8.65 ± 0.07^{bc}	2.16 ± 0.03^{d}	9.86 ± 0.25^{b}
	ANOVA effect	M	0.7	< 0.001	< 0.001
		N	0.001	< 0.001	0.004
		M x N	0.006	< 0.001	0.294

Table 2. Relative N uptake (RN) and N productivity (NP) of mycorrhizal (M) and non-mycorrhizal (NM) plants. The plants received 3.8 or 1.9 mM NH_4^+ as N source. Values are averages ± SE. A two-way ANOVA was used to test the main effects and interactions within each group, and was followed by a Tukey test to look for significant differences between treatments. The letters represent significant differences at $p \leq 0.05$.

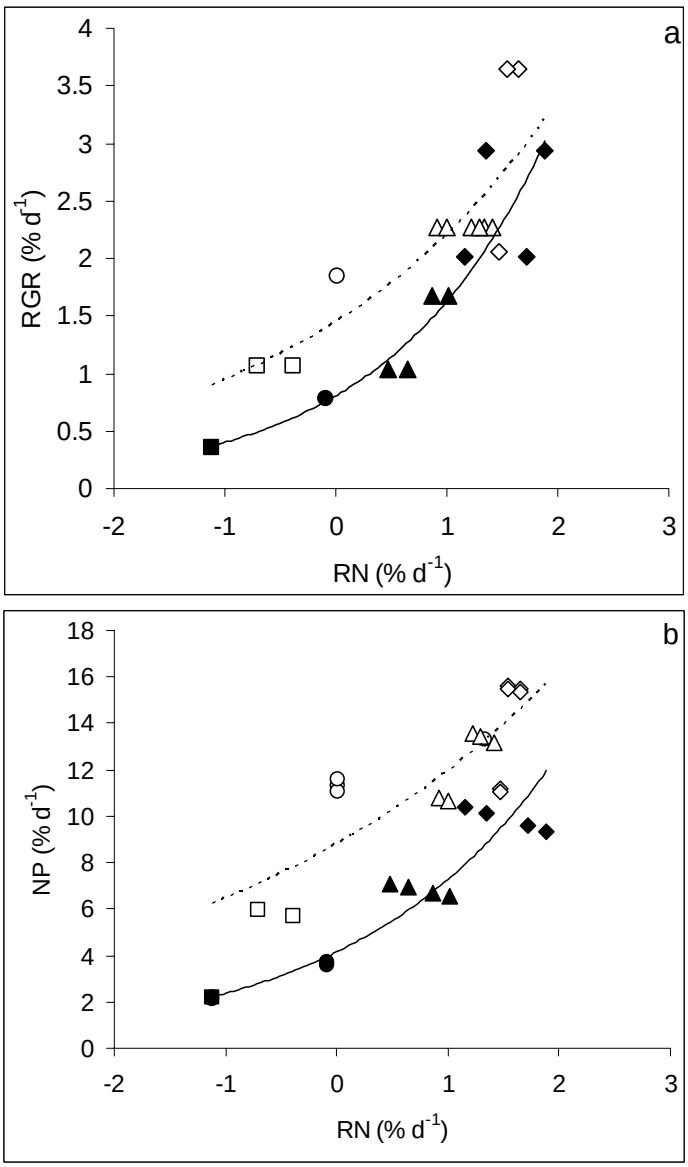

Figure 3. Correlation between relative nitrogen uptake rate (RN) and a) relative growth rate (RGR) and b) nitrogen productivity (NP) of G2 and G3 plants. The correlations between RN and RGR (NM plants: interrupted line, $y=1.45e^{0.42x}$, $r^2=0.80$, n=22, $p < 0.001$; M plants: solid line, $y=0.81e^{0.70x}$; $r^2=0.93$, n= 18, $p < 0.001$) and between RN and NP (NM plants: interrupted line, $y=8.00e^{0.37x}$, $r^2=0.81$, n= 22, $p < 0.001$; M plants: solid line, $y=4.40e^{0.52x}$, $r^2=0.88$, n= 18, $p < 0.001$) were best fitted by exponential equations. G2 with 1.9mM NH_4^+: squares; G2 with 3.8mM NH_4^+: circles; G3 with 1.9mM NH_4^+: triangles; G3 with 3.8mM NH_4^+: lozenges. M plants: closed symbols; NM plants: open symbols. The Pearson product moment correlation coefficient was used to evaluate the correlations between RN and RGR.

	N supply (mM NH_4^+)	Mycorrhizal status	G1	G2	G3
Whole plant RGR	1.9	NM	2.21 ± 0.29 [a]	1.85 ± 0.36 [a]	2.32 ± 0.16 [a]
		M	2.02 ± 0.13 [a]	0.78 ± 0.21 [bc]	1.29 ± 0.30 [b]
	3.8	NM	1.96 ± 0.23 [a]	1.06 ± 0.16 [ab]	3.11 ± 0.38 [a]
		M	1.96 ± 0.19 [a]	0.36 ± 0.14 [c]	2.47 ± 0.21 [a]
	ANOVA effect	M	0.661	0.000	0.005
		N	0.495	0.006	0.001
		M x N	0.666	0.372	0.488
Shoot RGR	1.9	NM	1.69 ± 0.32 [a]	1.29 ± 0.40 [a]	1.79 ± 0.23 [b]
		M	1.55 ± 0.26 [a]	0.52 ± 0.22 [ab]	0.91 ± 0.22 [b]
	3.8	NM	1.58 ± 0.34 [a]	0.85 ± 0.04 [ab]	2.96 ± 0.28 [a]
		M	1.23 ± 0.37 [a]	0.31 ± 0.13 [b]	2.40 ± 0.23 [a]
	ANOVA effect	M	0.153	0.002	0.017
		N	0.189	0.107	0.000
		M x N	0.338	0.568	0.341
Root RGR	1.9	NM	3.08 ± 0.53 [a]	2.60 ± 0.37 [a]	2.95 ± 0.28 [b]
		M	2.72 ± 0.54 [a]	1.40 ± 0.36 [ab]	1.84 ± 0.26 [c]
	3.8	NM	2.59 ± 0.45 [a]	1.39 ± 0.35 [ab]	4.23 ± 0.33 [a]
		M	3.44 ± 0.42 [a]	0.72 ± 0.22 [b]	2.99 ± 0.28 [b]
	ANOVA effect	M	0.632	0.008	0.007
		N	0.819	0.007	0.022
		M x N	0.234	0.425	0.850

Table 3. Relative growth rate of shoot, root and whole plant of mycorrhizal (M) and non-mycorrhizal (NM) plants. The plants received 3.8 or 1.9 mM NH_4^+ as N source. Values are averages ± SE. A two-way ANOVA was used to test the main effects and interactions within each group, and was followed by a Tukey test to look for significant differences between treatments. The letters represent significant differences at $p \leq 0.05$.

		N supply (mM NH_4^+)	Mycorrhizal status	G1		G2		G3	
				Day 0	Day 29	Day 0	Day 29	Day 0	Day 29
Shoot	%N	1.9	NM	2.32 ± 0.11	1.69 ± 0.03^{a}	1.61 ± 0.00	0.80 ± 0.02^{b}	1.92 ± 0.04 ^{b}	1.28 ± 0.03^{a}
			M		1.43 ± 0.02^{a}		1.19 ± 0.03^{a}		1.55 ± 0.14^{a}
		3.8	NM		1.77 ± 0.03^{a}		1.18 ± 0.08^{a}	2.23 ± 0.04 ^{a}	1.50 ± 0.02^{a}
			M		1.73 ± 0.03^{a}		1.22 ± 0.03^{a}		1.66 ± 0.20^{a}
		ANOVA effect	M		< 0.001		< 0.001		0.149
			N		< 0.001		< 0.001		0.253
			M x N		0.001		< 0.001		0.672
	C/N	1.9	NM	19.40 ± 0.81	27.06 ± 0.48^{a}	27.31 ± 0.09	56.53 ±1.49^{a}	23.27 ± 0.61^{a}	35.32 ± 0.41^{a}
			M		31.98±0.57^{a}		38.27 ± 1.01^{b}		29.76 ± 3.18^{a}
		3.8	NM		25.65 ± 0.46^{a}		39.51 ± 1.92^{b}	20.64 ± 0.54^{a}	30.68 ± 0.17^{a}
			M		26.60± 0.47^{a}		37.95 ± 1.00^{b}		28.12 ± 3.58^{a}
		ANOVA effect	M		< 0.001		< 0.001		0.166
			N		< 0.001		< 0.001		0.262
			M x N		0.001		< 0.001		0.566
Root	%N	1.9	NM	0.90 ± 0.20	1.40 ± 0.00^{a}	1.12 ± 0.09	0.95 ± 0.02^{b}	1.35 ± 0.07 ^{a}	1.09 ± 0.03^{ab}
			M		1.42 ± 0.00^{a}		1.14 ± 0.02^{a}		1.20 ± 0.06^{ab}
		3.8	NM		1.61 ± 0.00^{a}		0.66 ± 0.01^{c}	1.52 ± 0.07 ^{a}	1.01 ± 0.08^{b}
			M		1.52 ± 0.09^{a}		1.13 ± 0.02^{a}		1.35 ± 0.10^{a}
		ANOVA effect	M		0.183		< 0.001		0.016
			N		0.002		< 0.001		0.620
			M x N		0.065		< 0.001		0.150
	C/N	1.9	NM	28.10 ± 4.60	30.42 ± 0.46^{a}	34.86 ± 2.37	48.97 ± 1.24^{b}	29.76 ±2.16 ^{a}	36.08±0.94^{ab}
			M		30.05 ± 0.46^{a}		37.77 ± 0.96^{c}		32.00±1.59^{ab}
		3.8	NM		26.04 ± 0.40^{b}		68.23 ± 1.73^{a}	26.01 ±0.67 ^{a}	38.30 ± 1.54^{a}
			M		26.08 ± 0.78^{b}		38.03 ± 0.96^{c}		28.26 ± 3.18^{b}
		ANOVA effect	M		0.213		< 0.001		0.007
			N		0.001		< 0.001		0.680
			M x N		0.075		< 0.001		0.142
Whole plant	%N	1.9	NM	1.67± 0.01	1.56 ± 0.05^{ab}	1.40 ± 0.04	0.87 ± 0.01^{b}	1.68 ± 0.03^{b}	1.18 ± 0.01^{c}
			M		1.42 ± 0.04 ^{b}		1.17 ± 0.01^{a}		1.38 ± 0.05^{ab}
		3.8	NM		1.70 ± 0.06 ^{a}		0.94 ± 0.04^{b}	1.97 ± 0.03^{a}	1.28 ± 0.04^{bc}
			M		1.60 ± 0.04^{ab}		1.18 ± 0.01^{a}		1.53 ± 0.07^{a}
		ANOVA effect	M		0.060		< 0.001		< 0.001
			N		0.028		0.020		0.014
			M x N		0.798		0.069		0.508
	C/N	1.9	NM	22.08 ± 0.05	28.22 ± 0.97^{a}	29.86 ± 0.61	52.43 ± 0.90^{a}	25.43 ± 0.65^{a}	35.66± 0.29^{a}
			M		30.96 ± 1.02^{a}		38.05 ± 0.65^{b}		30.62 ±1.15^{bc}
		3.8	NM		25.66 ± 0.90^{a}		48.79 ± 1.72^{a}	22.17 ± 0.15^{a}	33.35±0.48^{ab}
			M		27.20 ± 0.43^{a}		38.01 ± 0.65^{b}		28.02 ± 1.49^{c}
		ANOVA effect	M		0.074		< 0.001		< 0.001
			N		0.024		0.064		0.022
			M x N		0.540		0.069		0.882

Table 4. N and C concentrations and C/N ratios of shoots, roots and whole mycorrhizal (M) and non-mycorrhizal (NM) plants. The plants received 3.8 or 1.9 mM NH_4^+ as N source. Values are averages ± SE. A two-way ANOVA was used to test the main effects and interactions within each group, and was followed by a Tukey test to look for significant differences between treatments. The letters stand for the significant differences at $p \leq 0.05$.

The NP (Table 2) and RGR (Table 3) were not affected by mycorrhization in plants of G1. In contrast, mycorrhization strongly affected these two parameters in G2 and G3, where they were lower for M plants, regardless of the N supply (Table 2 and 3).

N supply had no effect on RN on G1, but it had the strongest effect on G2 and the only statistically significant effect on G3 (Table 2). The N supply also had no statistically significant effects on the RGR of plants in G1 (Table 3), while in G2 and G3 significantly higher RGR was observed with higher N supply.

G1 plants had, in general, higher RN than in G2 and G3 (Table 2).

A significant correlation between RN and RGR (Fig. 3a) and NP (Fig. 3b) was found in G2 and G3, but not in G1. The correlations found were different for NM and M plants. M plants had lower NP and RGR than NM plants with similar RN values (Fig. 3), and this difference was more pronounced at lower RN.

C and N concentrations

In G1, although there were no significant differences in the shoot N concentration, the effect of mycorrhization was significant, and M plants had lower shoot N concentrations (Table 4). These lower N concentrations resulted in higher C/N ratios in the shoot of these M plants. At the root level, mycorrhization had no effect on either N concentrations or C/N ratios (Table 4).

In both G2 and G3, M plants had higher N concentrations in both shoot and root, and this effect of mycorrhization was always statistically significant except for the shoots of G3 plants (Table 4). As a result, the C/N ratios of these M plants were lower than in NM plants.

Plants receiving more N had, in general, higher N concentrations than those receiving less N.

Discussion

In this study, mycorrhization lowered the N uptake (RN) in all cases, although this effect was only significant in G1 and G2 plants (Table 2). In these two groups mycorrhization was faster and the levels of mycorrhizal colonization reached were higher than in G3 (Fig. 2), indicating that the effect of mycorrhization on N uptake depended on the degree of mycorrhization. This is in accordance with previous findings that periods of increased mycelial growth were associated with increased N retention by the fungal partner (Colpaert et al., 1992).

In younger plants at the time of inoculation (G1) the decreased N uptake was the most important effect of mycorrhization, but it did not lead to decreased RGR or to changes in NP, i.e., in the amount of biomass gained per N taken (Table 2, 3). As the N availability, and N uptake, decrease so does the percentage of N taken that is available for growth, and therefore NP (Lambers et al., 1998; Ingestad, 1979; Freijsen and Veen, 1990). Although the decrease in N uptake was not great enough to result in lower RGR and NP, it did, however, result in significantly lower shoot N concentrations in M plants, contrary to what was observed in G2 and G3 (Table 4). These G1 M plants had been previously found to have lower photosynthetic efficiency, which was mostly observed in plants receiving less N (Corrêa et al., 2006), indicating that mycorrhization represented a stress in these plants leading to decreased plant vitality (Strasser et al., 2004). This was considered to be a consequence of their smaller leaf area, and lesser ability to respond to the increased C drainage by the fungus (Corrêa et al., 2006). The present results, however, indicate that it was most likely a result of their decreased shoot N concentrations, and therefore a consequence of the N immobilization by the fungus, rather than of increased C drainage.

In contrast to G1, mycorrhization did have a significant effect on the host plants' NP and RGR in both G2 and G3, where these two parameters were lower in M plants (Table 2), indicating that the decreased N uptake due to mycorrhization resulted in a N limitation to growth. This was also indicated by the higher N concentrations observed in these M plants, which were more (G2) or only (G3) significant at the root level, indicating N retention at the roots, which is characteristic of stress caused by N limitation (Ingestad and Ågren, 1988). ECM fungi have a high potential to take up, accumulate (Wallenda and Kottke, 1998) and immobilize N (Colpaert *et al.*, 1992, 1996).

However, growth was not exclusively limited by N in these M plants, since they had lower NP values than NM plants with similar RN (Fig. 3) indicating a limitation to growth other than N (Ingestad *et al.*, 1986). The higher shoot N concentrations observed in these M plants were also indicative of such a limitation, leading to N accumulation (Table 2). This could be due to the immobilization by the fungus of a nutrient other than N (e.g., phosphorus), or to a C limitation. Mycorrhizal establishment has been generally found to increase the demand of carbohydrates for fungal maintenance and growth (Dosskey, et al., 1990; Hampp et al., 1999; Reid et al., 1983; Lambers et al., 1998;

Smith and Read, 1997), and clear evidence exists that *P. tinctorius* acts as a strong C sink at least during the early stages of the symbiosis (Cairney and Chambers, 1997).

Because G2 and G3 plants were older at the time of inoculation than G1, and therefore bigger, and starved in the case of G2, their N requirements were higher than those of younger plants (G1). This was confirmed by the significant correlation between RN and RGR or NP found in these plants, independently of the mycorrhizal status, indicating N limitation to growth, contrary to younger plants (Fig. 3). Their growth was therefore more likely to be affected by the fungal N immobilization. In fact, the RN of plants in G2 and G3 were lower than in G1 (Table 2), and therefore their N needs were being less satisfied. Furthermore, contrary to the younger plants of G1, these plants no longer had cotyledons, or they were mostly senesced, at the time of inoculation, making them more dependent on the N derived from the substrate and supplied by the fungus. The effects of the N supply on the parameters measured also indicated this to be the case, since G2 and G3 plants were strongly influenced by it contrary to G1 (Table 2, 3, 4).

The N immobilization by the fungus may also explain the growth limitation due to mycorrhization, observed only in older plants at the time of inoculation, that was not N related. Such an immobilization will decrease the N available to the plant, and therefore increase the C cost per unit N, since lower N availability will imply a bigger investment in root and fungal absorbing structures, which could explain a C limitation to growth. This is supported by the fact that the difference between M and NM plants was more pronounced at lower RN (Fig. 3). Both the extension of the mycorrhizal colonization and the development of extraradical mycelium have been found to be negatively correlated with the N availability and with the N concentrations at the host plant (Wallander and Nylund, 1991; Nilsson and Wallander, 2003; Wallenda and Kottke, 1998). These results are, however, in contrast with the proposal that mycorrhizal plants have superior cost efficiency, i.e., amount of nutrients acquired per C expended, in limiting nutrient conditions (Tuomi et al., 2001). Although in G3 the mycorrhizal degree was lower than in the other two groups, greater development of extra-matricial mycelium may still have taken place. A bigger investment in fungal growth could also explain a limitation due to a nutrient other than N.

The differences in response between older and younger plants were therefore a consequence of the first being N limited, contrary to the later. The limitation to the older plants' growth resulted from their bigger size, requiring higher availability of resources

to grow and making them more sensitive to resource limitation due to the presence of the fungus. It did not, however, result in a stress to the plant, contrary to G1, as their photosynthetic performance was not affected by mycorrhization (Corrêa et al., 2006). Seedlings can adjust growth on the basis of their internal N concentrations over quite a wide range and only show visible symptoms of deprivation when internal concentrations are changing or are extremely low (van den Driessche and van den Driessche, 1991), and therefore growth limitations do not necessarily imply a decrease in plant vitality (Ingestad & Lund, 1979).

Although the N status of the host plant and the amount of N supplied were less important than their age in the way the plant responded to mycorrhization, they were not devoid of influence. Overall, the effect of mycorrhization in all groups was stronger when the plants were fed with less N, and in G2 where the plants' nutritional status was lower at the time of inoculation (Table 2, 3, 4). This was, however, less likely a direct consequence of the N nutrition itself, and more of the higher degrees of colonization found in those conditions (Fig. 2). The effects of mycorrhization on plant productivity have been considered to depend on the degree of mycorrhization (Gange and Ayres, 1999; Douds et al., 1988; Tuomi et al., 2001), as the C demand and nutrient retention by the fungal partner increase with it.

In summary, the previously reported negative effects over the host plants' growth, in older plants, and vitality, in younger plants (Corrêa et al., 2006), were found to be mainly a consequence of decreased N uptake, and therefore of N retention by the fungus. Older plants also evidenced an additional limitation to growth, likely due to increased belowground C allocation, but this may also have been a consequence of the decreased N availability due to the fungal presence. The extent to which the plants were N limited was found to be more important in the way the plant reacted to mycorrhization than the N status.

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CHAPTER 4

Plants' response to ectomycorrhizae in N limited conditions: which factors determine its variation?

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CHAPTER 4. Plants' response to ectomycorrhizae in N limited conditions: which factors determine its variation?

Summary

Mycorrhizal interactions can have a wide range of effects on plant productivity. Which factors determine the type of response is still uncertain.

The effects of mycorrhization over a wide range of combinations between N availability (RAR_N), N concentration in plant tissues and degree of mycorrhizal colonization, were studied in *Pinus pinaster* L. mycorrhizal with *Pisolithus tinctorius*. Several plant productivity parameters, the seedlings' N status, chl *a* fluorescence (JIP test) and mycorrhizal colonization were measured.

N was limiting in all the conditions tested. The mycorrhizal effects on plants' growth and on N uptake were very strongly and positively correlated, and ranged from negative to positive. A new index, the mycorrhizal N demand/supply ratio (MDS_N), is suggested, and was found to efficiently explain this variation. The mycorrhizal effect on the host plants' growth was not related to a negative effect on the host plants' photosynthetic performance (PI_{abs}), and hence on its fitness.

Mycorrhizal effect on N uptake was concluded to determine the host plants' growth in N limited conditions, and to vary according to MDS_N. Differences in growth reflected changes in resource allocation between host plant and mycorrhizal fungus, and not in plant vitality. The results are interpreted in the light of several models and hypothesis.

Key words: ammonium, chlorophyll *a* fluorescence, colonization density, cost/benefit, demand/supply, ectomycorrhizae, mycorrhizal response, nitrogen, O-J-I-P test.

Introduction

The increasing number of reported negative and null responses of plant productivity to mycorrhization (e.g.: Dosskey *et al.* 1990, Colpaert *et al.* 1992, 1996, Conjeaud *et al.* 1996, Eltrop and Marschner 1996) has led to the growing belief that it's effects on the host plant can vary from the traditionally accepted mutualistic through to antagonistic in a continuum of responses (Jonhson *et al.* 1997, Jones and Smith 2004,

Mazancourt *et al.* 2005). Furthermore, it is believed that in any particular host-plant – fungus combination the response to mycorrhization can move along this continuum.

The outcome of a mycorrhizal relationship has been presumed to depend on the balance between the fungal demand for energy and the plant's needs for nutrients, and negative effects of mycorrhizal colonization are expected to occur when the net C costs for fungal maintenance and growth exceed the net benefits obtained from improved nutrient uptake (Jonhson *et al.* 1997, Tuomi *et al.* 2001). This balance seems to depend on the amount of nutrients available to the plant, and mycorrhizal plants have been proposed to perform better (increased nutrient concentrations, nutrient uptake capacity, increased growth) under nutrient poor conditions due to their superior cost efficiency (amount of nutrients acquired per C expended), whereas non-mycorrhizal plants should be more cost efficient and grow better at high nutrient levels (Shwartz and Hoeksema 1998, Tuomi *et al.* 2001).

In addition, reduced seedling growth in response to ectomycorrhizae has been considered to result from increased belowground carbon allocation (Colpaert *et al.* 1996, Conjeaud *et al.* 1996) or as a consequence of high nutrient retention by the mycobiont (Colpaert *et al.* 1992, 1996).

Some models have been proposed to explain the variation of mycorrhizal effects on plant productivity that considered it to depend on the degree of mycorrhization (Gange and Ayres 1999, Tuomi *et al.* 2001), on the nutrient availability (Janos 2007) or on the balance between C cost and nutrient gain (Schwartz and Hoeksema 1998, Tuomi *et al.* 2001, Neuhauser and Fargione 2004). These models have, however, been seldom, if ever, tested, and we are still far from understanding what determines a positive or negative response from the plant to the mycorrhizae.

On the other hand the parameters chosen to evaluate the mycorrhizal effect are also problematic. The response to mycorrhization has most often been evaluated through its effect on the growth or nutrient status of the host plant, but this is misleading as these are not always the best indicators of plant fitness (Johnson *et al.* 1997, Jones and Smith 2004). These are, however, easily and always measurable, as opposed to survival and fecundity which are generally acknowledged as the best parameters when evaluating plant fitness, and therefore the plants' response to mycorrhization.

In addition, the time window chosen for observation may also be a source of controversy. The C cost and N gain deriving from the symbiosis fluctuate in time, in a

non-synchronized way, as they vary with environmental changes, as well as with developmental stages of the plant, the fungus and the mycorrhizal structures.

The present work was designed to understand how the plant response to mycorrhization varies, and what factors determine this variance, namely the nitrogen (N) availability, the plants' N status and the level of mycorrhizal colonization. Because plant and fungus have their own growth and developmental rates, which may generate feedbacks on the interaction between symbionts, plants of different ages, together with sequential harvests, were used to help the integration of these feedbacks.

Parameters were chosen that were indicative of plant productivity and plant N status, and changes in chl *a* fluorescence were used as an indicator of stress/plant vitality.

Matherial and Methods

Plant and fungal material

The ectomycorrhizal fungus *Pisolithus tinctorius* was grown in pure culture in liquid modified Melin-Norkans (MMN) medium (Marx 1969).

For inoculation, mycelium of *P. tinctorius* was grown for 2 months in the dark at 24 ºC on a perlite/vermiculite (v/v) mixture moistened with liquid MMN medium.

P. tinctorius was also grown in Petri dishes, in the dark at 24ºC, with solid modified MMN medium, supplemented with $5gL^{-1}$ glucose, covered with cellophane. The mycelium was collected 3 weeks after inoculation.

Pinus pinaster L. seeds were surface-sterilized with 30 % calcium hypochlorite for 30 minutes, rinsed several times in distilled water, and soaked in distilled water at 4 ºC for 48 h. Sowing was carried out in gnotobiotic conditions on a sand/vermiculite (v/v) mixture sterilized at 120 ºC for 1 h. Seedlings were watered with distilled water as needed.

When the second set of leaves appeared, approximately one month after sowing, the seedlings were transferred to 350 mL root trainers (20 cm Fleet Roottrainers, Ronaash, Ltd., Roxburghshire, U.K.) with a perlite/vermiculite (v/v) mixture as substrate, that had been sterilized at 120 ºC for 1 h.

Experimental design

The seedlings were divided into three groups (Table 1). The first group (G1) was inoculated at the time of transfer from the sowing beds to the root trainers. The seedlings in the other two other groups (G2, G3) were inoculated 1 month after. Prior to inoculation the seedlings in G2 were fed the same nutrition that they were to receive after inoculation (non-starved), while in G3 they received only distilled water (starved).

Group	Pre-treatment	Inoculation time	Type of inoculation	N supply [(a)]	Harvest times
G1	None	First set of leaves reached half the cotyledon length [(b)]	M	3.8 mM NH_4^+	4, 8, 12 and 16 weeks after inoculation
			NM		
			M	1.9 mM NH_4^+	
			NM		
G2	1 month N supply as in (a)	1 month after (b)	M	3.8 mM NH_4^+	4, 8 and 12 weeks after inoculation
			NM		
			M	1.9 mM NH_4^+	
			NM		
G3	1 month distilled water	1 month after (b)	M	3.8 mM NH_4^+	4, 8 and 12 weeks after inoculation
			NM		
			M	1.9 mM NH_4^+	
			NM		

Table 1. Scheme of the culture conditions, inoculation and harvest times of the seedlings used.

Half the seedlings of each group were inoculated with alive (mycorrhizal - M) and half with dead (non-mycorrhizal control - NM) *P. tinctorius* mycelium. For the inoculation, 100 mL inoculum, previously washed with distilled water, was placed in contact with the roots. Dead mycelium was obtained by sterilizing the inoculum for 1 hour at 120 ºC.

From the moment of inoculation (and prior to inoculation on G2) the seedlings were watered twice a week with 25 mL of medium containing 3.8 mM NH_4^+ or 1.9 mM NH_4^+ as N source. The nutrient solution used was MMN medium, from which thiamine was omitted, which has an ammonium concentration of 3.8 mM. It was modified in order to obtain a medium with half this concentration (1.9 mM), containing 0.95 mM $(NH_4)_2HPO_4$ and 4.6 mM KH_2PO_4. The volume of nutrient solution given to the seedlings was never in excess of the substrate holding capacity, and therefore there was never loss of solution.

The experiment was performed in a growth chamber under a 16 h light / 8 h dark photoperiod at 24 / 18 ºC, approximate 70 % relative humidity and 250 µmol m^{-2} s^{-1} at plant height. The light intensity was chosen so that the lighting conditions were close to those found in the understorey of forest sites (George *et al.* 1999).

Following inoculation the seedlings were followed for 16 (1 month old seedlings) or 12 (2 month old seedlings) weeks. Between 8 and 12 seedlings were harvested every 4 weeks. Shoots and roots were weighed and collected separately. The roots were washed in running water and excess water was removed prior to weighing. The samples were immediately frozen after weighing, at -70ºC.

Samples were kept at -70ºC, freeze dried for 72 h and stored at -20ºC in vacuum. They were then homogenized in liquid N_2 using a ball mill, and freeze dried again for 24 h. All freeze dried samples were kept under vacuum at -20ºC.

The fungal material obtained in pure culture in Petri dishes was also collected, weighed and freeze-dried, as described for the plant samples.

For ergosterol, C and N concentration determinations, the shoots or roots of 2 to 3 seedlings of each treatment and harvest were pooled in one sample, making a total of 3 samples per treatment and per harvest.

Chlorophyll a *fluorescence measurements*

Full chlorophyll *a* (Chl *a*) fluorescence transients were recorded weekly throughout the duration of the experiment. The measurements were conducted in fully dark adapted leaves, before the onset of illumination in the culture chamber, with a Plant Efficiency Analyser (PEA, Hansatech Ltd., England). Light was provided by an array of six light-emitting diodes (peak 650 nm) focused on the sample surface. Chl *a* fluorescence signals were detected using a PIN photocell after passing through a long pass filter (50 % transmission at 720 nm). The fluorescence signal was recorded for 1s, starting from 50 µs after the onset of illumination. The data acquisition was every 10 µs for the first 2 ms, and every 1 ms until 1s. On a logarithmic time scale, the rising transient from minimal fluorescence, F_0 (fluorescence at 50 µs) to maximal fluorescence, F_M, had a polyphasic behaviour. Analysis of the transient took into consideration the fluorescence values at 50 µs (F_0), 100 µs (F_{100}), 300 µs (F_{300}), 2 ms (step J), 30 ms (step I) and F_M. This method is called the JIP test (Strasser *et al.* 2004). The values were grouped in 4 week packages for analysis.

The performance index (PI_{abs}) is a performance expression combining the three most functional components in photosynthetic activity: (1) concentration or density of reaction centres in the chlorophyll bed (expressed on an absorption basis as RC/ABS), (2) the performance of the light reactions (primary photochemistry), and (3) the performance of the dark reactions, i.e., the ability to feed electrons into the electron chain between photossystem II and I (Strasser *et al.* 2004) (Appendix 1).

Net photosynthesis rate

Gas exchange measurements were made with a compact CO_2 /H_2O porometer CQP-130 coupled with a NDIR gas analyser (Binus 100 Leybold Heraeus, D-6450 Hanau, Germany). Net photosynthesis rates were measured every 4 weeks at 250 μmol m^{-2} s^{-1}, the same light intensity at which the plants were kept. 8 to 12 plants of each sub-group (Fig. 1) were measured for each sampling time.

Extraction and determination of ergosterol

The metabolically active fungal biomass in mycorrhizal roots (mantel + Hartig net) was determined based on the ergosterol, a specific constituent of fungal membranes, and used as a measure of mycorrhizal colonization. Free ergosterol of approximately 5mg freeze-dried root or *P. tinctorius* mycelium were extracted in 1mL ice cooled absolute ethanol, for 15 minutes, with occasional vortexing. After centrifugation (10 min, 14000*g*), the supernatant was used for determination of ergosterol, without further purification.

25 μl of the supernatant were injected into the HPLC system and separated on a Spherisorb S5 ODS2 (4.6 x 250 mm) column (Phase Separations). The mobile phase was pure methanol at a flow rate of 1 ml min^{-1}. Ergosterol was detected at 280 nm with a UV detector (UV 2000, Spectra Physics). A standard curve was obtained with 0.1, 0.5, 1, 4, 8, 10, 20, 40 and 60 μg ml^{-1} of ergosterol dissolved in absolute ethanol.

The ergosterol content of *P. tinctorius* grown in pure culture in Petri dishes was used to convert the ergosterol in roots to fungal biomass.

Determination of C and N concentrations

C and N concentrations were determined in freeze-dried shoots, roots and fungal mycelium using an elemental analyser Euro EA 3000 (EuroVector CHNS-O Elemental

Analyser; Callidus Software Interface-Version 4.1). The quantification was made using an external pattern, through linear calibration. The pattern used was the reference material Wheat Flour (OAS) calibrated for NIST patterns. The separation was made using a gaseous chromatography column, and the detector was a thermal conductivity detector (TCD). The integration of the chromatographic peaks was made using the Callidus software, version 4.1 (EuroVector).

The N concentrations of *P. tinctorius* grown in pure culture, together with the calculated fungal dry weight per root dry weight (estimated through ergosterol measurements) and the N concentration of M roots, were used to estimate the percentage of fungal N on the root.

Plant Growth and Productivity Parameters

For each 4 week period the following parameters were calculated (Appendix 2): N relative addition rate (RAR_N), relative growth rate (RGR), N relative uptake rate (RN), N productivity (NP), N net uptake rate (U) and the percentage of N allocated to the shoot (S_N). All these parameters, except RGR, were calculated for N.

The RAR_N is associated with the concept of steady-state nutrition, which was not used in this work, since the N supply was kept constant over the duration of the experiment resulting in decreasing RAR_N. The RAR_N presented here are therefore averages for the time period they refer to.

The pairing method was used in the calculations of RGR and RN. RGR, NP and S_N were calculated using dry weights. U was calculated using the fresh weight, since this is better correlated with the root area.

Mycorrhizal profit

The mycorrhizal profit, or the net increase in total value generated by investing in mycorrhizae instead of in non-mycorrhizal roots, was calculated as the ratio between any given parameter of an individual mycorrhizal plant (m) relative to a mean value for non mycorrhizal plants (NM) grown in the same experimental conditions: m/NM.

The mycorrhizal profits in growth (RGR_m/RGR_{NM}), N uptake (RN_m/RN_{NM}), net N uptake (U_m/U_{NM}), photosynthetic performance ($PI_{abs\ m}/PI_{abs\ NM}$), and shoot N allocation ($S_{N\ m}/S_{N\ NM}$) were calculated.

Mycorrhizal N demand/supply ratio - MDS_N

The balance between the plants demand for N and its supply through the mycorrhizae can be expected to vary according to the mycorrhizal colonization (Myc; μg *P.tinctorius* DW / mg root DW) and the N availability (RAR_N). The mycorrhizal N demand/supply (MDS_N) was therefore estimated by calculating the ratio between these two variables.

Statistical analysis

A 2 by 2 between-groups analysis of covariance (two-way ANCOVA) was used to test for the effects of mycorrhization and N supply on shoot, root and whole plant N and C concentrations and C:N ratios, photosynthetic performance (PI_{abs}), and net photosynthesis rate (A), using the age of the plant as the covariate. The effects of mycorrhization and N supply on the percentage of N that was allocated to the shoot (S_N) were analysed with a two-way between-groups analysis of variance (two-way ANOVA).

The correlations between several variables was investigated. The Pearson product moment correlation coefficient was used to evaluate these correlations whenever they were found to be linear. When they were non-linear, a curve was fitted to the data. The validity of assumptions was checked, and the goodness of fit of the model was evaluated using a one-way ANOVA.

In all cases preliminary analyses were performed to ensure no violation of the assumptions regarding each test. SPSS software, version 13.0, was used for all tests.

Results

The experimental design allowed a wide variety of combinations of N availability in the root medium (RAR_N), N concentrations in tissues (Fig. 1) and mycorrhizal colonization degrees. This variety was essential in analysing what factors were behind the variation in the plants' response to mycorrhization. It was also successful in integrating possible feedbacks deriving from plant developmental stage since none of the mycorrhizal profits were significantly affected by the plants age.

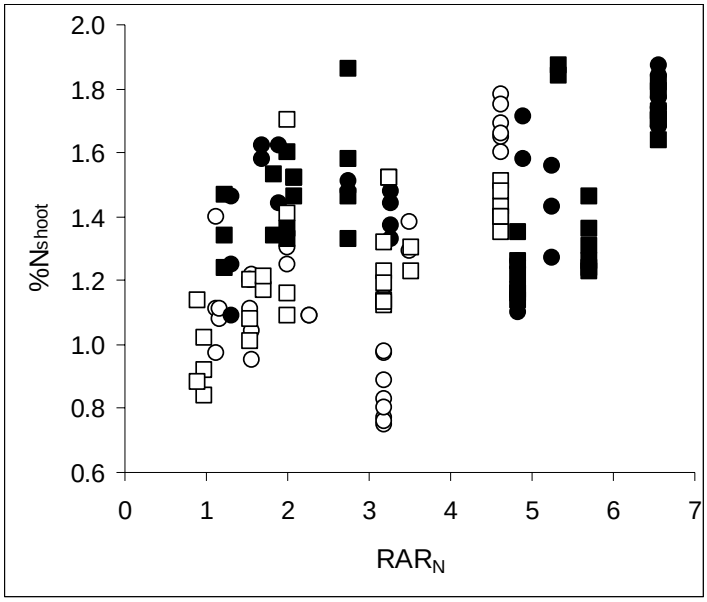

Figure 1. Shoot N concentrations of mycorrhizal (squares) and non-mycorrhizal (circles) plants that were fed either 1.9 (open) or 3.8 mM NH_4^+ (closed) plotted against the respective N relative addition rates (RAR_N).

Correlation between RAR_N and shoot N concentrations

Plants that were supplied with more N (3.9 mM NH_4^+) had higher shoot N concentrations than those supplied with less N (1.9 mM NH_4^+), at the same RAR_N (Fig. 1).

N and C concentrations

The effect of the variables tested (mycorrhization and N nutrition) on plant N and C concentrations and C/N ratios was the same in all the samplings. For this reason, all the measurements for each parameter were analysed together, and the values presented (Table 2) are averages of all the measurements.

From all the tested variables, mycorrhization had the only significant effect on root N and C concentrations (Table 2). M roots had higher N and lower C concentrations than NM roots at all harvests, and consequently lower C/N ratios.

In contrast, shoot N concentration was only significantly affected by the N supply. Plants receiving more N had higher N concentrations than those receiving less, at all harvests, leading to lower C/N ratios. There were no significant differences in shoot C concentration. There were no significant interactions between mycorrhization and nutrition.

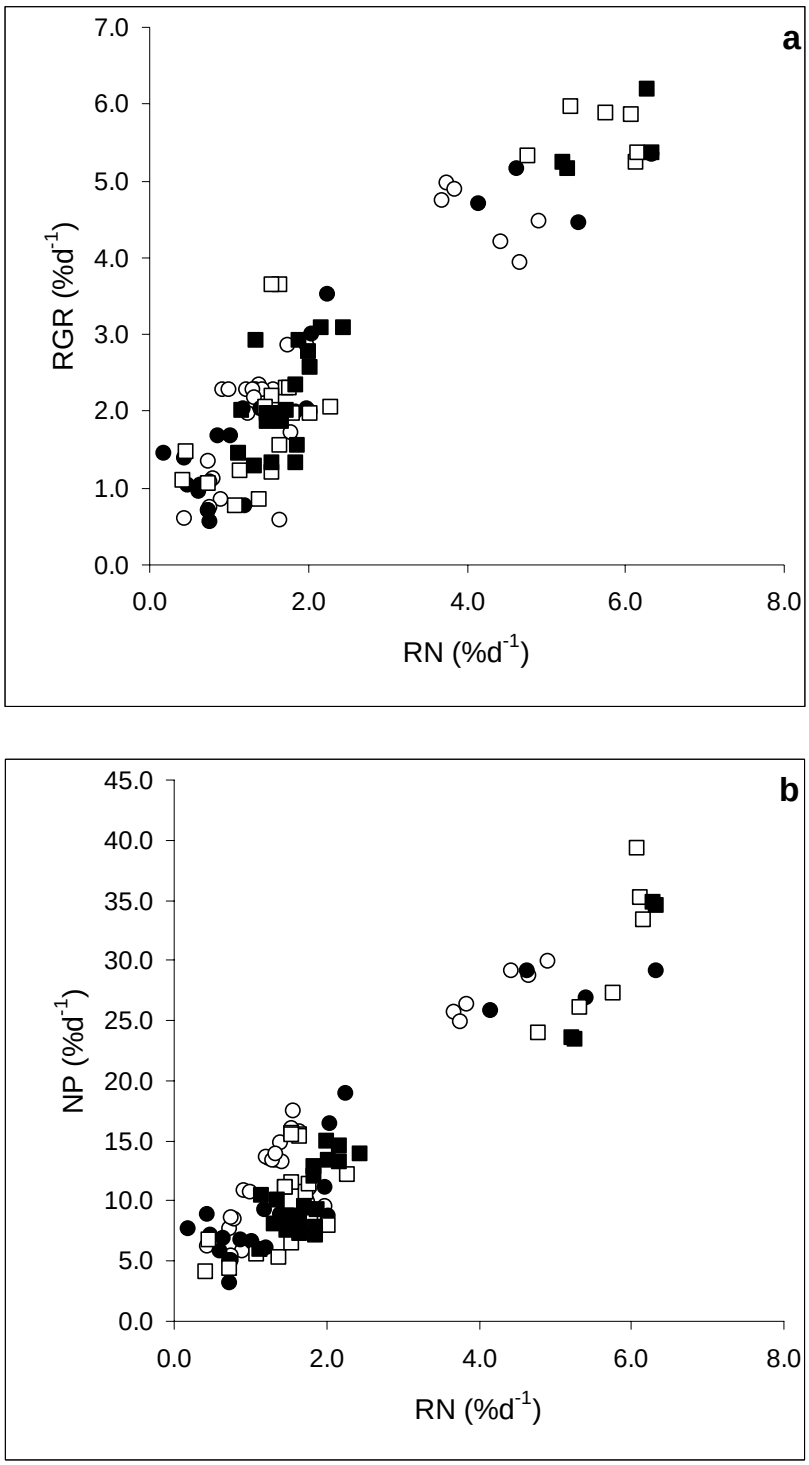

Figure 2. Correlations between the nitrogen relative uptake rate (RN) and (a) the relative growth rate (RGR) (r=0.92, n= 100, $p < 0.001$), or (b) the nitrogen productivity (NP) (r=0.90, n= 116, $p < 0.001$). The plants were mycorrhizal (closed) or non mycorrhizal (open), and fed either 1.9 (circles) or 3.8 mM NH_4^+ (squares) as N source. The Pearson product moment correlation coefficient was used to evaluate the correlations.

1.26 to 33% of the N of M roots were estimated to be fungal N, which amounted to 0.3 to 15.5% of the whole plant N concentration. In 33% of the cases this percentage was approximately the same (± stdev) as the difference in N concentration between M and NM roots, while it was lower on the remaining cases.

Correlations between RN, RGR and NP

Very strong correlations were found between RN and RGR ($r = 0.92$, $n = 100$, $p < 0.001$) (Fig. 2a), and RN and NP ($r = 0.90$, $n = 116$, $p < 0.001$) (Fig. 2b) values. The correlations were independent of mycorrhizal status or amount of N supplied.

Correlations between mycorrhizal growth and N uptake profits

The mycorrhizal plant growth profit (RGR_m / RGR_{NM}) was strongly and positively correlated with the profit on N uptake (RN_m / RN_{NM}) ($r = 0.58$, $n = 67$, $p < 0.001$) (Fig. 3a). A gradient of mycorrhizal growth and N uptake profits was observed, ranging from positive to negative.

The mycorrhizal N uptake profit (RN_m / RN_{NM}) was also strongly and positively correlated with the profit on N net uptake (U_m / U_{NM}) ($r = 0.973$, $n = 67$, $p < 0.001$) (Fig. 3b).

The concept of mycorrhizal N demand/supply ratio and its relation with the mycorrhizal growth profit

The mycorrhizal growth profit (RGR_m / RGR_{NM}) was found to be best and strongly correlated to the mycorrhizal N demand/supply ratio (MDS_N) than any other parameter measured or combination of parameters (Fig. 4). This correlation was best fitted by a second order polynomial and a third-order polynomial respectively for the plants receiving more ($r = 0.74$, $n = 34$, $p < 0.001$) and less N ($r = 0.60$, $n = 34$, $p = 0.006$) (Fig. 4).

A similar correlation was found when the shoot RGR was considered instead of the whole plant (results not shown). Mycorrhizal plants showed negative growth profits at lower MDS_N. As it increased, this profit became progressively more positive, and declined again above a certain value of MDS_N. The negative profit with higher values of MDS_N was, however, only observed in plants that received less N.

At low MDS_N all the negative growth profits observed belonged to plants collected one month after inoculation.

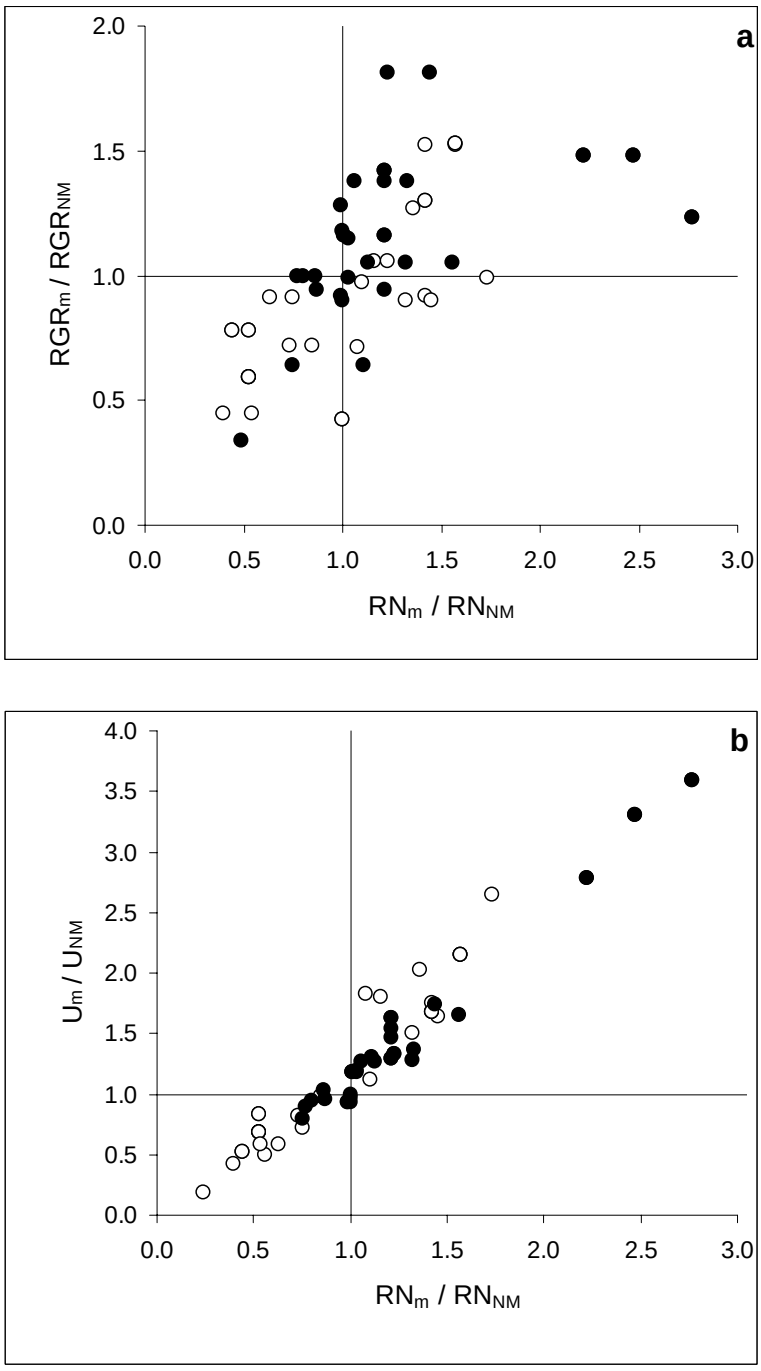

Figure 3. Correlations between the mycorrhizal profits on N uptake (RN_m/RN_{NM}) and **(a)** on growth (RGR_m/RGR_{NM}) (r=0.58, n= 67, $p < 0.001$), or **(b)** on N net uptake (U_m/U_{NM}) (r=0.97, n= 67, $p < 0.001$). The mycorrhizal profit, or the net increase in total value generated by investing in mycorrhizae instead of in non-mycorrhizal roots, was calculated as the ratio between the parameter for an individual mycorrhizal plant and the mean value for non mycorrhizal plants grown in the same experimental conditions. The plants were fed either 1.9 (open circles) or 3.8 mM NH_4^+ (closed circles) as N source. The lines dividing the graphic area make the division between the areas of negative (below 1) and positive (above 1) profits. The Pearson product moment correlation coefficient was used to evaluate the correlations.

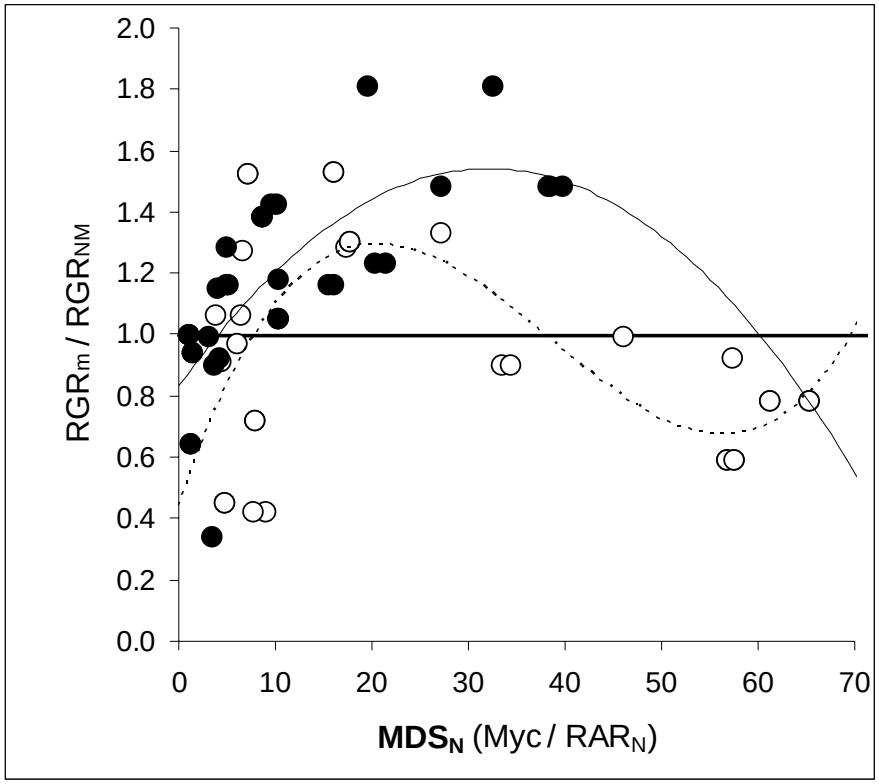

Figure 4. Correlations between the mycorrhizal growth profit (RGR_m/RGR_{NM}) and the mycorrhizal demand/supply ratio (MDS_N). MDS_N was calculated as the ratio between the level of mycorrhizal colonization (Myc; *P. tinctorius* μg DW root mg DW^{-1}) and the RAR_N (RAR_N). The plants were fed either 1.9 (open circles) or 3.8 mM NH_4^+ (closed circles) as N source. A second order (solid line; $y=0.83+0.04x-7^{-04}x^2$; r=0.74, n= 34, $p < 0.001$) and a third-order (interrupted line; $y=0.44+0.10x-0.003x^2+3^{-05}x^3$; r=0.60, n= 34, p = 0.006) polynomial were fitted respectively to the data sets for the plants receiving more and less N. The line dividing the graphic area makes the division between negative (below 1) and positive (above 1) mycorrhizal growth profit.

The RAR_N used in these calculations were the ones found for M plants. However, the RAR_N of the corresponding NM control plants were very similar, the difference being always less than 10% or $0.3\%d^{-1}$.

Mycorrhizal colonization

The first signs of mycorrhizae establishment were observed 6 to 12 days after inoculation. All mycorrhizal plants were colonized only by the inoculated mycobiont. Production of extra-radical mycelium was very abundant. No mycorrhizae formation was observed in NM plants.

	N supply (mM NH_4^+)	Mycorrhizal status	%N	%C	C/N
Shoot	3.8	NM	1.54 ± 0.04	45.32 ± 0.15	30.01 ± 0.79
		M	1.49 ± 0.04	45.53 ± 0.16	31.38 ± 0.85
	1.9	NM	1.27 ± 0.06	45.22 ± 0.14	38.31 ± 1.71
		M	1.29 ± 0.06	45.33 ± 0.10	36.73 ± 1.30
	ANCOVA effect	Myc	0.694	0.162	0.495
		Nut	< 0.001	0.269	< 0.001
		Myc x nut	0.490	0.676	0.248
		age	< 0.001	0.003	< 0.001
Root	3.8	NM	1.03 ± 0.06	40.77 ± 0.59	43.25 ± 2.56
		M	1.24 ± 0.04	38.59 ± 0.72	31.76 ± 1.02
	1.9	NM	0.96 ± 0.05	40.75 ± 0.60	45.08 ± 1.76
		M	1.19 ± 0.04	39.34 ± 0.79	34.00 ± 1.03
	ANCOVA effect	Myc	< 0.001	0.007	< 0.001
		Nut	0.083	0.583	0.260
		Myc x nut	0.831	0.572	0.909
		age	< 0.001	0.141	< 0.001
Whole Plant	3.8	NM	1.31 ± 0.03	42.93 ± 0.27	33.52 ± 0.98
		M	1.41 ± 0.03	41.81 ± 0.23	30.37 ± 0.70
	1.9	NM	1.06 ± 0.03	42.52 ± 0.27	41.55 ± 1.11
		M	1.21 ± 0.04	42.01 ± 0.29	35.71 ± 0.80
	ANCOVA effect	Myc	< 0.001	0.108	<0.001
		Nut	<0.001	0.222	<0.001
		Myc x nut	0.421	0.294	0.252
		age	< 0.001	0.297	< 0.001
P. tinctorius	3.8		4.62 ± 0.25	44.41 ± 2.57	9.74 ± 1.15
	1.9		4.95 ± 0.03	45.22 ± 0.75	9.14 ± 0.21

Table 2. N and C concentrations and C/N ratios of shoots, roots and whole mycorrhizal (M) and non-mycorrhizal (NM) plants, and of *P. tinctorius* mycelium grown in pure culture. The plants and the fungus received 3.8 or 1.9 mM NH_4^+ as N source. Values are overall averages (± SE) of all harvests. A two-way ANCOVA was used to test the main effects and interactions.

Correlation between mycorrhizal colonization and plant N concentrations

The degree of mycorrhizal colonization was best correlated with the shoot N concentration. This was a strong negative correlation ($r = -0.76$, $n = 71$, $p < 0.001$) (Fig. 5). No correlation was observed with root N concentration.

Net photosynthesis rate

There were no significant effects of mycorrhization in net photosynthesis rate, either per needle area (results not shown) or total shoot (A_{plant}; Table 3). The plants that were supplied with more N had higher A_{plant} ($p = 0.005$; Table 3) although the net photosynthesis rate per needle area was also not significantly different to the plants that were supplied with less N. No correlations were found between photosynthesis and any of the other parameters measured.

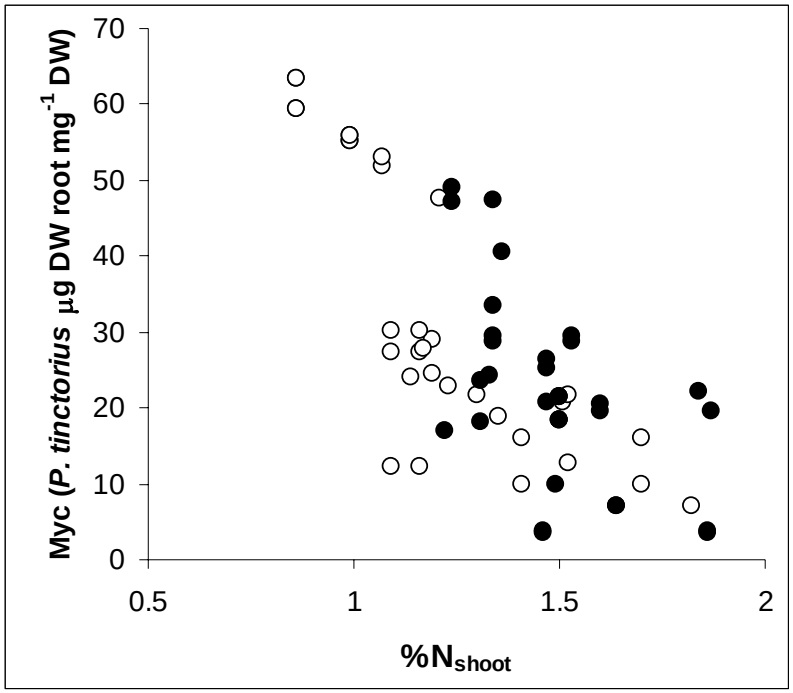

Figure 5. Correlation between mycorrhizal colonization, measured as fungal dry weight per root dry weight, and N shoot concentration ($r = -0.76$, $n = 71$, $p < 0.001$). The plants were fed either 1.9 (open circles) or 3.8 mM NH_4^+ (closed circles) as N source. The Pearson product moment correlation coefficient was used to evaluate the correlation. Fungal biomass was calculated from ergosterol concentrations measured in roots. These were converted to fungal dry weights using the average ergosterol concentration measured in *P. tinctorius* grown in pure culture (6.74 µg ergosterol mg^{-1}DW) as the conversion factor.

N supply ($mM\ NH_4^+$)	Mycorrhizal status	PI_{abs}	n	A_{plant} ($\mu mol\ CO_2\ plant^{-1}s^{-1}$)	n
3.8	NM	32,28 ± 0,62	411	357,84 ± 16,06	135
	M	31,59 ± 0,70	332	309,93 ± 14,74	136
1.9	NM	24,58 ± 0,48	367	266,53 ± 12,10	133
	M	24,78 ± 0,56	305	250,56 ± 10,69	137
ANCOVA effect	Myc	0.271		0.214	
	Nut	<0.001		0.005	
	Myc x nut	0.508		0.531	
	age	0.001		<0.001	

Table 3. Photosynthetic performance index (PI_{abs}), and net photosynthesis rate per plant (A_{plant}) of mycorrhizal (M) and non-mycorrhizal (NM) plants grown with 3.8 or 1.9 mM NH_4^+ as N source. Values are overall averages (± SE) of all measurements. A two-way ANCOVA was used to test the main effects and interactions.

Chl a fluorescence and N allocation

The photosynthetic performance (PI_{abs}) was only significantly affected by the N supply, and plants that were fed with higher N concentrations had higher PI_{abs} values (Table 3).

N allocation (S_N) was also only influenced by the N supply (Table 4). Plants that received more N allocated a significantly bigger percentage of the N taken to the shoot. Mycorrhization did not have a significant effect on either PI_{abs} or S_N.

Although mycorrhization did not have a statistically significant effect on PI_{abs} or on S_N, a gradient of effects of mycorrhization on these two parameters was observed that varied with the RAR_N. The correlations between RAR_N and the mycorrhizal profit on PI_{abs} ($PI_{abs\ m}/PI_{abs\ NM}$) (1.9 mM NH_4^+: $r = 0.79$, $n = 33$, $p < 0.001$; 3.8 mM NH_4^+: $r = 0.81$, $n = 34$, $p < 0.001$) and S_N ($S_{N\ m}/S_{N\ NM}$) (1.9 mM NH_4^+: $r = 0.75$, $n = 33$, $p < 0.001$; 3.8 mM NH_4^+: $r = 0.68$, $n = 34$, $p < 0.001$) were different for plants that were supplied with more or less N, but in all cases were curvilinear and best described by second order

polynomial equations (Figs. 6a, b). The effect of mycorrhization on both PI_{abs} and S_N was much more pronounced in plants that were fed with less N, with a narrower interval of RAR_N values in which mycorrhization led to positive profits (PI_{abs}), or more strongly positive or negative profits (S_N), than the plants that were fed with more N.

In all treatments, PI_{abs} was strongly and positively correlated to S_N ($r = 0.64$, $n = 109$, $p < 0.001$) (Fig. 7a). There was no correlation between these two parameters and RN or RGR. The mycorrhizal PI_{abs} profits were strongly correlated to mycorrhizal S_N profits. This last correlation was curvilinear and best described by a quadratic equation, and only observed in plants fed with less N ($r = 0.86$, $n = 36$, $p < 0.001$) (Fig. 7b).

N supply (mM NH_4^+)	Mycorrhizal status	S_N **(% total N)**	n
3.8	NM	66.37 ± 1.10	28
	M	60.51 ± 3.11	28
1.9	NM	56.08 ± 1.86	30
	M	55.82 ± 2.02	25
ANOVA effect	Myc	0.162	
	Nut	0.001	
	Myc x nut	0.201	

Table 4: N allocation to the shoot (S_N) of mycorrhizal (M) and non-mycorrhizal (NM) plants grown with 3.8 or 1.9 mM NH_4^+ as N source. Values are overall averages (± SE) of all measurements. A two-way ANOVA was used to test the main effects and interactions.

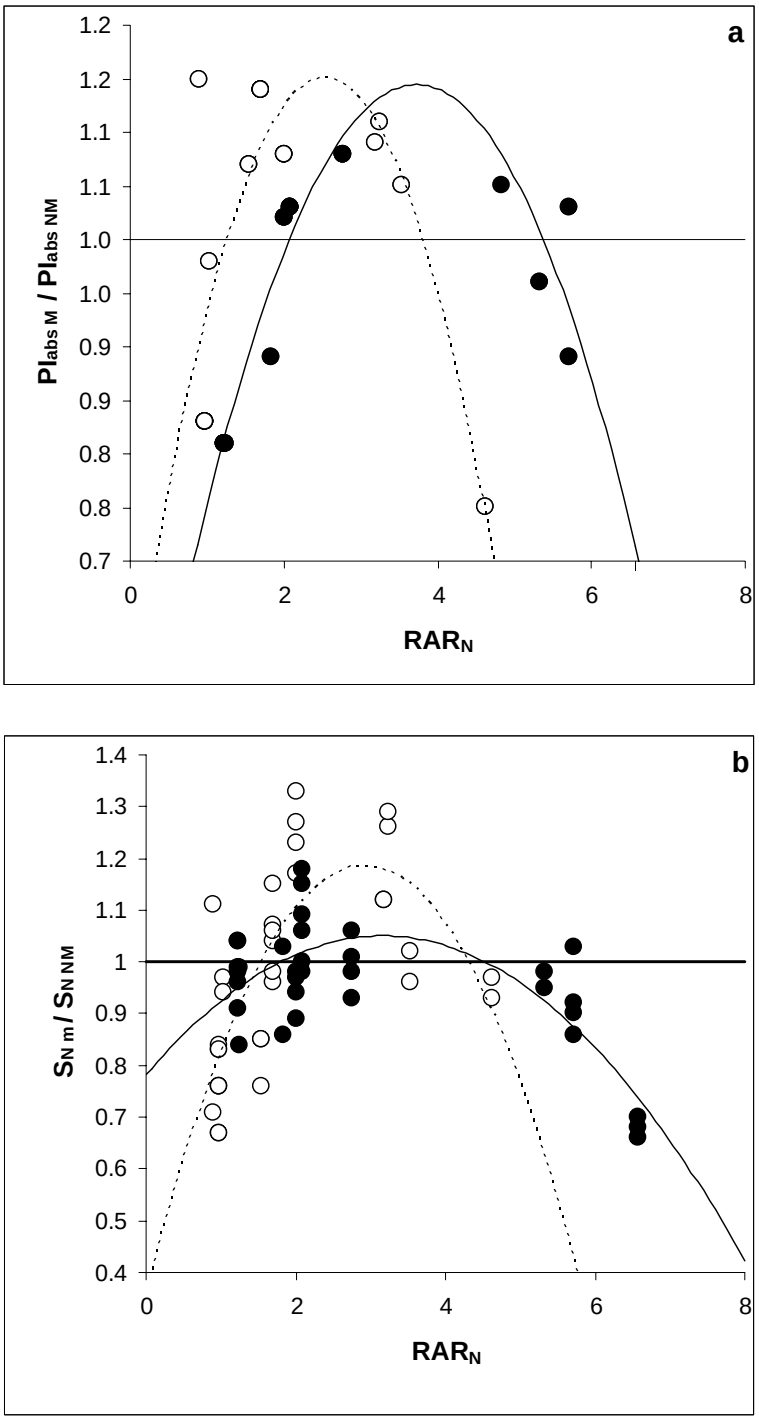

Figure 6. Correlations between **(a)** the photosynthetic performance index (PI_{abs}) and N shoot allocation (S_N; % total N) (r = 0.64, n = 109, p < 0.001), and between **(b)** the mycorrhizal profit on PI_{abs} ($PI_{abs\ m}$/ $PI_{abs\ NM}$) and on S_N ($S_{N\ m}$/ $S_{N\ NM}$). In (b) a correlation was only found for the plants receiving less N, and a second order polynomial was fitted to the data set (interrupted line; $y=-1.37x^2+3.24x-0.80$; r=0.86, n=36, p < 0.001). The plants were fed either 1.9 (open circles) or 3.8 mM NH_4^+ (closed circles) as N source. The Pearson product moment correlation coefficient was used to evaluate the correlation in (a). The lines dividing the graphic area in (b) make the division between negative (below 1) and positive (above 1) mycorrhizal profits.

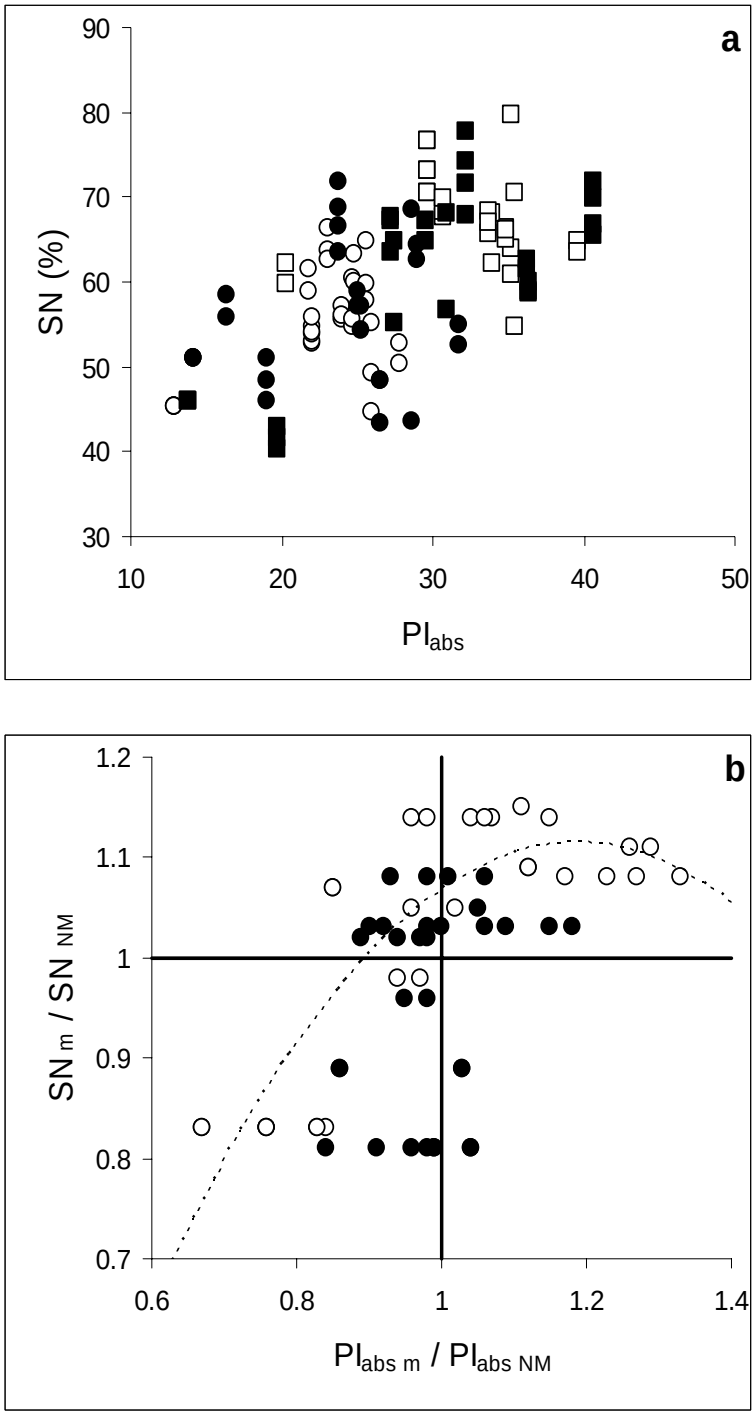

Figure 7. Correlations between the RAR_N and mycorrhizal profits on **(a)** the photosynthetic performance index ($PI_{abs\ m}$/ $PI_{abs\ NM}$) (1.9mM NH_4^+: r =0.79, n =33, p < 0.001; 3.8mM NH_4^+: r =0.81, n =34, p < 0.001) and **(b)** shoot N allocation ($S_{N\ m}$/ $S_{N\ NM}$) (1.9mM NH_4^+: r =0.75, n =33, p < 0.001; 3.8mM NH_4^+: r =0.68, n =34, p < 0.001). The plants were fed either 1.9 (open circles) or 3.8 mM NH_4^+ (closed circles) as N source. Second order polynomials were fitted to the data sets for the plants receiving less (interrupted line; (a) $y = -0.10x^2 + 0.57x + 0.35$; (b) $y = -0.09x^2 + 0.48x + 0.55$) or more N (solid line; (a) $y = -0.02x^2 + 0.12x + 0.85$; (b) $y = -0.04x^2 + 0.28x + 0.55$).

Discussion

The concept of mycorrhizal profit

In this work the concept of profit is introduced as an alternative to the concept of benefit in the evaluation of mycorrhization effects. Profits in the economists' sense are defined as a direct measure of the net increase in total value generated by employing scarce resources in one particular use rather than in their most valuable alternative use in some other undertaking. The ratio between, for example, M and NM plants RGR (RGR_M / RGR_{NM}) can therefore be considered as a measure of the mycorrhizal profit in growth. If instead of a net increase there is a decrease, this is termed cost, a concept that is complementary to profit, but it can also be referred to as negative profit. The concept of profit has the advantage of being a quantitative parameter while benefit is qualitative.

N uptake, growth and the gradient of mycorrhizal effects

The linear correlation between RGR and RN (Fig. 2a), indicates that under all the tested conditions, and independently of mycorrhizal formation, growth was dependent on the N uptake, and therefore N was limiting. This is in accordance with the fact that the measured N concentration in tissues (Table 2) ranged from values considered "adequate" to "very severely deficient" in published standards for lodgepole pine (Brockley 2001).

In sub-optimum N conditions the N availability (RAR_N) controls N uptake (RN), which in its turn controls growth (RGR), resulting in a linear correlation between RN and RGR (Ingestad and Lund 1979, Ericsson and Ingestad 1988, Lambers *et al.* 1998). The nitrogen productivity (NP), i.e the efficiency with which a unit of N was used to produce biomass, was also dependent on the N uptake (RN) (Fig. 2b) further confirming that N was limiting. Furthermore, the correlation between NP and RN did not differ between M and NM plants (Fig. 2b), similarly to what was found by Ingestad *et al.* (1986), indicating that the fungal partner did not result in a C limitation of growth. Lower NP with the same RN would be expected in M plants if that was the case (Ingestad *et al.* 1986). This explains why the net photosynthesis rate was not significantly affected by mycorrhization (Table 3).

Because of the impossibility of separating the plant from the fungal tissues (fungal mantle and Hartig net) within the root, fungal N was measured along with the root N, which may have affected the calculation of RN. However, although it would be possible

to estimate and subtract the fungal N content from the root N, it was considered that this would be to incur on a bigger error.

The mycorrhizal growth profit (RGR_m/RGR_{NM}), was also positively correlated with the mycorrhizal N profit (RN_m/RN_{NM}) (Fig. 3a), indicating that mycorrhization altered the host plant's growth by changing their N uptake, resulting in a gradient of mycorrhizal effects on the host plant's growth. While increased growth rates in mycorrhizal plants are most often attributed to increased nutrient uptake (e.g., Jones *et al*., 1990, 1991), which is in agreement with the present results, decreased growth rates have most often been considered to result from excessive fungal C drain (Colpaert *et al.* 1996, Conjeaud *et al.* 1996). The present results, however, show that in N limited conditions it is solely the N uptake profit, and therefore whether the mycorrhizal fungus retains or facilitates N, and not the amount of C allocated to the fungus, what determines the mycorrhizal growth profit (Fig. 3a). In fact, ECM fungi have a high potential to take up, accumulate (Wallenda and Kottke 1998) and immobilize N (Colpaert *et al.* 1992, 1996).

This explains why models based on the balance between nutrient gain and C cost (Schwartz and Hoeksema 1998, Tuomi *et al.* 2001, Neuhauser and Fargione 2004) are effective at predicting the negative effects on the host plant at high nutrient availabilities, but not situations of decreased growth when the nutrients are limiting. Although they are much less frequent, negative growth responses to mycorrhization in severely nutrient deficient plants have been previously observed (Ingestad *et al.* 1986, Colpaert *et al.* 1999, Hobie 2006).

The lower C and higher N root concentrations observed in M plants (Table 2), could indicate, respectively, increased C use at the root and increased N retention by the fungus present at the root. This last hypothesis was also indicated by the fact that the higher N concentrations in mycorrhizal roots were not accompanied by higher shoot N concentrations (Table 2), similarly to previous reports (Wallander and Nylund 1991, Colpaert *et al.* 1996). These observations were, however, made in all the cases and therefore neither accounts for the variance in growth profit observed in this case. Furthermore, in the majority of the cases (77%), the estimated fungal N at the root was lower than the difference between M and NM roots, indicating N accumulation that was not due to fungal retention. Such accumulations are characteristic of N stress (Ingestad and Ågren 1988).

In the present experiment a semi-hydroponic system was used allowing both mycorrhizal and non-mycorrhizal plants to have good access to the nutrients, and hence excluding the effect of improved exploitation of the substrate by the fungus (Colpaert *et al.* 1996, Eltrop and Marschner 1996). The very strong correlation found between the mycorrhizal profits in N uptake (RN_m / RN_{NM}) and in N net uptake rate (U_m / U_{NM}), which is a measure of root uptake efficiency, strongly indicates that mycorrhizae increased or decreased the N uptake profit by changing the plants' uptake capacity or efficiency per root unit when compared to NM plants (Fig. 3b). ECM must therefore have an effect on the host plants' nutrient uptake that is not dependent on, or a result of, the overcoming of spatial limitations to the uptake. Mycorrhizal roots and external hyphae have been found to have more transporters (Selle *et al.* 2005) and higher maximum influx rates (V_{max}) and higher affinity (lower K_m) for N uptake than non-mycorrhizal roots (Eltrop and Marschner 1996, Javelle *et al.* 1999). In the present experiment, however, the effect of mycorrhizae on the roots nutrient uptake capacity could also be negative. To our knowledge, this is the first time that a decreased ammonium uptake capacity has been reported. This is likely a consequence of the decreased access to N.

The mycorrhizal N demand/supply ratio - MDS_N

The observed variation in N uptake and growth profits by M plants was found to be best explained and to have a curvilinear relation with the mycorrhizal N demand/supply ratio (MDS_N, Fig. 4).

Both extremes of MDS_N can correspond to conditions of increased mycelial growth, and hence increased N use and retention by the fungus, which would explain the negative growth profits observed. This hypothesis is in accordance with the findings of negative correlations between plant biomass and the extension of fungal development, the mass of the fungal mycelium produced or the relative growth rate of the mycelium (Dosskey *et al.* 1990, Colpaert *et al.* 1992). In the present case, the abundant production of extra-radical mycelium observed indicate that mycelial growth could be a particularly important sink.

MDS_N is low when the N availability (RAR_N) is high relatively to the amount of colonization. This can correspond to a situation where the fungus is still colonizing the root and in some cases the substrate. This was presumably the case of the plants collected one month after inoculation (Table 1), in which negative growth profits were

observed at low MDS_N. It is important to notice that these negative growth profits were obtained both with very limiting and near to optimum RAR_N, so this negative effect was less dependent on the N available than on the degree of mycorrhizal colonization.

On the other hand, very high MDS_N are conditions of high colonization and low N availability, which may correspond to a situation where all resources are invested in increased extra-radical fungal growth, in an effort to reach new sources of N. At low rates of nitrogen addition to small seedlings shoot growth will become a weak sink for carbon and, consequently, more carbon can be allocated below ground to support root growth and associated fungi (Wallander 1995, Hobie 2006).

The mycorrhizal response has been considered to vary according to the nutrient availability (Schwartz and Hoeksema 1998, Tuomi *et al.* 2001, Neuhauser and Fargione 2004, Janos 2007) or the degree of mycorrhizal colonization (Gange and Ayres 1999, Tuomi *et al.* 2001), and models have been proposed that predicted this response to be curvilinear (Gange and Ayres 1999, Janos 2007). Furthermore, the correlation between degree of mycorrhizal colonization and host plant growth has been previously fitted by a third-order regression by Clapperton and Reid (1992), similarly to what was done for the plants that were fed less N in the present study. However, in the present work, it was the combination between these two variables, in the calculation of MDS_N, that was found to best describe the variance in response.

The model proposed by Gange and Ayres (1999) predicts that different environmental conditions would originate curves with different behaviours or characteristics, and hence a family of curves. This is in agreement with the results reported here (Fig. 4). However, it assumes that the maximum extent to which mycorrhizal infection can improve plant performance is a function of the nutritional deficit of the plant, and as a consequence the number of instances of the mycorrhizae being antagonistic increased at higher nutrient supplies. This is not in agreement with the results presented here, since negative effects were found sooner for plants that received less N (Fig. 4). This discrepancy can be explained by the variables used: the amount of nutrient supplied or its concentration in the Gange and Ayres model, or RAR_N in this work. Because the plants receiving more N had higher shoot N concentrations at the same RAR_N (Fig. 1), and the degree of mycorrhization was closely correlated to the shoot N concentration (Fig. 5), these plants had lower mycorrhization degrees for the same RAR_N. The critical value of MDS_N that determines a negative

response was therefore higher for plants receiving more N, since it was only reached at lower values of RAR_N.

Growth profit does not mean improved plant fitness

The mycorrhizal growth profits were not correlated with profits in terms of photosynthetic performance (PI_{abs}), indicating that the decreased growth of mycorrhizal plants was not related to decreased plant vitality, and hence plant fitness (ability to survive), and vice-versa, and that the observed changes in growth profits were simply a result of different strategies of resource investment. In fact, most plants can be expected to adapt to slow changes in N availability reducing growth and remaining healthy (Ingestad and Lund 1979).

Although the effect of mycorrhization on PI_{abs} was found not to be statistically significant (Table 3), a clear gradient of mycorrhizal PI_{abs} profits was observed, that was best correlated to the RAR_N (Fig. 6a). This indicates that it was the availability of N that determined whether or not the mycorrhizae had a detrimental effect on the host plant fitness.

The mycorrhizal profit on S_N, i.e., the net increase or decrease in the allocation of N to the shoot as a result of mycorrhization, was also best correlated to RAR_N, and in the same manner as the PI_{abs} profit (Fig. 6b). The mycorrhizal profit on S_N was only positive at intermediate RAR_N, indicating that mycorrhization led to higher N investment in the shoot as the N supply became more limiting, and only until it became too limiting.

Both these correlations were a lot stronger for the plants that received less N (Fig. 6). This indicates that either the plants N concentration, since plants receiving more N had higher shoot N concentrations at the same RAR_N (Fig. 1), or the mycorrhizal degree (Fig. 5), may also have played a part. However no correlation was found between these variables and PI_{abs} or S_N profits.

In all the plants studied, PI_{abs} was dependent on S_N (Fig. 7a) which reflects the photosynthetic apparatus dependence on N. However, the mycorrhizal PI_{abs} profit was only correlated to S_N profit in plants that were fed less N (Fig. 7b), indicating that the mycorrhizal effect on S_N was strong enough to influence the photosynthetic performance of the host plant only when the plants were being supplied less N.

Regulation of the symbiotic N/C exchange

Mechanisms linking both P (in AM; Fitter 2006) and N (in ECM; Nehls *et al.* 2007) supply to the plant with C transfer from the plant to the fungus, have been proposed. These mechanisms assume that the transport of phosphate or N into the plant-fungus interface either stimulates the supply of C by the plant (Fitter 2006) or decreases the competition between the fungus and the plant for apoplastic hexoses (Nehls *et al.* 2007), and that if the fungus fails to supply the plant with adequate amounts of nutrients, it will reduce the C supply to the fungus. This control of the carbohydrate efflux by the host plant in symbiosis has been considered essential to avoid fungal parasitism (Nehls *et al.* 2007).

In the present case, however, it was when the N status of the plants was lower that the higher mycorrhizal rates were observed (Fig. 5), indicating that the plants continued to provide C to the fungus although the N supplied by it was increasingly lower, to the point of negative N and growth profits (Fig. 4) (Hobie 2006).

This suggests that the regulation of the C/N exchange between symbionts must involve mechanisms other than the nutrient recognition by the plant at the interface. This regulation may respond to: (i) very low shoot N concentrations, (ii) C accumulation, or (iii) both. In support of the first hypothesis, the degree of mycorrhizal colonization was strongly negatively correlated with shoot N concentration (Fig. 5) similarly to previous reports (Wallander and Nylund 1991). The hypothesis that it is the N status of the trees that regulates growth of extra-radical EM mycelia, and hence the C supply to the fungus, had already been proposed by Nilsson and Wallander (2003).

It is however possible that the shoot N status is not the sole regulator in this response, since the C supply seems to have an important role in N uptake and translocation to the plant and evidence that those exchanges between partners are reciprocal has been found (Kitöviita 2005).

In conclusion, we found that, in conditions of limited N, it is the N retention or facilitation by the mycorrhizal fungus that determined the effect of mycorrhization on the host plants' growth, while no evidences of C limitation to growth were observed. In these conditions, it was the balance between the N supply by the fungus and the N demand by the plant that determined whether the host plant will profit in growth from the association, and the MDS_N was found to be explanatory of how this balance changes. We also found that the effects of mycorrhization on growth were not reflected in the host plants fitness, and were therefore only a result of changes in resource

allocation between host plant and mycorrhizal fungus. This work represents a step further in the understanding of what are the effects of mycorrhization on the host plant, and of their meaning.

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Technical fluorescence parameters	
F_0	F_{50}, fluorescence intensity at 50μs
F_M	Maximal fluorescence intensity
F_V/F_M	$(F_M-F_0)/ F_M$
V_J	Variable fluorescence at 2ms. $V_J = (F_J-F_0) / (F_M-F_0)$
Quantum efficiency or flux ratios	
φ_{P0} or TR_0/ABS	$\varphi_{P0} = (F_M-F_0) / F_M$
Ψ_0 or ET_0/TR_0	$1- V_J$
Density of reaction centres	
RC/ABS	$(RC/TR_0)\ (TR_0/ABS) = [V_J/(d_V/d_{t0})]\ (F_V/F_M)$
Performance index	
PI_{abs}	$(RC/ABS)\ [\varphi_{P0}/(1- \varphi_{P0})]\ [\Psi_0/(1- \Psi_0)]$

Appendix 1. Technical data of the O-J-I-P curves and the selected JIP test parameters used in this study.

Parameter	Formula	Dimension	References
N relative addition rate **RAR_N**	$N_s = N_0$ * (exp (**RAR** * t) -1) <=> **RAR** = ln ((N_s / N_0) -1) / t	Dimensionless	Ingestad and Lund, 1979
Relative growth rate **RGR**	**(1 / W) × (dW / dt)** = ($\ln W_2 - \ln W_1$) / ($t_2 - t_1$)	Weight weight^{-1} time^{-1}	van den Driessche and van den Driessche, 1991
Nitrogen relative uptake rate **RN**	**(1 / N) × (dN / dt)** = ($\ln N_2 - \ln N_1$) / ($t_2 - t_1$)	Weight weight^{-1} time^{-1} or, mole mole^{-1} time^{-1}	van den Driessche and van den Driessche, 1991
Nitrogen net uptake rate **U**	**(1 / R) × (dN / dt)** = [($N_2 - N_1$) × ($\ln R_2 - \ln R_1$)] / [($R_2 - R_1$) ×($t_2 - t_1$)]	Weight weight^{-1} time^{-1} or, weight area^{-1} time^{-1}	van den Driessche and van den Driessche, 1991
Nitrogen productivity **NP**	**(1 / N) × (dW / dt)** = [($W_2 - W_1$) × ($\ln N_2 - \ln N_1$)] / [($N_2 - N_1$) ×($t_2 - t_1$)]	Weight weight^{-1} time^{-1}	van den Driessche and van den Driessche, 1991; Ericsson and Ingestad, 1988; Lambers *et al.* 1998
N shoot allocation **S_N**	100 * (% N_{shoot} / % $N_{whole\ plant}$) * (W_{shoot} / $W_{whole\ plant}$)	% total N	Ingestad and Ågren, 1988

Appendix 2. Equations for the calculation of the plant growth analysis parameters used. N_s: amount of N added; N_0: the initial plant N content; W: plant weight; N: plant N content; t: time; R: root weight or area.

CHAPTER 5

Responses of plant C and N metabolism to ECM formation are correlated to C and N belowground allocation and fungal growth.

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CHAPTER 5. Responses of plant C and N metabolism to ECM formation are correlated to C and N belowground allocation and fungal growth.

Summary

In this study we address the question of how the readjustments in the C and N metabolism of an ectomycorrhizal plant adjust from the moment of inoculation and until a fully established mycorrhizal state, and how they depend on N supply.

Pinus pinaster L. seedlings were inoculated with alive or dead *Pisolithus tinctorius*, supplied with 1.9 or 3.8 mM ammonium as N source, and their development followed for 29 days. Concentrations of root ergosterol, activities of root sucrolytic enzymes, and of PEP carboxylase in roots, needles and cotyledons, as well as concentrations of soluble carbohydrates, starch, F26BP, N and free amino acids were assayed. In addition, rates of net photosynthesis, chlorophyll *a* fluorescence and dry weights were measured periodically, and relative rates of growth and N uptake calculated.

No qualitative alterations of N or C metabolism in mycorrhizal plants were observed, but only quantitative ones. Alternating periods of metabolic activation and deactivation were observed in mycorrhizal plants, which were related, respectively, to periods of decreased or increased belowground investment.

The present work indicates that fungal growth and root colonization rates may not be constant in time, but fluctuate corresponding to periods of higher or lesser C and N demand by the fungus. This is mirrored by transient changes in host plant metabolism.

Key words: N, C, ECM, resource allocation, C and N metabolic enzymes, amino acids

Introduction

The exchange of carbohydrates for nutrients is central in all mycorrhizal symbioses. So far, our understanding of the nature of these exchanges remains limited. It is, however, obvious that this trade implies a series of metabolic readjustments in both symbionts. In particular, the establishment of ECM must lead to deep changes in the C

and N metabolism of the host plant and of the mechanisms that balance them, including the regulation of the partitioning of photosynthetic C between plant growth, nutrient uptake and assimilation, and fungal growth.

The metabolisms of C and N have a central role in plant development and are closely linked in several ways. Uptake and assimilation of N need reducing power and carbon skeletons for amino acid assembly, making them totally dependent and closely regulated by the availability of C metabolites and their translocation from the shoot to the root. On the other hand, C metabolism is also dependent on N uptake by the root system and its allocation to the shoot. The integration of these two metabolisms must involve extensive co-regulation between the root and the shoot, and any change in activity of one of them implicates an adjustment in the other (Foyer & Noctor, 2002).

ECM interactions predominate in nitrogen-limited ecosystems (Smith & Read, 1997), and improvements in nitrogen uptake and N status as a result of ECM formation have been frequently observed (e.g.: Eltrop & Marschner, 1996; Javelle *et al*., 1999; Selle *et al*., 2005). ECM associations also have a considerable impact on plant C metabolism, generally increasing the C allocation to the roots, the biggest part of which is thought to be used to support growth and maintenance of the fungal partner (Wright *et al*., 2000; Wu *et al*., 2002).

However, the effects of mycorrhization are not limited to changes in demand and supply. A number of studies indicate that there is also a shift in partner-specific C and N metabolism in ECM tissues. Molecular and biochemical evidence has been obtained that indicate a deactivation of the root and an activation of the fungal metabolism (Wingler *et al*., 1996; Blaudez *et al*., 2001; Johansson *et al*., 2004; Duplessis *et al*., 2005; Frettinger *et al*., 2007; Herrmann & Buscot, 2007) in response to mycorrhizae formation. These results have led to the idea that ECM symbioses allow plants to slow-down regulative pathways, and that this may compensate the cost of photoassimilate transfer to the fungal partner.

Changes in the free amino acid profile and in enzyme expression and activities indicate that N metabolism is altered and readjusted in mycorrhizal roots (Vézina *et al*., 1989; Chalot *et al*., 1991;Botton & Dell, 1994). An important factor in these readjustments is the form in which N is received by the plant, which has for long been thought to be in the form of amino acids (Smith & Read, 1997). Recent results, however, have indicated the transfer of N as ammonia in AM interactions

(Govindarajalu *et al*., 2005; Cruz *et al*., 2007), and it has been hypothesized that the same may happen in ECM (Chalot *et al*., 2006).

On the other hand, little is known about the metabolic compartmentation of N assimilation in ECM. In ECM fungi, and in *P. tinctorius* in particular, this seems to be achieved through the GS/GOGAT cycle (Wright *et al*., 2005; Morel *et al*., 2006). This may take place entirely in the fungus, or be separated, with GS active in the fungus and GOGAT in the root, in which case there could be a glutamine-glutamate shuttle across the interface (Martin & Botton, 1993).

The reorganization of metabolic pathways has been repeatedly observed to occur at very early stages following contact (Johansson *et al*., 2004; Duplessis *et al*., 2005; Frettinger *et al*., 2007), but these initial changes may be transient, and later be replaced by different adjustments (Blaudez *et al*., 1998; Boiffin *et al*., 1998).

In this study we addressed the question of how the modifications in plant C and N metabolism occur from the moment of inoculation and until a fully established mycorrhizal state, and how they are dependent on N supply. The enzyme activities measured were chosen for their relevance in the C/N exchange between symbionts, but also because they are plant-specific, thus allowing us to isolate the plant response at the root level.

Material and methods

Plant and fungal material

Mycelium of *P. tinctorius* was grown, in pure culture, for 2 months in the dark at 24 ºC on a perlite/vermiculite (v/v) mixture moistened with liquid modified Melin-Norkans (MMN) medium (Marx, 1969), and used as inoculum.

P. tinctorius was also grown in Petri dishes, in the dark at 24ºC, with solid MMN medium covered with cellophane. This mycelium was collected after 3 weeks, separated from the cellophane, weighed and stored at -70ºC until freeze-drying.

Pinus pinaster L. seeds were surface-sterilized with 30 % calcium hypochlorite for 30 minutes, rinsed in several changes of distilled water, soaked in distilled water at 4 ºC for 48 h, and sown on a sand/vermiculite (v/v) mixture, sterilized at 120 ºC for 1 h. Seedlings were watered with distilled water as needed.

When the second set of leaves appeared, approximately one month after sowing, the seedlings were transferred to 350 mL root trainers (20 cm Fleet Roottrainers, Ronaash, Ltd., Roxburghshire, U.K.).

Experimental design

The plants were inoculated at the time of transfer from the sowing beds into the root trainers. Half of the seedlings were inoculated with alive (mycorrhizal - M) and half with dead (non-mycorrhizal control - NM) *P. tinctorius* mycelium, which had been sterilized for 1 hour at 120 ºC. For the inoculation, 100 mL inoculum, previously washed with distilled water, was placed in contact with the roots.

From the moment of inoculation the plants were fed with MMN medium, from which thiamine was omitted. Each sub-group, M or NM, was again divided. Half of the plants received 3.8 mM NH_4^+, and the other half 1.9 mM NH_4^+ as N source.

MMN medium has an ammonium concentration of 3.8 mM, and was modified in order to obtain a medium with half this concentration (1.9 mM), containing 0.95 mM $(NH_4)_2HPO_4$ and 4.6 mM KH_2PO_4. Each plant was watered twice a week with 25 mL of medium.

The experiment was performed in a growth chamber under a 16 h light / 8 h dark photoperiod at 24 / 18 ºC, approximate 70 % relative humidity and a light intensity of 250 µmol m^{-2} s^{-1} at plant height.

Between 8 and 12 plants were harvested before the transfer to root trainers and inoculation (time 0), and then every 2 days until day 14, and every 5 days until day 29. Cotyledons, leaves, stem and roots were collected separately, and frozen immediately at -70ºC. The roots were washed in running water as quickly as possible and excess water was removed prior to freezing.

A parallel experiment was conducted to evaluate the fungal ability to grow with different C sources. *P. tinctorius* was grown in Petri dishes as already described, but with 10 g L^{-1} of glucose, fructose or sucrose as C source, or no C source. In order to avoid its cleavage during autoclaving, the sucrose was filter-sterilized and added later to the medium. After 3 weeks, the mycelium was separated from the cellophane and stored at -70ºC.

All fungal and plant samples were stored at -70ºC, until freeze-drying for 72 h, and then stored in vacuum at -20ºC. Individual plant samples were grouped into 3 to 4

mixed samples per harvest time. They were then homogenized in liquid N_2 using a ball mill, and freeze dried again for 24 h. The dry weights were recorded after freeze-drying.

Relative growth rate (RGR) of root, shoot and whole plant was calculated using the pairing method (Hunt, 1982) and the dry weights, according to: $(\ln W_1 - \ln W_0) / (t_1 - t_0)$, where W_0 is the initial plant weight, at time 0 (t_0) and W_1 the plant weight at time 1 (t_1).

Chlorophyll a *fluorescence measurements and net photosynthesis rate*

Full chlorophyll *a* (Chl *a*) fluorescence transients were recorded, as described in Corrêa *et al*. (2006), and gas exchange measured, on day 2, 6, 8, 14 and 29. The fluorescence transient was analysed according to the JIP test. The performance index (PI_{abs}), a photosynthetic performance expression, was calculated (Strasser *et al.*, 2004).

Gas exchange measurements were made with a compact CO_2 /H_2O porometer CQP-130 coupled with a NDIR gas analyser (Binos 100 Leybold Heraeus, D-6450 Hanau, Germany), at light intensity of 250 μmol m^{-2} s^{-1}. Eight to 12 plants were used to determine net photosynthetic rate (A) for each sampling time.

Extraction and determination of soluble carbohydrates and starch

Soluble carbohydrates and starch were measured according to Magel *et al*. (2001). Approximately 20 mg PVPP were added to approximately 4 mg of freeze dried material, and heated to 105ºC for 20 minutes. After cooling, soluble carbohydrates were extracted in 500 μl bidistilled water, for 25 minutes at room temperature with occasional vortexing. The samples were centrifuged at 10,000 rpm for 10 min, and the supernatant used for soluble carbohydrate determination. For starch determination, the pellet was resuspended in 150 μl of 0.1 M acetate buffer, pH 4.6, and heated to 100 ºC for 15 min. After cooling, 9 Uml^{-1} amyloglucosidase were added to each sample. The samples were incubated overnight at 37 ºC. After centrifugation at 10,000 rpm for 10 minutes, the supernatant was used for determination of glucose formed from starch degradation.

The carbohydrates contained in a 10 μl aliquot of the supernatant were assayed enzymatically. Eighty μl of a buffer containing 500 mM TRA, pH 7.4, 6 mM $MgSO_4$, 4 mM ATP, 2 mM NADP and 0.28 Uml^{-1} glucose-6-phosphate dehydrogenase were added to the aliquot. The assay was performed by sequential addition of 2.5 Uml^{-1} hexokinase (glucose), 3 Uml^{-1} phosphoglucoseisomerase (fructose) and 0.4 $mgml^{-1}$ of β-

fructosidase (sucrose). The final assay volume was 150 µl. In the case of starch determination, only glucose was quantified, in a final volume of 70 µl. The respective formation of NADPH was followed at 340 nm in a microplate reader spectrophotometer (Tecan Thermo Spectra, Tecan, Switzerland), at 30ºC. A blank was set up to which no sample was added.

Extraction and determination of fructose 2,6 biphosphate (F26BP)

F26BP was assayed according to Einig and Hampp (1990), by its stimulation of PPi-dependent fructose-6-phosphate phosphotransferase (PFP). Approximately 4 mg freeze dried material were extracted in 500 µl 100 mM mercaptoethanol, pH 10, with 7% insoluble PVPP. After incubation for 10 min on ice, with occasional vortexing, the samples were centrifuged for 10 min at 10,000 g, and the supernatant used for F26BP determination.

Ninety µl of 200 mM Tris-acetate buffer, pH 5.9, with 4 mM Mg acetate, 0.04% BSA, 1 mM PPi, 2 mM fructose-6-phosphate, 500 µM NADH, 2 $\mu g ml^{-1}$ glucose-3-phosphate dehydrogenase, 17 $\mu g ml^{-1}$ aldolase, 0.3 $\mu g ml^{-1}$ triosephosphate isomerase, 0.1 $U ml^{-1}$ PFP and 50 mM mercaptoethanol were added to 10 µl extract. The respective consumption of NADH was followed at 340 nm in a microplate reader spectrophotometer (Tecan Thermo Spectra, Tecan, Switzerland), at 30ºC. A blank was set up to which no PFP was added.

Extraction and determination of ergosterol

The metabolically active fungal biomass in mycorrhizal roots (mantle + Hartig net) was determined from ergosterol, and used as a measure of mycorrhizal colonization. Free ergosterol was determined according to Schaeffer *et al.* (1995). Approximately 5 mg freeze-dried root material was extracted in 1 ml ice-cooled absolute ethanol, for 15 minutes, with occasional vortexing. After centrifugation (10 min, 14 000g), the supernatant was used for determination of ergosterol, without further purification.

Twenty five µl of the supernatant were separated by HPLC on a Spherisorb S5 ODS2 (4.6 x 250 mm) column (Phase Separations), using pure methanol as eluant. Ergosterol was detected at 280 nm with a UV detector (UV 2000, Spectra Physics).

The ergosterol concentration of *P. tinctorius*, grown in pure culture in Petri dishes, was also determined, and used to convert the ergosterol concentrations measured in roots to fungal dry weight.

An absorbance peak was observed, in all plant samples, which greatly overlapped with the ergosterol peak. This overlapping peak was fairly stable over time, and had little variation between samples. For this reason, and due to difficulties in the separation of the two peaks, its average absorbance in NM samples was subtracted from the absorbance of M samples.

Extraction and determination of enzyme activities

PEPc activity was determined according to Wingler *et al*. (1994). Approximately 10 mg freeze dried material were extracted in 1 ml 100/300 mM Tris-borate buffer, pH 7.6, with 5 mM EDTA, 4 µg ml^{-1} chymostatin, 14 mM mercaptoethanol, 1. µl ml^{-1} Sigma protease inhibitor cocktail II, and 7% insoluble PVPP. After incubation for 10 min on ice, with occasional vortexing, the samples were centrifuged for 10 min at 10,000 g, and the supernatant tested for PEPc activity.

PEPc activity was measured spectrophotometrically by coupling the reaction to NADH oxidation mediated by malate dehydrogenase (MDH; EC 1.1.1.37). The assay mixture contained, in a final volume of 250 µl: 100 µl supernatant, 50 mM Tris-HCl, 15 mM $NaHCO_3$, 5 mM DTT, 0.3 mM NADH, 20 mM $MgCl_2$, 9.6 Uml^{-1} MDH and 20 mM PEP (trisodium salt), with final pH 8. The reaction was initiated by the addition of PEP. The mixture was incubated in microplates, at 30ºC for 5 minutes, to allow temperature adaptation. The change in absorbance at 340 nm (A_{340}) was monitored for 40 min.

For determination of invertase activity, approximately 4 mg freeze dried material were extracted in the same extraction buffer as used for PEPc, but without protease inhibitor. After incubation for 10 minutes on ice, with occasional vortexing, the samples were centrifuged for 10 min at 10,000 g and 4 ºC. The supernatant was tested for soluble acid invertase (AI_s) and neutral invertase activities (NI). No activity peak was found for neutral invertase.

The pellet was washed twice with 1 mL extraction mixture, the supernatant discarded, and the pellet resuspended in 500 µL extraction mixture and tested for insoluble acid invertase (AI_{ins}) activity.

Acid invertase determination was adapted from Egger & Hampp (1993). AI_s was assayed in 80 mM citric acid, 5 mM EDTA, 4 µg ml^{-1} chymostatin and 400 mM sucrose, at pH 4. Ten µl of supernatant were tested in a final volume of 130 µl. The mixture was incubated in microplates, at 30 ºC. After 1 hour, the reaction was stopped by adding 3 µl 5M NaOH to each microplate well, and incubated at 100 ºC for 20 min.

AI_{ins} activity was tested by adding 50 µl of suspended pellet to 600 µl of the same reaction buffer as used for AI_s. The mixture was incubated in Eppendorf microtubes, with periodic agitation. After 1 h, the reaction was stopped by adding 15 µl 5 M NaOH, and heating the microtubes to 95 ºC for 5 min. The tubes were centrifuged for 10 min at 14 000 g, and the supernatant used for glucose quantification.

In both cases, the glucose liberated during incubation was determined as previously described.

Sucrose synthase (SS; E.C.2.4.1.13) activity was measured in the sucrose hydrolysis direction, according to Egger & Hampp (1993). Approximately 10 mg freeze-dried plant material were extracted in 1 ml 50 mM HEPES-KOH buffer, pH 7.6, with 5 mM EDTA, 4 µg ml^{-1} chymostatin, 14 mM mercaptoethanol and 7 % PVPP, for 10 min, on ice, with occasional vortexing, and then centrifuged for 10 min at 10,000 g and 4 ºC. The supernatant was tested for sucrose synthase activity.

Sixty µl extract were incubated in 50 mM Hepes-KOH, pH 7.6, with 600 mM sucrose and 10 mM UDP, in a final volume of 160 µl. The mixture was incubated in microplates, at 30 ºC. After 40 min, the reaction was stopped by heating the plate to 100 ºC for 20 min. The amount of UDP-glucose produced during the incubation was determined in 250 mM glycine-KOH, pH 10, with 2.5 mM NAD and 150 $mUml^{-1}$ UDP-glucose dehydrogenase, in a final volume of 250 µl.

In all cases, three types of blanks were set up: one without extract and substrate, one without substrate, and one without extract. In control experiments, it was checked that addition of fungal material extract did not cause an artificial reduction in enzyme activity. Absorbances were read in a microplate reader spectrophotometer (Tecan Thermo Spectra, Tecan, Switzerland).

Because the insoluble acid invertase activity was determined directly from the pellet, and thus could not be expressed per protein, and because all enzyme activities measured varied in the same way whether they were expressed per protein or per dry weight, all activities are given per dry weight.

Determination of C and N contents and relative N uptake rate (RN)

C and N contents were determined in freeze-dried shoots, roots and fungal mycelium in an elemental analyser Euro EA 3000 (EuroVector CHNS-O Elemental Analyser; Callidus Software Interface-Version 4.1). The quantification was made using the method of external pattern, through linear calibration, using Wheat Flour (OAS) calibrated for NIST patterns as reference material. The separation was made using a gaseous chromatography column, and the detector was a thermal conductivity detector (TCD). The integration of the chromatographic peaks was made using the Callidus software, version 4.1 (EuroVector).

The relative N uptake rate (RN) was calculated using the pairing method and the whole plant N concentrations, according to: $(\ln N_1 - \ln N_0) / (t_1 - t_0)$, where N_0 is the initial plant N content (at time 0; t_0) and N_1 is the plant N content at time 1 (t_1).

Extraction and determination of free amino acids (FAA).

Approximately 60 to 70 mg freeze-dried plant material were extracted in 4 mL of a 1:1 mixture of methanol and 0.5% lithium citrate / HCl, pH 2.3, for 24 h, at 5ºC, with agitation, and then centrifuged for 10 min at 10,000 g. The supernatant was evaporated in order to eliminate the methanol. The aqueous residue was extracted twice in 10 mL ether, frozen, the ether phase discarded, and evaporated. The solid residue was resuspended in lithium buffer (0.7% lithium acetate and 0.6% LiCl; Pickering Laboratories, Mountain View, CA) spiked with 0.2 mM norleucin. The samples were centrifuged 3 min at 14 000 g, and the supernatant filtered through a 0.2 µm Minisart RC 15 filter. Amino acids were separated by HPLC on a cation-exchange column (high efficiency fluid column, 3mm x 150 mm; Pickering Laboratories) using lithium buffer as eluant. Amino acids were derivatized with ninhydrine before photometric detection.

Data analysis

A two-way ANOVA, followed by a Tukey test, was used to test for significant differences in enzyme activities, metabolite and N concentrations, RN, RGR, A and PI_{abs}, at $p<0.05$, between M and NM plants.

A two-way ANCOVA was used to test for the effects of mycorrhization and N supply on root, needles and cotyledon soluble carbohydrates and starch, using the age of the plant as the covariate, at $p<0.05$.

In all cases preliminary analyses were performed to ensure no violation of the assumptions regarding each test. SPSS software, version 13.0, was used for all tests.

All metabolite contents and enzyme activities were expressed per dry weight of plant tissue. For graphic representation, the metabolite contents and enzyme activities were normalized by calculating the ratio between individual mycorrhizal plants (m) and the mean of the corresponding non mycorrhizal control plants (NM): m/NM.

Results

Fungal and plant growth

No statistically significant differences were found in the RGR of root, shoot or whole plant (Table 1).

The *P. tinctorius* isolate used was only able to grow when supplied with glucose as C source.

N concentration and relative N uptake rate (RN)

During the first 14 days following inoculation, M plants that were supplied with more N had higher RN than the corresponding NM plants (Table 2), while there was no difference with less N. Between day 14 and 29 M plants had lower RN than NM plants with both N supplies.

Root N contents were not significantly different in M and NM plants, but the shoot N was higher at day 14 in M plants with both N supplies (Table 2).

Mycorrhizal colonization:

Mycorrhizal colonization started on day 6, when the first signs of mantle formation were observed. Colonization was faster until day 14, and again between day 24 and 29. Between day 14 and 24 no changes were observed in mycorrhizal colonization in plants with less N, while in plants with more N it decreased (Fig. 1). This decrease was a result of fungal dilution due to root growth that was not accompanied by further mycorrhizal colonization (results not shown).

Mycorrhization was lower in plants that were supplied with more N, except on day 12 through 19 (Fig. 1). By the end of the experiment, in a visual assessment of the percentage of colonized root tips, the plants that were supplied with less N were found

to have a minimum of approximately 70% of colonized root tips, while plants supplied with more N were close to 50% colonization.

N supply ($mM\ NH_4^+$)	Mycorrhizal status	RGR		
		Shoot	Root	Whole plant
1.9	NM	1.69 ± 0.32 [a]	3.08 ± 0.53 [a]	2.21 ± 0.29 [a]
	M	1.55 ± 0.26 [a]	2.72 ± 0.54 [a]	2.02 ± 0.13 [a]
3.8	NM	1.58 ± 0.34 [a]	2.59 ± 0.45 [a]	1.96 ± 0.23 [a]
	M	1.23 ± 0.37 [a]	3.44 ± 0.42 [a]	1.96 ± 0.19 [a]
ANOVA effect	Myc	0.860	0.498	0.448
	Nut	0.496	0.098	0.478
	Myc x Nut	0.234	0.326	0.063

Table 1. Root, shoot and whole plant relative growth rate (RGR) of M and NM plants that were supplied with a nutrient solution containing 1.9 mM NH_4^+ or 3.8 mM NH_4^+ as N source. Values are averages ± S.E.. Letters stand for statistically significant differences at each time ($p<0.005$). A two-way ANOVA was used to test for statistically significant differences between activities of M and NM roots, followed by a Tukey test.

	N supply ($mM\ NH_4^+$)	Mycorrhizal status	Day 0	Day 14	Day 29
Shoot N (% DW)	3.8	NM	2.32 ± 0.07	1.67 ± 0.05 [b]	1.77 ± 0.05[a]
		M		2.15 ± 0.04[a]	1.73 ± 0.05[a]
	1.9	NM		1.73 ± 0.05[ab]	1.69 ± 0.05[a]
		M		2.09 ± 0.22[a]	1.43 ± 0.04[b]
RN ($\% d^{-1}$)	3.8	NM		1.22 ± 0.02[b]	2.60 ± 0.45[a]
		M		2.80 ± 0.10[a]	1.10 ± 0.24[b]
	1.9	NM		1.39 ± 0.03[b]	2.34 ± 0.43[a]
		M		1.34 ± 0.28[b]	1.24 ± 0.33[b]

Table 2. Shoot N contents (% DW) and relative nitrogen uptake rate (RN) of M and NM plants that were supplied with a nutrient solution containing 1.9 mM NH_4^+ or 3.8 mM NH_4^+ as N source. N contents were measured at inoculation time (day 0), after 14 and 29 days. RN was calculated for the periods between day 0 and 14 and between day 14 and 29. Values are averages ± S.E.. Letters stand for statistically significant differences at each time ($p<0.005$). A two-way ANOVA was used to test for statistically significant differences between activities of M and NM roots, followed by a Tukey test.

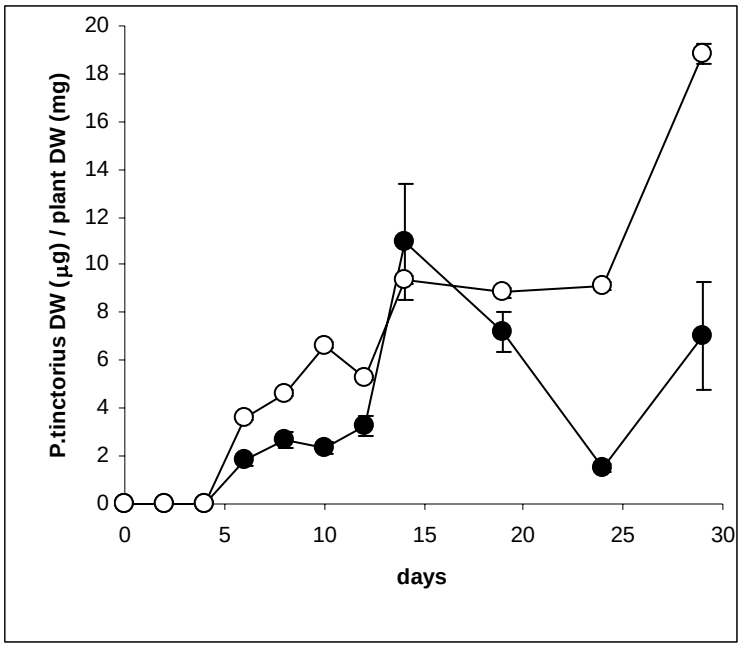


Figure 1. *P. tinctorius* biomass in mycorrhizal whole roots. Fungal biomass was estimated using the mean value of the ergosterol content of *P. tinctorius* mycelia grown in pure culture as a conversion factor (6.74 µg mg^{-1} DW). The plants were fed a nutrient solution containing 1.9 mM NH_4^+ (open circles) or 3.8 mM NH_4^+ (closed circles) as N source. Values are averages ± S.E. (n=4).

Carbohydrates

Glucose and fructose were detected in comparable concentrations in all samples.

A decrease in the shoot soluble carbohydrates contents of M plants was observed, between day 2 and 8, preceding and coinciding with the beginning of infection and mycorrhizal establishment (Fig. 2), and again between day 19 and 29. These decreases were more pronounced in plants that received more N.

Hexose contents of M roots also decreased with the beginning of infection and mycorrhizal establishment (day 6 - 8; Fig. 1 and 3). Two other decreases in root hexoses were observed (Fig. 3), between day 14 and 19, and 24 and 29.

In general, mycorrhization led to lower soluble carbohydrates and starch contents both in the shoot and in the root, independent of the amount of N supplied (Table 3). However, when the plants were supplied with more N, carbohydrates decreased mostly in the shoot, while with less N they decreased mostly in the root (Table 3).

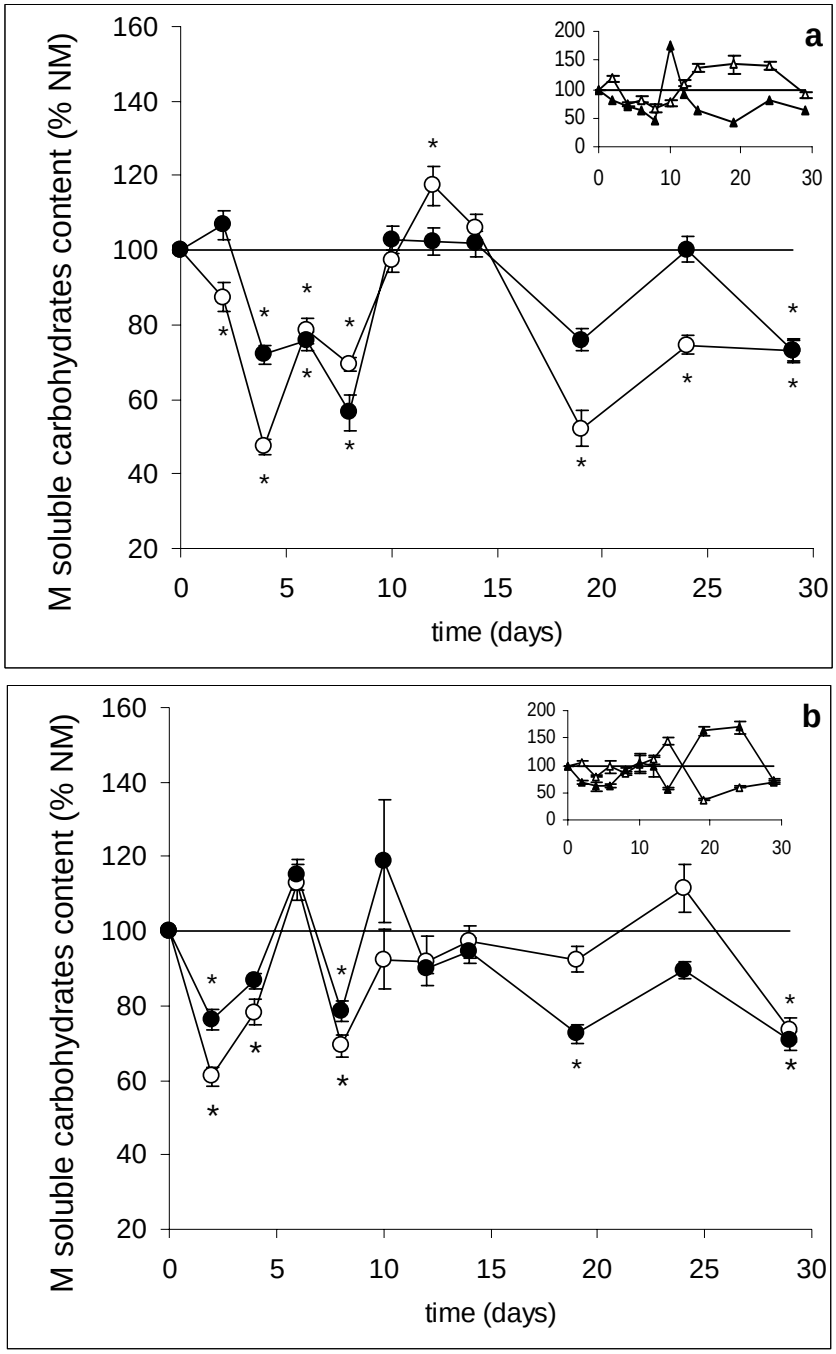


Figure 2. Total soluble carbohydrates in cotyledons (closed circles) and needles (open circles) of M plants as percentages of NM plants. The plants were fed a nutrient solution containing (a) 3.8 mM NH_4^+, or (b) 1.9 mM NH_4^+. Inserts: sucrose (closed triangles) and hexoses (open triangles) in cotyledons of M plants as percentages of NM plants. All values are averages ± S.E.. A two-way ANOVA was used to test for statistically significant differences between M and NM roots, followed by a Tukey test. Asterisks stand for significant differences on each day, at $p<0.05$.

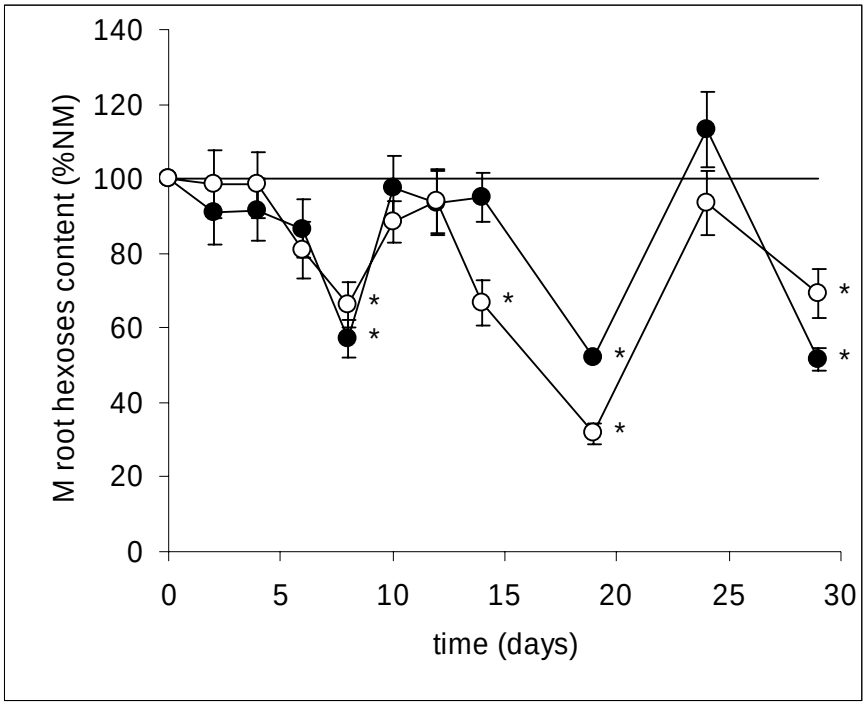


Figure 3. Root hexoses in roots of M plants as a percentage of NM roots. The plants were fed a nutrient solution containing 1.9 mM NH_4^+ (open circles), or 3.8 mM NH_4^+ (closed circles). All values are averages ± S.E.. A two-way ANOVA was used to test for statistically significant differences between activities of M and NM roots, followed by a Tukey test. Asterisks stand for significant differences on each day, at $p<0.05$.

The sucrose concentrations in needles were very low (5 to 12 times lower than hexoses), as were the starch concentrations in roots (approximately 4 to 20 times lower than in needles and cotyledons).

Enzyme activities

No activity of any of the enzymes tested was detected in fungal mycelium.

The root AI_s, AI_{ins} and PEPc activities of M plants simultaneously increased or decreased relatively to the NM control. With less N, the activity of SS varied in the opposite direction to the remaining enzymes (Fig.4).

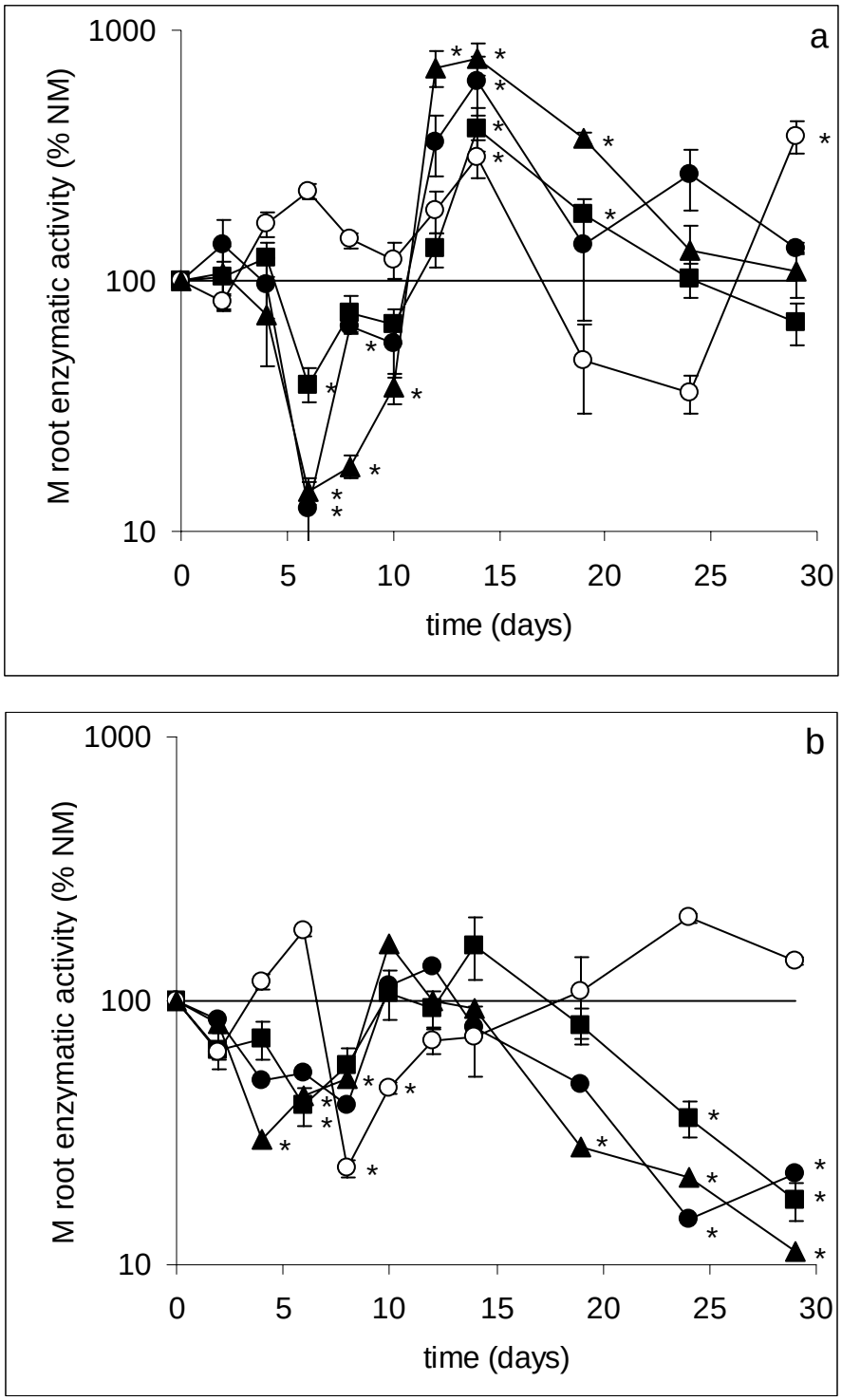


Figure 4. AI_s (closed circles), AI_{ins} (closed squares), PEPc (closed triangles) and SS (open circles) activities in M roots relative to those of NM plants (%NM). The plants were fed a nutrient solution containing (a) 3.8 mM NH_4^+, or (b) 1.9 mM NH_4^+. All values are averages ± S.E.. A two-way ANOVA was used to test for statistically significant differences between activities of M and NM roots, followed by a Tukey test. Asterisks stand for significant differences between M and NM plants on each day, at $p<0.05$.

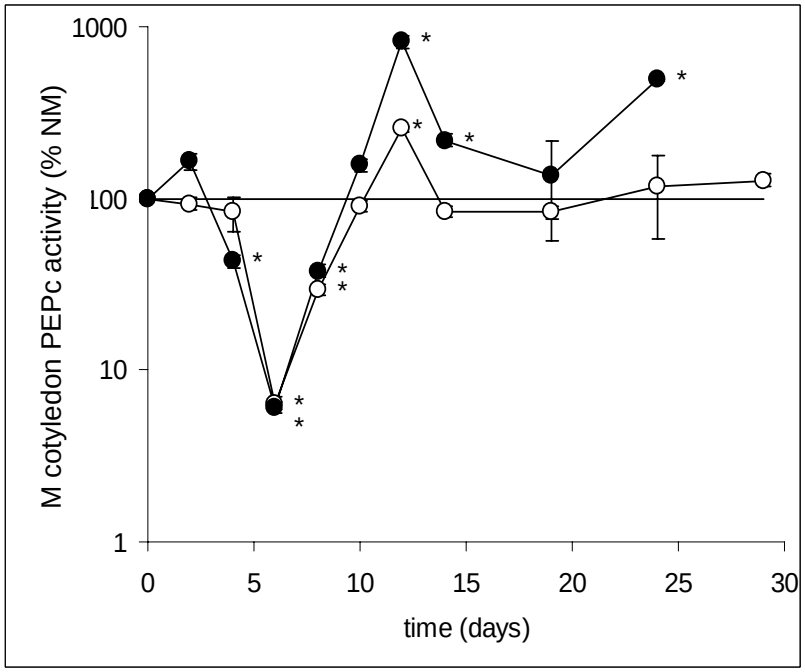


Figure 5. Cotyledon PEPc activity in M plants relative to those of NM plants. The plants were fed a nutrient solution containing 1.9 mM NH_4^+ (open circles), or 3.8 mM NH_4^+ (closed circles). All values are averages ± S.E.. A two-way ANOVA was used to test for statistically significant differences between activities of M and NM roots, followed by a Tukey test. Asterisks stand for significant differences between M and NM plants on each day, at $p<0.05$.

The responses of root enzyme activities to mycorrhization changed in parallel to the root hexoses and shoot soluble carbohydrates contents. With more N, decreases in the activities of root AI_s, AI_{ins} and PEPc were observed between day 6 and 10 that were accompanied by an increase in the activity of SS (Fig. 4a). The invertases and PEPc then started to recover at day 12, accompanied by an increase in SS activity, until they reached higher levels than the ones measured in NM plants, and decreased again at day 14. They remained, however, higher or similar to the ones observed in NM plants. The activity of SS increased again at day 29, once more exhibiting an opposite response to the remaining enzymes.

With less N, the first response of the root enzymes to mycorrhization (day 2-8) were similar to the ones observed with more N (Fig. 4b). The rise in the activities of the root acid invertases and PEPc at day 10, however, was only to similar or slightly higher levels than the ones measured in NM plants. The following decrease, at day 14, was

again to lower levels than in NM plants. Unlike the plants with more N, the activity of SS changed always in the opposite direction of the remaining enzymes.

F26BP

Decreases in the F26BP content of cotyledons and needles were observed, with both N supplies, coinciding with the beginning of infection and mycorrhizal establishment (day 2-8; Fig. 6). After this initial decrease, with more N, the variations in F26BP in the cotyledons continued to follow the same pattern like both the root enzyme activities (except SS) and the shoot carbohydrate contents. They increased between day 10 and 14 to a higher level than in NM plants, and decreased again between day 19 and 29 (Fig. 6a). In the needles of M plants, from day 10 until the end of the experiment, F26BP contents became increasingly higher than those in NM plants (Fig. 6b).

With less N, F26BP contents in both needles and cotyledons remained lower than in NM plants for the majority of the remainder of the experiment (Fig. 6a, b).

N supply ($mM\ NH_4^+$)		Root		Needles		Cotyledons	
		TSC	Starch	TSC	Starch	TSC	Starch
3.8	NM	111.6 ± 11.3	58.2 ± 10.4	175.2 ± 17.4	759.8 ± 165.8	134.0 ± 12.3	906.0 ± 126.1
	M	96.8 ± 10.9	52.1 ± 9.0	148.2 ± 17.7	392.9 ± 78.5	113.2 ± 8.5	636.2 ± 116.7
1.9	NM	130.0 ± 18.6	69.9 ± 14.3	165.6 ± 14.3	713.8 ± 119.3	138.1 ± 12.7	942.7 ± 140.0
	M	103.4 ± 9.6	58.1 ± 10.8	148.0 ± 12.5	626.7 ± 102.5	121.6 ± 12.4	957.7 ± 124.5
ANCOVA effect	M	< 0.001	0.103	< 0.001	< 0.001	< 0.001	0.002
	N	0.003	0.120	0.246	< 0.001	0.057	< 0.001
	M x N	0.384	0.835	0.267	< 0.001	0.519	0.001
	Time	0.019	0.160	< 0.001	< 0.001	< 0.001	0.334

Table 3. Total soluble carbohydrates (TSC) and starch contents (nmol hexose units $mg^{-1}DW$) in roots, needles and cotyledons of M and NM plants. The plants were fed a nutrient solution containing 1.9 mM NH_4^+ or 3.8 mM NH_4^+. Values are averages of all the sampling times ± S.E.. A two-way ANCOVA was used to test for the effects of mycorrhization (M) and N supply (N), with time as covariate.

In cotyledons, the PEPc activity responded to mycorrhization in a similar manner as the PEPc and acid invertases in roots, with both N supplies, while no significant differences were observed in needle PEPc (Fig. 5). The activity of PEPc in cotyledons (1.10 to 34.82 pmol min^{-1} mg dw^{-1}) and needles (0.68 to 52.86 pmol min^{-1} mg dw^{-1}) was approximately 10 times lower than in roots (7.16 to 527.53 pmol min^{-1} mg dw^{-1}).

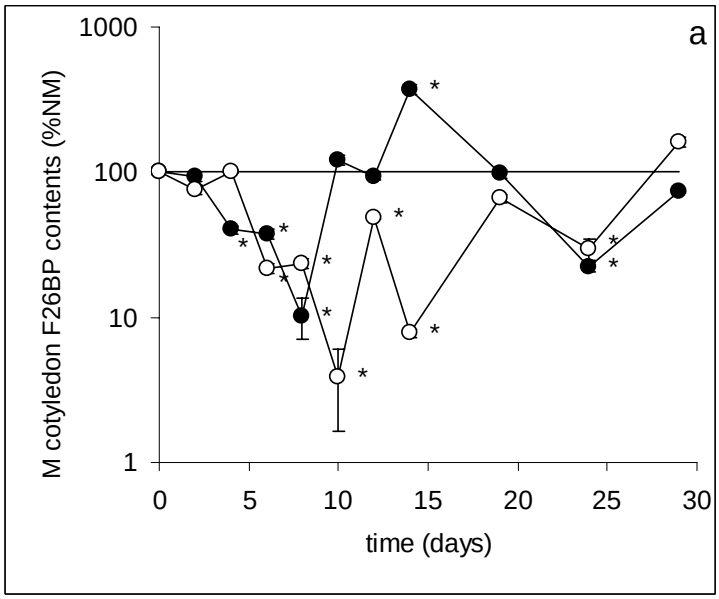
The only significant difference in root F26BP was on day 12, when there was a decrease in M plants, with either nutrition (Fig. 6c).

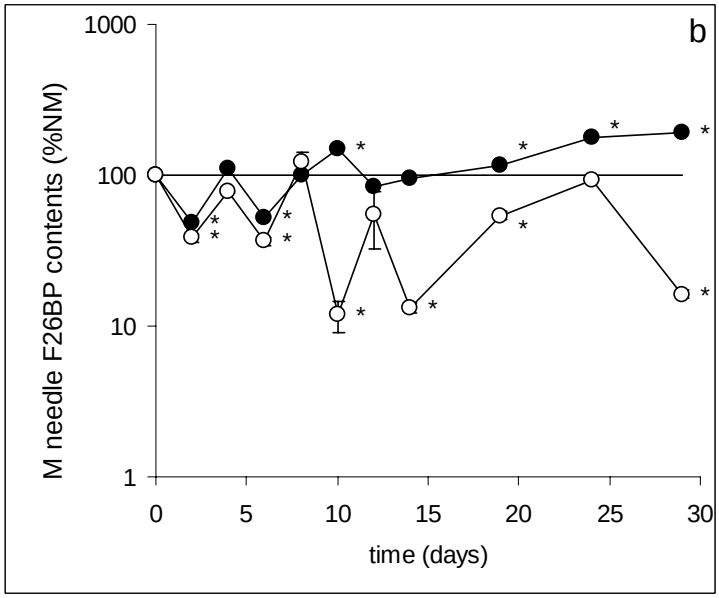
Amino Acids

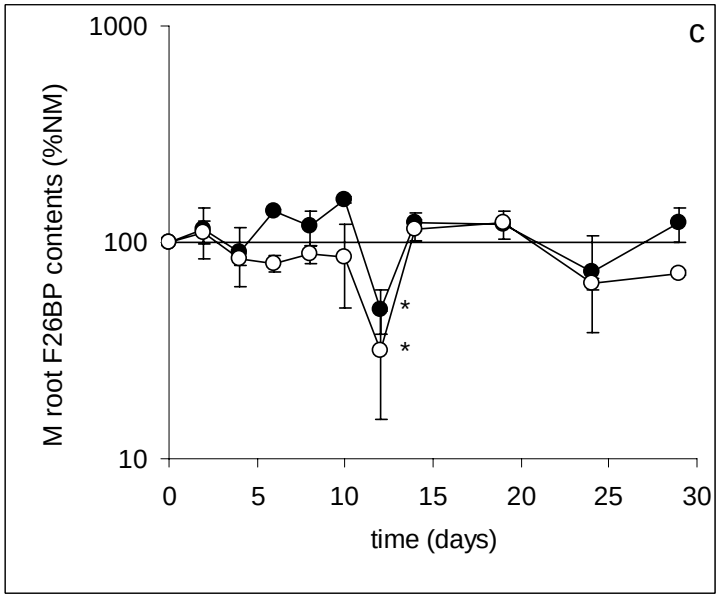
Significantly higher free amino acid (FAA) contents were observed in mycorrhizal roots on day 2, for the plants that were supplied more N, or day 6 and 29, for the ones that were supplied less (Fig. 7). This was a result of a general increase in most of the FAA detected, and therefore did not result in changes in their relative abundance (Fig. 8). On day 12 the FAA contents of mycorrhizal roots were significantly lower with both N supplies, and this was still the case on day 14 for the plants that were supplied less N (Fig. 7). This was due to a decrease in a group of 6 major amino acids: serine, asparagine, aspartate, glutamate, glutamine, and threonine. As a result, the relative abundance of all other amino acids increased (Fig. 8). In M plants that were fed more N, the glycine concentration was increased at day 12, but otherwise remained very low (Fig. 8b).

Asparagine contents were higher at the beginning of the experiment, amounting to approximately 30% of the total FAA, but progressively decreased during the first half of the experiment. From day 12 until the end of the experiment its levels stabilized, varying between 3 and 6% of the total FAA (Fig. 8).

Figure 6. F26BP contents in (a) cotyledons, (b) needles and c) roots, of M plants' as a percentage of NM plants. The plants were fed a nutrient solution containing 1.9 mM NH_4^+ (open circles) or 3.8 mM NH_4^+ (closed circles). All values are averages ± S.E.. A two-way ANOVA was performed to test for statistically significant differences between activities of M and NM roots, followed by a Tukey test. Asterisks stand for significant differences between M and NM plants on each day, at $p<0.05$.


a
1000
100
10
1
M cotyledon F26BP contents (%NM)
0
5
10
15
20
25
30
time (days)


b
1000
100
10
1
M needle F26BP contents (%NM)
0
5
10
15
20
25
30
time (days)


c
1000
100
10
M root F26BP contents (%NM)
0
5
10
15
20
25
30
time (days)

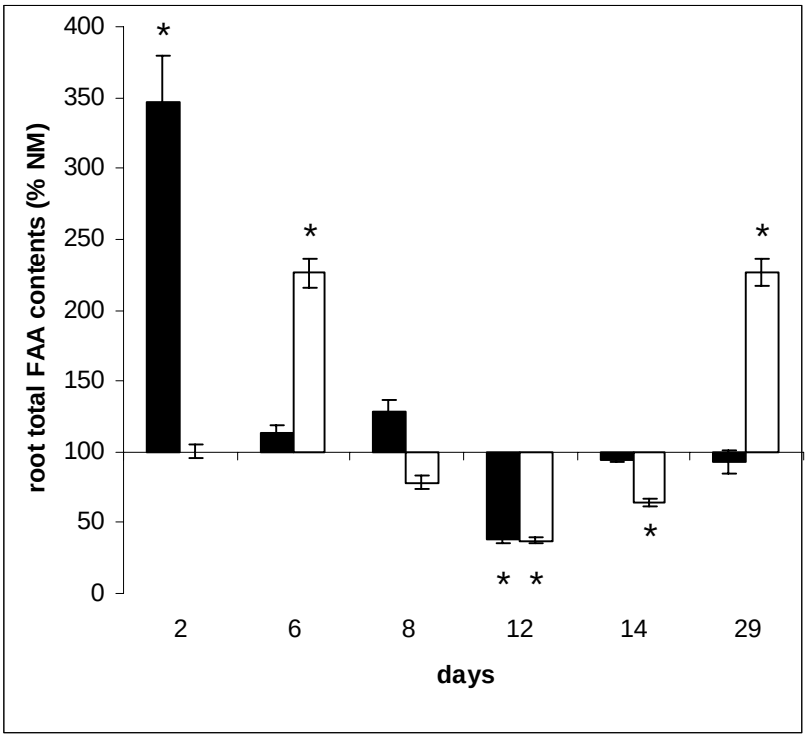


Figure 7. Total free amino acids (FAA) in M plants' roots, as a percentage of NM plants. The plants were fed a nutrient solution containing 1.9 mM NH_4^+ (white bars) or 3.8 mM NH_4^+ (black bars). All values are averages ± S.E.. A two-way ANOVA was performed to test for statistically significant differences between activities of M and NM roots, followed by a Tukey test. Asterisks stand for significant differences between M and NM plants on each day, at $p<0.05$.

Net photosynthesis rate and photosynthetic performance

Mycorrhization did not have a significant effect on the rate of net photosynthesis (Table 4).

Photosynthetic performance of M plants, as indicated by PI_{abs}, was significantly lower than that of NM plants when the plants were supplied with less N, starting on day 6 and until the end of the experiment. No difference was found between M and NM plants with more N (Table 4).

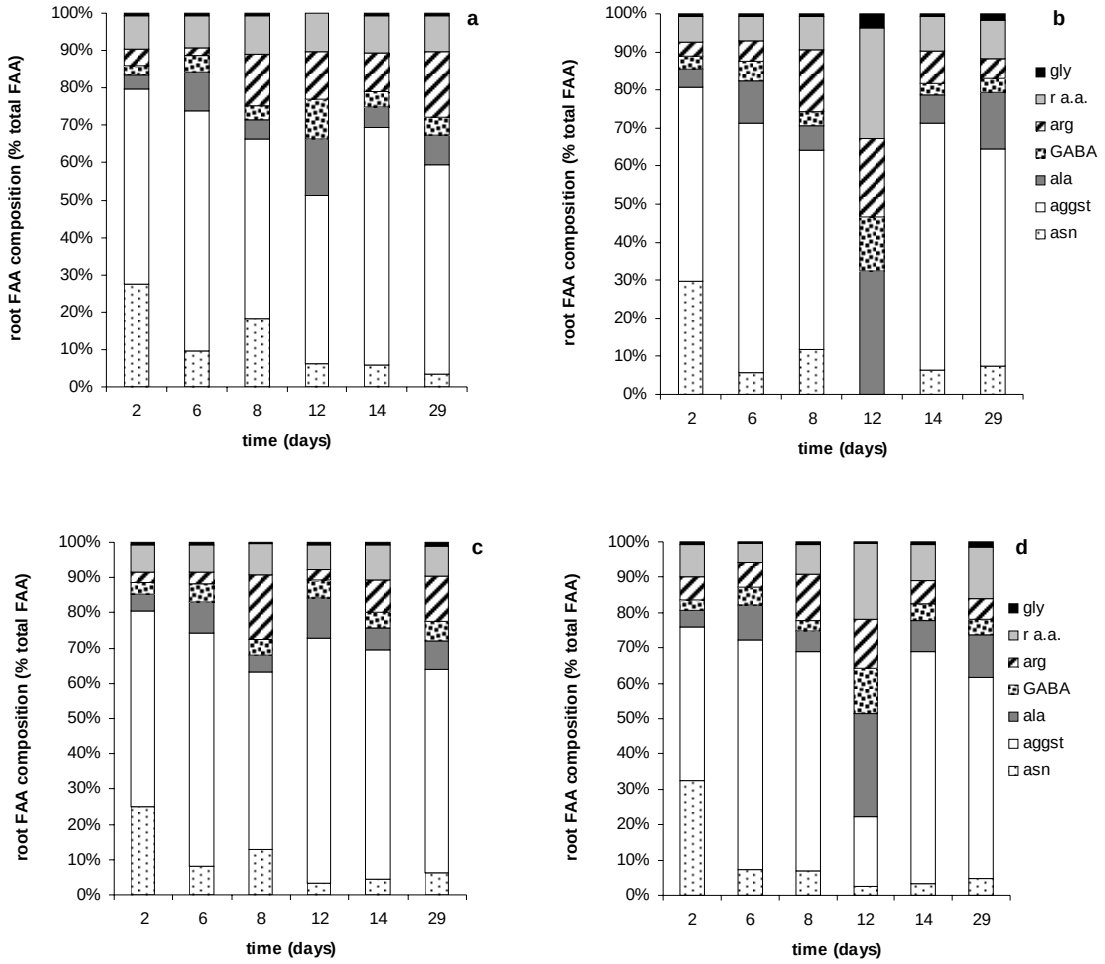


Figure 8. Relative composition of the root free amino acid (FAA) pool, as percentages of the total FAA. The plants were supplied with 3.8 mM NH_4^+ or 1.9 mM NH_4^+ as N source, and were either mycorrhizal (M) or non-mycorrhizal controls (NM). a) NM with 3.8 mM NH_4^+; b) M with 3.8 mM NH_4^+; c) NM with 1.9 mM NH_4^+; b) M with 1.9 mM NH_4^+. asn: asparagine; aggst: glutamate, glutamine, aspartate, serine and threonine; ala: alanine; arg: arginine; gly: glycine; r.a.a.: remaining detected amino acids.

	N supply (mM NH_4^+)	Mycorrhizal status	Day 2	Day 6	Day 8	Day 14	Day 29
A (μmol $CO_2 m^{-2} s^{-1}$)	3.8	NM	3.2 ± 1.0 [a]	3.9 ± 0.5[a]	5.2 ± 0.5[a]	7.3 ± 0.4[a]	7.3 ± 0.5[a]
		M	3. 9± 0.3[a]	3.9 ± 0.4[a]	4.4 ± 0.4[a]	7.0 ± 0.6[a]	9.6 ± 0.8[a]
	1.9	NM	4.5 ± 1.2[a]	3.8 ± 0.4[a]	5.7 ± 0.6[a]	6.9 ± 0.5[a]	7.0 ± 0.3[a]
		M	4.1 ± 0.4[a]	4.4 ± 0.5[a]	6.8 ± 0.5[a]	7.7 ± 0.5[a]	8.4 ± 0.6[a]
PI_{abs}	3.8	NM	18.3 ± 0.8 [a]	18.3 ± 0.7[a]	18.4 ± 0.7[a]	19.4 ± 0.5[a]	18.7 ± 1.0[a]
		M	19.0 ± 1.3[a]	15.9 ± 0.8[b]	18.2 ± 1.0[a]	18.3 ± 0.2[a]	17.7 ± 1.2[a]
	1.9	NM	16.1 ± 0.8[a]	19.4 ± 0.7[a]	21.5± 0.8[a]	22.5 ± 0.4[a]	18.7 ± 1.1[a]
		M	16.1 ± 0.7[a]	14.3 ± 0.7[b]	15.0± 0.7[b]	15.2 ± 0.3[b]	13.8 ± 1.1[b]

Table 4. Net photosynthesis rate (A) and the performance index (PI_{abs}) of M and NM plants that were supplied with a nutrient solution containing 1.9 mM NH_4^+ or 3.8 mM NH_4^+ as N source. Values are averages ± S.E.. Letters stand for statistically significant differences at each time ($p<0.005$). A two-way ANOVA was used to test for statistically significant differences between activities of M and NM roots, followed by a Tukey test.

Discussion

The response to mycorrhizal colonization observed in this study varied in time, and three phases could always be distinguished regardless of the amount of N the plants were receiving.

Between day 2 and 10, mycorrhizal establishment led first to a decrease in the soluble carbohydrate and F26BP contents at the shoot (needles and cotyledons) (Fig. 2, 6a, b). F26BP is a regulator molecule in the photosynthetic carbon partitioning between glycolysis and gluconeogenesis (Stitt, 1990; Lee *et al*., 2006). Its decrease indicates increased sucrose production and C allocation to the root, which resulted in lower carbohydrate contents at the shoot. This is in accordance with numerous previous findings of increased C allocation to the root in ECM plants (e.g.: Wright *et al*., 2000; Wu *et al*., 2002). Decreased F26BP contents in cotyledons and leaves of mycorrhizal spruce and aspen, respectively, accompanied by other indications of increased sucrose production (higher SPS activation, lower sucrolytic activity) were previously reported (Loewe *et al*., 2000).

The decrease in F26BP was more pronounced in cotyledons than in needles (Fig. 6a, b). The cotyledons were the main source organs in these seedlings, while the young needles were likely to be stronger sinks than sources. The seedlings used were inoculated when the first set of needles reached half the cotyledon length, and were therefore still in expansion. This was also indicated by their very low sucrose contents, which may have been due to the use of hexoses as osmolytes during needle expansion (Sturm, 1999), and/or to a higher rate of glycolysis.

The decrease in F26BP and carbohydrates in the shoot were observed before mycorrhizal formation. This supports the notion that both signalling processes and responses at the gene expression and physiological levels start in the pre-symbiotic stage (Martin *et al*., 2001; Duplessis *et al*., 2005; Frettinger *et al*., 2007; Herrmann & Buscot, 2007).

Following these changes at the shoot, a drop in the root hexoses contents (Fig. 3) and an increase in SS activity (Fig. 4) were observed in M roots that were also consistent with increased root sink strength. A role for SS in phloem unloading was suggested in several studies, namely in pine roots and needles (Schaeffer *et al*., 1995; Sung *et al*., 1996), and it has been suggested that its activity can be directly used as a marker for sink strength (Sebková *et al*., 1995). This higher root sink strength could be either due to activation of the root metabolism or to fungal consumption of carbohydrates. The simultaneous decrease in the activities of AI_s, AI_{ins} and PEPc, together with the increase in the free amino acid content (Fig. 4, 7), indicate a deactivation of the root metabolism instead of activation (Simon-Sarkadi *et al*., 2006). This is in agreement with several previous studies (Blaudez *et al*., 2001; Johansson *et al*., 2004; Duplessis *et al*., 2005; Frettinger *et al*., 2007; Herrmann & Buscot, 2007). In some cases, this has been considered a consequence of a metabolic shift, with some metabolic functions of the root being taken over by the fungus (Vézina *et al*., 1989; Johansson *et al*., 2004). Namely, decreased PEPc activity and amount of protein in mycorrhizal spruce were considered to indicate a switch of N assimilation from the plant to the fungus (Wingler *et al*., 1996). However, in the present case the decreased activity of cotyledon PEPc indicated this metabolic down-regulation to be a whole plant, as opposed to localized, response. Furthermore, such a metabolic shift to the fungus was unlikely in these early stages of mycorrhizal establishment, since the symbiotic interface was either not fully formed or very limited. This deactivation may thus have been a consequence of increased C allocation to the rhizosphere. The root's

exudates have been considered as interesting candidates for C transfer to the fungal partner (Hampp *et al*., 1999).

ECM fungi have so far been found to have no sucrolytic capacity (Salzer & Hager, 1991; Schaeffer *et al*., 1995; Nehls *et al*., 2007), and this was also the case of the *P. tinctorius* isolate used in the present study. The activity of the host plant's sucrolytic enzymes has, for this reason, been hypothesized to be essential for the cleavage of sucrose and supply of hexoses to the fungal partner. In particular, cell-wall bound AI is involved in this (Salzer & Hager, 1991; Schaeffer *et al*., 1995; Nehls *et al*., 2007), and could therefore be expected to increase in M plants. In AM interactions this has been observed repeatedly (e.g.: Blee & Anderson, 2002; Shaarschmidt *et al*., 2006), but not in ECM (Schaeffer *et al*., 1995; Wright *et al*., 2000). In the present investigation, the activities of AI_s and AI_{ins} decreased, in contrast to SS (Fig. 4).

Following the initial reaction, the response started to differ depending on the amount of N the plants received. With more N all sugar contents recovered to similar values to NM plants, between day 10 and 12 (Fig. 2, 3). This seemed to reflect a decrease in belowground C demand and allocation, since the F26BP contents of both cotyledons and needles increased to similar or higher levels than in NM plants (Fig. 6a, b). In parallel, all M root enzyme activities increased (Fig. 4a), indicating an activation of the M root metabolism, as did the decreased pool of root FAA observed on day 12 (Fig. 7). This decrease was due exclusively to a group of 6 amino acids (glutamine, glutamate, asparagine, aspartate, serine and threonine), all amino group donors, which is consistent with an acceleration of metabolism, and remobilization of protein N (Fig. 8). The large increase in glycine content observed at this time further indicates an increased importance of protein degradation in these plants (Bourguignon *et al.*, 1999). Mycorrhization has been previously found to change the composition of the free amino acid pool of the host root, but this effect varied from study to study (Vézina *et al*., 1989; Näsholm *et al*., 1991; Plassard *et al*., 2000; Blaudez *et al*., 2001). Interestingly, this N remobilization took place even though mycorrhization improved the N uptake of M plants (Table 2), which probably started to take place during this second phase of the response. Increased PEPc activities and F26BP contents in both needles and roots have been found in spruce upon improved N supply (Wingler *et al*., 1994).

Also on day 12, the only significant decrease in M root F26BP was observed (Fig. 6c), indicating sucrose production and C allocation to the shoot, likely using the C skeletons resulting from amino acid and protein degradation. M plants seemed therefore

to be investing more into the shoot than NM plants, which was reflected in the increased activity of cotyledon PEPc and shoot N contents (Fig. 5; Table 2).

Between day 14 and 19, root and shoot soluble carbohydrates, root enzymes activities, and cotyledon F26BP again became lower than in NM plants (Figs. 2, 3, 4, 6a), indicating a new increase in root C demand and deactivation of M plant metabolism. This time, however, this seemed to be less demanding to the plant, since the enzyme activities did not drop to lower levels than the ones measured for NM plants, except on day 29. Moreover, it did not result in increased sucrose production and export from the needles (Fig. 6b), indicating an adaptation to the increased belowground demand. Furthermore, the drop in TSC in this second stage was exclusively due to a drop in hexoses, but not of sucrose (Fig. 2a).

This coincided with a period of decreased mycorrhization (Fig. 1), indicating that the increased C being allocated to the root was not being used to establish more mycorrhizae. It may, however, have been channelled towards the growth of extra-matrical mycelia, and substrate colonization, which is an important sink for the C allocated belowground (Wu *et al*., 2002). A period of stronger investment in extra-matrical growth could be triggered by the need of reaching new N sources, once the ones available become depleted or insufficient to fulfil the plants' and fungal needs. Such a situation would be in agreement with the fact that, from day 14 until the end of the experiment, mycorrhization resulted in decreased N uptake (Table 3) (Colpaert *et al*., 1992; Colpaert *et al*., 1996). Furthermore, the decreasing asparagine contents until day 12 indicates a decrease in the contribution of cotyledon derived N reserves, and therefore increased plant dependence on the N taken from the medium, and on the mycorrhizal contribution to this uptake. Asparagine formation in high quantities is a consequence of the remobilization of cotyledon N reserves in pine seedlings (Cañas *et al*., 2006). Colonization did increase again between days 24 and 29, coinciding with a new decrease in root and shoot soluble carbohydrates, and an increase in SS activity (Fig. 1, 2, 3).

With less N, a similar pattern of changes was observed. There were, however, some differences. Contrary to the plants with higher N supply, the F26BP contents of cotyledons and needles remained lower in M plants until the end of the experiment (Fig. 6a, b), indicating that the increased belowground C allocation was both constant and stronger with less N. This was also indicated by the fact that the third phase in the response of enzyme activities was identical to the initial one (Fig. 4b), and that therefore

the belowground C demand resulted in a more severe metabolic restriction. This can be explained by the higher levels of colonization observed in these plants, demanding more C for fungal growth and maintenance. The degree of mycorrhization is known to be closely and negatively correlated with N availability (Wallenda & Kottke, 1998; Treseder, 2004), and negative responses to mycorrhization have been proposed to depend on the degree of mycorrhizal colonization (Gange & Ayres, 1999), and were observed in nutrient deficient plants (Colpaert *et al.*, 1999; Hobie, 2006). In this case, the recovery in carbohydrate contents observed at day 10 must have therefore been due to an adaptation to the increased belowground C demand. This was not achieved through increased carbohydrate production, since there was no significant change in the photosynthetic rate (Table 4).

The metabolic activation in M plants observed between day 10 and 12 was slighter with less N, with the increases in root enzyme activities and decrease in root FAA contents being less pronounced (Fig. 4, 7). This was likely due to the fact that, in this case, mycorrhization did not result in increased N uptake (Table 2). However, the same indications of increased N and C mobilization towards the shoot were observed as with more N, indicating that this was independent of the mycorrhizal effect on N uptake (Fig. 7, 8).

Overall, although the effects of mycorrhization were similar in plants receiving more and less N, there were differences that could be explained both by the fact that mycorrhization only resulted in improved N uptake with more N, and that plants receiving less N were forced to invest more in fungal growth and maintenance. All indications of metabolic activation due to mycorrhization were stronger in M plants receiving more N, while all signs of mycorrhizae constituting increased C sinks, and of metabolic deactivations, were more pronounced in M plants with less N. This resulted in mycorrhizae being advantageous to plants receiving more N, while with less N they were only able to balance fungal demands, which was reflected in decreased photosynthetic performance (PI_{abs}) (Table 3). This was also reflected by the fact that the lower carbohydrate contents of M plants were mostly observed at the shoot with more N, and at the root with less (Table 4), indicating that mycorrhization resulted in a higher investment in the shoot with more N, and in the root with less N.

Conclusions

Unlike previous reports (Vézina *et al*., 1989; Chalot *et al*., 1991; Botton & Dell, 1994), the changes in enzyme activities and FAA profiles did not indicate qualitative alterations of N or C metabolism in mycorrhizal roots, but only quantitative ones.

Mycorrhization resulted in a higher C investment into the root, but with more N this was transient, limited to the beginning of the establishment and to periods of increased colonization/fungal growth. This alternated with periods of decreased belowground C allocation when compared to NM plants. In plants receiving less N the increased belowground C demand was constant, indicating that they could not gain from the symbiosis.

The present work gives evidence that fungal growth and root colonization rates may not be constant with time, but alternate between faster and slower periods, corresponding to periods of higher or lesser C and N demand by the fungus. As a consequence, transient and alternate activations and deactivations of the host plant metabolism take place, which seem to be due to temporary shifts in resource allocation to the fungus. Similar temporary shifts in fluxes of P and C have been previously observed, and suggested to be related to different fungal growth (Jones *et al*., 1991).

In Fig. 9 we propose a scheme for the dynamics of such a response. During mycorrhizal establishment all resources are allocated belowground, regardless of the N supply (Fig. 9a). After this, the response starts to vary, according to the N supply and the rate of the fungal growth: i) when N is high and fungal growth is low, mycorrhization results in improved N uptake and most of the resources are channelled to the shoot (Fig. 9b); ii) when N is low mycorrhization does not result in improved N uptake and most of the resources are channelled to the root, but if the fungal growth is low there is also a return from the root to the shoot (Fig. 9c); iii) when fungal growth is high, most of the resources are channelled towards the root and the fungal partner, and this channelling can be more or less extreme depending on the N supply (Fig. 9d, e).

It is possible that this “flip-flop” effect will only take place during the earlier stages of mycorrhizal establishment and that at later stages there is a more continuous effect of mycorrhization. This, however, has still to be investigated.

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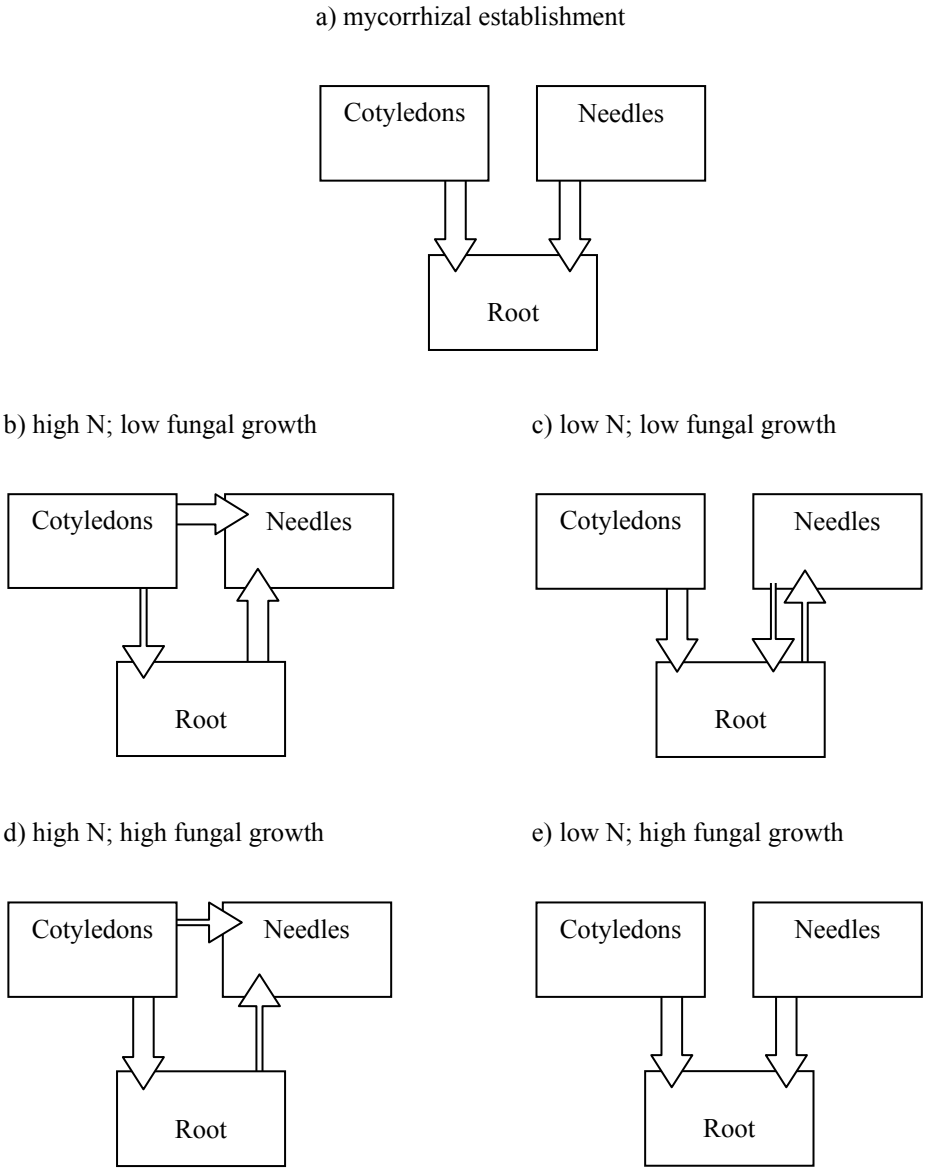

Figure 9. Scheme for the resource allocation dynamics in an ectomycorrhizal symbiosis. a) During mycorrhizal establishment all resources are allocated belowground, regardless of the N supply. Following establishment resource allocation varies with the N supply and the rate of the fungal growth: b) when N is high and fungal growth is low, most of the resources are channelled to the shoot; c) when N is low and fungal growth is low, most of the resources are channelled to the root, but there is also return from the root to the shoot; d, e) when fungal growth is high, most of the resources are channelled towards the root and the fungal partner, which is more or less pronounced depending on the N supply.

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CHAPTER 6

General discussion

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In this work the effects of mycorrhization over the plants productivity were tested in conditions that went from severely N limited to near optimum N supply. In these conditions the plants response to mycorrhization was consistently found to be determined by its effect on the plants' N uptake (chapter 2, 3, 4). This is in contrast to what had been proposed so far.

The outcome of a mycorrhizal relationship to the host plant is expected to depend on the balance between the C cost and the nutrient gain deriving from the association (Jonhson *et al.*, 1997; Schwartz and Hoeksema, 1998; Tuomi *et al.*, 2001; Neuhauser and Fargione, 2004). Negative growth responses to mycorrhization have consistently been considered a result of excessive fungal C drain (Colpaert *et al.*, 1996; Conjeaud *et al.*, 1996; Eltrop and Marschner, 1996). However it is also thought that under nutrient poor conditions the balance between C cost and N gain is positive, benefiting mycorrhizal over non-mycorrhizal plants because of their superior cost efficiency (amount of nutrients acquired per C expended) (Shwartz and Hoeksema, 1998; Tuomi *et al.*, 2001). The results of the present thesis, however, indicate that in N limited conditions the cost efficiency of its acquisition has only a limited, if any, importance in the outcome of the symbiosis because C is in excess, as it cannot be used in growth. Rather than the balance between C cost and N gain it is the balance between the N supply by the fungus and the N demand by the plant that becomes determinant. The new index proposed in this thesis, the mycorrhizal N demand/supply ratio (MDS_N), which considers this balance to vary with the mycorrhizal degree and the N availability (RAR_N) was found to accurately explain how this balance changes (chapter 3).

The results of the present thesis are in agreement with the idea that under low nutrient supply growth is more limited by nutrients than by C supply. Carbohydrates accumulate in plant organs, and it becomes more advantageous for plants to allocate more photosynthetic C belowground, and to the mutualistic partner, if by doing so they can acquire more of the nutrients they need (Kiers *et al.*, 2006). Furthermore, under nutrient limited conditions the accumulation of carbohydrates can lead to a down regulation of genes and enzymes responsible for photosynthesis, and the investment in the mycorrhizal symbiosis could even enhance the hosts' photosynthetic capacity (Kiers *et al.*, 2006). It is also in agreement with the suggestion that mycorrhizal associations

are based on the exchange of excess resources, or by-product benefits (Brundrett, 2002; Kiers *et al*., 2006). If this is the case, they could be more evolutionary stable, since there are fewer net investment costs incurred by the partners (Kiers *et al*., 2006), and the species imperative for cheating would not be a factor, as it would not be more advantageous.

During the first month following inoculation a limitation to growth by a factor other than N was observed, in plants that were older at the time of inoculation (chapter 2). This limitation, however, also exhibited a dependency on the N availability and effects of mycorrhization on N uptake (chapter 2). In the tested conditions, therefore, although particular circumstances may arise where C may have an additional growth limiting effect, it is the N retention or facilitation by the mycorrhizal fungus that ultimately determines the effect of mycorrhization on the host plants' growth (chapter 3).

Changes in the plants C and N metabolism could also result in different energetic balances with and without mycorrhizae, and determine an advantage or disadvantage in mycorrhization. Changes in the FAA profile and in enzyme expression and activities (Vézina *et al*., 1989; Chalot *et al*., 1991; Botton and Dell, 1994) indicate that the N metabolism is altered and readjusted in mycorrhizal roots, and this can have consequences to the C budget of the host plant. Evidences indicating that ECM symbioses allow plants to slow-down regulative pathways, and that at least part of the metabolic expenses may be transferred to the fungus, have been obtained in a number of studies (Wingler *et al.*, 1996; Blaudez *et al*., 2001; Johansson *et al*., 2004; Duplessis *et al*., 2005; Frettinger *et al*., 2007; Herrmann and Buscot, 2007). The group of plants that were inoculated younger was chosen to investigate this hypothesis because the plants supplied with less N in this group were the only ones where a loss in photosynthetic efficiency, and therefore plant vitality, was observed (chapter 1).

The present work indicates that fungal growth and root colonization rates may not be constant in time, but alternate between faster and slower periods, corresponding to periods of higher or lesser demand by the fungus of both C and N (chapter 4). These fluctuations are most likely a consequence of alternate periods of sufficient resources available with periods when further growth investment is necessary to reach new nutrient sources. As a result, temporary shifts in resource allocation to the fungus take place, instead of permanent metabolic shifts, resulting in transient deactivations of the host plant metabolism (chapter 4). Unlike previous reports (Vézina *et al.*, 1989; Chalot

et al., 1991; Botton and Dell, 1994), no qualitative alterations of N or C metabolism in mycorrhizal roots were observed, but only quantitative ones (chapter 4).

The outcome to the plant, in terms of productivity or vitality, was, however, not dependent on these shifts, but again on the N supply and the effect of mycorrhization over the N gain. Mycorrhization only resulted in an advantage for the host plant when supplied more N (decreased belowground C allocation and increased N gain; chapter 4), while with less N the increased belowground C demand was constant and the N uptake lower, leading to a negative effect on photosynthetic performance (chapter 2, 4).

It is important to notice that the situations of mycorrhizal disadvantage observed in this thesis were at least partly a consequence of the fact that the resources needed to support two organisms are larger than to support just one. This situation will likely only arise in laboratory conditions, since in field conditions the plant will coinhabit with numerous other organisms, and this factor will likely loose importance. Although the experimental approach adopted in the present work is widely used in mycorrhizal research, this is a question as a rule not considered by researchers.

The possibility of negative effects of mycorrhization on the plants productivity have led some authors to question the nature of the interaction (Johnson *et al*., 1997; Egger and Hibbet, 2004; Neuhauser and Fargione, 2004; de Mazancourt *et al*., 2005), and to consider the hypothesis that it may become a parasitism depending on the conditions.

This consideration, however, has several problems, some of which have been previously reviewed (Johnson *et al*., 1997; Jones and Smith, 2004):

i) The parameters used to evaluate benefit or detriment may not be the most adequate. The plant's photosynthetic performance was used in the present thesis as an indicator of plant vitality/stress, and was not reliably correlated with mycorrhizal effects on growth or N status (chapter 2 and 3), although mycorrhization may, in some instances, lower N status resulting in lower photosynthetic performance (chapter 2). This confirmed that neither growth nor the nutritional status are good indicators of stress or plant fitness, although they are generally used in the evaluation of the mycorrhizal effects (Johnson *et al.*, 1997; Jones and Smith, 2004).

ii) The observation time window is critical. In chapter 4 an alternation between periods of increased investment in fungal growth and of improved N uptake leading to

increased investment in the plant was observed, demonstrating that the time window chosen to observe a system is crucial. Furthermore, those results showed that investing more in fungal growth at one particular moment is not necessarily a disadvantage in the long term.

iii) Mycorrhization may be mostly helpful in periods of environmental stress to the plant (Tsimilli-Michael and Strasser, 2002; Jones and Smith, 2004), and this may be crucial in determining higher survival. In the present work mycorrhization was observed to have a buffer effect towards stress, helping the plant to recover from situations of mechanical and nutritional stresses, regardless of its effects on plant growth or nutrition (chapter 1).

The consideration that mycorrhizae may not be mutualisms, and may even become parasitisms, however, has additional problems.

The vast majority of the works focus on the plant, while the consequences to the fungus are generally overlooked. The evaluation of whether mycorrhizae are, or not, always mutualisms would require that the cost/benefit balance of the two sides were taken into account, since parasitism would imply that the fungus thrives at the expense of the plant. In particular, in situations of nutrient limitation the investment in fungal growth in detriment of the plant growth is more likely the best strategy available to the plant to reach new N sources, and should not be assumed to be an evidence of fungal parasitism. Furthermore, it has been considered more likely for the plants to exploit mycorrhizal fungi than for them to be exploited (Brundrett, 2002).

Although there are doubts about how much control over the development of the symbiosis is held by the plant, it seems undeniable that it can, at least partially, control the degree of the mycorrhization, making parasitism unlikely. Reduction in mycorrhizal colonization in conditions where mycorrhizae provide little or no nutritional benefit has been repeatedly observed (Wallenda and Kottke, 1998; Miller *et al*., 2002). In the present work, this was indicated by the strong correlation between mycorrhizal degree and the shoot N concentration (chapter 3). Furthermore, the ecological persistence of the mycorrhizal symbioses implies that some mechanisms exist that allow for the selection of the more strongly mutualist fungi by the plant, in detriment of weakly beneficial mutualists or cheaters (Hoeksema and Kummel, 2003). This could be achieved through sanctions, similarly to what happens in the legume-rhizobia interactions (West *et al*.,

2002). Namely, host plants may inhibit colonization, promote hyphal digestion (Kiers *et al*., 2006), reduce the C supply (Nehls *et al.*, 2007) or enhance the mortality of root tips colonized by less mutualistic fungi (Hoeksema and Kummel, 2003).

In addition, the experimental approach most often used in mycorrhizal research is not adequate to conclude whether or not mycorrhizae are mutualisms (de Mazancourt *et al*., 2005). Classically, the performance of a species before and after its partner has been removed, or has been kept at low density, is evaluated. This comparison defines what is called the proximate response of a species to the removal of its partner, and measurement of such a response is the criterion for identifying a proximate mutualism, characterized by negative proximate responses of each species to the removal of the other species (de Mazancourt *et al*., 2005). Such negative proximate responses can be due to "real" (i.e. ultimate) benefits, but may merely reflect evolved dependence resulting from adaptation to partner. An ultimate mutualism, i.e., an interspecific interaction in which each partner could never have performed as well without the other, even if it was adapted to the absence of the partner, is never tested for. This would require the long-term removal of the partner, allowing for adaptation to its absence, and the comparison of a genotype adapted to the presence of the partner in the presence of the partner with the performance of a genotype adapted to the absence of the partner in the absence of the partner (de Mazancourt *et al*., 2005).

In summary, under nutrient limited conditions the hypothesis that the plant controls the resource allocation to the fungus depending on the surplus of C it is producing, and that therefore there is an exchange of by-product instead of costly benefits, explains the majority of the reported results, here and elsewhere, better than two organisms in permanent competition for costly benefits. The assessment of the fungal side would be essential to truly understand the dynamics of the interaction, and future works should focus on this.

Finally, biological market models seem to be more accurate approximations to mycorrhizal symbiosis than Iterated Prisoner's Dilemma (IPD) based models, namely because they allow for by-product benefits. The results of the present thesis clearly indicate that, in the nutrient limited conditions tested, the C allocated to the fungal partner is a by-product and not a costly benefit, which is not contemplated in IPD models.

Furthermore, the IPD assumes that the plant will adapt the investment in the fungal partner according to the payoff received in the previous iteration. This is in

disagreement with the fact that the plants will allocate more C to the fungus as the nutrient availability decreases, even if the amount of nutrients supplied by the fungus to the plant also decreases (chapter 3). According to biological market models, the plant's investment in the fungal partner does not depend on the amount of nutrient obtained through it, but on the price of its acquisition through the fungus, and on the degree of specialization of the partners in the acquisition of the exchanged resources (Schwartz and Hoeksema, 1998; Hoeksema and Schwartz, 2002). Most interactions, however, probably exist somewhere between a pure biological market and a pure non-market (Hoeksema and Bruna, 2000).

The observation that the initial effects of mycorrhization vary depending on the age of inoculation (chapter 1, 2) indicates that this is a factor that should be considered in establishing mycorrhization protocols for agriculture and forestry.

The plant growth analysis approach used in this thesis was essential in the characterization and analysis of the situation studied, and in its understanding. Namely the use of RAR_N, which allowed for a measurement of the N availability in the root medium in relation to the plants needs, was of extreme importance. This type of analysis has fallen in disuse, but was demonstrated in the present work to be an extremely useful tool.

Also not so commonly used in mycorrhizal research, the chlorophyll *a* fluorescence analysis is perhaps the best tool available to evaluate stress and plant vitality, and therefore fitness, save from survival and fecundity, since it integrates the multiple effects of mycorrhization, including non-nutritional effects, which can be difficult to evaluate. In particular PI_{abs} is an extremely sensitive parameter that was proven to be of great value in identifying both situations of stress to the plant, and the stress buffering effect of mycorrhization (chapter 1, 3).

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