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Response of plants to ectomycorrhizae in N-limited conditions: which factors determine its variation?

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Abstract In the present work, the following hypotheses were tested: (1) the negative effects of mycorrhization over host plant productivity in N-limited conditions are due to N retention by the fungal partner and not due to excessive C drainage; (2) If mycorrhization results in decreased N uptake, the host plant decreases its C investment in fungal growth. The effects of mycorrhization over a wide range of combinations between N availability, 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 always limiting. A gradient of mycorrhizal effects over the host plant's growth and vitality was successfully obtained. The mycorrhizal effects on plant growth and N uptake were very strongly and positively correlated, and no evidence was found of a C limitation to growth, confirming hypothesis 1. Indications were found that the plants continued to provide C to the fungus although the N supplied by it was increasingly lower, denying hypothesis 2. A new index, the mycorrhizal N demand–supply balance, was found to efficiently explain, and to have a curvilinear relation with, the variation in response to mycorrhization. The mycorrhizal effect on host plant growth was not related to a negative effect on its photosynthetic performance and, therefore, reflected changes in resource allocation between host plant and mycorrhizal fungus, not in plant vitality.

Keywords Ammonium · Chlorophyll *a* fluorescence · Colonization density · Cost–benefit · Demand–supply · Ectomycorrhizae · Mycorrhizal response · Nitrogen · O-J-I-P test

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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 its effects on the host plant can vary from the traditionally accepted mutualistic through to antagonistic in a continuum of responses (Johnson et al. 1997; Jones and Smith 2004; de 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 (Johnson et al. 1997; Schwartz and Hoeksema 1998; Tuomi et al. 2001; Neuhauser and Fargione 2004). Furthermore, the variation of mycorrhizal effects on plant productivity has been proposed to depend on the degree of mycorrhization (Gange and Ayres 1999;

Tuomi et al. 2001) or on the nutrient availability (Janos 2007).

The balance between the fungal demand for energy and the plant's needs for nutrients seem, indeed, to depend on the amount of nutrients available to the plant (Douds et al. 1988; Dickson et al. 1999; Bücking and Heyser 2001). Mycorrhizal plants are presumed to perform better (increased nutrient concentrations, nutrient uptake capacity, increased growth) under nutrient poor conditions due to their superior cost efficiency, i.e., the amount of nutrients acquired per C expended, whereas nonmycorrhizal plants should be more cost efficient and grow better at high nutrient levels (Schwartz and Hoeksema 1998; Tuomi et al. 2001; Jones et al. 1998). In fact, models based on the balance between nutrient gain and C cost predict negative effects on the host plant at high nutrient availabilities (Schwartz and Hoeksema 1998; Tuomi et al. 2001; Neuhauser and Fargione 2004). However, although less frequently, negative growth responses to mycorrhization in severely nutrient deficient plants have also been observed (Ingestad et al. 1986; Colpaert et al. 1999; Hobbie 2006). Since nutrient limited conditions are the most likely to occur in nature and are simultaneously the conditions where mycorrhizae are thought to be an advantage to the host plant, it is of great interest to understand what makes the difference between a positive or negative response in such cases.

Decreased growth of mycorrhizal plants may result from the 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). However, while increased growth rates in mycorrhizal plants are most often attributed to increased nutrient uptake (e.g., Jones et al. 1990, 1991), decreased growth rates are mostly considered to result from excessive fungal C drain (Colpaert et al. 1996; Conjeaud et al. 1996) even though ectomycorrhizal (ECM) fungi have a high potential to accumulate (Wallenda and Kottke 1998) and immobilize N (Colpaert et al. 1992, 1996). If mycorrhizal plants are more cost efficient in nutrient limited conditions, then the reason for growth limitation in such conditions cannot be excessive C drainage and must, therefore, be N limitation.

Mechanisms linking both P (in arbuscular mycorrhiza; 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). If this is the case, then the host plant should transfer less C to the fungal partner in such situations when mycorrhization leads to decreased N absorption. However, the carbon cost of mycorrhizal colonization, as well as the fungal growth, has been found to be greater at lower nutrient supply and relative growth rates (Hobbie 2006; Treseder and Allen 2002).

In the present work, the response to mycorrhization in N-limited conditions was studied. Three factors were tested as possible determinants of the plant response to mycorrhization: the N availability, the N status of the plants, and the level of mycorrhizal colonization. An experiment was designed in order to obtain a wide range of combinations between these three variables. Which of these variables, or combination of variables, were responsible for the variation in mycorrhizal effects, and how, was tested. 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. A gradient of mycorrhizal effect over the host plant's growth and vitality was successfully obtained.

The following hypotheses were tested:

1. The negative effects of mycorrhization over host plant productivity in N-limited conditions are due to N retention by the fungal partner and not to excessive C drainage.
2. In situations when mycorrhization results in decreased N uptake, the host plant will decrease its C investment in fungal growth.

In addition, how the effects on plants' productivity and vitality are related was studied.

Matherial and methods

Plant and fungal material

The *Pisolithus tinctorius* (Pers.) Coker and 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°C on a perlite/vermiculite (*v*/*v*) mixture moistened with liquid MMN medium.

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

Pinus pinaster L. seeds were collected in Sines and Santarém, Portugal and provided by the National Centre of

Forest Seeds of the Portuguese Ministry of Agriculture. The seeds were surface-sterilized with 30% calcium hypochlorite for 30 min, rinsed several times in distilled water, and soaked in distilled water at 4°C for 48 h. Sowing was carried out in gnotobiotic conditions on a sand/vermiculite (*v*/*v*) mixture sterilized at 120°C for 1 h. Seedlings were watered with distilled water as needed.

When the second set of leaves appeared, approximately 1 month after sowing, the seedlings were transferred to 350 mL root trainers (20 cm Fleet Roottrainers, Ronaash, Ltd., Roxburghshire, UK) with a perlite/vermiculite (*v*/*v*) mixture as substrate that had been sterilized at 120°C for 1 h. The root trainers consist of polypropylene fold-up liners, each comprising four individual plant holding cells that are held upright by polypropylene trays. Each tray contained 10×4 cells.

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).

Half the seedlings of each group were inoculated with alive (mycorrhizal—M) and half with dead (nonmycorrhizal 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 h at 120°C.

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, 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. Thiamine and glucose were omitted from the medium. The volume of nutrient solution given to the seedlings was never in excess of the substrate holding capacity; therefore, there was never a loss of solution.

The four plants contained in each root trainer liner were inoculated and collected at the same time and received the same treatment. Liners containing plants receiving different treatments were randomly distributed within the same tray. Mycorrhizal and nonmycorrhizal plants were kept in separate trays to prevent unwanted mycorrhization.

The experiment was performed in a growth chamber under a 16 h light/8 h dark photoperiod at 24/18°C, 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). The position of the trays within the growth chamber was changed daily.

The plant culture system described was semi-hydroponic. This technique ensures nutrient delivery to the root surface, not allowing the creation of nutrient depletion zones, which could be overcome by the presence of the mycorrhizal fungus (Colpaert et al. 1996; Eltrop and Marschner 1996). This allows for a better evaluation of the N acquisition costs through the mycorrhizae, as well as a more accurate comparison between M and NM plants at similar N availabilities.

Following inoculation, the seedlings were followed for 16 (1-month-old seedlings) or 12 (2-month-old seedlings) weeks. Between eight and 12 seedlings were harvested every 4 weeks after inoculation. In addition, eight plants were harvested 8 and 14 days after inoculation for mycorrhizal colonization monitoring. Shoots and roots were weighed and collected separately. The roots were

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

Group	Pretreatment	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		

washed in running water, and excess water was removed prior to weighing. The samples were immediately frozen after weighing at −70°C.

Samples were kept at −70°C, freeze-dried for 72 h, and stored at −20°C 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 −20°C.

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 and C and N concentration determinations, the shoots or roots of two to three seedlings of each treatment and harvest were pooled in one sample, making a total of three 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 analyzer (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 1 s, 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 1 s. 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 behavior. 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 centers 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 photosystem II and I (Strasser et al. 2004; "Appendix").

Net photosynthesis rate

Gas exchange measurements were made with a compact CO_2/H_2O porometer CQP-130 coupled with a NDIR gas analyzer (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. Eight to 12 plants of each subgroup (Table 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 5-mg freeze-dried root or *P. tinctorius* mycelium was extracted in 1 mL ice-cooled absolute ethanol for 15 min, with occasional vortexing. After centrifugation (10 min, 14,000×*g*), the supernatant was used for determination of ergosterol without further purification.

Twenty-five microliters of the supernatant were injected into the high performance liquid chromatography system and separated on a Spherisorb S5 ODS2 (4.6×250 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 an ultraviolet (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 analyzer Euro EA 3000 (EuroVector CHNS-O Elemental Analyzer; 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. 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 mycorrhizal 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 (Table 2): N relative addition rate (RAR_N),

Table 2 Equations for the calculation of the plant growth analysis parameters used

Parameter	Formula	Dimension	References
N relative addition rate (RAR_N)	$N_s = N_0 * (\exp(RAR_N * t) - 1)$	% $time^{-1}$	Ingestad and Lund 1979
Relative growth rate (RGR)	$(1/W) \times (dW/dt) = (\ln W_2 - \ln W_1)/(t_2 - t_1)$	Weight $weight^{-1}$ $time^{-1}$	van den Driessche and van den Driessche 1991
Nitrogen relative uptake rate (RN)	$(1/N) \times (dN/dt) = (\ln N_2 - \ln N_1)/(t_2 - t_1)$	Weight $weight^{-1}$ $time^{-1}$ or mole $mole^{-1}$ $time^{-1}$	van den Driessche and van den Driessche 1991
Nitrogen net uptake rate (U)	$(1/R)\times(dN/dt) = [(N_2 - N_1) \times (\ln R_2 - \ln R_1)]/[(R_2 - R_1) \times (t_2 - t_1)]$	Weight $weight^{-1}$ $time^{-1}$ or weight $area^{-1}$ $time^{-1}$	van den Driessche and van den Driessche 1991
Nitrogen productivity (NP)	$(1/N)\times(dW/dt) = [(W_2 - W_1) \times (\ln N_2 - \ln N_1)]/[(N_2 - N_1) \times (t_2 - t_1)]$	Weight $weight^{-1}$ $time^{-1}$	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

N_s Amount of N added, N_0 initial plant N content, W plant weight, N plant N content, t time, R root weight or area

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).

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 produce a bigger error.

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 (Hunt 1982) 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

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. 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 mycorrhizal profit, or the net increase in total value generated by investing in mycorrhizae instead of in nonmycorrhizal roots, was calculated as the ratio between any given parameter of an individual mycorrhizal plant (m) relative to a mean value for nonmycorrhizal 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 balance

The balance between the plants' demand for N and its supply through the mycorrhizae can be expected to vary according to the mycorrhizal colonization (in microgram *P. tinctorius* DW/mg root DW) and the N availability (RAR_N). The mycorrhizal N demand–supply balance (MDS_N) was, therefore, estimated by calculating the ratio between these two variables. Because the RAR is an average value for a given period of time, as is the case of the plant growth analysis parameters used in this study, average values of mycorrhizal colonization for the considered periods of time were used in the calculation of MDS_N.

Statistical analysis

A two-by-two-between-groups analysis of covariance was used to test for the effects of mycorrhization and N supply on all the parameters measured using the age of the plant as covariate.

The correlation between several variables was investigated. The Pearson product moment correlation coefficient was used to evaluate these correlations whenever they were found to be linear. Correlation coefficients were obtained for different groups of data (e.g., M and NM; 1.9 and 3.8 mM NH_4^+), and the statistical significance of the difference between them was tested. When they were nonlinear, a curve was sometimes fitted to the data. The validity of assumptions was checked, and the goodness of fit of the model was evaluated using a one-way analysis of variance.

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

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 analyzing what factors were behind the variation in the response of the plants to mycorrhization. The experimental design 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.

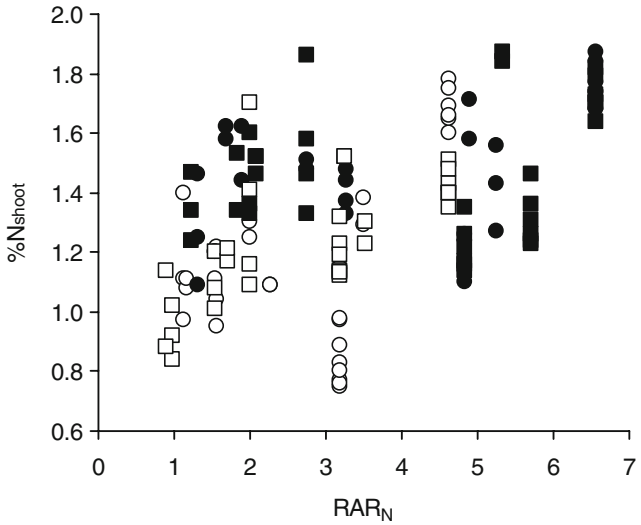

Fig. 1 Shoot N concentrations of mycorrhizal (*squares*) and nonmycorrhizal (*circles*) plants that were fed either 1.9 (*open*) or 3.8 mM NH_4^+ (*closed*) plotted against the respective N relative addition rates (RAR_N)

N and C contents

The effect of the variables tested (mycorrhization and N nutrition) on plant N and C contents and C/N ratios was the same in all the samplings. For this reason, all the measurements for each parameter were analyzed together, and the values presented (Table 3) are averages of all the measurements.

From all the tested variables, mycorrhization had the only significant effect on root N and C contents (Table 3). Mycorrhizal roots had higher N and lower C concentrations than nonmycorrhizal 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.

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

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

Correlations between RN, RGR, and NP

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) were very strongly and positively correlated. The correlations were independent of mycorrhizal status or amount of N supplied when all samples were considered (Fig. 2a,b).

Significantly different correlations between RN and NP for mycorrhizal plants (r=0.91, n=11, p<0.001) and respective nonmycorrhizal controls (r=0.89, n=12, p< 0.001) were found only for the subgroup of plants that presented negative growth profits at low MDS_N (Fig. 2c; see ahead for further details).

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

Table 3 N and C concentrations and C/N ratios of shoots, roots, and whole mycorrhizal (M) and nonmycorrhizal (NM) plants, and of *P. tinctorius* mycelium grown in pure culture

	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 × 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 × 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 × 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

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.
ANCOVA Analysis of covariance

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 mycorrhizal N demand–supply balance and its relation with the mycorrhizal growth profit

The mycorrhizal growth profit (RGR_m/RGR_{NM}) was found to be better correlated to the MDS_N than any other parameter measured or combination of parameters (Fig. 4). This correlation was curvilinear for both plants receiving more and less N. Negative growth profits were observed for mycorrhizal plants 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. A similar correlation was found when the shoot RGR was considered instead of the whole plant (results not shown).

The RAR_N used in these calculations were the ones calculated for mycorrhizal plants. However, the RAR_N of the corresponding nonmycorrhizal control plants were very similar, the difference being always less than 10% or 0.3% day^{-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 extraradical mycelium was very abundant. No mycorrhizae formation was observed in nonmycorrhizal plants.

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.

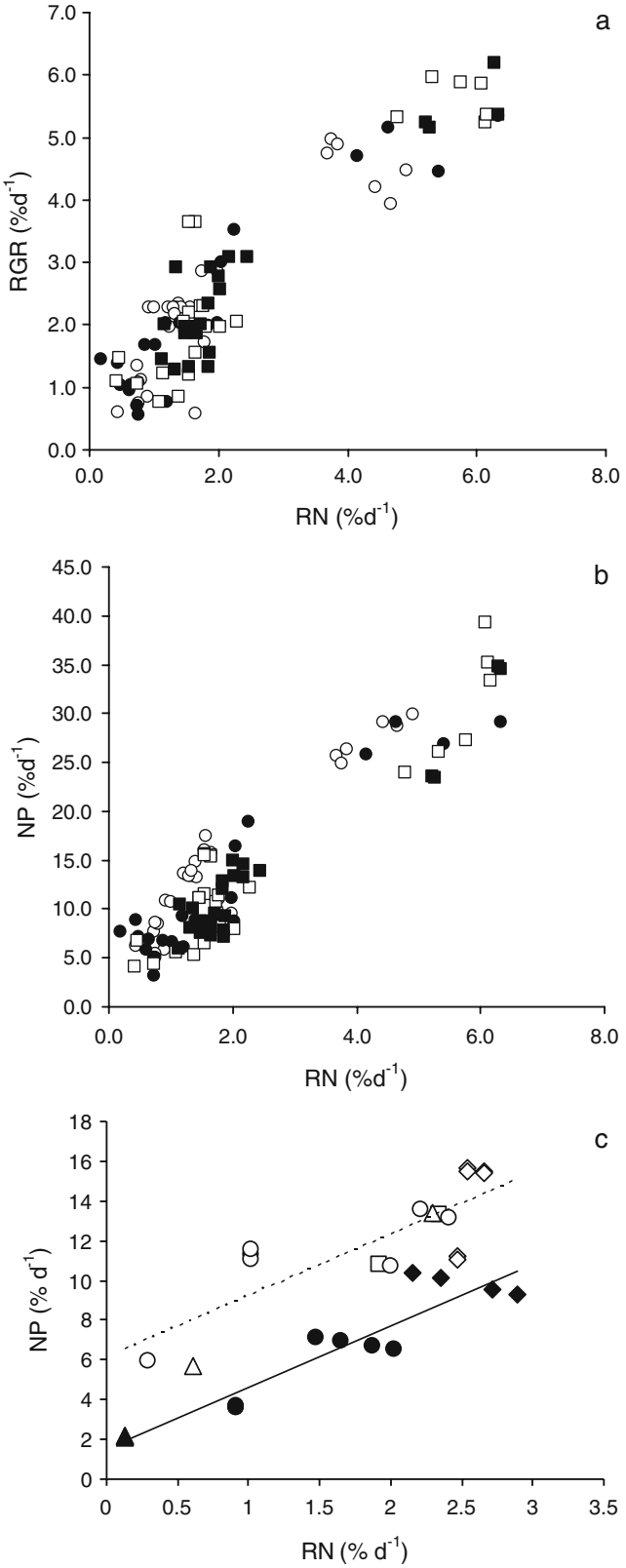

Fig. 2 Correlations between the nitrogen relative uptake rate (RN) and **a** the relative growth rate (RGR), or **b** the nitrogen productivity (NP) for all the samples, and **c** between RN and NP for the sub-group of plants that presented negative growth profits at low MDS_N. The plants were mycorrhizal (*closed*) or nonmycorrhizal (*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

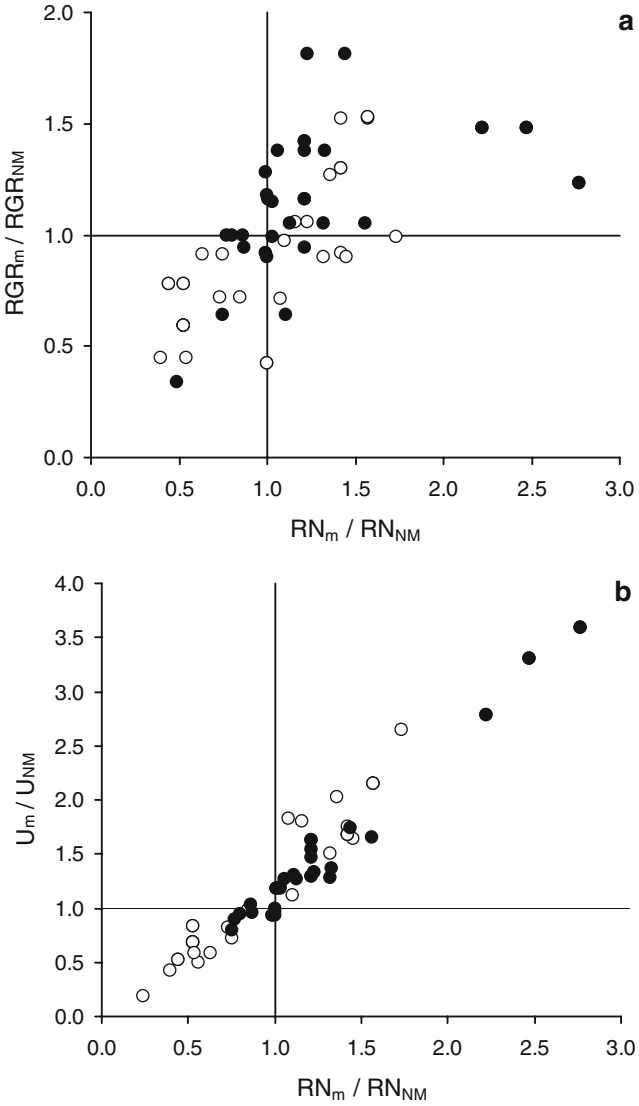

Fig. 3 Correlations between the mycorrhizal profits on N uptake (RN_m/RN_{NM}) and **a** on growth (RGR_m/RGR_{NM}), or **b** on N net uptake (U_m/U_{NM}). The mycorrhizal profit, or the net increase in total value generated by investing in mycorrhizae instead of in non-mycorrhizal roots, was calculated as the ratio between the parameter for an individual mycorrhizal plant and the mean value for nonmycorrhizal 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

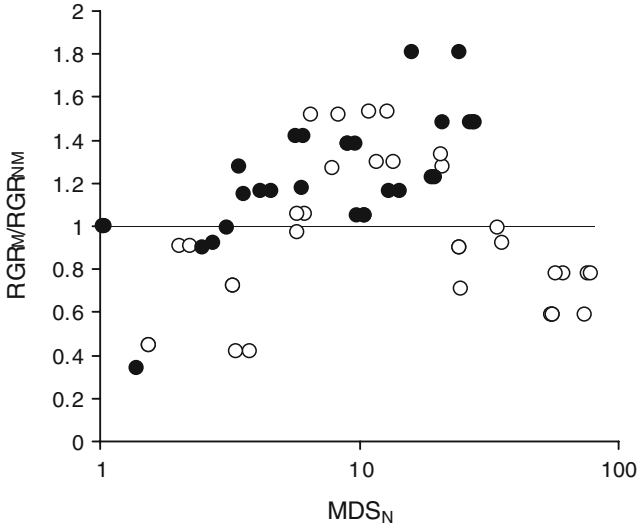

Fig. 4 Correlations between the RGR_m/RGR_{NM} and MDS_N. MDS_N was calculated as the ratio between the average level of mycorrhizal colonization for the considered period (Myc; *P. tinctorius* μg DW root mg DW^{-1}) and the RAR_N (RAR_N). The plants were fed either 1.9 (*open circles*) or 3.8 mM NH_4^+ (*closed circles*) as N source. The line dividing the graphic area makes the division between negative (below 1) and positive (above 1) mycorrhizal growth profit

Net photosynthesis rate

There were no significant effects of mycorrhization on net photosynthesis rate, either per needle area (results not shown) or total shoot (A_{plant}; Table 4). The plants that were supplied with more N had higher A_{plant} (p=0.005; Table 4) although the net photosynthesis rate per needle area was not significantly different compared to the plants that were supplied with less N.

No correlations were found between photosynthesis and any of the other parameters measured.

Chl *a* fluorescence and N allocation

The 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 4).

S_N was also only influenced by the N supply (Table 5). 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 (Fig. 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).

Discussion

The conditions tested were confirmed to be N-limited by the linear correlation between RGR and RN (Fig. 2a), indicating that growth was dependent on the N uptake, independently of mycorrhizal formation. In suboptimum N conditions, the N availability (RAR_N) controls N uptake (RN), which in its turn

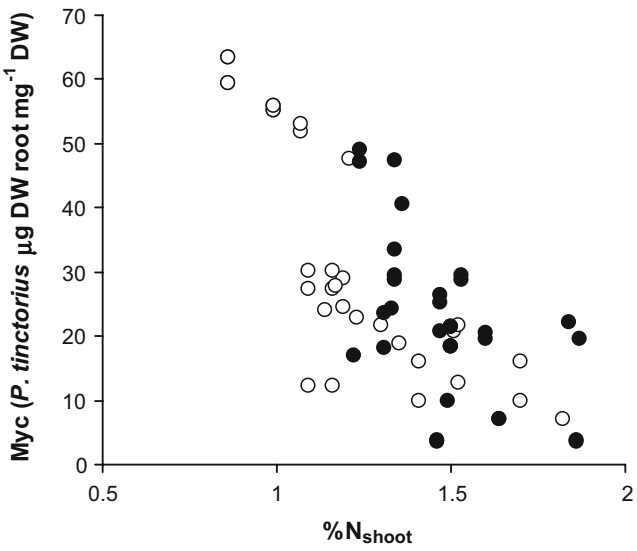

Fig. 5 Correlation between mycorrhizal colonization, measured as fungal dry weight per root dry weight, and N shoot concentration. The plants were fed either 1.9 (*open circles*) or 3.8 mM NH_4^+ (*closed circles*) as N source. The Pearson product moment correlation coefficient was used to evaluate the 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

Table 4 PI_{abs} and net photosynthesis rate per plant (A_{plant}) of M and NM plants grown with 3.8 or 1.9 mM NH_4^+ as N source

N supply (mM NH_4^+)	Mycorrhizal status	PI_{abs}	Number	A_{plant} (μmol CO_2 $plant^{-1}s^{-1}$)	Number
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	

Values are overall averages (± SE) of all measurements. A two-way ANCOVA was used to test the main effects and interactions.
ANCOVA Analysis of covariance, *M* mycorrhizal, *NM* nonmycorrhizal, PI_{abs} photosynthetic performance index

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 NP, i.e., the efficiency with which a unit of N was used to produce biomass, was also dependent on the RN (Fig. 2b), further confirming that N was limiting. This is in accordance with the fact that the measured N concentration in tissues (Table 3) ranged from values considered "adequate" to "very severely deficient" in published standards for Lodgepole Pine (Brockley 2001).

With the exception of a specific subgroup of plants (Fig. 2c), the correlation between RN and RGR or NP did not differ between mycorrhizal and nonmycorrhizal plants (Fig. 2a,b) similarly to what was found by Ingestad et al. (1986), indicating that mycorrhization did not result in a C or any other additional limitation to growth or in an alleviation of any existent C limitation, confirming our first hypothesis. In accordance to this, RGR_m/RGR_{NM} was positively correlated with RN_m/RN_{NM} (Fig. 3a), indicating that mycorrhization altered the host plant's growth by changing its N uptake. The observed negative effects on the host plants' growth were, therefore, due to N retention by the fungal partner and not a consequence of excessive C drainage, further confirming our first hypothesis.

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 that determines the mycorrhizal growth profit (Fig. 3a).

The lower C and higher N root concentrations observed in mycorrhizal plants (Table 3) 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 supported by the fact that the higher N concentrations in mycorrhizal roots were not accompanied by higher shoot N concentrations (Table 3) similarly to previous reports (Wallander and Nylund 1991; Colpaert et al. 1996). These observations were, however, made in all cases and, therefore, do not account for the variance in growth profit observed in this case. Furthermore, after discounting the estimated fungal N at the root, in the majority of cases (77%), mycorrhizal roots still had higher N content than nonmycorrhizal roots, indicating N accumulation that was not due to fungal retention. Such accumulations are characteristic of N stress (Ingestad and Ågren 1988).

A major implication of these observations is that in N-limited conditions, changes in the cost efficiency of mycorrhizal plants are not the reason for increased or decreased growth. This would only be the case if C was in limited supply, in which case mycorrhization could result in, or alleviate, a C limitation to growth, which was never observed (Fig. 2a,b). This explains why the net photosynthesis rate was not significantly affected by mycorrhization (Table 4). If C is limiting, on the other hand, growth can be expected to depend on its partitioning between N (and other nutrients)

Table 5 S_N of M and NM plants grown with 3.8 or 1.9 mM NH_4^+ as N source

N supply (mM NH_4^+)	Mycorrhizal status	S_N (% total N)	Number
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 × nut	0.201	

Values are overall averages (± SE) of all measurements. A two-way ANOVA was used to test the main effects and interactions.
S_N N allocation to the shoot, *M* mycorrhizal, *NM* nonmycorrhizal

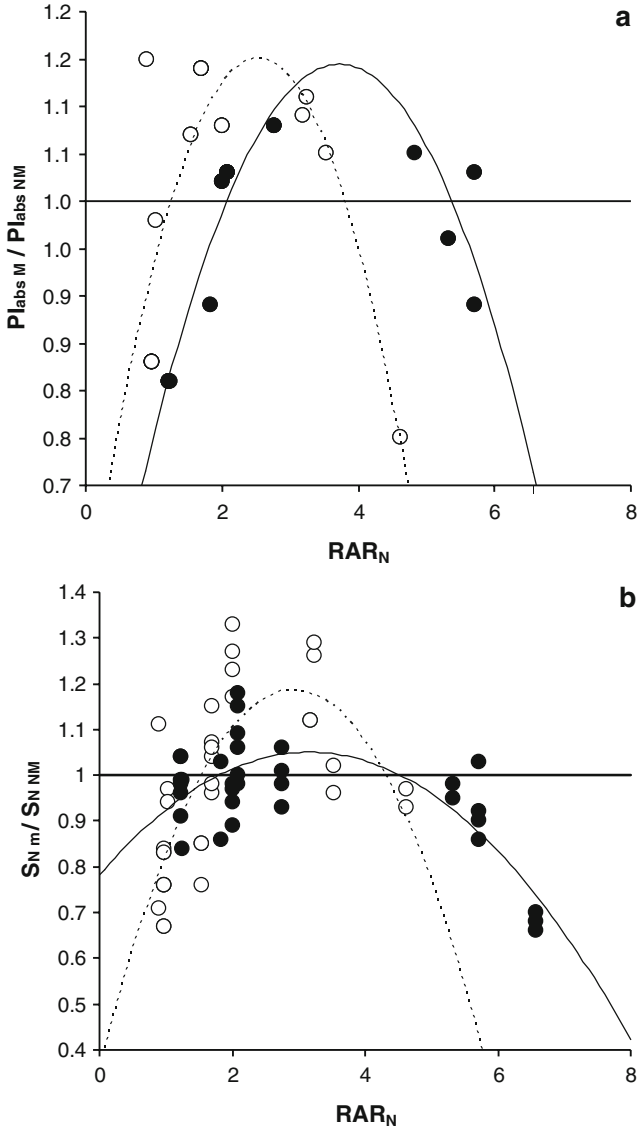

Fig. 6 Correlations between **a** the PI_{abs} and S_N (% total N), and between **b** the $PI_{abs\ m}/PI_{abs\ NM}$ and $S_{N\ m}/S_{N\ NM}$. In (**b**), a correlation was only found for the plants receiving less N, and a second order polynomial was fitted to the data set (*interrupted line*; $y=-1.37\times^2+3.24\times-0.80$). 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

acquisition and plant growth, and the cost efficiency becomes important.

In these conditions, the balance between the N supply by the fungus and the N demand by the plant, therefore, determines whether the host plant will profit from the association. This balance was accurately reflected by the MDS_N (Fig. 4), which was found to best explain the observed variation in mycorrhizal N uptake and growth profits. 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). However, in the present work, it was the combination of these two variables, in the calculation of MDS_N, which was found to best describe the variance in response. The fact that the observed response was curvilinear is in agreement with the results of Clapperton and Reid (1992) that fitted a third-order regression to the correlation between degree of mycorrhizal colonization and host plant growth. Curvilinear responses have also been proposed in several models (Gange and Ayres 1999; Janos 2007).

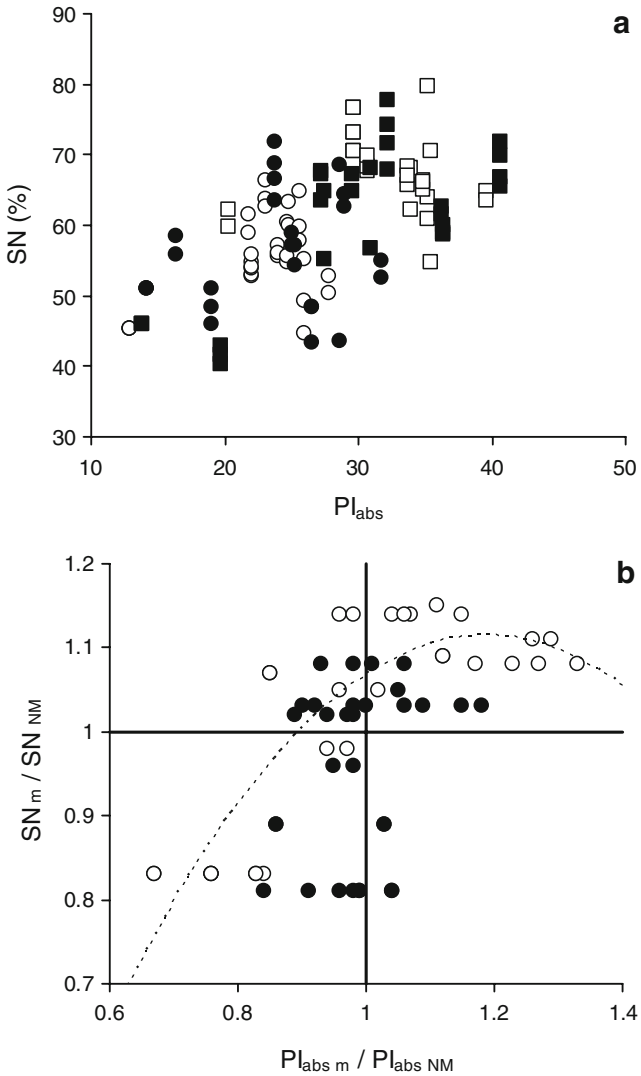

Fig. 7 Correlations between the RAR_N and mycorrhizal profits on **a** the $PI_{abs\ m}/PI_{abs\ NM}$ and **b** $S_{N\ m}/S_{N\ NM}$. The plants were fed either 1.9 (*open circles*) or 3.8 mM NH_4^+ (*closed circles*) as N source. Second order polynomials were fitted to the data sets for the plants receiving less (*interrupted line*; (**a**) $y=-0.10\times^2+0.57\times+0.35$; (**b**) $y=-0.09\times^2+0.48\times+0.55$) or more N (*solid line*; (**a**) $y=-0.02\times^2+0.12\times+0.85$; (**b**) $y=-0.04\times^2+0.28\times+0.55$)

Very high MDS_N are conditions of high colonization and low N availability (RAR_N), which may correspond to a situation where all resources are invested in increased extraradical 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; Hobbie and Colpaert 2003; Hobbie 2006). On the other hand, MDS_N is low when the N availability is high relatively to the amount of colonization. This can correspond to a situation where the fungus is still colonizing the root, in some cases the substrate, and, therefore, also of increased fungal growth. The negative mycorrhizal growth profits, observed at both extremes of MDS_N, may, therefore, be a consequence of increased mycelial growth and, hence, increased N use and retention by the fungus. This would be 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 extraradical mycelium was not measured, but its abundant production observed indicates that mycelial growth could be a particularly important sink. However, the plants presenting negative growth profits at low MDS_N had significantly lower NP at similar RN than the respective nonmycorrhizal control plants (Fig. 2c), indicating an additional limitation to growth by a factor other than N (Ingestad et al. 1986). N and this other factor alternately limited growth at a shorter time scale than the one recorded, and the integrated results of both limitations were observed. This other factor could be the immobilization by the fungus of a nutrient other than N (e.g., phosphorus) or a C limitation. It is important to notice that these negative growth profits were obtained both with very limiting and near to optimum RAR_N and, hence, were more dependent on the degree of mycorrhizal colonization than on the N available.

The model proposed by Gange and Ayres (1999) predicts that "benefit" will increase with mycorrhizal density, reach a plateau, and then decline and that different environmental conditions would originate a family of curves with different behaviors or characteristics. 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 may 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.

Our results indicate that mycorrhizae increased or decreased the host plant N uptake by changing the uptake capacity of the plant or efficiency per root unit when compared to nonmycorrhizal plants. This was indicated by 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 (Fig. 3b). In the present experiment, a semi-hydroponic system was used, allowing both mycorrhizal and nonmycorrhizal plants to have good access to the nutrients, excluding the effect of improved exploitation of the substrate by the fungus (Colpaert et al. 1996; Eltrop and Marschner 1996). ECM must, therefore, have an effect on the nutrient uptake of the host plant 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 and higher affinity for N uptake than nonmycorrhizal roots (Eltrop and Marschner 1996; Javelle et al. 1999). In the present experiment, however, the effect of mycorrhizae on the roots nutrient uptake could also be negative. To our knowledge, this is the first time that a decreased ammonium uptake has been reported. This is likely a consequence of the decreased access to N.

Our second hypothesis, which predicted that when mycorrhization results in decreased N uptake, the host plant decreases its C investment in fungal growth, was contradicted. In the present case, it was when the N status of the plants was lower that the higher mycorrhizal rates were observed (Fig. 5), indicating that 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). This is in accordance with previous reports (Hobbie 2006) and with the model proposed by Treseder and Allen (2002), according to which under low N availability mycorrhizal growth will be increasingly higher as the plant growth is increasingly more N limited, until the fungal growth becomes N limited itself.

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 (Fitter 2006; Nehls et al. 2007). This regulation may respond to:

(1) very low shoot N concentrations, (2) C accumulation, or (3) both. In support of the first hypothesis, the degree of mycorrhizal colonization was strongly negatively correlated with shoot N content (Fig. 5) similarly to previous reports (Wallander and Nylund 1991). The hypothesis that it is the N status of the trees that regulates the C supply to the fungus and, hence the growth of extraradical EM mycelia had already been proposed by Nilsson and Wallander (2003). This regulation may follow the concept of functional equilibrium: a low import of nutrients to the leaves triggers increased carbohydrate export to the root (Lambers et al. 1998) and, in this case, to the fungal partner. 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 (Kytöviita 2005).

The response to mycorrhization has most often been evaluated through its effect on the growth or nutrient status of the host plant. However, this can be misleading as these are not always the best indicators of plant fitness (Johnson et al. 1997; Jones and Smith 2004). For this reason, we were also interested in studying how the effects on plant growth and vitality were correlated. The mycorrhizal growth profits were not correlated with profits in terms of photosynthetic performance, indicating that the decreased growth of mycorrhizal plants was not related to decreased plant vitality and, hence, the plants' ability to survive and vice versa. The observed changes in growth profits were, therefore, simply a result of different strategies of resource investment and did not reflect the plants' health. In fact, most plants can be expected to adapt to slow changes in N availability, reducing growth and remaining healthy (Ingestad and Lund 1979).

However, mycorrhization also had an effect over the host plants' vitality. Although the effect of mycorrhization on PI_{abs} was not statistically significant (Table 4), 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 vitality.

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 content, 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.

In conclusion, we confirmed our hypothesis that, in conditions of limited N, it is the N retention or facilitation by the mycorrhizal fungus that determines the effect of mycorrhization on the growth of the host plant, while no evidences of C limitation to growth were observed. In these conditions, it was not the cost efficiency of the plant, but 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. The MDS_N was found to be explanatory of how this balance changes. The C investment by the host plant in fungal growth was found to increase as the N uptake decreased, contrary to what was hypothesized, indicating that it is not the N supply by the fungus what regulates this investment. We also found that the effects of mycorrhization on growth were not reflected in the host plants vitality and were, therefore, only a result of changes in resource allocation between host plant and mycorrhizal fungus.

The measurement of extraradical mycelium would have been essential for an evaluation of the total C and N sink strength of the fungal partner, but the very good correlations found using only the root associated mycelium indicate that, in this case, the colonization of the root was likely proportional to the development of extraradical mycelium. It would, however, be interesting to know how these correlations behave when the extraradical mycelium is accounted for and with other fungal partners.

This work represents a step further in the understanding of the effects of mycorrhization on the host plant and of their meaning. Based on the present findings, it would be of interest to focus future work on the analysis of the C and N economy of the interaction in closer detail at selected values of MDS_N and using direct measurements such as ^{15}N and ^{14}C isotope labeling or ^{15}N natural abundance.

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Appendix

Table 6 Technical data of the O-J-I-P curves and the selected JIP test parameters used in this study

Technical data of the O-J-I-P curves and the selected JIP test parameters	
Technical fluorescence parameters	
F_0	F_{50}, fluorescence intensity at 50 μs
F_M	Maximal fluorescence intensity
F_V/F_M	$(F_M–F_0)/ F_M$
V_J	Variable fluorescence at 2 ms. $V_J=(F_J–F_0)/(F_M–F_0)$
Quantum efficiency or flux ratios	
ϕ_{P0} or TR_0/ ABS	$\phi_{P0}=(F_M–F_0)/F_M$
Ψ_0 or ET_0/TR_0	$1-V_J$
Density of reaction centers	
RC/ABS	(RC/TR_0) $(TR_0/ABS)=[V_J/(d_V/d_{t0})]$ (F_V/F_M)
Performance index	
PI_{abs}	(RC/ABS) $[\phi_{P0}/(1-\phi_{P0})]$ $[\Psi_0/(1-\Psi_0)]$

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