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ORIGINAL PAPER

Carbon allocation in ectomycorrhizal plants at limited and optimal N supply: an attempt at unraveling conflicting theories

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Abstract With regard to mycorrhiza, conflicting theories try to explain how the balance between fungal demand for carbohydrates and the plant's needs for nutrients varies, resulting in conflicting predictions. In order to evaluate current concepts, we investigated some metabolic parameters, which are indicative for plant carbon allocation in response to mycorrhization at limited and optimal N supply. *Pinus pinaster* seedlings were inoculated with living or dead (control) cultures of *Pisolithus tinctorius*, supplied with ammonium at 4 (limiting) or 7% d^{-1} (non-limiting) N relative addition rate (RAR_N), and followed development for 29 days. Mycorrhizal colonization of roots was quantified by the determination of ergosterol. A series of enzymes (sucrose and trehalose metabolism, anaplerosis) and metabolites (soluble carbohydrate, including trehalose; fructose 2,6 bisphosphate, free amino acids) relevant in the C/N exchange between symbionts, and in the carbon allocation and sink strength within the plant were assayed for 2-day-intervals for up to 14 days, and at 5-day-intervals for the rest of the experiment. The first 10 days reflected the establishment of mycorrhizal interaction, and the carbon allocation to the root was higher in M plants independent of N supply. Following this period, carbon allocation became N-related, higher at low, and lower at high N supply. The belowground C investment of M plants was dependent on N availability, but not on N gain. Finally, increased belowground C allocation was accompanied by a shift from plant to fungal metabolism.

Keywords C sink · C allocation · ECM · N · Cost:benefit · Trehalose

Abbreviations

ECM	ectomycorrhizae
PEPc	phosphoenolpyruvate carboxylase
SS	sucrose synthase
TSC	total soluble carbohydrates
FAA	free amino acids
M	mycorrhizal
NM	non-mycorrhizal

Introduction

Mycorrhizae are symbiotic interactions between soil fungi and roots. The benefit of mycorrhiza formation is generally considered to depend on the balance between the fungal

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demand for energy and the plant's needs for nutrients. Negative effects of mycorrhizal colonization on the host plant are expected when the net C costs for fungal maintenance and growth exceed the net benefits obtained from improved nutrient supply (Jonhson et al. 1997; Schwartz and Hoeksema 1998; Tuomi et al. 2001; Neuhauser and Fargione 2004). Several studies have indicated that the effect of mycorrhiza on the host plant productivity depends on the amount of nutrients available, and on the host plant nutrient status (Douds et al. 1988; Dickson et al. 1999; Bücking and Heyser 2000; Janos 2007; Corrêa et al. 2008). An evaluation of the belowground C allocation and its correlation with nutritional benefits and fungal metabolism is therefore important to understand the cost–benefit balance in this association.

Ectomycorrhizal roots have generally been found to be stronger C sinks than non-mycorrhizal roots (for an overview see Table 1). The influence of nitrogen availability on carbon allocation, however, has not been thoroughly addressed, and N supply is often not detailed, nor is the plant N status quantified (Table 1). Screening the literature, we were only able to find four studies where the effect of N availability was investigated. Of these, two did not analyze non-mycorrhizal plants. In the remaining two studies, the differences in growth between mycorrhizal (M) and non-mycorrhizal (NM) plants were used as indicators of fungal C sink strength. In both cases, however, the effects could have been caused by differences in N uptake instead of C expenditure (Table 1; Corrêa et al. 2008). It is therefore still poorly understood how the balance between fungal demand for energy and the plant's needs for nutrients varies. In addition, conflicting observations have led to the proposal of a number of different hypotheses which lead to conflicting predictions and expectations. Consequently, a review of current knowledge on the interactions between C allocation and N availability in mycorrhizae results in a patchwork of mismatched observations and theories obtained and proposed in independent studies, mostly lacking integration with each other. Major assumptions are as follows: (1) Mycorrhization causes a carbon sink, which decreases with decreased N availability, due to increasing cost efficiency, i.e., higher nutrient acquisition per C expended, when compared to non-mycorrhized plants (Jones et al. 1998; Schwartz and Hoeksema 1998; Tuomi et al. 2001; Neuhauser and Fargione 2004). (2) Mycorrhization causes a carbon sink, which increases with decreased N availability, due to increased fungal growth / soil exploration. This is suggested by the fact that both plant growth reductions due to mycorrhization and fungal growth have been found to be greater at lower nutrient supply and plant relative growth rates (Corrêa et al. 2008; Hobbie 2006; Högberg et al. 2003; Ingestad et al. 1986; Treseder and Allen 2002). (3) The mycorrhizal C sink is dependent on N gain by the plant, as it has been proposed that the plant reduces the C supply to the fungus if it fails to supply adequate amounts of nutrients (Nehls et al. 2007; Fitter 2006). This is supported by the report that increased N supply caused 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), and was proposed to explain the reduced growth and root colonization of ECM fungi at increased N availabilities (Wallenda and Kottke 1998; Nilsson and Wallander 2003; Treseder 2004; Nilsson et al. 2005). (4) The mycorrhizal C sink is dependent on N availability, but not on N gain. Recent evidence has been found that as the N availability decreases, mycorrhization may result in decreased instead of increased N gain, but that the plant will nonetheless invest increasingly more in fungal growth (Corrêa et al. 2008). (5) Mycorrhization results in a shift of metabolic functions from the plant to the fungus, which may compensate carbon allocated to fungal maintenance and growth, resulting in decreased or equal C expenditure in the presence of mycorrhiza. This has been suggested in a number of studies where molecular and biochemical evidence indicate a deactivation of the root and an activation of the fungal metabolism in response to mycorrhizae formation (Schaeffer et al. 1996; Wingler et al. 1996; Blaudez et al. 1998, 2001; Johansson et al. 2004; Duplessis et al. 2005; Frettinger et al. 2007; Herrmann and Buscot 2007).

In this study we followed different steps of mycorrhiza development between *Pisolithus tinctorius* and *Pinus pinaster*, under two different N regimes, and analyzed C allocation by metabolic parameters, in an attempt to confirm or invalidate the existing hypothesis.

In addition we hypothesize that:

a) Initially, as the fungus colonizes the root and mycorrhizae get established, the fungus will represent a C sink, but it will not yet be supplying N to the plant. Furthermore, although the growth of extra-radical mycelium can be expected to vary with the N availability, and the mycorrhizal C sink strength to vary accordingly, this will only become a factor after the mycorrhizae becomes established. The C sink strength of the mycorrhizal fungus will therefore be independent of the N availability.
b) Following the initial mycorrhizal establishment phase, the balance between N supply and C demand will determine the belowground C sink strength and vary with N availability.

We measured a series of enzymes and metabolites that were chosen not only for their relevance in the C/N

exchange between symbionts and in the carbon allocation and sink strength within the plant, but also because they are plant-specific, thus allowing us to isolate the plant response at the root level: Fructose 2,6 bisphosphate (F26BP), a regulator molecule in the photosynthetic carbon partitioning between glycolysis and gluconeogenesis by acting as an inhibitor of sucrose phosphate synthase (Stitt 1990; Lee et al. 2006); the activity of phosphoenolpyruvate carboxilase (PEPc) as an indicator of C channeling towards N assimilation; and the activity of the sucrolitic enzymes sucrose synthase (SS) and invertases. 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 directly be used as a marker for sink strength (Sebková et al. 1995). Fungal metabolism was accessed through the measurement of trehalose contents and the activity of acid trehalase.

Materials 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, for 2 months in the dark at 24°C 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 24°C, with solid MMN medium covered with cellophane. This mycelium was collected after 3 weeks, separated from the cellophane, weighed and stored at −70°C until freeze drying.

P. 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 min, rinsed in several changes of distilled water, soaked in distilled water at 4°C for 48 h, and sown on a sand/vermiculite (1:1) mixture, sterilized at 120°C for 1 h. Seedlings were watered with distilled water as needed.

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, sterilized at 120°C for 1 h, when the second set of leaves appeared, approximately 1 month after sowing. This guaranteed that the seedlings used were of similar sizes, and at the same developmental stage, at the beginning of the experiment.

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 h at 120°C. 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 liquid MMN medium, from which thiamine and glucose were 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 amount of N supplied during the experimental period corresponded to a nitrogen relative addition rate (RAR_N; Ingestad and Lund 1979) of seven, in the case of the 3.8 mM NH_4^+, which was determined to be optimal N supply for *P. pinaster*, and four in the case of the plants receiving 1.9 mM NH_4^+, and therefore N limited.

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 a light intensity of 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 experiment was repeated for four independent times, during the course of 1 year.

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, stems, and roots were collected separately, and frozen immediately at −70°C. The roots were washed in running water as quickly as possible, and excess water was removed prior to freezing. At each of the four experiment repetitions, between two and three plants were harvested per treatment and time, resulting in a total of 8 to 12 plants.

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 −70°C.

Table 1 C sink strength of mycorrhizal roots and shoot N content (% shoot DW) as quantified in previous studies, and N supply used

Plant	Fungus	N supply	% N shoot DW^{-1} (end of experiment)	Mycorrhizal C sink strength	Parameters measured	Reference
Only one N supply used. Comparison with non-mycorrhizal plants.						
Eucalyptus pilularis	*Pisolithus tinctorius*	Not described	Not quantified	+	Allocation of ^{14}C	Cairney et al. 1989
Pinus pinaster	*Hebeloma cylindrosporum*	2 mM NH_4^+ and 2 mM NO_3^-, 150 mL twice a week	NM: 1.69±0.16 M: 1.21±0.15	+	Root respiration, CO_2 assimilation, plant growth, root fungal biomass, plant nutrient concentration	Conjeaud et al. 1996
Pseudotsuga menziesii	1. *Rhizopogon vinicolor*	Not described	1. NM: 0.64±0.03; M: 0.71±0.03	1. +	Net photosynthesis rate, plant growth, plant nutrient concentration	Dosskey et al. 1990, 1991
	2. *Hebeloma crustuliniforme*		2. NM: 0.63±0.03; M: 0.74±0.04	2. +		
	3. *Laccaria laccata*		3. NM: 0.63±0.03; M: 0.57±0.02 (Concentrations per FW)	3. 0		
Salix viminalis	*Telephora terrestris*	Not described	Not quantified	+	Allocation of ^{14}C	Durall et al. 1994a
Pinus ponderosa	*Hebeloma crustuliniforme* *Laccaria bicolor*	25 μg N mL^{-1}	Not quantified	+	Allocation of ^{14}C	Durall et al. 1994b
Picea abies	*Pisolithus tinctorius*	0.8 mM N as: 1. NH_4^+ 2. NO_3^-	1. NM: 1.48±0.22; M: 1.74±0.12 2. NM: 1.45±0.04; M: 1.38±0.04 to 1.48±0.05 (Concentrations per needle DW^{-1})	1. + 2. 0	Root respiration, CO_2 assimilation, plant growth, root fungal biomass, plant nutrient concentration	Eltrop and Marschner 1996
Salix viminalis	*Telephora terrestris*	Not described	Not quantified	+	Allocation of ^{14}C	Jones et al. 1991
Eucalyptus coccifera	*Laccaria bicolor* *Telephora terrestris*	Not described	Not quantified	0	Allocation of ^{14}C	Jones et al. 1998
Picea abies	*Amanita muscaria*	3.8 mM NH_4^+	Not quantified	+	C metabolism enzyme activities and metabolite pools, CO_2 assimilation, plant growth.	Loewe et al. 2000
Populus tremula × *P. tremuloides*	*Paxillus involutus*					
Larix	*Suillus grevillei* Native fungus	Not described	Not quantified	+	Allocation of ^{14}C	Qu et al. 2004
Pinus taeda	*Pisolithus tinctorius*	Not described	NM: 0.8 to 1.2 M: 1.1±2.2	+	Allocation of ^{14}C	Reid et al. 1983
Pinus ponderosa	*Hebeloma crustuliniforme*	Not described	Not quantified	+	Allocation of ^{14}C	Rygiewicz and

Table 1 (continued)

Plant	Fungus	N supply	% N shoot DW^{-1} (end of experiment)	Mycorrhizal C sink strength	Parameters measured	Reference
						Andersen 1994
Betula pendula	*Paxillus involutus*	1.6 mg N L^{-1} as NO_3^-	NM: 1.56±0.01 M: 1.42±0.01	+	Root respiration, CO_2 assimilation, plant growth, plant nutrient concentration, C metabolism enzyme activities and metabolite pools.	Wright et al. 2000
Pinus densiflora	unidentified	Not described	Not quantified	+	Allocation of ^{14}C	Wu et al. 2002
More than one N supply used. Comparison with non-mycorrhizal plants.						
Pinus sylvestris	*Suillus bovinus*	2, 3, 4, 5, 5.5% d^{-1} (N relative addition rate)	0.94±0.012 to 2.20±0.025	Growth deficit of mycorrhizal plants increases as N decreases, but this could be due to increased C sink or to decreased N uptake	Plant growth, plant nutrient concentration, plant growth analysis.	Ingestad et al. 1986
Pinus sylvestris	*Suillus bovinus*	1. 2.6% d^{-1}	1. NM: 1.05±0.04; M: 0.8±0.02 to 0.95±0.03	+	Respiration, CO_2 assimilation, plant and fungal growth, plant and fungal nutrient concentration.	Colpaert et al. 1996
	Telephora terrestris *Scleroderma citrinum*	2. 5.2% d^{-1} (N relative addition rate)	2. NM: 1.45±0.04; M: 1.38±0.04 to 1.48±0.05	Mycorrhizal effect is similar with more or less N		
More than one N supply used. No comparison with non-mycorrhizal plants.						
Betula pendula	*Paxillus involutus*	1. 1.8 mM N (NH_4NO_3), adding to a total of 1 mg N	1. 1.78±0.03	Higher C sink at higher N supply	Allocation of ^{14}C and ^{15}N	Kytöviita 2005
		2. 9 mM N (NH_4NO_3), adding to a total of 7 mg N	2. 1.97±0.8			
Picea abies	Unidentified	1. 0.7 g N kg^{-1} 2. 0.85 g N kg^{-1} 3. 1 g N kg^{-1}	Not quantified	Higher C sink at higher N supply	C metabolism enzyme activities and metabolite pools, root fungal biomass.	Wallenda et al. 1996

+: higher C sink strength in mycorrhizal than in non-mycorrhizal roots; 0: no differences found between mycorrhizal and non-mycorrhizal roots. *M* mycorrhizal plants, *NM* non-mycorrhizal plants

All fungal and plant samples were stored at −70°C, until freeze drying for 72 h, and then stored in vacuum at −20°C. The dry weights were recorded after freeze drying. The individual plant samples that corresponded to a given treatment, harvest time, and experimental repetition were grouped into mixed samples. They were then homogenized in liquid N_2 using a ball mill and freeze dried again for 24 h.

Plant metabolite pools and enzyme activities were assayed in leaves and cotyledons (shoot), and roots, whereas weights and N concentrations were measured in leaves, cotyledons and stems (shoot), and roots.

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).

Net photosynthesis rate

Gas exchange measurements were made on days 2, 6, 8, 14, and 29, 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 a light intensity of 250 μmol m^{-2} s^{-1}. To determine net photosynthetic rate (A) for each sampling time, 8 to 12 plants were used.

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 min, with occasional vortexing. After centrifugation (10 min, 14,000 *g*), the supernatant was used for determination of ergosterol, without further purification.

Using pure methanol as eluant, 25 μl of the supernatant was separated by HPLC on a Spherisorb S5 ODS2 (4.6× 250 mm) column (Phase Separations). 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 establish a correlation ergosterol/dry weight. This was used to convert the ergosterol concentrations measured in roots to fungal dry weight.

An absorbance peak was observed, in all plant samples, which overlapped with the ergosterol peak. This overlapping peak had little variation over time and between samples (±9.4%). Its average absorbance in NM samples was subtracted from the absorbance of M samples.

Extraction and determination of soluble carbohydrates and starch

Plant soluble carbohydrates and starch in leaves and cotyledons (shoot), and roots were assayed according to Magel et al. (2001). Approximately 20 mg of PVPP were added to approximately 4 mg of freeze-dried material and heated to 105°C for 20 min. After cooling, soluble carbohydrates were extracted in 500-μl bidistilled water, for 25 min at room temperature with occasional vortexing. The samples were centrifuged at 14,000 *g* for 10 min, and the supernatant was 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°C for 15 min. After cooling, 9 U ml^{-1} amyloglucosidase was added to each sample. The samples were incubated overnight at 37°C. After centrifugation at 14,000 *g* for 10 min, the supernatant was used for the determination of glucose formed from starch degradation.

The carbohydrates contained in a 10-μl aliquot of the supernatant were measured. Eighty microliters of 500 mM TRA buffer, pH 7.4, containing 6 mM $MgSO_4$, 4 mM ATP, 2 mM NADP, and 0.28 U ml^{-1} glucose-6-phosphate dehydrogenase was added to the aliquot. The assay was performed by sequential addition of 2.5 U ml^{-1} hexokinase (glucose), 3 U ml^{-1} phosphoglucoseisomerase (fructose), and 0.4 mg ml^{-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, at 30°C. A blank was set up to which no sample was added.

Trehalose concentration was assayed according to Winkler et al. (1991). A 12-μl aliquot of the supernatant was incubated in 28 μl of 200 mM citrate/NaOH buffer, pH 4.5, containing 2.5 mU acid trehalase, at 30°C, for 1 h. The glucose concentration was then measured in a 7.5-μl aliquot of the reaction mix as already described.

Extraction and determination of fructose 2,6 biphosphate

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 was 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 was used for F26BP determination.

Ninety microliters 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 μg ml^{-1} glucose-3-phosphate dehydrogenase, 17 μg ml^{-1} aldolase, 0.3 μg ml^{-1}

triosephosphate isomerase, 0.1 Uml^{-1} PFP, and 50 mM mercaptoethanol was added to a 10-µl extract. The respective consumption of NADH was followed at 340 nm, at 30°C. A blank was set up to which no PFP was added.

Extraction and determination of enzyme activities

PEPc (E.C. 4.1.1.31) activity was determined according to Wingler et al. (1994). Approximately 10-mg freeze-dried material was 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 was 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 U ml^{-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 30°C for 5 min, to allow temperature adaptation. The change in absorbance at 340 nm (A_{340}) was monitored for 40 min.

For determination of invertase (E.C. 3.2.1.26) activity, approximately 4-mg freeze-dried material was extracted in the same extraction buffer as used for PEPc, but without protease inhibitor. After incubation for 10 min on ice, with occasional vortexing, the samples were centrifuged for 10 min at 10,000 *g* and 4°C. The supernatant was tested for soluble acid invertase (AI_s) and alkaline invertase activities. No activity peak was found for alkaline 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 cell wall acid invertase (AI_{cw}) activity.

Acid invertase determination was adapted from Egger and 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 microliters of supernatant was tested in a final volume of 130 µl. The mixture was incubated in microplates, at 30°C. After 1 h, the reaction was stopped by adding 3 µl 5 M NaOH to each microplate well and incubated at 100°C for 20 min.

AI_{cw} 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°C for 5 min. The tubes were centrifuged for 10 min at 14,000 *g*, and the supernatant was used for glucose quantification.

Acid trehalase (E.C. 3.2.1.28) activity was assayed as for AI_s. No activity peak was found for alkaline invertase. The glucose liberated during incubation in the invertases and trehalase assays 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 and Hampp (1993). Approximately 10-mg freeze-dried plant material was 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°C. The supernatant was tested for sucrose synthase activity.

Sixty-microliter extract was 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°C. After 40 min, the reaction was stopped by heating the plate to 100°C 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 mU ml^{-1} UDP-glucose dehydrogenase, in a final volume of 250 µl.

In all enzyme activity assays 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.

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

Determination of protein

Protein extracts used for enzyme activity determination were stored at −20°C until assayed for protein concentration. The soluble protein concentration was determined by the method of Bradford (1976), using the Bio-Rad reagent and BSA as standard.

All absorbances in enzyme activity and metabolite determination assays were determined in 96-well microplates (Costar, Cambridge, MA, USA) in a microplate reader spectrophotometer (Tecan Thermo Spectra, Tecan, Switzerland).

Extraction and determination of free amino acids

Approximately 60 to 70 mg freeze-dried plant material was extracted in 4 mL of a 1:1 mixture of methanol and 0.5%

lithium citrate / HCl, pH 2.3, for 24 h, at 5°C, 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, 3× 150 mm; Pickering Laboratories) using lithium buffer as eluant. Amino acids were derivatized with ninhydrine before photometric detection.

Determination of N concentrations and relative N uptake rate calculations

N concentrations were determined in freeze-dried leaves, cotyledons, stems, and roots of plants collected at inoculation time (day 0), after 8, 14 and 29 days, and fungal mycelium grown in pure culture, using an elemental analyser Euro EA 3000 (EuroVector CHNS-O Elemental Analyser; Callidus Software Interface-Version 4.1). The quantification was made through linear calibration, using the reference material Wheat Flour (OAS) calibrated for NIST patterns as external pattern. 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 the fungal mycelium, 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, which was then subtracted from the measured root N content. The whole plant N content was calculated using this corrected value for root N content and used to calculate RN.

The RN was calculated for the periods from days 0 to 8, 8 to 14, and 14 to 29, using the equation: $RN = (1/N) \times (dN/dt) = (\ln N_2 - \ln N_1)/(t_2 - t_1)$,where N is the whole plant N content, and t is time (van den Driessche and van den Driessche 1991).

Data analysis

A two-way ANCOVA was used to test for the effects of mycorrhization and N supply on the different parameters measured in this study, using the age of the plant as covariate, at $p<0.05$.

A one-way ANOVA, considering as independent variable the combination of the variables mycorrhization, N supply and time, followed by a Tukey test, was used to test for significant differences between treatments at each time, 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 concentrations and enzyme activities were expressed per dry weight of plant tissue. For graphic representation, the metabolite concentrations and enzyme activities were normalized by calculating the ratio between individual mycorrhizal plants and the mean of the corresponding non-mycorrhizal control plants: M/ $\bar{x}$ (NM).

Results

Fungal and plant growth, and rate of net photosynthesis

No statistically significant differences were found in the dry weights or RGR of roots, shoots or whole plants, between M and NM plants or high and low N, either when calculated for the whole experimental period or for shorter time intervals (not shown).

Mycorrhizal colonization started on day 6, when the first signs of mantle formation were observed. Colonization was faster until day 14, when it reached a plateau and stabilized. A second increase in colonization was observed in plants receiving limiting N between days 24 and 29 (Fig. 1). Mycorrhization was generally lower in plants that were supplied with sufficient N (Fig. 1).

There were no significant differences between M and NM plants net photosynthesis (not shown), and the degree of mycorrhization (fungal DW/root DW) did not have a significant effect on the rate of net photosynthesis.

N shoot concentration and relative N uptake rate

Between 8 and 14 days after inoculation, M plants that were supplied with sufficient N had significantly higher RN values than the corresponding NM plants. With limiting N there was no difference (Table 2). Between days 14 and 29, M plants had lower RN than NM plants with both N supplies. Shoot N concentration was significantly higher at day 14 in M plants with more N (Table 2).

Enzyme activities and metabolite pools quantifications

All the enzyme activities and metabolite pools quantified changed in a similar manner in cotyledons and leaves. The data for cotyledons and leaves was therefore combined, and presented as shoot.

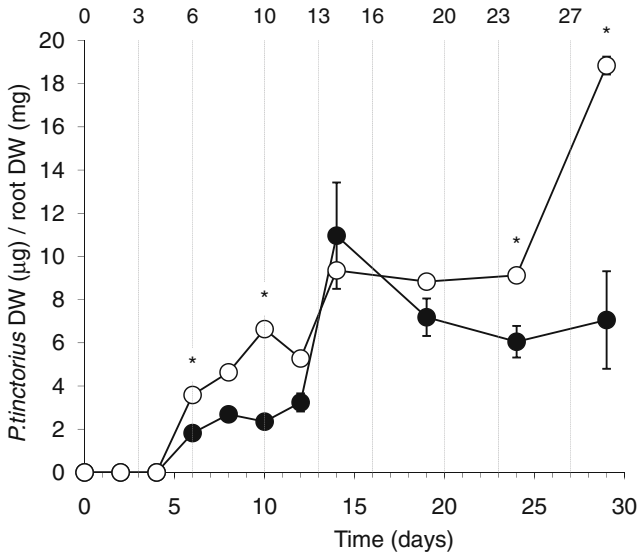

Fig. 1 *P. tinctorius* biomass in mycorrhizal whole roots. Fungal biomass was estimated using the mean value of the ergosterol concentration of *P. tinctorius* mycelia grown in pure culture as a conversion factor (6.74 µg mg^{-1} DW). The plants were fed a nutrient solution with NH_4^+ as N source, at 7%d^{-1} (*closed circles*) or 4%d^{-1} (*open circles*) RAR_N. Values are averages ± S.E. (n=4×2–3). The *dashed lines* indicate the days of watering

Carbohydrates

Decreases in shoot (leaves and cotyledons) soluble carbohydrates (days 2–8; Fig. 2a) and root hexose concentrations (day 6–8; Fig. 2b) of M plants were observed preceding and coinciding with the beginning of infection and mycorrhizal establishment (Fig. 1), and with a peak in root trehalose concentration (day 6; Fig. 2c). This was followed by a recovery of both shoot and root carbohydrates at day 10 to similar levels as in NM plants (Fig. 2a, b). A second decrease in carbohydrate concentration of both shoot and root was observed between days 14 and 29. All decreases in shoot soluble carbohydrates were more pronounced in plants that received more N. Following the initial peak, root trehalose gradually increased until the end of the experiment (Fig. 2c).

At the shoot, starch concentrations varied in a similar manner to soluble carbohydrates (See Supplementary Figure S1), whereas no significant differences were found in root starch concentrations. Shoot starch concentrations (311–1120 nmol glucose eq. mg^{-1} DW) were approximately ten times higher than soluble carbohydrates (90.8–204.5 nmol glucose eq. mg^{-1} DW), whereas at the root starch (23.4–103.8 nmol glucose eq. mg^{-1} DW) was present in lower concentrations than soluble carbohydrates (70.1–149.1 nmol glucose eq. mg^{-1} DW). Glucose and fructose were detected in comparable concentrations in all samples. Trehalose was not detected in non-mycorrhizal roots.

F26BP

F26BP is a regulator of sucroneogenesis. Increased concentrations inhibit fructose 1,6-bisphosphatase and thus sucrose formation. Decreases in shoot F26BP concentrations (= higher capacity for sucrose formation) were observed in M plants with both N supplies, coinciding with the beginning of infection and mycorrhizal establishment (days 2–8; Fig. 3a). With limiting N, amounts of F26BP remained lower than in NM plants throughout the

Table 2 Shoot and whole plant N contents (% DW) and relative nitrogen uptake rate (RN) of M and NM plants that were supplied with a nutrient solution with NH_4^+ as N source, at 7%d^{-1} or 4%d^{-1} RAR_N. N contents were measured at inoculation time (day 0), and after 8, 14 and 29 days. RN was calculated for the periods from days 0 to 8, 8 to 14, and 14 to 29. Values are averages ± S.E. A one-way ANOVA, considering as independent variable the combination of the variables mycorrhization, N supply and time, followed by a Tukey test, was used to test for significant differences between treatments at each time, at $p<0.05$. Letters stand for statistically significant differences at each time

	N supply (mM NH_4^+)	Mycorrhizal status	Day 0	Day 8	Day 14	Day 29
Shoot %N	3.8	NM	2.32±0.07	1.80±0.09^a	1.67±0.05^b	1.77±0.05^a
		M		1.79±0.09^a	2.15±0.04^a	1.73±0.05^a
	1.9	NM		1.80±0.09^a	1.73±0.05^{ab}	1.69±0.05^a
		M		1.73±0.09^a	2.09±0.22^a	1.43±0.04^b
Whole plant %N	3.8	NM	1.29±0.10	1.68±0.09^a	1.71 ± 0.07^a	1.70±0.06^a
		M		1.72±0.09^a	2.00±0.04^a	1.60±0.04^{ab}
	1.9	NM		1.80±0.09^a	1.71±0.07^a	1.56±0.05^{ab}
		M		1.69±0.09^a	1.97±0.18^a	1.43±0.04^b
RN (% d^{-1})	3.8	NM		1.32±0.05^a	1.22±0.02^b	2.60±0.45^a
		M		1.33±0.05^a	2.80±0.10^a	1.10±0.24^b
	1.9	NM		1.44±0.05^a	1.39±0.03^b	2.34±0.43^a
		M		1.39±0.05^a	1.34±0.28^b	1.24±0.33^b

experiment, while with more N they increased first to similar (day 10) and later to significantly higher values than in NM plants (Fig. 3a).

The only significant difference in root F26BP was on day 12, when there was a decrease in M plants with either nutrition (Fig. 3b).

Enzyme activities

With the exception of acid trehalase, no activity of the tested enzymes was detected in fungal mycelium.

The root AI_s, AI_{cw} and PEPc turnover rates of M plants changed simultaneously in relation to the NM control (Fig. 4), and they did so in parallel to the observed changes in root and shoot carbohydrates. The rates decreased between days 4 and 10, which was accompanied by an increase in the activity of SS (Fig. 4a). The rate of turnover of invertases and PEPc then started to recover at day 12, accompanied by a further increase in SS activity. With more N, the rates reached higher levels than the ones measured in NM plants, and decreased again after day 14. They remained, however, higher or similar to the ones observed in NM plants. With limiting N, the rise in the activities of the root acid invertases and PEPc at day 10 was to values comparable with NM plants. After day 14, they again decreased to levels lower than in NM plants (Fig. 4b).

In the shoot, the PEPc activity responded to mycorrhization similar to roots (Fig. 5). The activity of PEPc in shoots (3.6–34.9 pmol min^{-1} mg dw^{-1}) was much lower than in roots (7.1–527.5 pmol min^{-1} mg dw^{-1}).

Acid trehalase activity in mycorrhizal roots increased between days 6 and 14 (Fig. 6), a period when the root colonization by the fungus was enhanced (Fig. 1). Later on, it gradually decreased until the end of the experiment. Acid trehalase activity showed high rates in fungal mycelium (486.0±17.5 nmol trehalose min^{-1} mg^{-1}DW), and there was also some activity in non-mycorrhizal roots (7.8±0.2–13.8±0.1 nmol trehalose min^{-1} mg^{-1}DW).

Amino acids

Significantly higher amounts of FAA were observed in mycorrhizal roots on days 6 and 29 in plants that were supplied limiting N (Fig. 7). This was a result of a general increase in most of the FAA detected, and therefore did not

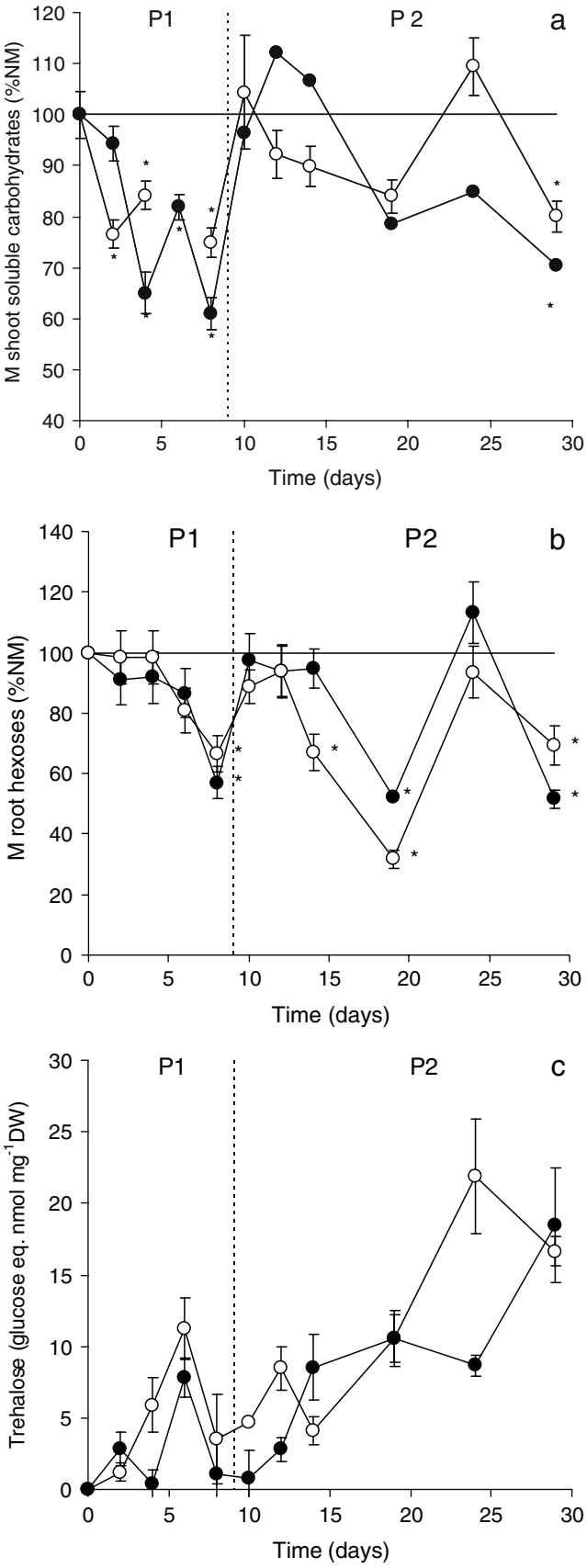

Fig. 2 **a** Total shoot soluble carbohydrates and **b** root hexose concentration of M plants as a percentage of NM plants, and **c** trehalose concentration of M roots. The plants were fed a nutrient solution with NH_4^+ as N source, at 7%d^{-1} (*closed circles*) or 4%d^{-1} (*open circles*) RAR_N. P1: early mycorrhizal establishment phase; P2: post-establishment phase. All values are averages ± S.E. (n=4×2–3). *Asterisks* stand for significant differences on each day, at $p<0.05$

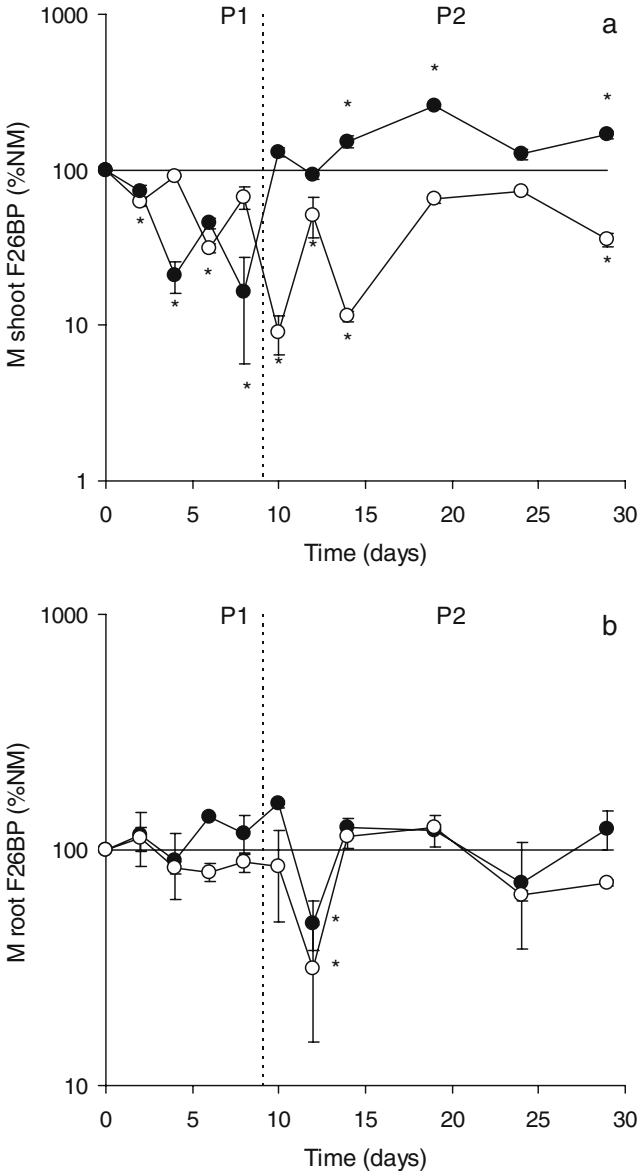

Fig. 3 F26BP concentrations in **a** shoot and **b** roots, of M plants as a percentage of NM plants. The plants were fed a nutrient solution with NH_4^+ as N source, at 7%d^{-1} (*closed circles*) or 4%d^{-1} (*open circles*) RAR_N. *P1* early mycorrhizal establishment phase, *P2* post-establishment phase. All values are averages ± S.E. (n=4×2–3). *Asterisks* stand for significant differences between M and NM plants on each day, at $p<0.05$

result in changes in their relative abundance (Fig. 8). On day 12, the FAA concentration of mycorrhizal roots was significantly lower than in NM plants with both N supplies, and this was still the case on day 14 for N-limited plants (Fig. 7). This was due to a decrease in a group of six major amino acids: serine, asparagine, aspartate, glutamate, glutamine, and threonine. As a result, the relative abundance of all other amino acids increased, with gamma aminobutyric acid (GABA), arginine, and alanine becoming the most abundant (Fig. 8). In M plants with sufficient N supply, the glycine concentration was increased at day 12, but otherwise remained very low (Fig. 8b). The concentration of asparagine gradually decreased from the beginning of the experiment in both M and NM plants.

Discussion

During the first 10 days following inoculation, an initial response to mycorrhizal establishment was observed, that points towards increased belowground C allocation according to decreased levels of shoot F26BP (favoring sucrose

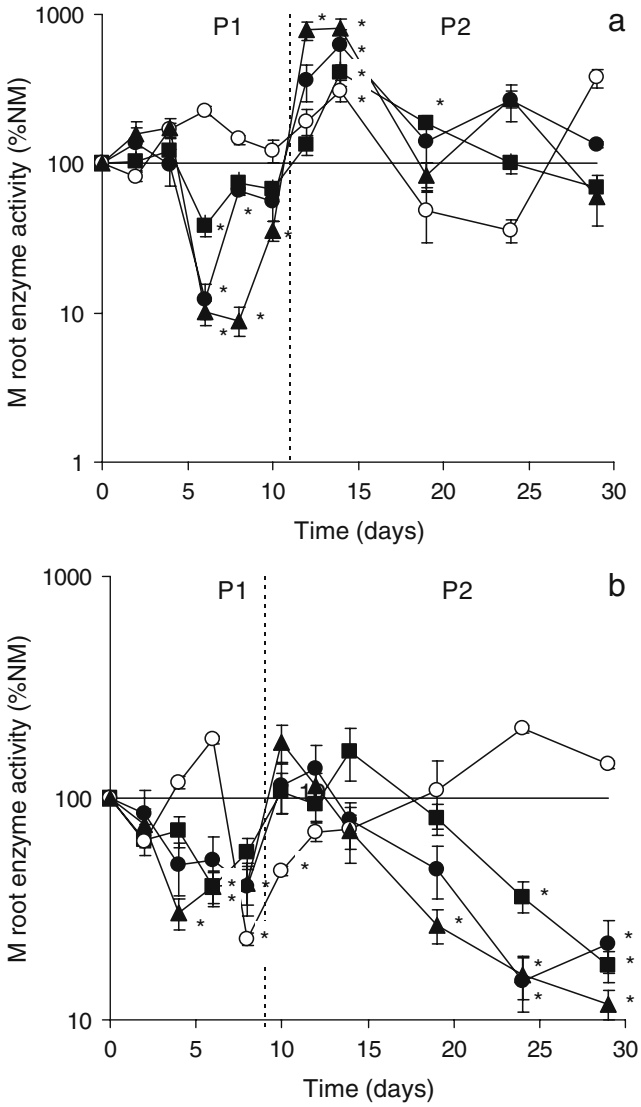

Fig. 4 AI_s (*closed circles*), AI_{cw} (*closed squares*), PEPc (*closed triangles*), and SS (*open circles*) activities in M roots as a percentage of NM roots. The plants were fed a nutrient solution with NH_4^+ as N source, at **a** 7%d^{-1} or **b** 4%d^{-1}. *P1* early mycorrhizal establishment phase, *P2* post-establishment phase. All values are averages ± S.E. (n=4×2–3). *Asterisks* stand for significant differences between M and NM plants on each day, at $p<0.05$

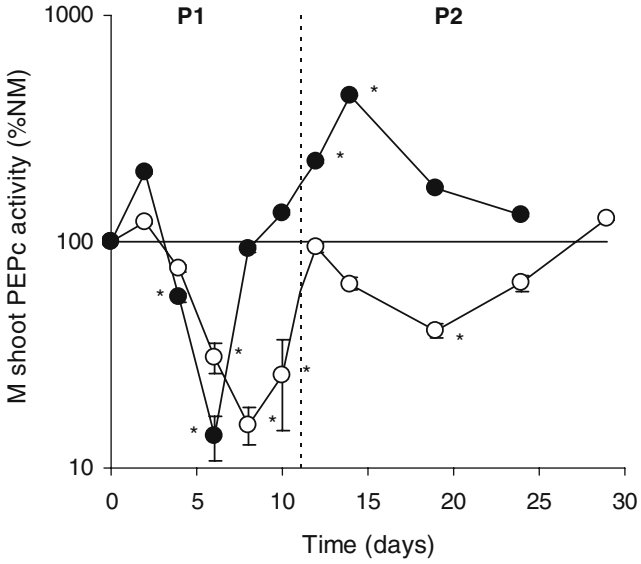

Fig. 5 Shoot PEPc activity in M plants as a percentage of NM plants. The plants were fed a nutrient solution with NH_4^+ as N source, at 7%d^{-1} (*closed circles*) or 4%d^{-1} (*open circles*) RAR_N. *P1* early mycorrhizal establishment phase, *P2* post-establishment phase. All values are averages ± S.E. (n=4×2–3). *Asterisks* stand for significant differences between M and NM plants on each day, at $p<0.05$

production), lower amounts of soluble carbohydrates, and increased SS activity in mycorrhized roots. This was independent of N supply, and is in support of our hypothesis that the initial response to mycorrhization is independent of N availability, and reflects only an increase in belowground allocation of C.

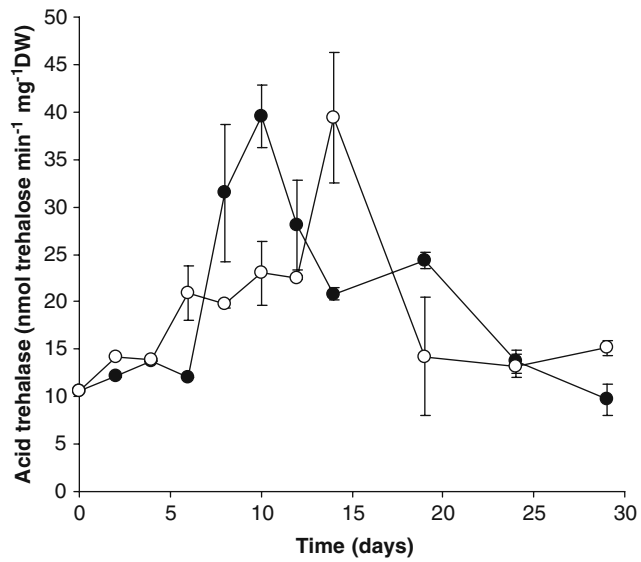

Fig. 6 Root acid trehalase activity in M plants. The plants were fed a nutrient solution with NH_4^+ as N source, at 7%d^{-1} (*closed circles*) or 4%d^{-1} (*open circles*) RAR_N. All values are averages ± S.E. (n=4×2–3)

As host metabolic responses were visible before root colonization, this supports the notion that the interaction between partners begins already in the pre-symbiotic stage (Martin et al. 2001; Duplessis et al. 2005; Frettinger et al. 2007; Herrmann and Buscot 2007). Following the changes in the shoot (Figs. 2a and 3a), a drop in hexoses (Fig. 2b) and an increase in SS activity (sucrolysis; Fig. 4) were observed in M roots. SS has an important role in phloem unloading (Schaeffer et al. 1995; Sung et al. 1996), and it has been suggested that its activity can directly be used as a marker for sink strength (Sebková et al. 1995). This is in parallel to an increase in the fungal sugar, trehalose, and thus consistent with increased mycorrhization-caused sink strength (for the role of trehalose in establishing a partner-specific sink see Aeschbacher et al. 1999; Müller et al. 1999); Fajardo López et al. 2007). In addition, high rates of acid trehalase were detected in mycorrhizal roots up to day 14, which should be due to an enhanced trehalose turnover, needed for fungal growth (Figs. 1 and 6). Plants also have a trehalose metabolism, and in accordance to this, some activity of acid trehalase was detected in NM roots (about 2% of the fungal activity). The trehalase activity measured in M roots could therefore be of plant origin. In plants, trehalase is thought to have a defensive role in removing externally produced trehalose (Müller et al. 1995, 1999; Bae et al. 2005; Goddijn and Van Dun 1999). In the present case, however, this is unlikely, since the acid trehalase activity was only related to trehalose concentrations during this early colonization stage (Figs. 2c and 6).

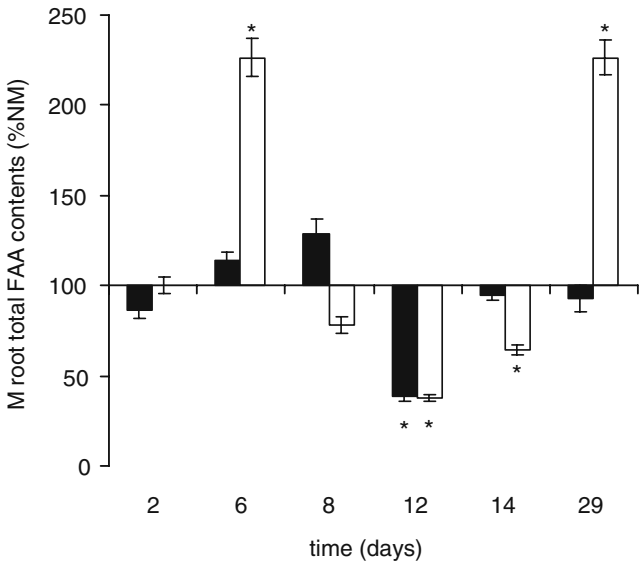

Fig. 7 Total free amino acids (*FAA*) in M plants' roots, as a percentage of NM roots. The plants were fed a nutrient solution with NH_4^+ as N source, at 7%d^{-1} (*black bars*) or 4%d^{-1} (*white bars*) RAR_N. All values are averages ± S.E. (n=4×2–3). *Asterisks* stand for significant differences between M and NM plants on each day, at $p<0.05$

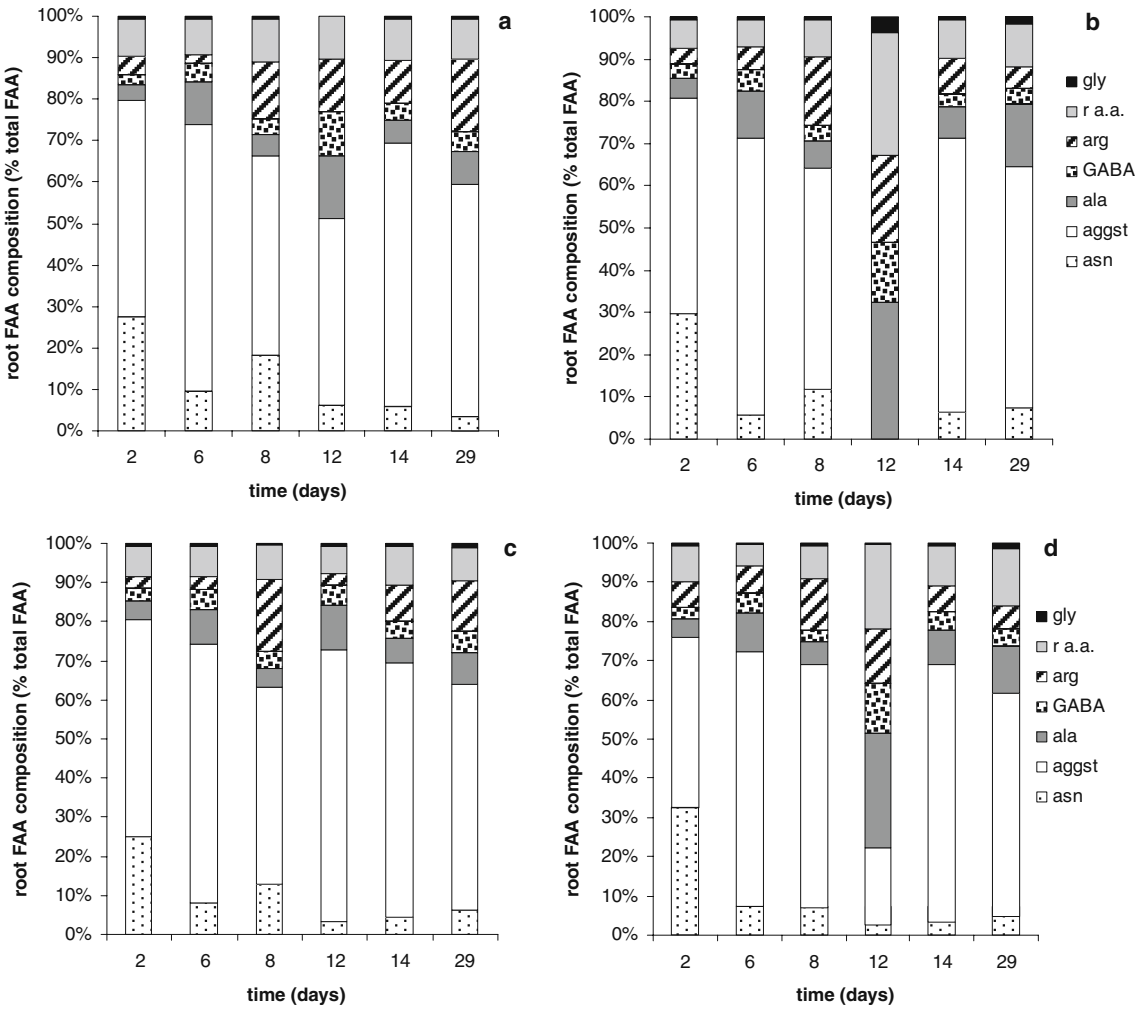

Fig. 8 Relative composition of the root free amino acid (*FAA*) pool, as percentages of the total FAA. The plants were supplied with 3.8 mM NH_4^+ or 1.9 mM NH_4^+ as N source, and were either mycorrhizal (*M*) or non-mycorrhizal controls (*NM*). **a** NM with 7%d^{-1} RAR_N; **b** M with 7%d^{-1} RAR_N; **c** NM with 4%d^{-1} RAR_N; **d** M with 4%d^{-1} RAR_N. *asn* asparagine; *aggst* glutamate, glutamine, aspartate, serine and threonine; *ala* alanine; *arg* arginine; *gly* glycine; *r.a.a.* remaining detected amino acids; *GABA* gamma aminobutyric acid

The dependence on N supply of mycorrhization and root carbon demand

Starting on day 12, N-dependent responses became apparent. In plants receiving sufficient N, the shoot F26BP concentration of M plants increased first to similar and later to higher levels than in NM plants (Fig. 3a). This could indicate decreased rates of sucrose formation, and consequently, reduced belowground allocation of C in M plants. With limiting N, however, amounts of F26BP remained lowered in M plants until the end of the experiment (Fig. 3a). This indicates that under these conditions more sucrose is produced and allocated to the mycorrhizal roots in order to support fungal growth and maintenance, and that therefore the C sink due to mycorrhization increases. This is also reflected by an increased fungal colonization of these plants (Fig. 1), and is in accordance with reports of negative correlation between the degree of mycorrhization and N availability (Wallenda and Kottke 1998; Treseder 2004).

This increased belowground investment was, however, not compensated with increased N uptake. Whereas M plants with sufficient N had a superior N gain (RN) between days 8 and 14, with limiting N this was never the case (Table 2). The C belowground investment of M plants seemed therefore to be dependent on N availability, but not on N gain. This is according to the findings of Corrêa et al. (2008), and to the model by Landis and Fraser (2007), that proposes that the C investment in fungal growth is independent of P gain from the fungus, and depends only on the plants nutrient (in their case, P) status and needs. This would be the basis for the greater C investment in fungal growth at low nutrient availabilities, even when the plant is not gaining more nutrients through the fungus than it would if it was non-mycorrhizal.

Other responses were observed which were not constant with time and which indicated a fluctuation in C allocation to the fungus.

Between days 10 and 12, and independent of the N supply, the carbohydrate concentrations of both shoots and roots (Fig. 2a, b) recovered to similar levels as in NM plants. In parallel, root trehalose decreased. Probably this reflects a decrease of carbohydrate supply to the fungus (Fig. 2c).

From day 14 onwards, root and shoot soluble carbohydrates in M plants again became lower than in NM plants, while the trehalose concentration increased (Fig. 2). Obviously, this indicates a new period of increased fungal C demand and C supply to the fungus. As the amount of ergosterol suggests an unchanged degree of mycorrhization (limiting N) or even a decline (sufficient N) (Fig. 1), we assume that in this period, the extra C is used for the growth of extra-matrical mycelia, and substrate colonization, which is an important sink for the C allocated belowground (Wu et al. 2002). In accordance to this, the rise in trehalose was also not accompanied by acid trehalase activity (Fig. 6), which could mean that trehalose was not used at, or close to, the root. To confirm this, we would need data for extra-radical mycelia, which we do not have.

A period of stronger investment in extra-matrical growth could be triggered by the need of accessing new N sources, once the ones close to the root surface become depleted. Such a situation would be in agreement with the fact that, after day 14 until the end of the experiment, mycorrhization resulted in decreased N uptake, independent of N supply (Table 2, RN, day 29) (Colpaert et al. 1992, 1996). Furthermore, the decreasing concentration of asparagine until day 12 could indicate a decrease in the contribution of cotyledon-derived N reserves, and therefore increased plant dependence on N taken up from the medium and the mycorrhizal contribution to this uptake (Fig. 8). Asparagine formation in high quantities is a consequence of the remobilization of cotyledon N reserves in pine seedlings (Cañas et al. 2006).

Fungal colonization of the roots increased again between days 24 and 29, coinciding with a new decrease in root and shoot soluble carbohydrates (Figs. 1 and 2).

Shifts between root and fungus metabolism in mycorrhized plants

In each period of increased C allocation towards mycorrhized roots we found indications of a down-regulation of root metabolism. During the initial phase of mycorrhizal establishment, the activities of AI_s, AI_{cw} and PEPc decreased and, in plants with limited N supply, the concentration of free amino acids increased (Figs. 4 and 7), indicative of a deactivation of root metabolism (Simon-Sarkadi et al. 2006). This effect was largely independent of N supply and similar to the other responses at this early phase.

From day 14 onwards, as the fungal demand increased again, root enzyme activities in M plants again became lower than in NM plants (Fig. 4). During this second period, however, all indications of root metabolic deactivation were more pronounced in M plants with limited N supply: here the enzyme activities again dropped to lower levels than in NM plants (Fig. 4b), while the amount of total free amino acids increased (Fig. 7). With sufficient N, the enzyme activities did not drop below those measured for NM plants (Fig. 4a).

Such fluctuations in root metabolic activity may be due to a takeover of root metabolic functions by the fungal partner (Vézina et al. 1989; Johansson et al. 2004), but did not result in a reduction of the belowground C sink strength, or belowground C allocation, since they coincided with periods of increased belowground C allocation.

It has previously been suggested that steps of N assimilation are shifted from roots to fungal cells under mycorrhization (down-regulation of PEPc and upregulation of fungal carboxylases; Wingler et al. 1996). In our study we found a down-regulation of both shoot and root PEPc under N-limitation, showing a whole plant response (Figs. 4b and 5).

The reverse was also observed. The decreased belowground C allocation between days 10 and 14 was accompanied by evidences of an activation of the host plant metabolism. All enzyme activities of M roots and shoots increased to similar (limiting N) or higher (sufficient N) levels than in NM plants (Figs. 4 and 5). A decrease in root FAA was also observed on day 12 (Fig. 7), which was exclusively due to a group of six amino acids (glutamine, glutamate, asparagine, aspartate, serine, and threonine; Fig. 8), all amino group donors, and starting points in a variety of biosynthetic pathways (Bourguignon et al. 1999; Lea and Ireland 1999). This is consistent with an acceleration of metabolism. All indications of metabolic activation in response to mycorrhization were stronger in plants with sufficient N (Figs. 4 and 8). In this group of plants, the large increase in glycine observed at this time further indicates an increased importance of protein degradation and remobilization of protein nitrogen (Fig. 8; Bourguignon et al. 1999).

Mycorrhization has been previously found to change the composition of the FAA 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). Also on day 12, the only significant decrease in M root F26BP was observed, independent of N supplies (Fig. 3b). This could indicate a shift from glycolysis to gluconeogenesis (Hampp et al. 1994; Schaeffer et al. 1996). At this stage, M plants seem therefore to invest more into the shoot than NM plants, which is reflected by the increased activity of shoot PEPc (Fig. 5). Increased PEPc activities and F26BP

concentrations in both needles and roots have been found in spruce upon improved N supply (Wingler et al. 1994).

ECM fungi have so far been found to have no sucrolytic capacity (Salzer and Hager 1991; Schaeffer et al. 1995; Nehls et al. 2007), and this was also the case for the *P. tinctorius* isolate, used in the present study. The activity of the host plant's sucrolytic enzymes (in particular the cell-wall bound AI) has thus been hypothesized to be essential for the cleavage of sucrose and the supply of hexoses to the fungal partner (Salzer and Hager 1991; Schaeffer et al. 1995; Nehls et al. 2007). An increase in the activity of these enzymes is therefore expected in M plants. In AM interactions, such a response has been reported repeatedly (e.g.,: Blee and Anderson 2002; Schaarschmidt et al. 2006). In ECM, however, this has not been the case (Schaeffer et al. 1995; Wright et al. 2000), and has also not been detected in our study (Fig. 4). This is an obvious difference in carbon acquisition between AM and ECM.

In summary, we present evidence for our hypothesis that the initial response to mycorrhizae formation is independent of N availability and reflects only the C needs of mycorrhizae establishment. Only later a balance between N supply and C demand is established, which will determine the belowground C sink strength, and vary with N availability. In addition, we found evidence that (a) the C sink due to mycorrhization increases at lower N availability, (b) that the C belowground investment of M plants is dependent on N availability, but not on N gain, and (c) that, although we observed decreased root metabolic activities in M plants, this does not result in decreased belowground C allocation. Our results therefore invalidate assumptions 1 and 3, and are consistent with assumptions 2 and 4.

Finally, our data confirm that trehalose metabolism plays an important role in the mycorrhizal association, both as plant carbon scavenger and intermediate of fungal carbohydrate metabolism. These functions vary as can be deducted from periodic changes in trehalose pool sizes and activities of the degrading enzyme, acid trehalase.

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