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Are mycorrhiza always beneficial?

A. Corrêa[1,3], R.J. Strasser[2] & M.A. Martins-Loução[1]
[1]*Faculdade de Ciências, Centro de Ecologia e Biologia Vegetal, Universidade de Lisboa, Campo Grande C2, Piso 4, 1749-016, Lisboa, Portugal.* [2]*Laboratoire de Bioénergétique, Université de Genève, Chemin des Embrouchis 10, CH-1254, Jussy Genève, Switzerland.* [3]*Corresponding author**

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Abstract

In this work we evaluate whether the effect of ectomycorrhiza in the early developmental stages of symbiosis establishment is detrimental or beneficial to plant productivity and whether this effect is dependent on either N nutrition or plant age. Groups of *Pinus pinaster* L. plants with different ages and nutritional status were inoculated with alive or dead *Pisolithus tinctorius*. The plants were fed with either 1.9 m*M* or 3.8 m*M* ammonium as N source. Ectomycorrhiza establishment was monitored until 1 month after the inoculation through daily chlorophyll *a* fluorescence measurements and the analysis of fast fluorescence kinetics O-J-I-P, biomass increment and photosynthesis. Our results show that plants react differently to ectomycorrhiza formation depending on their age (stage of development, leaf area), their initial nutritional status, and the amount of nitrogen supplied. Mycorrhiza formation was found to constitute a stress depending on the plants' age. Increased availability of N softened or eliminated the negative impact of mycorrhiza formation. Only younger plants eventually developed a higher net photosynthesis rate when mycorrhizal. It is concluded that ectomycorrhiza formation may have a detrimental rather than a beneficial effect on plants' productivity during their establishment and early developmental stages, and that this depends on the amount of N available to the plant, on the nutritional status and on the age of the plant. Chlorophyll *a* fluorescence measurements proved to be a non-destructive, non-invasive and reliable tool able to identify the first signals of plant-mycorrhiza fungi interactions.

Introduction

Although the beneficial role of mycorrhizal symbiosis has been frequently observed, there have been reported cases where mycorrhizal inoculation has led to a decrease in plant productivity, particularly in arbuscular mycorrhizal plants (AM) (e.g., Baon et al., 1994; Jifon et al., 2002; Koide, 1985; Schroeder and Janos, 2004). This decrease has often been found to be transient and recovered later, being followed by a positive growth response. Few such growth depressions are referred to in literature regarding ectomycorrhiza (ECM), which are generally found to enhance plant growth (e.g., Burgess et al., 1994; Lu et al., 1998). However, a number of studies have found no differences in growth between ectomycorrhizal and non-mycorrhizal plants (Bâ et al., 1999; Thomson et al., 1994) and cases of reduced growth have also been found (Colpaert et al., 1992; Eltrop and Marschner, 1996a).

Association with ectomycorrhizal fungi can improve nitrogen acquisition by plants, playing a key role in nitrogen nutrition of forest trees. Ammonium is the predominant form of mineral nitrogen in most of the forest ecosystems that support a profuse ectomycorrhizal development

* FAX No: +351 21 7500048.
E-mail: a.correa@iol.pt

(Prima-Putra et al., 1999; Wingler et al., 1996). Ectomycorrhizal fungi are also known to increase ammonium uptake and ammonium assimilation rates in woody plants (Eltrop and Marschner, 1996a; Javelle et al., 1999; Rudawska et al., 1994; Wingler et al., 1996).

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

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

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

In this study we tried to address the following questions:

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

Material and methods

Plant and fungal material

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

For inoculation, mycelium of *P. tinctorius* was grown for 2 months in the dark at 24 °C on a perlite/vermiculite (v/v) mixture moistened with liquid MMN medium supplemented with 5 g/L glucose.

Pinus pinaster L. seeds were surface-sterilized with 30% calcium hypochlorite for 30 min, rinsed in several changes of 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).

Experimental design

At the time of transfer to the root trainers the plants were divided into three groups. Group 1 was inoculated at the time of transfer from the sowing beds into the root trainers (see Figure 1 for detailed experimental design). Half of the seedlings were inoculated with live (mycorrhizal – M) and half with dead (non-mycorrhizal control – NM) *P. tinctorius* mycelium. For the inoculation, 100 mL inoculum, previously washed with distilled water, was placed in contact with the roots. For the dead mycelium, the inoculum was sterilized for 1 h at 120 °C.

Group 2 was not inoculated at the time of transfer. The plants were watered twice a week with 25 mL distilled water, for 1 month after the transfer to the root trainers. They were then divided into two sub-groups, and inoculated with alive or dead *P. tinctorius* mycelium, as already described for group 1.

In both group 1 and 2, from the moment of inoculation the plants were fed with MMN medium, from which thiamine was omitted. Each sub-group, M or NM, was again divided. Half of the plants received 3.8 m*M* NH_4^+, and the other half 1.9 m*M* NH_4^+ as N source.

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

Group 3 was also not inoculated at the time of transfer. The plants were divided into 2 sub-groups and started being fed with either

3.8 m*M* NH_4^+ or 1.9 m*M* NH_4^+ as N source. After 1 month each of the 2 sub-groups were again divided and inoculated with either alive or dead inoculum. They continued being fed as before.

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

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

The timing of mycorrhiza formation was established by collecting 6 to 8 plants every 2 days from the day of inoculation until day 14, and every 5 days until day 29. Visual assessment of the mycorrhizal status and root ergosterol quantification were performed.

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

Chlorophyll a fluorescence measurements

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

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

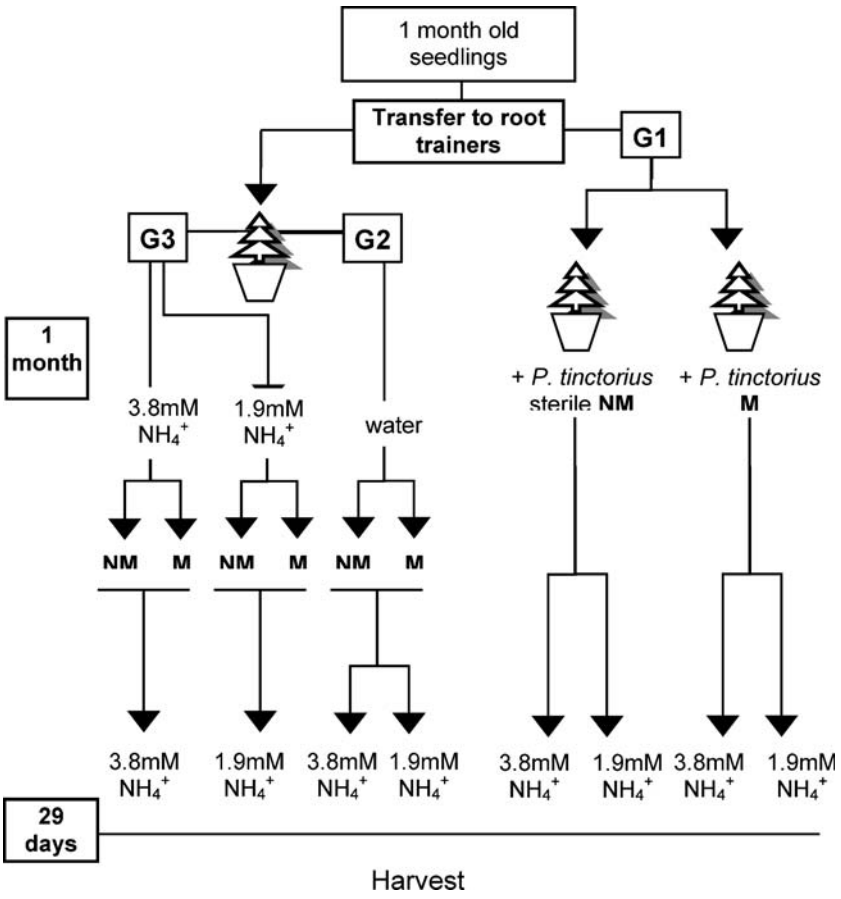

Figure 1. Scheme of the experimental design.

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

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

Recently, the performance index (PI) has been introduced (Srivastava et al., 1999, Strasser et al., 1999; Strasser et al., 2000; Tsimilli-Michael et al., 2000). PI_{abs} combines three parameters favourable to photosynthetic activity: (1) the density of reaction centres (expressed on an absorption basis), (2) the quantum yield of primary photochemistry, and (3) the ability to feed electrons into the electron chain between photosystem II and I (Srivastava et al., 1999). Table 1 summarizes the technical parameters used in calculations as well as a selection of the JIP parameters used in this study.

Net photosynthesis rate

Gas exchange measurements were made with a compact CO_2/H_2O porometer CQP-130 coupled with a NBIR gas analyser (Binus 100 Leybold

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

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

Heraeus, D-6450 Hanau, Germany). Whole shoot net photosynthesis was measured 4, 6, 10, 14, 21 and 29 days after inoculation (day 0) in plants from group 1. The groups 2 and 3 were measured 8, 14 and 29 days after inoculation. The light intensity was 250 μmol m^{-2} s^{-1}. 8–12 plants were measured for each day and group.

Data analysis

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

Results

In plants from groups 1 and 2 the first signs of mycorrhiza establishment were observed 6 days after inoculation. In group 3, this was only observed 12 days after inoculation (data not shown). No mycorrhiza formation was observed in control plants.

Chlorophyll a fluorescence

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

In plants from group 1, that were younger at the time of inoculation, a significant drop in PI_{abs} was observed simultaneously with the first signs of mycorrhiza formation, with both N nutrition regimes (day 6; Figure 2). With 1.9 m*M* NH_4^+, M plants kept a lower level of PI_{abs} than NM throughout the rest of the experiment. With 3.8 m*M* NH_4^+ this drop was recovered to similar levels to those found in NM plants (Figure 2).

The initial drop, simultaneous with the first signs of mycorrhiza formation, was not observed in plants of groups 2 and 3, older at the time of inoculation (Figures 3, 4).

Group 2 plants presented very low PI_{abs} values at the time of inoculation, indicating that they were severely stressed after a month of starvation (Figure 3). With 1.9 m*M* NH_4^+ no significant differences between M and NM plants were observed in this group. With 3.8 m*M* NH_4^+ a rise of PI_{abs}, indicating a recovery from the starvation stress, was observed in both M and NM plants. This recovery was observed sooner in M plants, which had significantly higher PI_{abs} values during the first 12 days of treatment. After day 14 there were no significant differences between M and NM plants (Figure 3).

On group 3, a pronounced drop in PI_{abs} following inoculation was observed in both M and NM plants (Figure 4). This drop was clearly less pronounced in M plants, and the difference between M and NM was higher with 3.8 m*M* NH_4^+. No other differences between M and NM plants were observed throughout the experiment.

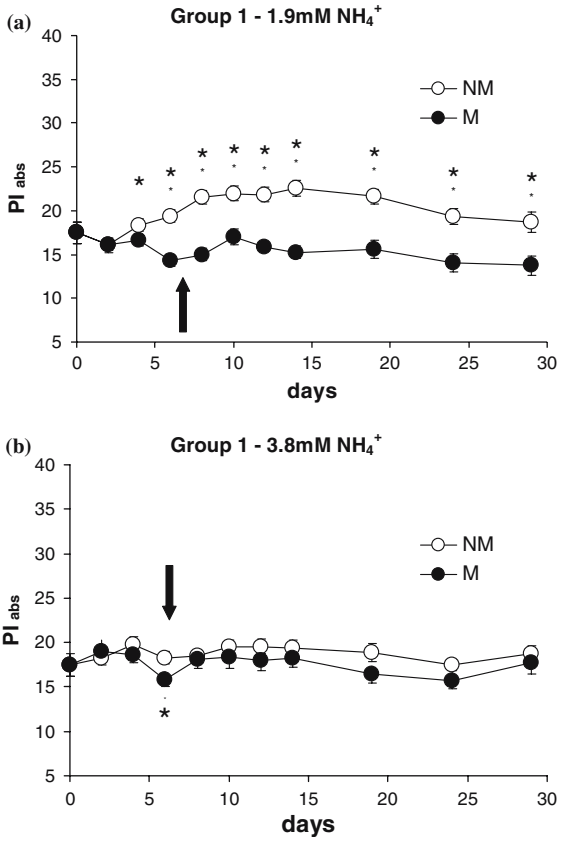

Figure 2. a, b. Photosynthetic Performance Index (PI_{abs}) variation during 30 days after inoculation of plants of group 1. At day 0 the plants were inoculated with live (M; closed circles) or dead (NM; open circles) *P. tinctorius*. The plants were fed with (a) 1.9 m*M* or (b) 3.8 m*M* NH_4^+. The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

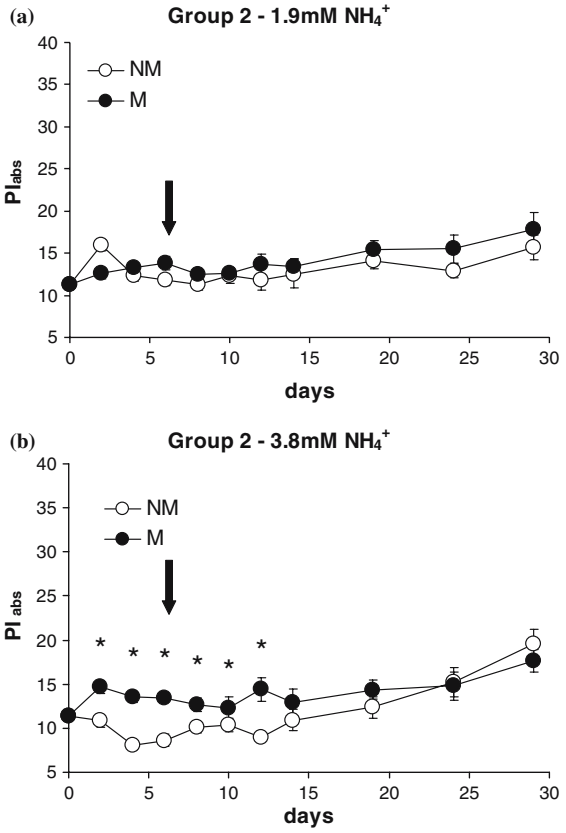

Figure 3. a, b. Photosynthetic Performance Index (PI_{abs}) variation during 30 days after inoculation of plants from group 2. At day 0 the plants were inoculated with live (M; closed circles) or dead (NM; open circles) *P. tinctorius*. The plants were fed with (a) 1.9 m*M* or (b) 3.8 m*M* NH_4^+. The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

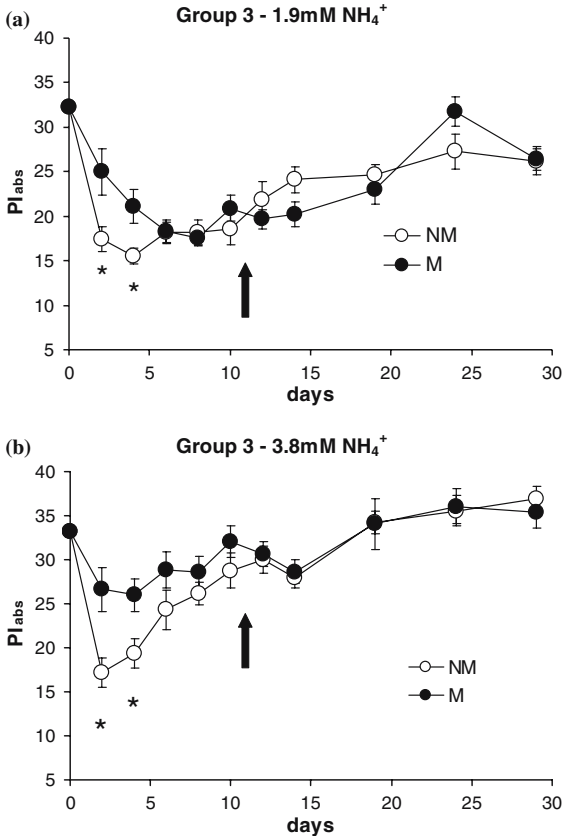

Figure 4. a, b. Photosynthetic Performance Index (PI_{abs}) variation during 30 days after inoculation of plants from group 3. At day 0 the plants were inoculated with live (M; closed circles) or dead (NM; open circles) *P. tinctorius*. The plants were fed with (a) 1.9 m*M* or (b) 3.8 m*M* NH_4^+. The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

Biomass increment

Mycorrhiza formation never had a positive effect in plant biomass gain (Figure 5). On group 1 there were no significant differences between treatments. On group 2, M plants grew less than NM. There were no differences between 1.9 and 3.8 m*M* NH_4^+ fed plants. Group 3 plants fed with 3.8 m*M* NH_4^+ were bigger than those fed with 1.9 m*M* NH_4^+. No differences were found between M and NM plants.

Net photosynthesis rate

Of group 1 plants, M plants exhibited higher net photosynthesis rate than NM plants after 29 days. However, this difference was only significant for 3.8 m*M* NH_4^+ fed plants. M plants of group 2 had lower photosynthesis than NM with both N nutritions. However, these differences were not significant. No significant differences were observed in group 3 (Figure 6).

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

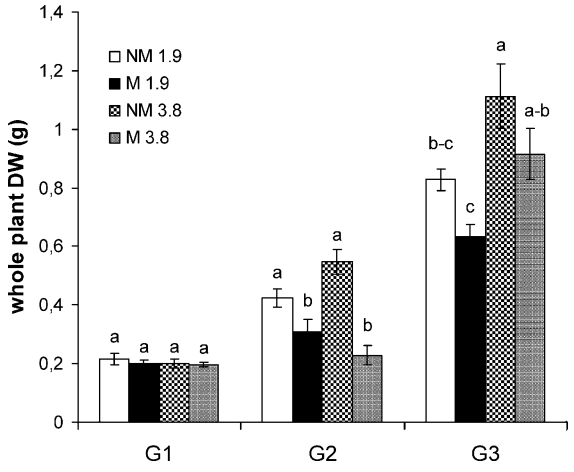

Figure 5. Biomass accumulation in *P. pinaster* plants 29 days after inoculation, in each of the groups of plants studied: group 1 (G1), 2 (G2) and 3 (G3) fed with 1.9 m*M* (1.9; white and black) or 3.8 m*M* NH_4^+ (3.8; chequered and grey). The plants were inoculated with dead (NM) or live (M) *P. tinctorius*.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

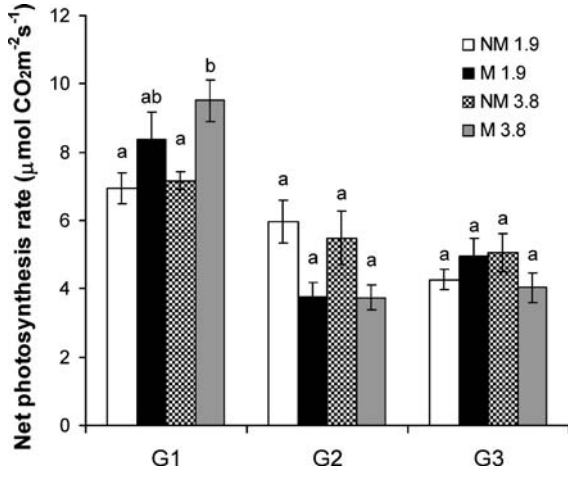

Figure 6. Net photosynthesis rate (μmol CO_2 m^{-2} s^{-1}) in *P. pinaster* plants 29 days after inoculation, in each of the groups of plants studied: group 1 (G1), 2 (G2) and 3 (G3) fed with 1.9 m*M* (1.9; white and black) or 3.8 m*M* NH_4^+ (3.8; chequered and grey). The plants were inoculated with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

Discussion

In younger plants at the time of inoculation (group 1) mycorrhiza had a detrimental effect on the plants' photosynthetic performance (PI_{abs}). This was either confined to the very beginning of mycorrhization (3.8 m*M* NH_4^+) or present throughout the duration of the experiment (1.9 m*M* NH_4^+), depending on the amount of N the plants were fed (Figure 2). A similar detrimental effect of mycorrhiza establishment on the host plants photosynthetic performance has been previously observed in vine plants with AM (Calantziz, 2002). In this previous study, the detrimental effect was transient, and followed by a recovery to a higher level than non-mycorrhizal plants. In our study recovery was only observed in plants that received more N, and never to a higher level than non-mycorrhizal plants. ECM formation can therefore have a negative impact on the host plant, similarly to what has been observed in AM interactions. The amount of N provided to the plant determined if this negative impact was transient or not, or at least influenced its duration.

In plants older at the time of inoculation (groups 2 and 3) a negative impact of mycorrhiza on the plants photosynthetic performance was never observed (Figures 3 and 4). This indicates that age determines the impact mycorrhiza will have on the host plant, and whether this will be a negative one. The smaller total leaf area of younger plants may decrease their capacity to cope with the increased C demand due to fungus colonization. This has been suggested in previous studies where transient growth depressions in response to AM formation have been observed (Schroeder and Janos, 2004). In addition, faster root colonization was observed in younger plants (data not shown) probably due to their smaller root system, which can be colonized faster and more uniformly by an equal amount of inoculum. This faster colonization may have led to a higher initial impact on the host plant.

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

negative (Jifon et al., 2002) effects have been observed.

In this study, no gain in plant biomass was observed as a result of mycorrhiza formation (Figure 5). The increased photosynthesis of M plants of group 1 was therefore not reflected in a higher biomass production (Figure 5). It has been reported that increased photosynthetic rates in mycorrhizal plants with *P. tinctorius* can be associated with increases (Cairney and Chambers, 1997; Reid et al., 1983) or decreases (Eltrop and Marschner, 1996b) in host biomass. Higher shoot growth has also been observed with no changes in photosynthesis in AM associations (Gavito et al., 2000).

Mycorrhiza formation led to decreased growth in starved plants (group 2) and had no effect on growth of non-starved plants of both ages (groups 1 and 3) This indicates that the nutritional status of the plant at the time of inoculation determined the way mycorrhization affected plants' biomass production. Growth depressions following mycorrhizal inoculation are generally attributed to the carbohydrate drain of the mycorrhizal fungus (Thomson et al., 1994). Clear evidence exists that *P. tinctorius* acts as a strong C sink at least during the early stages of the symbiosis (Cairney and Chambers, 1997). Positive growth effects of mycorrhiza are thought to occur when the benefits of increased nutrient uptake exceed the carbon cost of the association (Schroeder and Janos, 2004). In our study, group 2 M plants must not have been able to compensate for this increased C cost, because of their nutritional stress. These plants also had lower photosynthesis, although the differences were not statistically significant, confirming the negative effect of mycorrhiza establishment (Figure 6).

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

A buffer effect due to the presence of the live fungus was observed in groups 2 and 3. The pronounced drop in PI_{abs} following inoculation observed in plants of group 3 seemed to be due to the mechanical stress of the plants' transfer and inoculation process, and independent of mycorrhiza formation (Figure 4). This was less pronounced in M plants, indicating that the inoculation with living mycelium may help them to cope with this stress (Figure 4). This effect was present before any mycorrhiza had formed, and seems therefore to be due only to the presence of the fungus. A similar buffer effect was observed in group 2 plants with 3.8 m*M* NH_4^+ (Figure 3): M plants recovered from starvation quicker than NM plants, showing an immediate rise in PI_{abs} following the onset of the feeding. Again, this effect is visible prior to any mycorrhiza formation. The presence of live fungus seems therefore to have a stress buffering effect.

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

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

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

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

This study also shows the importance of the parameters used for mycorrhiza benefits evaluation.

Their selection is determinant to whether mycorrhiza formation will have a negative impact on the host plant or not. Chlorophyll *a* fluorescence measurements were a non-destructive, non-invasive and reliable tool able to identify the first signals of plant–mycorrhiza fungi interaction.

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