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Ectomycorrhizal fungi as an alternative to the use of chemical fertilisers in nursery production of *Pinus pinaster*

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ABSTRACT

Addition of fertilisers is a common practice in nursery production of conifer seedlings. The aim of this study was to evaluate whether ectomycorrhizal (ECM) fungi can be an alternative to the use of chemical fertilisers in the nursery production of *Pinus pinaster*. A greenhouse nursery experiment was conducted by inoculating seedlings obtained from seeds of *P. pinaster* plus trees with a range of compatible ECM fungi: (1) *Thelephora terrestris*, (2) *Rhizopogon vulgaris*, (3) a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum*, and (4) a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus*, using forest soil as substrate. Plant development was assessed at two levels of N–P–K fertiliser (0 or 600 mg/seedling). Inoculation with a mixture of mycelium from *S. bovinus*, *L. laccata* and *L. deterrimus* and with a mixture of spores of *P. tinctorius* and *S. citrinum* improved plant growth and nutrition, without the need of fertiliser. Results indicate that selected ECM fungi can be a beneficial biotechnological tool in nursery production of *P. pinaster*.

1. Introduction

Pinus pinaster Ait. (maritime pine) represents approximately 23% of the Portuguese forest area and is widely used on reforestation practices (Autoridade Florestal Nacional, 2009). Reforestation efficiency relies on the ability of seedlings to adjust to unfavourable conditions. By increasing their resistance, not only seedlings have a higher growth performance, but also post-transplantation mortality can be reduced. Reforestation using container-grown seedlings of *P. pinaster* produced in nurseries is a common practice in many countries.

Fertilisers are often used in nurseries since they enhance seed germination and root growth and development, resulting in a faster transplantation as desired in management practices for reforestation (Rincón et al., 2007; Walker, 2001). However, problems can arise from transplanting fertilised seedlings into forest soil as they can resent the dramatic change of nutrient availability and may not be able to adjust to the new and often adverse conditions (Castellano and Molina, 1989). Moreover, the use of chemical fertilisers can constitute a threat to the environment. A significant share of nutrients applied may be left in the soil, altering its ecology, and can be lost by leaching leading to eutrophication of surface waters (Entry and Sojka, 2007; Steinfeld et al., 2006; Syers et al., 2008). Another relevant aspect is the fact that some of the nutrients used in fertilisers, such as phosphorus, are not renewable sources and its use must therefore be well managed (Syers et al., 2008).

Ectomycorrhizal (ECM) fungi are known to form symbiotic relations with *P. pinaster* (Nieto and Carbone, 2009; Pera and Alvarez, 1995). Root colonisation by ECM fungi often has a beneficial effect on plant survival and growth. Their network of exploring hyphae or rhizomorphs brings water and nutrients from distant sites to the sites of utilisation by the host plant, in exchange for its photosynthetic carbohydrates (Chalot et al., 2002; Conjeaud et al., 1996). However, the association of host–ECM fungi is not always beneficial for the plant, since the demand for carbohydrates is increased when colonisation occurs, resulting in less carbon for its development (Conjeaud et al., 1996). Fertilisation practices can also have different effects on the establishment of ectomycorrhizas, as nutrient demand and response to nutrient supplements vary among fungi (Rincón et al., 2007). Fertilisers may enhance fungal associations with benefit for the plant (Liu et al., 2008) or, on the other hand, inhibit colonisation (Castellano and Molina, 1989; Vaario et al., 2009). Other studies have also reported that fertilisation does not affect ECM formation (Castellano and Molina, 1989; Conjeaud et al., 1996; Rincón et al., 2007). These observations point to a high specificity between host and ECM fungal species or isolates and also to a high sensitivity of the fungi regarding fertiliser and soil properties.

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The aim of this study was to evaluate whether selected ECM fungi can enhance the growth of *P. pinaster* seedlings, without the use of chemical fertilisers, for reforestation purposes. The work was conducted in a forest nursery greenhouse.

2. Materials and methods

2.1. Experimental design

In a forest nursery greenhouse, in Amarante, Northern Portugal, trays with 210 cm^3 cells were filled with non-sterile homogenised forest soil (10 $mg\,l^{-1}$ N, 325 $mg\,l^{-1}$ P_2O_5, 10,600 $mg\,l^{-1}$ K, 6600 $mg\,l^{-1}$ Mg, 4620 $mg\,l^{-1}$ Ca, 260 $mg\,l^{-1}$ Na, pH (H_2O) 6.2, electrical conductivity 0.2 $mS\,cm^{-1}$) collected from a forest site in Arcos de Valdevez, Northern Portugal. Fungi were added to the substrate as mycelial suspensions or spores. The fungal isolates used in these experiments belong to the collection of Escola Superior de Biotecnologia, and are referenced in the collection as: ref. TT-00, *Thelephora terrestris* Ehrh; ref. RH-01, *Rhizopogon vulgaris* (Vitt.) M. Lange; ref. SB-00, *Suillus bovinus* (Pers.) Roussel; ref. LL-02, *Laccaria laccata* (Scop.) Cooke; and ref. LD-02, *Lactarius deterrimus* Gröger. The isolates were maintained by successive transfers in Potato Dextrose Agar (PDA, Sigma) and in modified Melin Norkans agar (MMN, Marx, 1969). Spores of *Pisolithus tinctorius* (Pers.) Coker & Couch and *Scleroderma citrinum* Pers were collected in from a forest site in Caminha, Northern Portugal. For each treatment, different fungal inocula were used: mycelium of *T. terrestris* Ehrh. (treatment designated as T), mycelium of *R. vulgaris* (Vitt.) M. Lange (treatment designated as R), a spore mixture of *P. tinctorius* (Pers.) Coker & Couch and *S. citrinum* Pers. (treatment designated as PS), and a mixture of mycelium from *S. bovinus* (Pers.) Roussel, *L. laccata* (Scop.) Cooke and *L. deterrimus* Gröger (treatment designated as SLL). These ECM fungal isolates and mixtures were chosen for their compatibility with *P. pinaster* in previous laboratory studies (Oliveira et al., personal communication). The ECM fungal isolates were isolated from forest ecosystems of Northern Portugal. Only one isolate of each ECM fungal species was used in these experiments. Inoculation was performed either by injecting 6 ml of three weeks old mycelial suspensions (ca. 200 mg of fresh weight) or 10 ml of spore suspension (10^7 and 10^6 spores per seedling of *P. tinctorius* and *S. citrinum*, respectively) to the substrate of each cell. The spore concentration was assessed with a haemocytometer. A control treatment with non-inoculated seedlings was also established. All treatments were replicated six times.

P. pinaster seeds collected in the area of Ponte de Lima, Northern Portugal, from five adult trees classified as plus were rinsed overnight in running tap water, surface sterilised with 10% bleach solution for 15 min and washed three times with deionised sterile water. Two disinfected seeds were placed in each root tray. All cells were covered with autoclaved vermiculite (Verlite, Vermiculita y Derivados S.L., Asturias, Spain). One month after placing seeds and inoculum, plants were trimmed to one seedling per cell and two fertilisation treatments were applied: no fertilisation (0 mg/seedling) and fertilisation (600 mg/seedling). The nutrients were supplied as N–P–K slow release fertiliser (12% N, 12% P_2O_5, 17% K_2O, 2% MgO, 15% SO_3, 0.02% B, 0.1% Fe, 0.01% Zn) (BASF, Germany). Seedlings were watered everyday and maintained under an average photoperiod of 8 h. Greenhouse temperature varied between 1.9 and 41.0 °C and relative humidity between 10 and 80%. Trays of different treatments were periodically rotated to different bench positions to minimise differences due to their location in the greenhouse. With the exception of fungal inoculation, all the above mentioned procedures are currently used in forest nursery production in Portugal.

2.2. Plant sampling and analysis

After six months, all seedlings were gently removed from the trays and transported to the laboratory for further analyses. The shoot height was measured. The root system was separated from the shoot and washed to remove adhered substrate. The % of ECM fungal colonisation and the number of ECM root tips per root length were assessed using a stereomicroscope (SZ30, Olympus, Japan) according to Brundrett et al. (1996). Representative ECM root tips were characterised on the basis of colour, branching, shape, presence of emanating hyphae and inner and outer mantle patterns under a stereomicroscope and by differential interference contrast microscopy (BX60, Olympus, Japan) according to Agerer (1998). The fresh weight of the plants was determined by weighing plant material. Needles were dried at 70 °C for 48 h. Oven-dried needles were finely ground and 0.2 g of material were digested according to Novozamsky et al. (1983). The digested samples were used to determine the total nitrogen (N) concentration in needles by colorimetry (Unicam, Helios Gamma, Cambridge, UK) (Walinga et al., 1989).

2.3. Statistical analysis

The data were tested for normality and analysed using one-way analysis of variance (ANOVA). When a significant F-value was obtained ($P < 0.05$), treatment means were compared using the Duncan's multiple range test. Regression analyses were conducted at a significance level of 0.05. All statistical analyses were performed using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Plant parameters

The effect of fertilisation on the shoot height of *P. pinaster* seedlings varied with fungal inoculation. Fig. 1 shows that fertilisation led to an increase in shoot height of non-inoculated plants. In inoculated plants, the use of fertiliser increased shoot height in plants inoculated with the individual fungi *T. terrestris* and *R. vulgaris*, whereas the opposite effect was verified with the fungal mixtures *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*, where fertilised plants were significantly smaller than non-fertilised ones. Moreover, fertilised *T. terrestris* and *R. vulgaris* treatments and non-fertilised treatments using fungal mixtures,

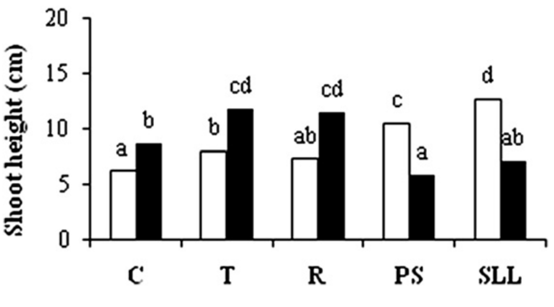

Fig. 1. Shoot height of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

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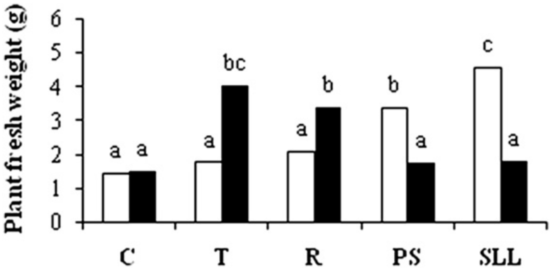

Fig. 2. Plant fresh weight of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

resulted in significantly higher plants than in non-inoculated controls.

There was no apparent influence of fertilisation in plant fresh weight of control seedlings. In inoculated plants, fertilisation enhanced plant biomass when *T. terrestris* and *R. vulgaris* were inoculated, whereas in the presence of mixtures of fungi (*P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*), non-fertilised plants showed a significantly higher biomass. Also, fertilised *T. terrestris* and *R. vulgaris* and non-fertilised plants inoculated with fungal mixtures, produced greater biomass than non-inoculated ones (fertilised and non-fertilised) (Fig. 2).

In non-inoculated plants and in plants inoculated with *T. terrestris*, fertilisation had no significant effect in N needle concentration. However, a different response was obtained for other fungal treatments. Fertilised plants inoculated with *R. vulgaris* presented a higher N concentration than the non-fertilised ones, whereas the opposite effect was verified for plants inoculated with the fungal mixtures (*P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*). Regarding non-fertilised treatments, the N concentration in plants inoculated with *T. terrestris* or *R. vulgaris* was similar to that of non-inoculated plants. The needles of plants inoculated with fungal mixtures, however, showed significantly higher N concentration. Regarding fertilised plants, the opposite effect was obtained. Plants inoculated with fungal mixtures had similar N concentration as control plants whereas the single fungal treatments (*T. terrestris* and *R. vulgaris*) presented higher N concentration (Fig. 3).

3.2. Fungal parameters

Fertilisation did not affect the percentage of root colonisation by ECM fungi (Fig. 4). Seedlings inoculated with fungal mixture *S. bovinus* + *L. laccata* + *L. deterrimus* showed a significantly higher ECM colonisation than non-inoculated controls (both fertilised and non-fertilised). The same was observed in the treatment *P. tinctorius* + *S. citrinum* without fertiliser. Non-fertilised *S. bovinus* + *L. laccata* + *L. deterrimus* and fertilised *R. vulgaris* were the treatments where a significantly higher number of ECM root tips per root length was observed (Fig. 5). The application of fertiliser decreased the number of ECM root tips in seedlings inoculated with *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*.

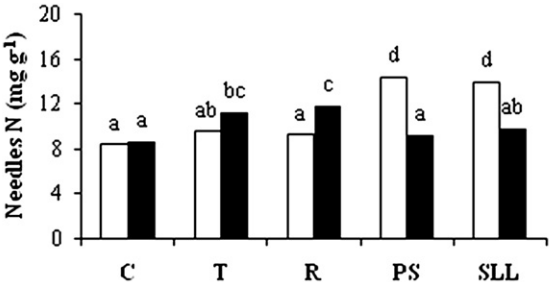

Fig. 3. Needles nitrogen concentration of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Values are expressed in mg of N per g of oven-dried needles. Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

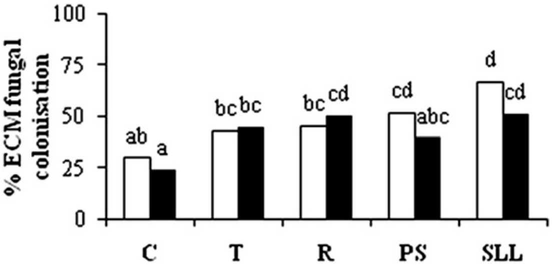

Fig. 4. Percentage of ectomycorrhizal fungal colonisation of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$. ECM, ectomycorrhizal.

The ECM morphotypes identified for each fungal treatment under the two fertilisation regimes are presented in Table 1. Non-inoculated plants had the lowest number of different ECM morphotypes while the fungal mixture *S. bovinus* + *L. laccata* + *L. deterrimus* presented the highest. In plants inoculated with *R. vulgaris* and *S. bovinus* + *L. laccata* + *L. deterrimus*, fertilisation decreased the number of morphotypes whereas in the other fungal treatments no difference occurred. Moreover, in non-inoculated plants and in those inoculated with *T. terrestris* and *P. tinctorius* + *S. citrinum*, the same morphotypes were observed in non-fertilised and in fertilised plants. The only three morphotypes occurring in non-inoculated plants were present in all fungal treatments. With the exception of the morphotype EM4, which appears simultaneously in plants inoculated with *R. vulgaris* and *S. bovinus* + *L. laccata* + *L. deterrimus*, the remaining morphotypes were specific to each fungal treatment.

3.3. Correlation between plant development and fungal colonisation

The plant fresh weight and shoot height of *P. pinaster* showed a significantly positive correlation with the percentage of ECM colonisation when no fertiliser was applied (Figs. 6a and 7a).

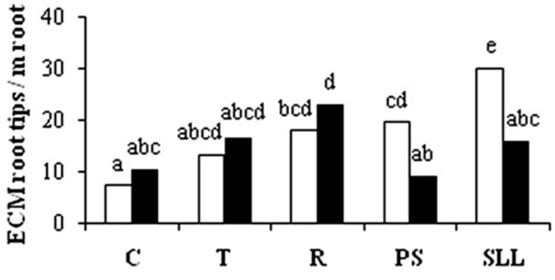

Fig. 5. Number of ectomycorrhizal root tips per root length of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

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Table 1
Ectomycorrhizal morphotypes found on the roots of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised or fertilised.

Morphotype code	Non-fertilised					Fertilised				
	C	T	R	PS	SLL	C	T	R	PS	SLL
EM1	+	+	+	+	+	+	+	+	+	+
EM2	+	+	+	+	+	+	+	+	+	+
EM3	+	+	+	+	+	+	+	+	+	+
EM4	−	−	+	−	+	−	−	−	−	+
EM5	−	+	−	−	−	−	+	−	−	−
EM6	−	−	−	+	−	−	−	−	+	−
EM7	−	−	+	−	−	−	−	+	−	−
EM8	−	−	−	−	+	−	−	−	−	+
EM9	−	−	−	−	+	−	−	−	−	−
Total number	3	4	5	4	6	3	4	4	4	5

+, presence; −, absence.
Morphotype description: EM1 – Dark brown, unbranched, long tortuous tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells and mounds of flattened cells, few emanating hyphae; EM2 – Dark brown, dichotomous branching, tortuous tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells, few emanating hyphae; EM3 – Dark orange, unbranched, straight tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells; EM4 – Dark grey, unbranched, smooth mantle surface; EM5 – Brown and whitish, dichotomous branching, tortuous tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells and irregularly arranged hyphae, few emanating hyphae; EM6 – Light brown, dichotomous branching, long tortuous tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells, few emanating hyphae; EM7 – Dark orange, dichotomous branching, straight tips, grainy mantle surface, pseudoparenchymatous outer and inner mantle with angular cells and mounds of flattened cells; EM8 – Golden yellow, dichotomous branching, straight hairy tips, pseudoparenchymatous outer mantle, plectenchymatous inner mantle with epidermoid cells bearing a delicate hyphal net, abundant emanating hyphae; EM9 – Light grey, monopodial pinnate branching, tortuous tips, smooth mantle surface.

The plant fresh weight and shoot height of non-fertilised *P. pinaster* were also significantly positive correlated with the number of ECM fungal tips per m of root (Figs. 6b and 7b). In fertilised plants there was no correlation between any plant and fungal parameters (data not shown).

4. Discussion

Many studies performed in nurseries have been carried out with single ECM fungal species (Duñabeitia et al., 2004; González-Ochoa et al., 2003; Walker, 2001). However, in nature, forest trees are often colonised by multiple ECM fungi (Parladé et al., 1999). It is suggested that these seedlings are more resistant than those colonised by single species (Parladé and Alvarez, 1993) as fungi can have complementary behaviour, with benefit to the host plant (Reddy and Natarajan, 1997). In this study two single species (*T. terrestris* and *R. vulgaris*) and two mixtures (*P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*) were tested as an alternative to the use of fertiliser in nurseries. The development of *P. pinaster*, including biomass and height, was highly affected by fungal inoculation. The highest plant growth was obtained with fertilised *T. terrestris* and *R. vulgaris* and non-fertilised fungal mixtures. All these treatments were more effective in promoting plant growth when compared with non-inoculated controls (both fertilised and non-fertilised).

Without the application of fertiliser, seedlings inoculated with *R. vulgaris* presented no significant growth difference from controls. Similar results were reported for *Rhizopogon* spp. by Rincón et al. (2005) and may be due to their high demand of carbohydrates, which does not allow the plant to obtain the carbon it needs for its growth. All other inoculation treatments (non-fertilised) promoted plant growth as also reported in other studies with *Pinus* spp. (Reddy and Natarajan, 1997; Rigou et al., 1995; Rincón et al., 2007).

When fertiliser was applied, there were also differences in plant development amongst the inoculation treatments. While inocula addition greatly enhanced plant height and weight on treatments with the individual fungi *T. terrestris* and *R. vulgaris*, the opposite was verified with the fungal mixtures *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*. *T. terrestris* is a common nursery seedling colonising ECM fungus. It is considered an early-stage fungus with medium-distance exploration (Agerer, 2001), which does not form a dense mycelium and is not the most efficient in nutrient uptake when external nutrient concentrations are low (Colpaert et al., 1999). These characteristics may explain the improvement in plant performance verified with the addition of fertiliser, since nutrients are more accessible to the fungus and consequently to the plant. Nevertheless, no significant difference was obtained in N needle concentration between fertilised and non-fertilised *T. terrestris* inoculated plants, suggesting an uptake of other nutrients. Fertilised plants in treatments *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus* showed a decrease in plant development compared with the non-fertilised seedlings, which may be related to the significant decrease in the number of ECM fungal tips per m of root. Negative effects or no effect of fertilisation on plants inoculated with ECM fungi have been reported in studies with *L. laccata*, *P. tinctorius*, *Rhizopogon* spp. (Castellano and Molina, 1989) and *Hebeloma cylindrosporum*

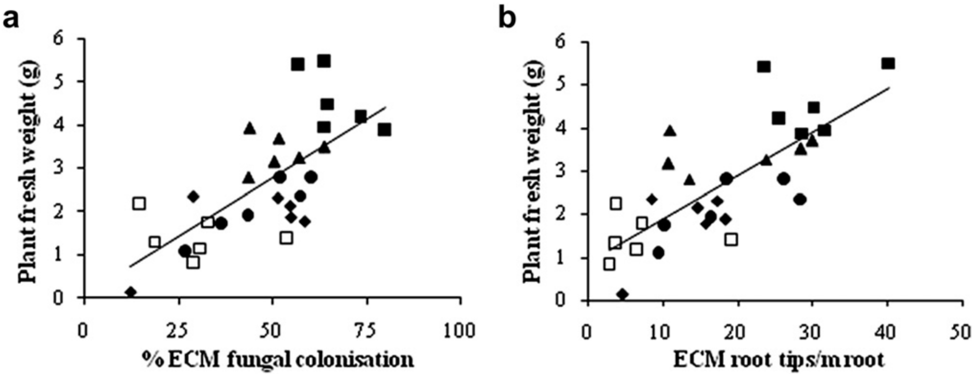

Fig. 6. Relationship between the plant fresh weight of non-fertilised *Pinus pinaster* seedlings and (a) the percentage of ectomycorrhizal fungal colonisation ($y = 0.054x + 0.084$, $R^2 = 0.489$, $P < 0.001$); and (b) the number of ectomycorrhizal root tips per meter of root ($y = 0.101x + 0.872$, $R^2 = 0.579$, $P < 0.001$). Seedlings inoculated with *Thelephora terrestris* (black diamonds), *Rhizopogon vulgaris* (black circles), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (black triangles), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (black squares) and non-inoculated control (open squares).

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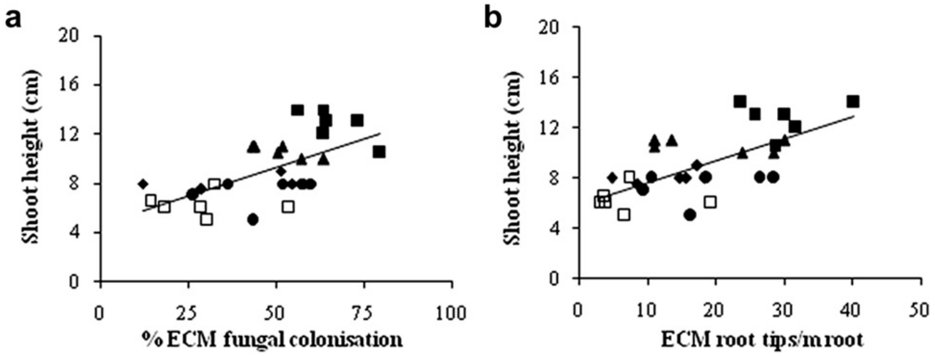

Fig. 7. Relationship between shoot height of non-fertilised *Pinus pinaster* seedlings and (a) the percentage of ectomycorrhizal fungal colonisation ($y = 0.093x + 4.607$, $R^2 = 0.384$, $P < 0.001$) and (b) the number of ectomycorrhizal root tips per meter of root ($y = 0.173x + 5.943$, $R^2 = 0.457$, $P < 0.001$). Seedlings inoculated with *Thelephora terrestris* (black diamonds), *Rhizopogon vulgaris* (black circles), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (black triangles), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (black squares) and non-inoculated control (open squares).

(Conjeaud et al., 1996). ECM fungi are especially important for the host plant on soils with low fertility, where the need for their exploring ability promotes plant–fungi association (Castellano and Molina, 1989). Adding nutrients to the soil can implicate a dramatic change on fungal behaviour and also an increase of plant independence towards fungi, not promoting the symbiotic association with a negative effect of fertilisation in plant development.

The majority of morphotypes occurred in both fertilisation treatments. However, plants inoculated with *R. vulgaris* and *S. bovinus* + *L. laccata* + *L. deterrimus* presented one less morphotype than the correspondent non-fertilised ones, indicating that fertiliser might have inhibited ECM formation. These results and the fact that fertilisation had no influence in the percentage of ECM colonisation corroborate the perception that different species respond very differently to fertilisation.

Variation in the percentage of ECM colonisation accounted for 48.9% of the variation in plant fresh weight and 38.4% in shoot height of non-fertilised *P. pinaster*, while variation in the number of ECM fungal tips accounted for 57.9% of the variation in plant fresh weight and 45.7% in plant shoot height of non-fertilised *P. pinaster*. The present data indicate that ECM fungal colonisation influences the growth of *P. pinaster* under nursery conditions. The fact that no correlation was found in fertilised seedlings suggests that when fertiliser is applied growth is more dependent on plant than on fungal mechanisms, since nutrients are more accessible, whereas when no fertiliser is applied, plant development is more dependent on the ECM fungal symbiosis. The overall N needle concentration was strongly consistent with fungal colonisation and plant biomass. Non-fertilised fungal treatments *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*, which presented significantly higher percentage of ECM fungal colonisation than the control plants, also presented higher N needle concentration and higher plant biomass, suggesting that N deficiency was suppressed by the inoculated fungi. Non-fertilised plants inoculated with *T. terrestris* and *R. vulgaris*, although had higher number of morphotypes, presented similar fungal colonisation percentage, similar N needle concentration and similar biomass when compared with the non-inoculated plants. Analogous cohesiveness between the three parameters was observed in fertilised plants, with the exception of the treatment *S. bovinus* + *L. laccata* + *L. deterrimus*, indicating that this fungal mixture is not as efficient in N uptake.

The major advantage of the use of fertilisers in nursery seedlings relies on the supply of nutrients, which speeds their production. However, there are some associated environmental threats, due to the leaching of nutrients, and economical disadvantages, since fertiliser can be an important financial fraction in production costs. In this study plants inoculated with selected ECM fungi had a greater biomass without the application of fertiliser under nursery conditions.

It is also important to investigate whether the behaviour of seedlings will be maintained after transplantation. Field studies conducted on this research topic presented distinct outcomes. Selosse et al. (2000) reported that inoculated *Laccaria bicolor* persisted at least ten years after outplanting and greatly enhanced plant development, whereas the inoculation of Sitka spruce with *L. laccata*, *Hebeloma crustuliniforme* and *Cenococcum geophilum* performed by Shaw et al. (1987) did not promote any nutrient benefits in comparison with non-inoculated seedlings. Quoreshi et al. (2008) and Vosátka et al. (2008) reported that a successful inoculation can be obtained by using more host-specific and site-specific plant–fungal combinations.

5. Conclusion

The application of chemical fertilisers should be minimised as its long-term consequences are unknown. The results from this study show that it is possible to replace chemical fertilisers by ECM fungi in the nursery production of *P. pinaster*, with a significant increase in plant development, and thus the use of selected ECM fungi can be an effective and more environmental friendly approach to plant management in the nursery. Nevertheless, due to the specificity of the ECM associations, further nursery and field studies should be undertaken in order to assess which fungal inoculum and conditions are adequate to produce more resistant and healthier outplanted seedlings.

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