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Combined use of *Pinus pinaster* plus and inoculation with selected ectomycorrhizal fungi as an ecotechnology to improve plant performance

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A B S T R A C T

Pinus pinaster is an important forest species for environmental and economic reasons. Due to its importance, tree improvement plans aimed at the selection and use of phenotypically superior trees, designated by plus trees, have been established. It is known that ectomycorrhizal (ECM) fungi can improve tree survival and growth. Seeds obtained from *P. pinaster* plus trees have been used in forest nurseries. However, the effect of inoculation with ECM fungi on the performance of these plants has not been studied. We compared the performance of *P. pinaster* plants obtained from seeds of plus and non-plus trees to inoculation with different selected ECM fungi under conventional forest nursery conditions. In plants obtained from seeds of non-plus trees only those inoculated with *Suillus bovinus* + *Laccaria laccata* + *Lactarius deterrimus* had a significantly greater biomass and needles nitrogen concentration, while in plants obtained from seeds of plus trees this effect was seen not only in those receiving that same ECM inoculation, but also in those inoculated with *Rhizopogon roseolus* or *Pisolithus tinctorius* + *Scleroderma citrinum*. The best performance was that of plants obtained from seeds of plus trees and inoculated with *R. roseolus* or *S. bovinus* + *L. laccata* + *L. deterrimus*, with an increase in biomass of 2.2 and 2.0 times, respectively. This significant improvement was achieved without extra input of agrochemicals. The combined use of seeds obtained from plus trees and inoculation with selected ECM fungi can be an advantageous ecotechnological approach to improve nursery production of *P. pinaster*.

1. Introduction

Pinus pinaster Ait. (maritime pine) is a relevant forest species with broad distribution in the western Mediterranean Basin, in Southern Europe and Africa, and in the Atlantic coast of Portugal, Spain and France. This species was also introduced by forestation in other European countries, Australia, New Zealand, South Africa and South America (Oliveira et al., 2000). It is the main coniferous species in France in terms of planted area and harvest yield (Pot et al., 2002) and it is the main forest species in Portugal occupying ca. 27% of the total forest area (Autoridade Florestal Nacional, 2010). The *P. pinaster* forest is important for environmental and economic reasons. *P. pinaster* has amazing performance as a pioneer tree and has been used for protection plantations and for restoration of degraded and impoverished soils (Barčić et al., 2006). Also, it is used as a main source for multiple industrial applications (e.g. wood, paper, resin) (Baptista et al., 2008; Louzada and Fonseca, 2002).

In Portugal, a tree improvement programme of *P. pinaster* has started in the early 1980s (Roulund et al., 1988). Since then, superior trees, designated as plus trees, have been selected in natural stands. The selection criteria of plus trees include characteristics such as growth rate, stem and timber quality, crown shape, resistance to diseases, to insect attack and to adverse environmental abiotic conditions, and the ability to produce fertile seeds (Lee, 2002).

Field transplantation of container-grown seedlings produced from seeds in nurseries is the most common method to establish *P. pinaster* plantations. The use of seeds from plus trees in the production of *P. pinaster* can be advantageous and contribute to obtain better plants for forestry industries. This approach to improve production avoids genetic modification of plants and is generally better accepted by the public and decision makers than other approaches involving gene transfers in plant material (Koski and Rousi, 2005).

Although seeds from plus trees have been used to increase forestry production of species from different genera (e.g. *Betula*, *Pinus*, *Pseudotsuga*) (Koski and Rousi, 2005; Lee and Connolly, 2004; Prat and Caquelard, 1995), studies are needed on the effect of nursery practices on the growth performance of seedlings obtained from seeds of plus trees. The application of ectomycorrhizal (ECM) fungi inocula on forest nursery production is becoming to be

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regarded as a good-practice management due to its potential for increasing growth and vigour of seedlings under nursery conditions (Brundrett et al., 2005; Chen et al., 2006; Duponnois et al., 2007) and for improvement in quality and performance of outplanted seedlings (Duponnois et al., 2007; Quoreshi et al., 2008; Rincón et al., 2007a).

ECM fungi can improve plant survival and growth by stimulating the uptake of soil nutrients and water, and by increasing plant resistance against biotic (e.g. plant pathogens) and abiotic (e.g. presence of toxic elements) stresses (Chalot et al., 2002; Garbaye, 2000; Hall, 2002; Smith and Read, 2008). Thus, the use of ECM fungi in forest nursery production can be considered an environmentally friendly approach, as it may help to reduce the input of chemical fertilisers and pesticides, preventing the contamination of soil and water resources (Khasa et al., 2001; Oliveira et al., 2010; Sousa et al., 2012). Although potentially beneficial, nursery inoculation is not always straightforward, and requires the selection of compatible and efficient ECM fungal isolates tuned for specific target plant and growth conditions (Oliveira et al., 2011; Vosátka et al., 2008). ECM fungi are known to form symbiotic associations with *P. pinaster* (Nieto and Carbone, 2009; Rincón and Pueyo, 2010). However, their effect in *P. pinaster* plus trees has not been studied.

The aims of the present study were to compare the response of *P. pinaster* plants obtained from seeds of plus and non-plus trees to inoculation with different selected ECM fungi under conventional forest nursery conditions; and to assess the potential of the combined use of seeds obtained from plus trees and inoculation with ECM fungi as a new ecological approach to improve the production of this important tree species.

2. Materials and methods

2.1. Experimental design

In a forest nursery greenhouse, in Amarante (41°16′11″N 8°04′40″W), Northern Portugal, trays with 210 cm^3 cells were filled with non-sterile homogenised peat (1636 mg kg^{-1} N-NO_3^-, 1833 mg kg^{-1} P_2O_5, 12,528 mg kg^{-1} K_2O, 31,000 mg kg^{-1} Mg, 16,400 mg kg^{-1} Ca, 400 mg kg^{-1} Na, pH 6.47, electrical conductivity 14.4 mS cm^{-1}). *P. pinaster* seeds were collected in the area of Ponte de Lima (41°52′30″N 8°50′24″W), Northern Portugal, from five adult trees classified as plus and from five adjacent adult non-plus trees. The classification criteria of the plus trees was based on volume, stem morphology, spiral grain and branch habits (Perry and Hopkins, 1967). Experimental units were arranged in a fully randomized manner using a 2 × 5 factorial design where one factor was seedling type (ST) (from plus and non-plus trees) and the second factor was fungal inoculation (FI) [non-inoculated controls (C), plants inoculated with mycelium of *Thelephora terrestris* Ehrh. (T), mycelium of *Rhizopogon roseolus* (Corda) Th. Fr. (R), a spore mixture of *Pisolithus tinctorius* (Pers.) Coker & Couch and *Scleroderma citrinum* Pers. (PS), and a mixture of mycelium of *Suillus bovinus* (Pers.) Roussel, *Laccaria laccata* (Scop.) Cooke and *Lactarius deterrimus* Gröger (SLL)]. Each treatment combination was replicated 10 times. These ECM fungal isolates and mixtures were chosen for their compatibility with *P. pinaster* in previous laboratory and greenhouse studies (Oliveira, R.S.; Franco, A.R.; Castro, P.M.L., unpublished; Sousa et al., 2012). Inoculation with a variety of ECM fungi including single/multiple species and spore/mycelium inoculum was adopted in order to try to assess a range of plant responses where possible differences in growth performance of *P. pinaster* seedlings obtained from seeds of plus and non-plus trees could be detected. The fungal isolates were isolated from forest ecosystems of Northern Portugal and were maintained by successive transfers in modified Melin Norkans agar (MNM, Marx, 1969). All 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, *T. terrestris*; ref. RH-01, *R. roseolus*; ref. SB-00, *S. bovinus*; ref. LL-02, *L. laccata* and ref. LD-02, *L. deterrimus*. Spores of *P. tinctorius* and *S. citrinum* were collected from a *P. pinaster* forest site in Caminha (41°46′04″N 8°35′03″W), Northern Portugal. Inoculation was performed either by injecting 6 ml of three weeks old mycelial suspensions (ca. 170 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. Seeds 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. The experiment was initiated at the time of placing seeds and inoculum (June 2004). One month after that, plants were thinned to one seedling per cell, guaranteeing that there were 10 seedlings in each treatment. Plants were watered everyday and maintained under an average photoperiod of 8 h. Greenhouse temperature varied between 5.0 and 40.0 °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 and the use of seeds from plus trees, all the above mentioned procedures are currently used in forest nursery production.

2.2. Plant and fungal parameters

All plants survived and six months after the beginning of the experiment, they were gently removed from the trays and transported to the laboratory for further analyses. The root collar diameter and shoot height were measured. The root system was separated from the shoot and washed to remove adhered substrate. The percentage of ECM fungal colonisation was 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 dry weights of roots and shoots were determined after drying the plant material at 70 °C for 48 h and the total plant dry weight obtained as the sum of shoot and root dry weights. 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 phosphorus (P) and nitrogen (N) concentrations in needles by colorimetry (Unicam, Helios Gamma, Cambridge, UK) (Walinga et al., 1989).

2.3. Statistical analysis

The data were analysed using two-way analysis of variance (ANOVA) for each dependent variable (plant and fungal parameters) versus the independent variables [seedling type (ST) and fungal inoculation (FI)]. When a significant *F*-value was obtained ($P < 0.05$), treatment means were compared using Duncan's multiple range test. All statistical analyses were performed using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Plant growth and nutrition

ECM inoculation and seedling type improved the growth of plants. The factor fungal inoculation showed a significant effect on