Reforestation of burned stands: The effect of ectomycorrhizal fungi on *Pinus pinaster* establishment

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A B S T R A C T

The area occupied by *Pinus pinaster* in Portugal is rapidly diminishing because of forest fires. Ectomycorrhizal fungi form obligate, mutually beneficial associations with *P. pinaster* which improve plant growth and resistance to adverse conditions. The aim of this work was to assess whether native ectomycorrhizal fungi could be a useful tool in the reforestation of burned areas. The work was conducted in a forest nursery greenhouse, where *P. pinaster* seedlings were inoculated with compatible ectomycorrhizal fungal isolates: *Suillus bovinus*, *Pisolithus tinctorius*, *Rhizopogon roseolus*, and a mixture of the three fungi, using burned and unburned forest soil as substrate. Inoculation significantly enhanced the growth of *P. pinaster*, with *R. roseolus* proving to be the most effective in burned soil, with an 8-fold increase in plant fresh weight. Overall, inoculation stimulated growth most in burned than in unburned soil.

This study suggests that inoculation with selected ectomycorrhizal fungi in containerised nurseries can be an advantageous approach for the successful establishment of *P. pinaster* in burned soil. The obtained results point out to the interest of extending these studies into fire-impacted areas, using ectomycorrhizal fungi as a biological tool.

1. Introduction

Maritime pine (*Pinus pinaster* Ait.) covers a large area of Southwest Europe and is one of the most economically important forest species (Fernandes and Botelho, 2004). However, primarily because of its high fuel content, Mediterranean maritime pine stands are highly flammable and prone to wildfires. The mean annual area burned in Portugal is 1070 km^2 which is, by far, the highest fire incidence in Europe (Nunes et al., 2005). In the 1990s, 48% of the Portuguese burned forest area belonged to *P. pinaster* plantations (Pereira and Santos, 2003). Although this species is considered one of the most resistant to low and moderate severity burns (Fernandes et al., 2008), its natural capacity to regenerate is often suppressed by high-severity fires. To circumvent such events, low to medium intensity burns are advised (Fernandes and Rigolot, 2007). Nevertheless, destructive high-severity wildfires are still common, particularly during the hot and dry summers of the Iberian Peninsula and are a major cause of vegetational changes in Portugal.

There are numerous studies addressing the impact of fire/heat on the physical, chemical and mineralogical properties of soil and its microbial community (Bárcenas-Moreno and Baath, 2009; Certini, 2005; Dahlberg et al., 2001; Esquilín et al., 2007; González-Peres et al., 2004; Hamman et al., 2007; Hart et al., 2005; Kipfer et al., 2010). The extent of such change is determined by several factors such as the severity of the burn, climate, surrounding vegetation, topography and soil moisture content (Certini, 2005).

P. pinaster forms ectomycorrhizas which commonly have significant effects on nutrient uptake, growth and plant survival and are, therefore, important components (Conjeaud et al., 1996) of all forest ecosystems, including the Mediterranean climatic regions (Courty et al., 2010). Ectomycorrhizal (ECM) fungi provide nutrients that otherwise would not be available to the host plant and, in exchange, the fungi receive photosynthates (Conjeaud et al., 1996; Pera and Alvarez, 1995). It is not easy to draw conclusions on the impact fire has on ECM fungal communities. An immediate consequence is a heat-induced mortality (Hart et al., 2005; Kipfer et al., 2010). Nevertheless, it is likely that changes which influence the photosynthetic production of the host tree, such as forest fire, will also affect the abundance and composition of ECM fungal populations (Baar et al., 1999; Dahlberg et al., 2001; Esquilín et al., 2007; Hart et al., 2005). Literature on fire-impacted fungal communities is strongly focused on the natural regeneration of the ecosystem (Baar et al., 1999; Esquilín et al., 2007; Hamman et al., 2007; Torres and Honrubia, 1997). However, reforestation

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processes are often required to mitigate severe burns and studies show that seedlings inoculated with ECM fungi at the nursery stage can enhance plant development in the field (Vosátka et al., 2008).

Due to the importance of *P. pinaster* in the spectra of Portuguese forest economy, a breeding program was initiated in the 1980s (Miguel et al., 2004). Plus pines trees are trees carefully selected taking into account growth rate and form traits. Given that plus trees are proven to have higher capacity of resistance to illnesses and adverse conditions they ought to be considered for reforestation purposes.

The aim of this work was to assess whether ECM fungi can enhance *P. pinaster* growth in burned soil. The study was conducted in a forest nursery greenhouse and the substrate used was forest soil, burned in a muffle furnace. This approach had the aim to isolate the soil burn effect from other environmental factors whilst simulating the reforestation process of a stand affected by a severe fire.

2. Materials and methods

2.1. Soil samples

Soil was collected from a forest area located in Arcos de Valdevez, Northern Portugal. It was sieved through a 5 mm mesh, thoroughly mixed and stored until the beginning of the experiment. Burned soil was obtained by heating samples in a muffle furnace (Nabertherm, Controller B170) for 20 min at 500 °C (Bárcenas-Moreno and Baath, 2009). The heated soil was allowed to cool to room temperature. Later it was mixed with a little fresh soil (6:1) to simulate the gradual build-up of a normal soil flora during an interval between burning and replanting. Unheated samples were used as controls. The main composition of both substrates is shown in Table 1.

2.2. Mycorrhization of P. pinaster seedlings

The fungal isolates used in these experiments belong to the collection of Escola Superior de Biotecnologia and have been maintained by successive transfers in Potato Dextrose Agar (PDA, Sigma) and in modified Melin Norkans agar (MMN, Marx, 1969). They are referenced in the collection as: ref. RH-10, *Rhizopogon roseolus*; ref. PT-00, *Pisolithus tinctorius* (Pers.) Coker & Couch; ref. SB-00, *Suillus bovinus* (Pers.) Roussel. These isolates were chosen for their compatibility with *P. pinaster* in previous laboratory studies (unpublished data) and their intrinsic characteristics, such as ability to readily colonise roots of *P. pinaster*, occurrence in post-fire sites and field persistence. *P. pinaster* seeds were collected in the area of Ponte de Lima, Northern Portugal, from five adult trees classified as plus and were disinfected as described by Pera and Alvarez (1995). Seedlings were germinated in water-agar (10%) and transferred to individual cells of PVC tubes containing 45 cm^3 of substrate composed by sieved peat, 2 mm mesh, vermiculite (n°3, Neoquímica) and unburned forest soil in a ratio of 1:1:1. The substrate mixture was autoclaved twice over two consecutive days. A soil bacterial suspension was added to the substrate mixture (25 mL bacterial suspension/l L substrate) to restore soil bacterial community. Soil was first homogenised and then added to sterile deionised water in a 1:2 proportion (1 kg of soil/2 L water). The resulting suspension was homogenised by vigorous mixing for 1 h. Following this treatment, soil particles were allowed to settle and the supernatant was filtered 5 times through Whatman N° 1 filter paper (Hall, 1978).

Fungal inoculation was performed by injecting 5 ml of three weeks old mycelial suspensions (ca. 170 mg of fresh weight) to the substrate of each cell. Only one isolate of each ECM fungal species was used in these experiments. The fungal mixture was prepared with one third of each fungal isolate. A control treatment with non-inoculated seedlings was also established. All treatments were replicated 9 times. This stage was performed in a growth chamber at 20 °C, 70% humidity and a 12 h light photoperiod.

2.3. Greenhouse experimental design

The experiment was conducted in a forest nursery greenhouse, in Amarante, Northern Portugal, between April and October 2009. Seedlings were transferred from the PVC tubes to trays with 250 cm^3 cells, filled with either burned or unburned forest soil. Because of the risk that mycorrhization had not been fully achieved during the first two months, the fungal inocula were added a second time to the substrate as mycelial suspension as described above. Two hundred mg of 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) were added to each seedling in June. Greenhouse temperature varied between 1.9 and 41.0 °C and relative humidity between 10 and 80%.

2.4. Plant sampling and analysis

After six months, all seedlings were gently removed from the trays and transported to the laboratory where shoot height was measured. The root system was then separated from the shoot and washed to remove adhered substrate. The percentage 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 separated on the basis of colour, branching, shape, and presence of emanating hyphae, according to Agerer (1998). Plant fresh weight was determined by weighing the plant material. Shoots were dried at 70 °C for 48 h. Oven-dried shoots were finely ground and 0.3 g of material was digested according to Novozamsky et al. (1983). The digested samples were used to determine the total nitrogen (N) and phosphorus (P) concentration in the shoots by colorimetry (Unicam, Helios Gamma, Cambridge, UK) (Walinga et al., 1989).

2.5. Identification of the inoculated fungi

Representative infected tips of each fungal treatment were kept frozen (−20 °C) in 2 × CTAB (cetyltrimethyl-ammonium bromide) until analysis. Prior to DNA extraction, ECM root tips were individually vortexed for 10 s in sterile deionised water and any residual soil particles were gently removed with fine forceps and a brush. DNA from the root tips and from pure fungal cultures was

Table 1
Concentrations of mineral N, extractable P, organic C, total N, Na, Ca, K, Mg, and pH and conductivity values of unburned and burned soils.

Substrate	pH (H_2O)	Cond. (ms/cm)	N_m (ppm)	P_e (ppm)	C_o (%)	N_t (ppm)	Na (ppm)	Ca (ppm)	K (ppm)	Mg (ppm)
Unburned	5.1	0.55	63	18	5.3	9765	300	236	2891	6237
Burned	4.5	1.40	144	243	3.8	8365	345	326	3373	6225

N_m represents the mineral nitrogen, P_e is the extractable phosphorus determined by the Egner–Riehm method, N_t represents the total nitrogen of soil, respectively.

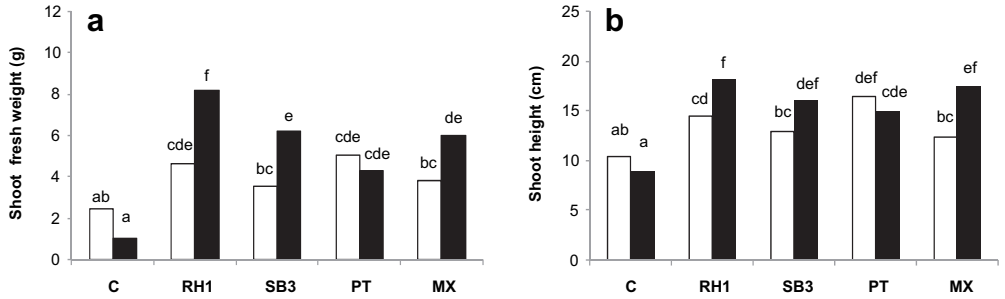

Fig. 1. Shoot fresh weight (a) and height (b) of *Pinus pinaster* seedlings inoculated with *Rhizopogon roseolus* (RH1), *Suillus bovinus* (SB3), *Pisolithus tinctorius* (PT), a mixture of the three isolates (MX) and non-inoculated control (C) under two substrates: unburned (open bars) or burned (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

extracted using the CTAB–chloroform–isopropanol extraction method. PCR amplification of the internal transcribed spacer (ITS) region of ribosomal DNA was performed using the primer combination ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCC GCTTATTGATATGC) on Bio-Rad Termociclador MJ Mini. Amplification conditions were the following: denaturation at 95 °C for 5 min, amplification for 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 10 min.

All PCR products were restricted with the enzymes Hinf-I and Taq-I. RFLP patterns of selected root tips were compared with RFLP gels of the pure cultures and those presenting the same pattern were selected for sequencing.

The selected amplified products were purified by GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced using both primers ITS1F and ITS4. The ITS sequences of selected morphotypes were compared with those of the pure cultures.

2.6. Statistical analysis

The data were tested for normality and a two-way ANOVA was performed for each dependent variable (growth and fungal parameters) versus the independent variables ECM fungal inoculation (Fungal) and the substrate used (Burn). The individual effect of each fungus was 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 17.0 software package (SPSS Inc., Chicago, IL, USA). The *t*-Student test was applied to verify possible statistical differences between growth averages within the control plants at a level of significance of $P < 0.05$.

3. Results

3.1. Plant parameters

3.1.1. Growth

Development of *P. pinaster* seedlings, measured as shoot fresh weight and height, is shown in Fig. 1. Control seedlings growing in unburned substrate had a 2.4-fold higher fresh weight than in the burned soil, whereas a 1.2-fold increase was registered in shoot height. Given that statistical differences in control seedlings were being veiled by the superior development of inoculated plants, a *t*-Student test was performed for non-inoculated seedlings. In both parameters, differences were significant ($P < 0.05$).

Different fungal associations led to different outcomes in *P. pinaster* growth. In unburned soil, *P. tinctorius* and *R. roseolus* were the most favourable associations with an average 2-fold increase in shoot fresh weight and 1.5-fold increase in shoot height whereas no significant increase was observed with *S. bovinus* or the fungal mixture. In burned soil, all fungal isolates enhanced plant growth and, overall, the ECM fungal isolates had a better performance in this substrate. *R. roseolus* was the most efficient species, with an 8-fold increase in plant fresh weight and a 2-fold increase in shoot height.

3.1.2. Nitrogen and phosphorus

Non-inoculated seedlings had a significantly higher N shoot concentration in burned soil and all fungal treatments resulted in lower N shoot concentration, regardless of the substrate (Fig. 2a). Substrate also had no influence in P concentration of control plants (Fig. 2b). Overall, no significant difference in P shoot concentration was observed between inoculated and non-inoculated growing in unburned soil seedlings whereas in burned

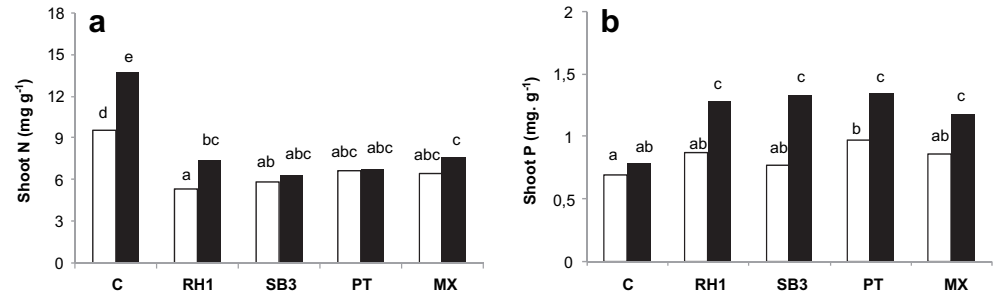

Fig. 2. Shoot nitrogen (a) and phosphorus (b) concentration of *Pinus pinaster* seedlings inoculated with *Rhizopogon roseolus* (RH1), *Suillus bovinus* (SB3), *Pisolithus tinctorius* (PT), a mixture of the three isolates (MX) and non-inoculated control (C) under two substrates: unburned (open bars) or burned (black bars). Values are expressed in mg of nutrient per g of oven-dried shoot. Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

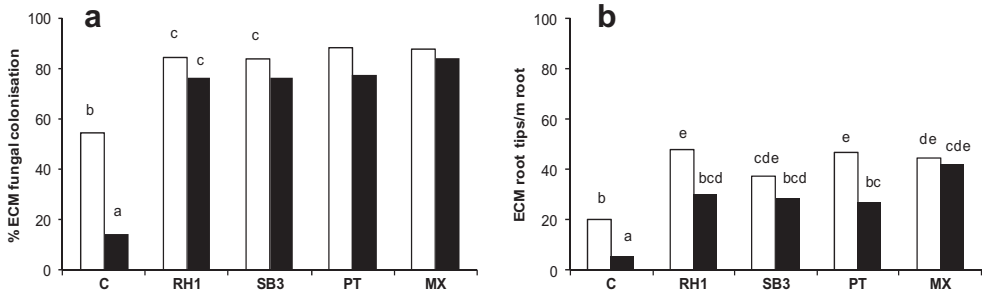

Fig. 3. Percentage of ectomycorrhizal fungal colonisation (a) and the number of ectomycorrhizal root tips per root length (b) of *Pinus pinaster* seedlings inoculated with *Rhizopogon roseolus* (RH1), *Suillus bovinus* (SB3), *Pisolithus tinctorius* (PT), a mixture of the three isolates (MX) and non-inoculated control (C) under two substrates: unburned (open bars) or burned (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$. ECM, ectomycorrhizal.

soil, shoot P concentrations of inoculated seedlings were 1.5- to 1.7-fold higher than the corresponding controls.

3.2. Fungal parameters

Inoculated seedlings presented significantly higher ECM root colonisation and higher number of ECM root tips per root length (Fig. 3) however it was more pronounced in the burned soil, with an average 3.8-fold increase. No significant differences were found within fungal treatments, in each substrate.

Plants inoculated with *R. roseolus* and *P. tinctorius* showed a significantly higher number of ECM root tips per root length in unburned soil than in burned soil, whereas there were no significant differences for *S. bovinus* and the fungal mixture between the two substrates.

Sequencing of the selected representative ECM root tips of each treatment revealed the presence of the fungal inoculants.

3.3. Relationships between plant development and fungal colonisation

Shoot fresh weight and height of *P. pinaster* seedlings revealed a significantly positive correlation with the percentage of ECM colonisation in both substrates (Fig. 4).

A negative correlation was found between the percentage of ECM colonisation and shoot N concentration. In burned soil, the percentage of ECM colonisation and shoot P concentration were significantly correlated, whereas in unburned soil it was not observed (data not shown).

4. Discussion

Control seedlings grown in the burned soil were smaller and less than half the fresh weight of those growing in unburned soil ($P < 0.05$, Fig. 1a and b). This was most likely caused by changes in soil physical and chemical properties by burning (Shaoqing et al., 2010) such as the lower pH, higher conductivity (Table 1) and a possible decrease in the rate of water infiltration and/or aeration. These factors may also have impeded microbial establishment (González-Peres et al., 2004; Hart et al., 2005) with a consequent impact in plant development.

Inoculation of plants with all three ECM fungi growing in the burned soil restored the mycorrhizal inoculum potential in the soil (Fig. 3), produced a significant 8-fold increase in growth (Fig. 1) and significantly increased shoot P concentrations (Fig. 2b). Significant growth increments to inoculation were also observed in plants growing in the unburned soil although these were less pronounced (Fig. 1a and b).

As expected burning the soil raised the available P concentration (Table 1) probably through the mobilisation of organic P and movement of P from the fixed inorganic P pool into the labile pool (Galang et al., 2010; White et al., 1973). In contrast, the total N concentration was lower in the burned than in the unburned soil. This was most likely caused by the volatilisation of N-containing organic compounds during burning (e.g. DeBano, 1991). However, the percentage of mineral N, a form more easily assimilated by plants, was higher in the burned than in unburned soil. Also, overall the N concentration in the inoculated seedlings in burned and unburned soil was not significantly different (Fig. 2a) which strongly suggests that N was not a factor limiting plant growth.

The inoculant fungi used in our experiments have been widely used in the past in mycorrhizal greenhouse experiments (Sousa et al., in press) and in one set of field trials (Vosátka et al., 2008). However, in the current study *R. roseolus* and *P. tinctorius*, which have been regarded as resilient early-stage symbionts (Kipfer et al., 2010; Torres and Honrubia, 1997), were superior to *S. bovinus* and a mixed inoculum containing all three species, with the latter producing no beneficial growth responses in the unburned soil

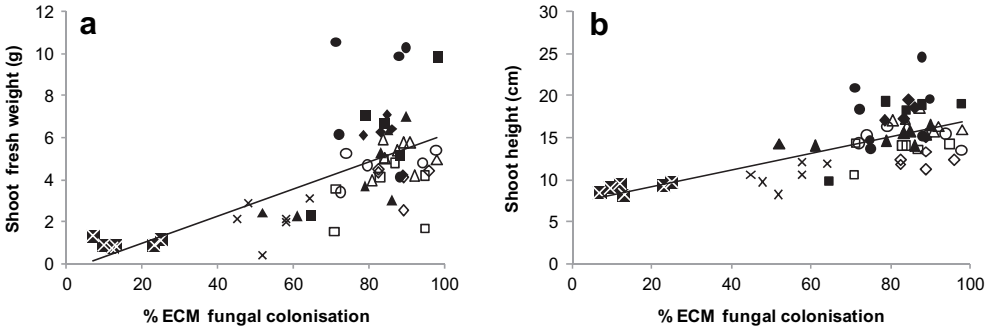

Fig. 4. Relationship between the percentage of ectomycorrhizal fungal colonisation of *Pinus pinaster* seedlings growing in burned (black figures) and unburned (open figures) substrates and (a) the shoot fresh weight ($y = 0.063x - 0.292$; $R^2 = 0.400$; $P < 0.001$); and (b) the shoot height ($y = 0.100x + 7.181$; $R^2 = 0.437$, $P < 0.001$). Seedlings inoculated with *Rhizopogon roseolus* (circles), *Pisolithus tinctorius* (triangles), *Suillus bovinus* (squares), the fungal mixture (diamonds), and non-inoculated control (crosses). ECM, ectomycorrhizal.

Table 2
ANOVA *F*-value results (two-way factorial analyses) for shoot, mycorrhizal development, nitrogen and phosphorus for *P. pinaster* seedlings subjected to four ectomycorrhizal fungal treatments and grown on two different substrates, in a forest nursery greenhouse in Portugal.

Sources	Shoot fresh weight	Shoot height	% ECM colonisation	ECM root tips per root length	Nitrogen	Phosphorus
Fungal (F)	13.973 (***)	16.763 (***)	30.375 (***)	7.672 (***)	20.724 (***)	9.979 (***)
Burn (B)	10.684 (**)	9.486 (**)	14.84 (***)	11.365 (**)	11.745 (**)	60.458 (***)
F x B	6.701 (***)	7.014 (***)	2.398 (NS)	0.846 (NS)	1.910 (NS)	2.886 (NS)

(*) Significant at $P < 0.05$; (**) Significant at $P < 0.01$; (***) Significant at $P < 0.001$. ECM, ectomycorrhizal.

(Fig. 1) despite the increased number of ECM root tips per root length (Fig. 3b).

Trees are commonly colonised with multiple ECM fungi (Parladé et al., 1999), mixtures of ECM fungi have been used in nursery practice (Duñabeitia et al., 2004; González-Ochoa et al., 2003; Hall and Perley, 2008; Sousa et al., in press), and a combination of mycorrhizal fungi has been shown to be more effective than a single species (Parladé and Alvarez, 1993; Reddy and Natarajan, 1997). It is possible that our mixed inoculum did not contain sufficient propagules of the most effective species but a more likely explanation is that *P. tinctorius* competed with the other two fungi (Reddy and Natarajan, 1997; Sousa et al., in press). Consequently, it might be better to use single species inoculum for field use rather than risk failure from mixed inoculum.

While mycorrhizal formation might also be achieved in bare root nurseries, generally in these situations little control can be exerted over which fungus forms mycorrhizas and ineffective, resident mycorrhizal fungi may form mycorrhizas instead of the inoculant species (Hall pers. comm.). Similarly, the forest soil used in our experiment had a profound P deficiency whereas a bare root nursery soil is likely to have a relatively high extractable P concentration which in turn may encourage fungi other than the inoculant species to infect.

All inoculated plants had lower N shoot concentrations regardless of soil treatment and there was a negative correlation between the percentage of ECM colonisation and shoot N concentration. Similar results were obtained by Conjeaud et al. (1996) and corroborate the fact that fungal mycelium development is an N requiring process. In burned soil, the percentage of ECM colonisation and shoot P concentration were significantly correlated, whereas in unburned soil this was not observed (data not shown). Both of these features are likely to have been caused by the increased P supplied by the mycorrhiza stimulating plant growth and possibly diluting N concentrations within the plants.

In this work, inoculation with different ECM fungal isolates and the type of substrate both significantly affected all studied variables, whilst the interaction between both factors was significant in shoot development (Table 2). The high accountability of ECM colonisation in the variation of plant development (Fig. 4) and the persistence of the inoculated ECM fungi in the root system emphasise the importance of mycorrhization at nursery stage.

5. Conclusions

Inoculating *P. pinaster* with ECM fungi stimulated plant growth in both burned and unburned ground. It is, therefore, recommended that *P. pinaster* for the reforestation of burnt areas should be inoculated with ECM fungi in the nursery. Of the three ECM fungi tested *R. roseolus* was found to be superior to the others in promoting plant growth in burned soil, a species often referred to as the post-fire fungus for its persistence and its ability to establish quickly after a stand replacing fire.

The next phase in this research will be the implementation of our findings in commercial nurseries and monitoring their performance on a burned site.

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