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Short communication. Physiological effects of *Rhizopogon roseolus* on *Pinus halepensis* seedlings

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Abstract

Aim of study: The inoculation of forest seedlings with ectomycorrhizal fungi can improve the morphological and physiological qualities of plants, especially those used for regeneration of arid areas. *Rhizopogon roseolus* is an ectomycorrhizal fungus (ECM) commonly used for reforestation.

In this study, the specific objectives were to know some morphophysiological effects of *Rhizopogon Roseolus* on *Pinus halepensis* seedlings under standard nursery conditions.

Area of study: ETSI Montes and EUIT Forestal, Madrid.

Material and methods: In nursery, under well watered conditions and peat growing substrates, Aleppo pine seedlings were inoculated with *R. roseolus*. Five months after the inoculations, we examined the growth, water parameters (osmotic potential at full turgor [$\psi\pi_{full}$], osmotic potential at zero turgor [$\psi\pi_0$], and the tissue modulus of elasticity near full turgor [E_{max}]), mycorrhizal colonization, and concentration and content of macronutrients in the seedlings. Subsequently, a trial was conducted to assess the root growth potential.

Main results: The mycorrhization decreased the height and diameter of mycorrhizal seedlings but increased the root weight and root branching. *R. roseolus* did not cause any significant effect on the regeneration of new roots or on any of the tested hydric parameters, but it did improve N uptake of the seedlings.

Research highlights: The mycorrhizal inoculation increased the N uptake.

The mycorrhizal inoculation caused opposite effects on some growth parameters.

Key words: osmotic adjustment; elastic adjustment; mineral nutrition; root growth potential; nursery; *Rhizopogon roseolus*; *Pinus halepensis*.

Introduction

In reforestation, numerous studies have been conducted with the objective of improving the quality of seedlings produced in nurseries (Caravaca *et al*., 2005). Among the cultural practices utilized, inoculation with ectomycorrhizal fungi (ECM) and plant growth promoting rhizobacteria (PGPR) has shown promise for improving the quality of seedlings and for increasing their survival in plantations, especially in soils with low microbial activity (Chanway, 1997).

There are many reports of positive mycorrhizal effects on the water uptake and water relations of seedlings during drought (Duddridge *et al*., 1980; Read and Boyd, 1986). However, it cannot be argued that ectomycorrhizae would always have a beneficial role in water uptake and water relations; it is still unresolved whether ectomycorrhizae influence the components of water potential or osmotic adjustment in shoots or roots (Lehto and Zwiazek, 2011). The capacity to adjust osmotic potential and increase the elasticity of the cell wall (elastic adjustment) is traditionally associated with the increased ability of plants to withstand water stress.

Rhizopogon roseolus is an ectomycorrhizal fungal species very common in *Pinus halepensis* forests; early ECM root colonizers that produce large quantities of rhizomorphs, and enhance water and nutrient uptake of plants (Gobert and Plassard, 2007). The spore inoculum is relatively easy to prepare from field-collected material and is a cheap inoculum with low labor costs (Massicotte *et al*., 1994). For these reasons, *R. roseolus* is an ectomycorrhizal fungus that has been frequently studied for use in forest nurseries and reforestation in Spain (Parladé *et al*., 2004; Ortega *et al*., 2004, Rincon *et al*., 2005, 2007).

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The objective of this study is to incorporate new information on the effects of *R. roseolus* on the physiology of *P. halepensis* (Aleppo pine, Pino Carrasco) seedlings grown in nurseries, especially on the water relations of seedlings. For this purpose, Aleppo pine seedlings were inoculated with the mycorrhizal fungus *R. roseolus*. We analyzed the osmotic potential at full turgor ($\psi\pi_{full}$) and zero turgor ($\psi\pi_0$), and the modulus of elasticity near full turgor (E_{max}). Additionally, we studied the effect of the inoculation on the growth, nutrient uptake, mycorrhizal colonization, and root growth potential of the seedlings.

Material and methods

Seeds of *P. halepensis* (Valencia) were collected in 2008 and stored in sealed polyethylene bags at 4°C until planting in Forest Pot 300® containers. A mixture of light and dark peat *Sphagnum* type (sterilized by autoclaving at 120°C for 2 h) plus vermiculite (3:1) were used for the substrate. Before sowing, all seeds were sterilized by immersion in 30% H_2O_2 for 15 min, which was followed by several rinses with distilled water. In mid-April 2008, *P. halepensis* seeds were sown in 300 cells (6 containers, 50 cells per container). The containers were conducted in a greenhouse in the School of Forestry of Madrid, and the seedlings were watered daily to saturation at temperatures ranging from 20 to 30°C until the inoculations were performed. No fertilizers were applied.

The *Rhizopogon roseolus* inoculum (North Gerona, Spain) was purchased from *Micología Forestal y Aplicada*©. Liquid spore inoculum was prepared by diluting the spore inoculum in distilled water. A concentration of 5-7 × 10^4 spores / ml liquid inoculum was estimated. A two-level (*R. roseolus* inoculation and non-inoculated control), unifactorial design that was distributed randomly in three blocks (1 × 2 × 3) was utilized. The inoculum was applied to the seedlings (1 month old) at two time points (20 ml/injection/plant), that were separated by a period of 15 days (in June and early July). 50% of the plants were injected in the substrata, with a total of 40 ml/plant (2-3 × 10^6 spores/plant). After performing the inoculations, the plants were taken outside to a shade house (E.U.I.T. Forest) and were watered daily to saturation. The average temperature ranged from 6°C to 30°C, and the environmental relative humidity average varied from 20% to 100% during the experimental period.

Nine pressure-volume curves (nine seedlings per treatment) were calculated (Tyree and Hammel, 1972) during November 2008, using shoot xylem pressure potentials (Scholander *et al.*, 1965). From each graph, the following water-relation parameters were obtained: 1) the osmotic potential at full turgor ($\psi\pi_{full}$), 2) the osmotic potential at zero turgor ($\psi\pi_0$), and 3) the modulus of elasticity near full turgor (E_{max}) (Cheung *et al.*, 1975). Subsequently, the roots of these inoculated seedlings were later analyzed and confirmed to have the mycorrhizal *Rhizopogon roseolus* fungus (Agerer, 1987-98).

Also, a random sample of 9 plants per treatment was chosen. Shoot heights and basal diameters were recorded, and the mycorrhizal colonization in the roots by the characterization and identification of the mycorrhizae (Agerer, 1987-98) was analyzed. After drying at 70°C for 48 h, the dry weights of shoots and total root mass were measured.

30 whole new plants per treatment were randomly selected, pooled, and the whole plant was finely ground and homogenized. For each group (n = 3) of 10 plants, the concentrations and content of N, P, K, Ca and Mg in whole plant tissues were determined, using ICP-Spectroscopy) (Perkin-Elmer 400) except N, which was measured by a Leco CHN 600 analyzer.

Subsequently, in early February 2009, nine new plants per treatment were randomly chosen, the height and basal diameter were measured previously, and subsequently, each plant was transplanted into a three-liter prismatic pot that was filled with white perlite. The pots were arranged randomly in the greenhouse and were grown for 21 days under optimal environmental conditions (16-22°C temperature; 95% relative humidity) to facilitate their root growth (Burdett, 1987); the plants were irrigated daily. Finally, each plant was extracted, and the number and total length of new roots (>1 cm) per plant were measured.

One-way ANOVA and Duncan's multiple-range test ($p < 0.05$) were performed for all parameters measured (Statgraphics Plus-Statistical software). For statistical analysis of the root growth potential, the height and diameter were selected as covariates.

Results and discussion

Despite well watering conditions, *R. roseolus* caused a significant decrease in height (5.7 cm versus 6.3 cm) and diameter (1 mm versus 1.13 mm) of seedlings, but

Table1. Mean values and signification of water-relations parameters, growth parameters, mycorrhizal colonization, nutrient concentration and contents, and root growth potential of *Pinus halepensis* seedlings

Treatment	Non- Inoculated	Inoculated	*Pvalue*[+]
Water-Relations parameters			
$\psi\pi_{full}$[1] (MPa)	–1.04 (±0.1)	–1.13 (±0.15)	0.6247
$\psi\pi_0$ (MPa)	–1.66 (±0.13)	–1.63 (±0.2)	0.8824
E_{max} (MPa)	5.94 (±0.81)	9.32 (±1.62)	0.0792
Growth			
Height (cm)	6.31 (±0.15)	5.69 (±0.12)	0.0047*
Basal Diameter (mm)	1.13 (±0.04)	1.01 (±0.02)	0.0192*
Shoot (g)	0.086 (±0.006)	0.095 (±0.004)	0.2363
Root (g)	0.082 (±0.008)	0.104 (±0.005)	0.0288*
Mycorrhizal colonization			
***Rhizopogon* (%)**	0	42 (±4)	0.0000*
E-Strain (%)	0	0.5 (±0.2)	0.0534
Total root tips [2] (Nº g^{-1} dw)	1867 (±124)	2707 (±259)	0.0100*
Total root tips plant^{-1}	149 (±12)	281 (±30)	0.0008*
Nutrients concentration			
N (mg g^{-1})	5.89 (±0.26)	7.34 (±0.28)	0,0196*
P (mg g^{-1})	0.91 (±0.05)	0.74 (±0.04)	0,0582
K (mg g^{-1})	9.71 (±0.64)	8.14 (±0.04)	0,0715
Ca (mg g^{-1})	7.13 (±1.65)	7.38 (±0.77)	0,8986
Mg (mg g^{-1})	4.11 (±0.67)	4.23 (±0.89)	0,9170
Nutrients content			
N (mg plant^{-1})	0.99 (±0.04)	1.46 (±0.06)	0.0027*
P (mg plant^{-1})	0.15 (±0.01)	0.15 (±0.01)	0,6978
K (mg plant^{-1})	1.63 (±0.11)	1.62 (±0.01)	0.9013
Ca (mg plant^{-1})	1.20 (±0.28)	1.47 (±0.15)	0.4453
Mg (mg plant^{-1})	0.69 (±0.11)	0.84 (±0.18)	0.5126
Root Growth Potential			
Nº Roots[3]	1 (±0)	1 (±0)	0.4637
Length[3] (cm)	1.01 (0.51)	1.69 (±0.51)	0.3583

[+] ANOVA one-way (*Rhizopogon* Inoculation Factor) for all the parameters except for Root Growth Potential parameters (ANOVA two-way). * Indicate significant differences ($p < 0.05$). [1] $\psi\pi_{full}$: osmotic potential at full turgor, $\psi\pi_0$: osmotic potential at zero turgor and E_{max}: modulus of elasticity near full turgor. [2] Total root tips: number of total root tips, is referred to as grams of root dry weight. [3] Number and total length of new roots per plant; covariate using the height parameter. Values in parentheses represent the standard error. $N = 9$ (Water-Relations, Growth, Mycorrhizal colonization and Root Growth Potential parameters); $N = 3$ (Nutrients parameters).

the root dry weight increased significantly in mycorrhizal seedlings (0.104 g) with respect to non-mycorrhizal seedlings (0.082 g), perhaps by providing phytohormones (Amaranthus and Perry, 1989). The mycorrhizae were morphological identified as *R. roseolus* (42%) and *E-Strain* (0.5%) in the inoculated plants; no other mycorrhizal morphotypes were founded in non-inoculated seedlings; *R. roseolus* generated an increase in root branching (2707 versus 1867 tips.g^{-1}dw) and an increase in the total number of root tips (281 versus 149 tips plant^{-1}) of seedlings (Table 1).

A nutrient-poor substrate induced a small growth of all seedlings in general. *R. roseolus* inoculation caused a significant improvement in N uptake (7.34 versus 5.89 mg g^{-1}; 1.46 versus 0.99 mg.plant^{-1}). By contrast, the mycorrhization did not cause significant effects on the absorption of the other macronutrients analyzed (Table 1).

Some authors have shown the ability of *R. roseolus* to improve growth and nutrient uptake of seedlings, especially N and P, of pines other than *P. halepensis* under nursery conditions (Rincon *et al*., 2005). On the contrary, other authors have observed limiting effects (Rincon *et al*., 2007). Casarin *et al*. (2004) showed in *P. pinaster* that *R. roseolus* facilitates the mobilization of P from soils with low available P. Gobert and Plassard (2007) showed that *R. roseolus* has a positive effect on the absorption of N in *P. pinaster* when the supply is low and fluctuating.

The capacity to adjust osmotic potential and increase the elasticity of the cell wall (elastic adjustment) is related to the resistance of the plants to drought. Through both mechanisms, plants are able to maintain turgor potential, the capacity for growth and photosynthesis, and the ability to tolerate more negative water potential and lower water availability (Villar-Salvador *et al*., 1997). According to the results obtained in this study, under well watering conditions, *R. roseolus* caused no significant effect on any hydric parameters (Table 1); *R. roseolus* seems to have caused an apparent rigidity of cell walls (p-value = 0.0792), although results were not significant. Other studies have shown that some mycorrhizal fungi, including *R. roseolus*, are able to improve the water availability of their host plants, especially under drought conditions because of strong development of external mycelium (Parke *et al*., 1983). Ortega *et al*., (2004) found that the hydraulic conductance of *R. roseolus* mycorrhizal seedlings was improved in sites under low water availability.

The ability to maintain open stomata and photosynthesis during the early stages of a drought could increase the supply of carbon for growth, particularly new root growth, which requires current photosynthates (van den Driessche, 1987). Moreover, an increased rate of photosynthesis supplies additional carbon for osmotic adjustment, thereby promoting turgor maintenance (Tan *et al*., 1992) and permitting higher water uptake from drier soils. In this study, the test to analyze the root growth potential showed that the *R. roseolus* did not cause any significant effects on the regeneration of new roots in Aleppo pine seedlings. This lack of response in the test of root regeneration could be related to the absence of significant differences in the osmotic potential analysis of seedlings under well watering conditions. Additionally, it may have been appropriate to increase the number of plants sampled and the duration of the radical regeneration trial such that the treatment effects would be manifested in the seedlings.

Conclusions

Under well watering conditions, *R. roseolus* did not provide osmotic adjustment mechanisms and regeneration of new roots in seedlings. However, under these conditions, throughout the first year of nursery cultivation, we demonstrated that *R. roseolus* caused effects such as increased weight and branching of the root, or increases in N uptake (on a substrate poor in macronutrients) in the seedlings, so these effects do not appear to be related to water availability. Further research could study the effect of *R. roseolus* and other ectomycorrhizal fungi under conditions of water stress on the osmotic adjustment, elastic adjustment, root growth potential, and other lesser-known mechanisms that affect the water relationships of their host forest plants.

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