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Effects of *Pseudomonas fluorescens* on the Water Parameters of Mycorrhizal and Non-Mycorrhizal Seedlings of *Pinus halepensis*

José A. Domínguez-Núñez^{1,*}, Daniel Muñoz¹, Ana de la Cruz² and José A. Saiz de Omeñaca¹

¹ Departamento de Silvopascicultura. E.T.S.I. Montes and E.U.I.T. Forestal. Universidad Politécnica de Madrid Av/Ciudad Universitaria s/n. 28040, Madrid, Spain;

E-Mails: danielmuozmate@yahoo.es (D.M.); joseantonio.saizdeomenaca@upm.es (J.A.S.O.)

² Departamento de Protección Forestal. CIFOR-INIA (Instituto Nacional de Investigaciones y Tecnología Agrarias). Ctra. Coruña, Km. 7, 28040, Madrid, Spain; E-Mail: calleja@inia.es

* Author to whom correspondence should be addressed; E-Mail: josealfonso.dominguez@upm.es; Tel.: +34-913-367-103; Fax: +34-913-365-568.

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Abstract: Inoculation of forest seedlings with mycorrhizal fungi and rhizobacteria can improve the morphological and physiological qualities of plants, especially those used for regeneration of arid areas. In this paper, under standard nursery conditions, Aleppo pine seedlings were inoculated with *Pseudomonas fluorescens* CECT 5281 rhizobacteria. Some of these seedlings were also inoculated with the ectomycorrhizal fungus *Pisolithus tinctorius*. 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 colonisation, and concentration of macronutrients (N, P, K, Ca and Mg) in the seedlings. Subsequently, a trial was conducted to assess the root growth potential. *P. fluorescens* CECT 5281 decreased the cellular osmotic potential of *P. halepensis* seedlings but increased its elasticity. *P. tinctorius* + *P. fluorescens* caused osmotic adjustment at zero turgor and increased tissue elasticity, which improved tolerance to water stress. All inoculations improved the growth and nutrition of the seedlings but caused non-significant effects on root growth potential. The co-inoculation *Pisolithus tinctorius* + *Pseudomonas fluorescens* at the nursery may be a suitable technique for producing improved seedling material for restoration purposes.

Keywords: rhizobacteria; osmotic adjustment; elastic adjustment; *Pisolithus tinctorius*; *Pinus halepensis*

1. Introduction

In semiarid Mediterranean ecosystems, water and nutrient availability are the primary limiting factors for plant productivity and for the diversity of microflora associated with the roots of plants [1]. Consequently, forest species of the Mediterranean area often exhibit different strategies of water use in response to drought [2].

Aleppo pine is one of the most common tree species in the Mediterranean area, and it has been one of the most frequently used species in the reforestation of degraded areas [3]. Numerous studies have been conducted with the aim of improving the quality of seedlings produced in nurseries [4]. Among the cultural practices utilised, inoculation with ectomycorrhizal fungi and plant growth promoting rhizobacteria (PGPR) has shown promise for improving the quality of seedlings and increasing their survival in plantations, especially in soils with low microbial activity [5].

Pisolithus tinctorius is an ectomycorrhizal fungus that has been widely used in reforestation programs since the 1970s. Its potential for cultivation and inoculation, along with the great diversity of plant species it associates with, are promising features for its study and application in improving the quality of forest plants. There have been a wide range of studies on the positive effects of *Pisolithus tinctorius* on the growth of plants; of particular interest are studies that examine its ability to enhance access to certain nutrients, such as phosphorus [6] and nitrogen [7,8], and its ability to reactivate soil microbiological activity [9]. The interaction of *Pisolithus* spp. with angiosperms and gymnosperms may reduce a host's water deficit under drought conditions [10]. Therefore, *P. tinctorius* is viewed as a suitable fungus for restoring vegetation in arid or semiarid environments, especially in combination with organic soil amendments [11].

Pseudomonas fluorescens is a PGPR bacterium that is capable of colonising a wide range of ecological niches, especially the rhizosphere of plants [12]. *P. fluorescens* promotes plant growth by producing phytohormones such as auxin (IAA), gibberellins and cytokinins, as well as specific amino acids and other growth promoters [13]. It also has a high capacity for phosphate solubilization, and is able to produce siderophores [14]. Also, certain strains of *Pseudomonas fluorescens* promoting the ACC deaminase activity, helping the plant to better resist the stress, including drought [15]. Adherence and colonisation of MHB (mycorrhizal helper bacteria) such as *P. fluorescens* on the surface of some ectomycorrhizas can affect and improve the symbiotic relationship between the plant and the ectomycorrhiza, and thus benefit the host plant [16]. Potential MHB mechanisms affecting the establishment and function of mycorrhizal symbiosis include the stimulation of spore germination, mycelial growth, promotion of root-fungus contact or stimulation of potential mycorrhizal short root production [17].

Our initial hypothesis was that *P. fluorescens* CECT 5281 would have synergistic effects and could positively influence physiology of *P. tinctorius* mycorrhizal seedlings. In the present study, Aleppo pine seedlings were co-inoculated with the mycorrhizal fungus *Pisolithus tinctorius* and the rhizobacterium

Pseudomonas fluorescens CECT 5281. The aim was to determine the specific effects of *Pseudomonas fluorescens* on certain water parameters that characterize the quality of mycorrhizal and non-mycorrhizal seedlings of *Pinus halepensis* used for reforestation. 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 inoculations on growth, nutrient uptake, mycorrhizal colonisation, and root growth potential of the seedlings.

2. Results and Discussion

Pseudomonas fluorescens is a plant growth stimulator that efficiently promotes seed germination, accelerates growth in early stages, induces root initiation, increases the formation of roots and root hairs, and controls pathogens in some forest species [18]. The mycorrhizal fungus *Pisolithus tinctorius* is also known to improve plant growth and nutrition in some cases, particularly for *Pinus halepensis* [19]. In general, the association of mycorrhizal fungi with *Pseudomonas* sp. can promote plant growth [16], especially in roots. However, *Pisolithus* sp. and PGPR are not always positively synergistic in the promotion of plant growth [20]. Previous research has highlighted the importance of the interaction between *Pseudomonas fluorescens* and *Pisolithus tinctorius* [21] and illustrated its positive effect on the growth of *Pinus halepensis* [22]. This effect occurs due to the mobilisation of phosphorus by the fungus, combined with the mineralising activity, activity of growth-promoting hormones, and anti-pathogenic activity of the bacteria.

In the present study, both *Ps* inoculation and *Ps* × *Pt* co-inoculation improved most of the growth parameters (Table 1). The *Ps* inoculation significantly increased the root dry weight to a greater extent than the *Ps* × *Pt* co-inoculation; however, the *Ps* × *Pt* co-inoculation significantly increased the total number of root tips to a greater extent than the *Ps* inoculation. It is possible that there was a synergistic effect, as *Pseudomonas fluorescens* stimulated root growth and MHB activity promoted the formation and establishment of *Pisolithus tinctorius* mycorrhizae. In turn, the fungus (in association with the rhizobacteria) stimulated growth and branching of the root system. Nevertheless, the root growth potential test (Table 1) illustrated that neither inoculation significantly increased the length of new roots, although a non-significant increase due to the inoculations was observed (3.3 cm/plant vs. 1.9 cm/plant). Nevertheless, the low sample size selected ($N = 9$) or the low Root Growth Potential test duration (21 days) could cause these non-significant effects on root growth. Even so, other studies have shown the capacity for root regeneration and root protection in plants inoculated with *Pseudomonas* sp. [23].

Generally, the benefits that the mycorrhizae bring to a host plant are often seen under nutrient-limiting conditions [24]. In several Mediterranean areas, phosphorus is a limiting nutrient during the early stages of growth of *P. halepensis* [25]; therefore, inoculation with *Ps* could improve the plants' ability to solubilise phosphorus, and thus improve their rate of phosphorus uptake.

In this study, P concentration was significantly decreased by both *Pseudomonas* and co-inoculation treatments, and K concentration was significantly decreased by the co-inoculation treatment (Table 1). However, Rincon *et al.* [26] have observed increases in P and K concentrations when Aleppo pine seedlings were inoculated with *Pseudomonas fluorescens*. However, in this study, the soil did exhibit low nutrient availability due to the use of unfertilised peat as a substrate. We suggest that it was not

possible to illustrate the effects of P solubilisation or mobilisation from *Pisolithus tinctorius* and *Pseudomonas fluorescens* under these conditions. We suggest that the fungus has a negative effect on the uptake of K when it is associated with *Pseudomonas fluorescens* CECT 5281; is it possible that the positive effect of the *Pseudomonas* strain was lost due during the biotic interaction between the two micro-organisms.

Table 1. Water-relations parameters, growth parameters, nutrient concentration and root growth potential in *Pinus halepensis* seedlings.

Treatment	Control	<i>Pseudomonas</i>	<i>Pisolithus</i> × <i>Pseudomonas</i>
Water-relations parameters			
$\Psi\pi_{\text{full}}^1$ (MPa)	−1.24 (±0.1) a	−0.43 (±0.1) b	−1.04 (±0.14) a
$\Psi\pi_0$ (MPa)	−1.72 (±0.13) b	−1.02 (±0.33) b	−2.74 (±0.33) a
E_{max} (MPa)	11.55 (±1.91) a	5.75 (±1.06) b	3.24 (±0.85) b
Growth			
Height (cm)	6.74 (±0.25) b	8.94 (±0.4) a	9.43 (±0.5) a
Basal Diameter (mm)	1.33 (±0.07) b	1.47 (±0.06) ab	1.64 (±0.09) a
Shoot (g)	0.137 (±0.008) b	0.268 (±0.019) a	0.3 (±0.036) a
Root (g)	0.1 (±0.01) c	0.251 (±0.02) a	0.18 (±0.014) b
Root growth potential			
Length ² (cm)	1.9 (±1.1) a	3.4 (±1) a	3.3 (±0.9) a
Mycorrhizal colonisation			
<i>Pisolithus</i> (%)	0 b	0 b	43 (±6) a
Total ³ (N/plant)	195 (±28) c	471 (±43) b	983 (±170) a
Nutrient concentration			
N (mg/g)	6.8 (±0.18) a	7.71 (± 0.33) a	7.85 (±0.34) a
P (mg/g)	0.59 (±0.01) a	0.41 (±0.01) b	0.4 (±0.03) b
K (mg/g)	6.22 (±0.07) a	6.07 (±0.06) a	5.46 (±0.16) b
Ca (mg/g)	7.4 (±0.51) a	6.83 (±0.1) a	7.06 (±0.22) a
Mg (mg/g)	4.33 (±0.15) a	3.72 (±0.15) a	3.83 (±0.22) a

¹ $\Psi\pi_{\text{full}}$: osmotic potential at full turgor, $\Psi\pi_0$: osmotic potential at zero turgor and E_{max} : modulus of elasticity near full turgor; ² Total length of new roots/plant; covariate using the height parameter. Values in parentheses represent the standard error. $N = 9$ (water-relations, growth, mycorrhizal colonisation and root growth potential parameters); $N = 3$ (nutrient parameters). Values in the same row with different letters differ significantly ($P < 0.05$) according to the Duncan test; ³ Total = number of total root tips/plant.

In some cases, the mycorrhizal fungus × *P. fluorescens* co-inoculation could increase root colonisation by *Pseudomonas fluorescens* [22] and mycorrhizal fungus. However, the co-inoculation did not affect colonisation of mycorrhizal fungus in other cases, and synergistic effects were observed on plant growth [27]. In this study, it is possible that *Pseudomonas fluorescens* CECT 5281, in association with Pt, could raise the mycorrhizal colonisation percentage of *Pisolithus tinctorius* up to 43%, which is a good percentage of mycorrhizae for a seedling nursery. However, in this trial, the data could not be compared with a simple Pt inoculation treatment, although some authors have noted that one MHB can benefit the mycorrhization of certain fungi and negatively impact others [28]. Moreover,

the mycorrhizae can seriously impact the composition of bacterial communities [29]. Additionally, only *Pisolithus tinctorius* morphotypes were observed in this analysis.

Based on the results obtained (Table 1), *Ps* × *Pt* co-inoculation caused an osmotic adjustment at zero turgor ($\Psi\pi_0$), although inoculation with *Ps* caused the opposite effect, which was a decrease of osmotic potential at full turgor ($\Psi\pi_{full}$). Finally, both inoculations increased the elasticity of the cell tissues, causing an elastic adjustment.

The capacity for osmotic adjustment and the increase in cell wall elasticity (low modulus) are mechanisms traditionally associated with the increased ability of plants to withstand water stress. Through both mechanisms, plants are able to maintain turgor potential, and thereby maintain the capacity for growth and photosynthesis and the ability to tolerate more negative water potentials and lower water content [30]. Specifically, it is possible that *P. halepensis* is unable to make these changes in response to water stress, as some authors have already demonstrated in other species [31]. Other authors have reported changes in the elasticity of cell walls, but not osmotic adjustment [32]; however, based on our results, it appears that *Pisolithus tinctorius* (in association with *Pseudomonas fluorescens* CECT 5281) can facilitate osmotic adjustment in *Pinus halepensis* seedlings. Additionally, two inoculations improved the elasticity of the tissue cells.

Many authors have emphasised that, under semi-arid environmental conditions, the interaction of *Pisolithus tinctorius* with angiosperms and gymnosperms can reduce the water deficit of the host plant [10,33]. Hormonal effects may also be involved in the water stress tolerance of plants inoculated with PGPR, as some bacteria can produce abscisic acid (ABA), which is a phytohormone directly related to plant drought responses [34]. In this study, it appears that *Pisolithus tinctorius* (in association with *Pseudomonas fluorescens* CECT 5281) could increase the concentration of active cellular solutes (osmolytes), as evidenced by osmotic adjustment at zero turgor $\Psi\pi_0$, thus improving water stress tolerance. However, the effect of *Pseudomonas fluorescens* CECT 5281 inoculation was the opposite because there was a significant decrease in osmotic potential at full turgor ($\Psi\pi_{full}$), which may have been due to the dilution of cellular osmolytes. In this regard, opposing cell osmotic effects could be seen between *Pisolithus tinctorius* and *Pseudomonas fluorescens*. Rincon *et al.* [26] found that *Pseudomonas fluorescens* can enhance the Aleppo pine water utilisation when plants undergo a water stress period, although the seedlings were well watered during our study.

Regarding the possible relationship between nutrient concentration and osmolytes, none of the inoculations in this study improved the nutrient concentrations of seedlings; nevertheless, co-inoculation caused an osmotic adjustment, which could have occurred because of *Pisolithus tinctorius* or possibly because of the synergistic effect of both inocula. In contrast, other authors [35] did not observe any osmotic effect of *Pisolithus tinctorius* in other forest species.

Potassium is an important solute associated with the regulation of cell turgor and stomatal opening [36]. We observed that co-inoculation decreased K uptake; however, although the fungus *P. tinctorius* could reduce the absorption of K, *P. tinctorius* (in co-inoculation) also facilitated the regulation of cell turgor by osmotic adjustment, perhaps through other mechanisms not dependent on K. Additionally, although *Pseudomonas fluorescens* CECT 5281 did not affect the K uptake, it may have caused dilution of active solutes ($\Psi\pi_{full}$) in the presence of the fungus (Table 1). Thus, according to the results of this work, it seems that there is no clear relationship between the regulation of cellular osmotic potential and of the K uptake by plants.

The simple *Ps* inoculation and the *Ps* × *Pt* co-inoculation increased cell wall elasticity, causing an elastic adjustment and improving plant tolerance to water stress, thereby allowing the plant to improve its ability to react to possible water changes in the soil ecosystem. However, some authors have suggested that a rigid cell wall may be more effective for maintaining cell and tissue integrity upon re-hydration after a period of stress in species that achieve osmotic adjustment through the accumulation of large amounts of organic solutes [37].

3. Experimental Section

3.1. Plant Production

The study was conducted at the IFAPA (Centre for Agricultural Research and Training) in La Mojonera, Almeria, Spain. Seeds of *Pinus halepensis* were collected in 2008 and stored in sealed polyethylene bags at 4 °C until planting in Forest Pot 300[®] containers (300 mL, 4.6 × 4.8 cm at the top section and 1.9 × 1.9 cm at the bottom section, 19 cm depth). For the growth medium (substrate), a mixture of light and dark peat was used for the organic component (*Sphagnum* moss at pH 6), and vermiculite was used for the inorganic component at a 3:1 ratio (peat:vermiculite). The peat was sterilised by autoclaving at 120 °C for 2 h.

The seeds were selected after flotation and submergence in distilled water for 24 h before sowing. Before sowing, all *Pinus* seeds were surface-disinfested by immersion in 30% hydrogen peroxide (H₂O₂) for 15 min, followed by several rinses with distilled water.

In mid April 2008, *P. halepensis* seeds were sown in 600 cells (12 containers, 50 cells/container). Each cell received 3–8 pine seeds; however, after germination, the plants were thinned so that only one pine seedling remained in each cell. The plantings were conducted in an IFAPA open shade house, and the seedlings were watered daily to saturation at temperatures ranging from 6 to 45 °C (28 °C mean) until the inoculations were performed. No fertiliser was added.

3.2. Fungal and Bacterial Inoculum

The lyophilized fungal inoculum of *Pisolithus tinctorius* was purchased from Micología Forestal Aplicada[®]. The spore inoculum was collected in December 2007 from *Pinus pinea* forests in North Gerona, Spain. After collection, the inoculum was stored at 4 °C until inoculation. Liquid spore inoculum was prepared by diluting the spore inoculum in distilled water. A concentration of 2.5×10^4 spores mL⁻¹ liquid inoculum was estimated. A lyophilised pre-culture of *Pseudomonas fluorescens* CECT 5281 (obtained from CECT, Spanish Type Culture Collection, University of Valencia) was stored at 10–15 °C until inoculation, and it was subjected to 3 successive incubations. A lyophilised pre-culture vial was first suspended in 0.3 mL of nutritive medium (1 g L⁻¹ beef extract, 5 g L⁻¹ peptone, 5 g L⁻¹ NaCl and 1 L distilled water, pH 7.2). A drop (0.02 mL) of that suspension was added to 5 mL of nutritive medium and incubated at 30 °C for 12 h; then it was transferred to 75 mL of nutritive medium and incubated on an orbital shaker (200 rpm) at 30 °C for 12 h. After the incubation period, this 80 mL was added to 720 mL of nutritive medium and incubated again as before. This final preparation of medium was used as the inoculum. We estimated about 2×10^9 C.F.U.s mL⁻¹ of liquid inoculum [38].

3.3. Experimental Design and Bio-Inoculations

A three-level (*Pseudomonas fluorescens* inoculation [*Ps*]; *Pseudomonas fluorescens* × *Pisolithus tinctorius* co-inoculation [*Ps* × *Pt*]; and non-inoculated control) unifactorial design distributed randomly in three blocks (1 × 3 × 3) with 50 plants per block was utilised (A total of 450 seedlings).

In plants of 2.5 months old, the inocula were applied in the substrata of plants at two time points separated by a period of 15 days (in late June and early July). The substrate of each plant was injected (1 to 5 cm deep) with *Pisolithus tinctorius* inoculum (10^6 spores/plant) at 40 mL/plant (20 mL/injection) and *Pseudomonas fluorescens* inoculum (2×10^{10} C.F.U.s/plant) at 10 mL/plant. Finally, 33% of the seedlings were maintained as controls (non-inoculated).

3.4. Measurements

3.4.1. Pressure-Volume Analysis and Plant Water Measurements

The pressure-volume curves of the plants (7 months old) were calculated [39,40] during November 2008. These curves were determined using shoot xylem pressure potentials (shoot water potentials) as measured in a pressure chamber [41]. From these graphs, 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}) [42–45]. Nine seedlings per treatment (three/block) were analyzed, and the plants inoculated with *Pisolithus tinctorius* were mycorrhizated with *Pisolithus tinctorius*.

3.4.2. Growth and Mycorrhizal Colonisation

Nine plants per treatment (three/block) were randomly chosen. Shoot heights and stem basal diameters were recorded, and mycorrhizal fungal colonisation in the roots was analyzed. After drying at 70 °C for 48 h, the dry weights of shoots and total root mass were measured.

The ectomycorrhizal fungal colonisation in the roots was analyzed by characterization and identification of the mycorrhizal morphotypes. To achieve this, the whole root system was analysed; the rooted “soil ball” of each of the nine plants was immersed in water several times so that the seedling roots could be carefully freed from most of the substrate in which they had been grown. All roots were cut into pieces approximately 2–3 cm in length and divided into ectomycorrhizal and non-mycorrhizal tips under a stereomicroscope. All roots showing any of the characteristics indicating ectomycorrhizal infection (blunt tips, altered branching patterns, pigmented mantles, emanating hyphae) were removed for morphotyping. Different features of mycorrhizae were the basis for the identification of ectomycorrhizal morphotypes [46]. The results are given as a percentage of mycorrhizal root tips and total number of root tips/plant.

3.4.3. Nutrient Analysis

The concentration of nitrogen, phosphorus, potassium, calcium, and magnesium in whole plant tissues were determined. In November of 2008, a new random sample of 30 whole plants (7 months old) per treatment was divided into three groups (10 plants/block). For each group, the concentrations of phosphorus, potassium, calcium, and magnesium were analyzed using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Perkin-Elmer model 400) after humid digestion in a microwave with a closed system using concentrated HNO₃. Nitrogen was measured with a CHN-600 autoanalyzer.

3.4.4. Root Growth Potential

A month before the start of spring (time of onset of forest plantations), in February 2009, nine new plants (10 months old) per treatment (three/block) were randomly chosen. For each plant, the height and basal diameter were measured. Subsequently, each plant was transplanted carefully into a three-litre prismatic pot filled with white perlite. The pots were arranged randomly in the greenhouse and grown for 21 days under optimal environmental conditions in order to facilitate their root growth [47]. For optimal root growth, plants were irrigated daily and the air temperature was maintained between 16 and 22 °C. The relative humidity was maintained at approximately 95%. In March 2009, after 21 days of growth, each plant (11 months old) was extracted, the new roots longer than 1 cm were counted, and the total length of new roots was measured.

3.5. Data Analysis

An analysis of variance (ANOVA) was performed, the means of all the study parameters were calculated, and Duncan multiple-range test was performed at the 0.05 confidence level. In cases where the variance was non-homogeneous, a non-parametric Kruskal-Wallis test was applied. All the statistical analyses were performed using the Statgraphics Plus computer software package (StatPoint Technologies, Inc.). For root growth potential statistical analysis, height and diameter were selected as covariates of the parameters analysed. Similar results were obtained for all covariates, such that only the results obtained against the initial height are presented. Similarly, the results for both parameters examined (number and root length) were similar, so only the results for the length of new roots are presented.

4. Conclusions

Despite the complexity of the analysis of microbial interactions in the rhizosphere of forest seedlings, a few major concepts can be deduced. When seedlings of *P. halepensis* are grown under non-limiting nursery conditions, inoculation with *Pseudomonas fluorescens* CECT 5281 rhizobacteria can promote growth. In some cases, growth promotion occurs without an improvement in P uptake. The *Pseudomonas fluorescens* CECT 5281 rhizobacteria may have negative osmotic effects on water stress, but it can also improve cell elasticity. In contrast, *Pisolithus tinctorius* can cause an improvement in cell elasticity and osmotic adjustment when it is associated with *Pseudomonas fluorescens*.

These results could be promising for the establishment of Aleppo pine seedlings in reforestation and for enhancing their tolerance to water stress via bio-inoculation of soil microorganisms.

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Conflicts of Interest

The authors declare no conflict of interest.

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