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ORIGINAL PAPER

The combined effects of *Pseudomonas fluorescens* and *Tuber melanosporum* on the quality of *Pinus halepensis* seedlings

J. A. Dominguez · A. Martin · A. Anriquez · A. Albanesi

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Abstract The ecological, economic and social values of the ectomycorrhizal fungi of the black truffle found in the rural Mediterranean are well known. The inoculation of *Pinus halepensis* seedlings with mycorrhizal fungi and rhizobacteria can improve the morphology and physiology of the seedlings and benefit the regeneration of arid regions and the reintroduction of inocula of mycorrhizal fungi into these areas. Some rhizobacteria can improve the establishment and functioning of ectomycorrhizal symbiosis. In this study, seedlings of *P. halepensis* were inoculated with the mycorrhizal fungus *Tuber melanosporum* and the rhizobacteria *Pseudomonas fluorescens* CECT 844 under non-limiting greenhouse conditions. Five months after inoculation, we analysed the growth, water parameters (osmotic potential at saturation, osmotic potential at turgor loss and modulus of elasticity), concentrations of mycorrhizal colonies, nutrient concentration and nutrient contents (N, P, K, Ca, Mg and Fe) in roots and aerial parts of the seedlings. Subsequently, tests were performed to estimate the root growth potentials. None of the treatments changed the water parameters or growth potentials of the roots. The inoculations improved the growth and nutrient uptake of the seedlings, although the combination of *P. fluorescens* CECT 844 and *T. melanosporum* did not generally lead to a significant improvement over the positive effects of a simple inoculation of *T. melanosporum*; however, the addition of *P. fluorescens* CECT 844 did double the rate of the mycorrhization of *T. melanosporum*. These results may be promising for enhancing the cultivation of truffles.

Keywords Rhizobacteria · Black truffle · *Pinus halepensis* · Water parameters · Mycorrhiza · MHB

J. A. Dominguez (✉) · A. Martin
E.T.S.I Mountains and E.U.I.T Forestry,
Polytechnic University of Madrid,
Av/Ciudad Universitaria s/n.,
28040 Madrid, Spain
e-mail: josealfonso.dominguez@upm.es

A. Anriquez · A. Albanesi
Faculty of Agronomy and Agroindustries,
National University of Santiago del Estero,
Av/Belgrano (S) 1912,
4200 Santiago del Estero, Argentina

Introduction

In a semi-arid Mediterranean ecosystem, the availabilities of water and nutrients are the main constraints to plant productivity and the preservation of the diversity of mycoflora that are associated with plant roots (Marulanda et al. 2006). Forest species in the Mediterranean region often exhibit different strategies for water usage in response to drought (Martínez-Ferri et al. 2000).

Pinus halepensis is one of the most common tree species in the Mediterranean and is also among the most frequently used species in the reforestation of damaged areas (Maestre and Cortina 2004). Numerous studies have focused on improving the quality of seedlings produced in nurseries (Caravaca et al. 2005). Among cultivation practices, it has been shown that inoculation with ectomycorrhizal fungi and plant growth-promoting rhizobacteria (PGPR) is an effective strategy for improving the quality of seedlings and increasing plant survival, particularly in soils with low levels of microbial activity (Chanway 1997; Probanza et al. 2001).

Pseudomonas fluorescens is a PGPR that is easily cultivated in vitro and can colonise a wide range of ecological niches, especially the rhizospheres of plants (Bolton et al. 1993); *P. fluorescens* genomes are highly diverse (Silby et al.

2009). The ubiquity of bacteria from the genus *Pseudomonas* and their ability to exploit a wide variety of nutrients reflect a system of environmental adaptation superior to those employed by other genera of bacteria. *P. fluorescens* can promote plant growth by producing phytohormones, such as auxins (IAA), gibberellins and cytokinins, specific amino acids and other growth promoters. This species also has a high capacity for phosphorus solubilisation and is able to produce siderophores (Matthijs et al. 2007).

Information on the productivity of ectomycorrhizal fungi, their ecological functions and their contributions to the productivity and recovery of altered agroecosystems is increasingly valuable in agroforestry. In Spain, the black truffle (*Tuber melanosporum* Vitt.) is of substantial economic and social value in rural areas of the Mediterranean (Reyna 2007), though specific studies of contributions by *T. melanosporum* to the growth and physiology of forest plants are scarce (Domínguez 2002). The ecological value of this symbiosis in the recovery of Mediterranean ecosystems has not been well characterised.

Inoculation of the black truffle-producing species (including *Quercus ilex*, *Quercus faginea* and *Corylus avellana*) with *T. melanosporum* is an important practice for the support of truffle silviculture in natural areas (Reyna 2007); however, preliminary experiments with other non-ascocarp-producing species are just now being initiated. These non-black truffle ascocarp-producing species (including *Pinus nigra* and *P. halepensis*) are components in mixed stands of the natural ecosystems in which the Mediterranean truffle is found (Domínguez et al. 2003).

The association of *T. melanosporum* with other indigenous microorganisms may improve growth and increase nutrient concentrations, thereby protecting the host plant from drought, which is common in the Mediterranean region. The adhesion and colonisation of mycorrhizal helper bacteria (MHB) such as *P. fluorescens* on the surfaces of some ectomycorrhizae can improve the symbiotic relationship, the pre-symbiotic stages and benefit the host plant (Frey-Klett et al. 2007; Deveau et al. 2007). Co-inoculation with mycorrhizal fungi and *P. fluorescens* may, in some cases, increase the colonisation of *Pseudomonas* (Ouahmane et al. 2009) and the mycorrhizal fungus. Although co-inoculation does not affect fungal colonisation in some cases, synergistic effects can be observed on plant growth (Rincón et al. 2005).

Several authors have linked the presence of *P. fluorescens* to different stages of maturation of the ascocarps of the genus *Tuber* (Citterio et al. 2001; Barbieri et al. 2007), especially *T. melanosporum* (Rivera et al. 2010).

Our initial hypothesis was that the combined inoculation of both microorganisms—*P. fluorescens* CECT 844 and *T. melanosporum*—would have synergistic effects and could positively influence seedling physiology, thereby improving the quality of the plant and its rate of mycorrhization of *T. melanosporum* from an early developmental stage, even in plants grown in less suitable substrates for black truffle. In the present study, inoculations (both combined and single) were performed using *T. melanosporum* and *P. fluorescens* CECT 844 in *P. halepensis* seedlings. Seedling growth, water relations and nutrient uptake were studied; mycorrhizal colonisation was analysed, and the effects of the inoculations on the root growth potentials of the seedlings were also investigated.

Materials and methods

Plant materials

Tests were conducted on the forest species *P. halepensis* (originating from Carboneras, Almería). Seeds, collected in March 2009, were kept in closed polyethylene bags at 4°C until planting. We used Forest Pot ® containers (300 ml per cell)) and culture substrates with vermiculite and a mixture of *Sphagnum* peat moss (black type), pH 6, in a 3:1 ratio of peat/vermiculite, prepared in mid-April. The peat was sterilised in an autoclave at 120°C for 2 h. No carbonate or other types of pH modifiers were added to the substrates to correct the pH levels to allow for identification of the effects of the treatments under pH conditions that were less suitable for the formation of black truffle mycorrhizae. The seeds were selected by floating and were submerged in water for 24 h before planting. Prior to planting in mid-April, the seeds were immersed in 30% H_20_2 for 15 min for disinfection and washed several times in distilled water. The trials were conducted in the E.T.S.I. Mountains and E.U.I.T. Forest of Madrid, and a total of 20 containers (1,000 cells) were planted. Three to eight seeds were planted in each cell, and a single pine seedling was allowed to germinate in each cell. Plantings were conducted in a greenhouse, and the plants were watered daily to saturation at temperatures ranging from 20°C to 30°C until the inoculations were performed.

Fungal inoculum

The black truffle inoculum was prepared from ascocarps from Molina de Aragón (Guadalajara, Spain) that were collected in February 2009 and from which *T. melanosporum* fruiting bodies were selected, cleaned superficially and flame sterilised. The samples were then stored in closed polyethylene bags at 4°C until the liquid spore inoculum was prepared, several days before inoculation. The fruiting bodies were ground, diluted in distilled water and stored at 4°C until inoculation. The fungal inoculum was estimated to contain approximately 3.4×10^4 spores/ml.

Bacterial inoculum

The inoculum of *P. fluorescens* CECT 844 was supplied by the CECT (Spanish Type Culture Collection), University of Valencia. Upon arrival, the lyophilised inoculum was stored at 10–15°C until inoculation. The liquid inoculum was prepared by suspending in a standardised nutrient medium that was suitable for *P. fluorescens* CECT 844 growth (1 g beef extract, 2 g yeast extract, 5 g peptone, 5 g NaCl and 1 L distilled water, pH 7.2). Using a Pasteur pipette, a drop of suspension was seeded in 5 ml of nutrient medium and incubated at 30°C for 12 h in the dark. Next, 5 ml of inoculum was replanted into 75 ml of fresh nutrient medium and incubated and shaken at 200 rpm and 30°C for 12 h. Finally, the combined 80 ml of the inoculum solution was reseeded again into 720 ml of liquid nutrient medium and incubated with stirring at 200 rpm and 30°C for 12 h until inoculation. Bacterial concentration was recorded after 1 day at 30°C in the dark. Dilutions yielding 30–100 colonies per plate were used for colony-forming unit (CFU) determination; 4×10^8 CFU/ml were estimated.

Trial design and inoculations

We used a four-level univariate design [inoculation of *P. fluorescens* CECT 844 (*Pf*), *T. melanosporum* (*Tm*), *T. melanosporum*×*P. fluorescens* CECT 844 (*Tm*×*Pf*) and control] distributed into four randomised blocks (1×4×4) with 50 plants per experimental unit. All of the inocula were applied at the same time (early July 2009). The substrate was injected with a dose of 4.5 g fresh carpophore/20 ml distilled water/plant (6.8×10^5 spores/plant) to half of the total plants produced (eight containers, 400 plants). Using 10 ml/plant of liquid *P. fluorescens* CECT 844 inoculum (4×10^9 CFUs/plant), the substrate was inoculated via injection to half of the plants that were produced (one fourth of which were also inoculated with *T. melanosporum*). Finally, one fourth of the seedlings were used as controls. No fertiliser was added.

After performing the inoculations, the plants were taken outside to a shade house (E.U.I.T. Forest) and watered daily to saturation. The temperatures ranged from 11°C to 29°C, and the average relative humidity varied from 28% to 80% during the experimental period.

Measurements

Pressure–volume curve and analysis of water parameters

During November 2009, corresponding pressure–volume curves were built (Tyree and Hammel 1972; Robichaux 1984) from measurements of stem water potentials that were calculated using a Scholander pressure chamber (Scholander et al. 1965). From each pressure–volume curve, three water parameters were calculated: the osmotic potential at saturation ($\Psi\pi_{full}$), the osmotic potential at the turgor loss point ($\Psi\pi_0$) and the modulus of elasticity (E_{max}) (Cheung et al. 1975; Jones and Turner 1980; Tyree and Jarvis 1982; Bowman and Roberts 1985).

We analysed 12 randomly chosen plants per treatment (three per block). Treatments that were inoculated with *T. melanosporum* were later analysed and confirmed to have the mycorrhizal fungus.

Growth

Twelve plants per treatment (three per block) were selected at random. We measured the height and diameter of each plant. After the samples were dried in an oven at 65–70°C for 48 h, we measured the dry weights of the shoots and roots of each sample.

Mycorrhizal colonisation

Eight plants per treatment (two per block) were randomly selected to analyse the colonisation of the mycorrhizal fungi in the roots by the characterisation and identification of the mycorrhizae (Agerer 1987–1998). Subsequently, mycorrhizal counts of *T. melanosporum* and other naturally occurring mycorrhizal fungi were performed. We also analysed the total roots; the root ball of each plant was submerged in water, and most of the substrate was removed to clean the roots. The roots were then chopped into 1–2-cm pieces that were cleaned, rinsed in distilled water and placed into a Petri dish with water for analysis. We calculated the percentages and number of root tips in both the inoculated and uninoculated plants.

Nutrient analysis

The concentrations and contents of key nutrients (N, P, K, Ca, Mg and Fe) were analysed in the seedlings at the end of the growing season. A new random sample of 48 plants per treatment was divided into four groups (12 plants per block).

Analyses of P, K, Ca, Mg and Fe were performed using inductively coupled plasma optical emission spectrometry after open digestions in nitric acid. The analysis of N was conducted using a LECO mass analyser (CHN-600).

Radical growth potential

In February 2010, we took a new random sample of 12 plants per treatment (three per block). We measured the height and basal diameter of each plant. Subsequently, each plant and its respective root ball were carefully transplanted

to prismatic pots (3 L) that were then filled with inert white perlite. The pots were placed randomly in a greenhouse for 21 days under optimum environmental conditions to promote growth (Burdett 1987). Irrigation was applied daily so that root growth would not be limited. The air temperature was maintained between 16°C and 22°C, and the relative humidity remained at approximately 95%. At 21 days (March), each plant was carefully removed, the number of new roots that were longer than 1 cm (distinguishable by their greater thicknesses and colour differentiations) was measured and the total length of the new roots/plant (greater than 1 cm) was also measured.

Data analysis

An analysis of variance was performed, means were calculated for the proposed parameters and a Duncan's comparison test was applied at a confidence level of 0.05%. We applied a nonparametric Kruskal–Wallis correction to nonhomogeneous variances. All statistical analyses were performed using the Statgraphics Plus programme (StatPoint Technologies, Inc.). For statistical analysis of the root growth potentials, the heights and basal diameters were selected as covariates. Results were similar with the selection of either covariate, and therefore, only the results pertaining to the initial height are presented. In addition, the results of both parameters (number and length of new roots) were similar, and therefore, only those describing the new roots are presented.

Results and discussion

Several authors have argued that the microflora associated with *Tuber* sp. is stable and selective and is mainly represented by the genus *Pseudomonas* and the aerobic spore-forming bacteria, *actinomycetes* and *rhizobacteria* (Citterio et al. 2001; Barbieri et al. 2007). Rivera et al. (2010) found that the predominant species in the ascocarps of *T. melanosporum* was *P. fluorescens*.

In the present study, we analysed the interaction of the ectomycorrhizal fungus *T. melanosporum* with the strain *P. fluorescens* CECT 844. Inoculation (*Tm*) and co-inoculation (*Tm*×*Pf*) improved the majority of the growth parameters (Table 1); in addition, inoculation (*Pf*) improved the height and root weight of the seedlings.

Some authors have emphasised the importance of fungus–bacterium interactions and their positive effects on the growth of *P. halepensis* (Ouahmane et al. 2009).

P. fluorescens can be a plant growth stimulator that efficiently promotes seed germination, accelerates growth in the early stages, induces root initiation, enhances the formation of roots and root hairs, facilitates root regeneration and helps to control pathogens in some forest species (Heinonsalo et al. 2004). These effects have been observed specifically in *P. halepensis* inoculated with *P. fluorescens* Aur6 (Rincón et al. 2008). However, the isolated effects of *P. fluorescens* CECT 844 on root initiation were not apparent in our work, although throughout the year, the root dry weights of seedlings were increased as a result of the inoculations [both (*Pf*) and (*Tm*×*Pf*)]. These inoculations did not cause the regeneration of new roots the following spring (root growth potential), in contrast to observations made in previous studies (Karabaghli et al. 1998; Heinonsalo et al. 2004); this may suggest different effects on the root regeneration for different strains of *P. fluorescens*; also, 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. However, we did not observe any positive synergistic effects of the fungus–MHB partnership on root growth or the regeneration of new roots.

The improvement of host plant vigour by mycorrhizae is often observed under limiting nutrient supplies (Smith and Read 1997). In other studies, Rincon et al. (2007) observed different effects of other ectomycorrhizal fungi on nutrient uptake in the seedlings of *P. halepensis*, which may have been the result of nutritional demands that were dependent on the species of fungus (Olsson et al. 2002). In previous studies that analysed the fungus *T. melanosporum*, this inoculation was shown to improve the growth and nutrition of the seedlings of *P. halepensis* (Domínguez et al. 2004; 2008). In the same way in the present study, we observed that the seedlings with the most optimal growth that absorbed the most nutrients were inoculated with *T. melanosporum* (*TM*; Table 1); however, *P. fluorescens* CECT 844 did not enhance the positive effects of *T. melanosporum* on the growth or the absorption of nutrients [it had a negative effect on (*Tm*×*Pf*) compared to (*Tm*)]. All treatments improved N uptake, although the best absorption was observed in treatment (*Tm*) in both the shoot and root.

In areas of the Mediterranean, P is a limiting nutrient in the early stages of growth of *P. halepensis* (Sardans et al. 2006). In the present study, the inoculation (*Tm*) improved the P content in both the aboveground and root plant parts, which is in agreement with previous studies (Domínguez et al. 2008) but had no effect on the P concentration. Although (*Tm*×*Pf*) increased the P content in the aboveground plant parts, it did not affect the roots. Rincon et al. (2008) observed that seedlings from *P. halepensis* inoculated with *P. fluorescens* Aur6 showed increased P concentrations following the inoculation. However in the present study, *P. fluorescens* CECT 844 did not improve the concentration and content of P, and in fact, it may have a limiting effect; perhaps, different strains cause different effects on the P uptake; on the other hand, the substrate used in our plant cultivation provided a low level of nutrients availability

Table 1 The water relation parameters, growth parameters, mycorrhizal colonisations, nutrient concentrations and contents and root growth potentials of *Pinus halepensis* seedlings

Treatment	Control	*Pseudomonas*	*Tuber*	*Tuber*×*Pseudomonas*
Water relation parameters				
$\Psi\pi_{full}$ (MPa)	0.81 (±0.06) a	0.93 (±0.09) a	0.95 (±0.08) a	0.89 (±0.04) a
$\Psi\pi_0$ (MPa)	1.21 (±0.11) a	1.34 (±0.09) a	1.38 (±0.10) a	1.29 (±0.07) a
E_{max} (MPa)	4.07 (±0.31) a	4.66 (±0.68) a	4.55 (±0.58) a	4.55 (±0.48) a
Growth				
Height (cm)	8.64 (±0.629) c	11.29 (±0.27) b	14.62 (±1.39) a	14.58 (±0.36) a
Basal diameter (mm)	1.19 (±0.08) b	1.35 (±0.04) b	1.62 (±0.11) a	1.60 (±0.07) a
Shoot (g)	0.19 (±0.04) c	0.25 (±0.01) bc	0.34 (±0.07) ab	0.39 (±0.03) a
Root (g)	0.10 (±0.02) b	0.15 (±0.01) a	0.16 (±0.02) a	0.17 (±0.01) a
Mycorrhizal colonisation				
Tuber (%)	–	–	15 (±2) b	28 (±3) a
Total[a] (*N*/plant)	279 (±73) a	375 (±45) a	369 (±47) a	313 (±37) a
Shoot nutrient concentration				
N (mg/g)	5.92 (±0.23) b	8.67 (±0.55) a	9.55 (±0.54) a	8.62 (±0.41) a
P (mg/g)	1.97 (±0.23) a	0.52 (±0.02) c	1.77 (±0.09) a	1.27 (±0.06) b
K (mg/g)	12.77 (±0.51) b	10.85 (±0.45) c	16.15 (±0.52) a	14.95 (±0.65) a
Ca (mg/g)	4.8 (±0.11) a	3.2 (±0.04) c	3.67 (±0.02) b	3.37 (±0.06) c
Mg (mg/g)	3.87 (±0.17) a	2.72 (±0.23) b	2.92 (±0.06) b	2.72 (±0.11) b
Fe (mg/g)	0.29 (±0.10) a	0.22 (±0.10) a	0.16 (±0.02) a	0.15 (±0.03) a
Root nutrient concentration				
N (mg/g)	5.5 (±0.22) c	7.0 (±0.37) ab	7.72 (±0.30) a	6.62(±0.24) b
P (mg/g)	1.75 (±0.22) a	0.55 (±0.03) c	1.6 (±0.15) a	1.05 (±0.03) b
K (mg/g)	10.45 (±0.36) d	12.8 (±0.24) b	13.67 (±0.19) a	11.9 (±0.12) c
Ca (mg/g)	6.55 (±0.05) a	6.3 (±0.17) a	6.35(±0.24) a	6.17 (±0.33) a
Mg (mg/g)	4.82 (±0.37) b	6.7 (±0.93) ab	7.7 (±0.64) a	6.3 (±0.63) ab
Fe (mg/g)	1.18 (±0.13) b	2.56 (±0.51) a	2.87 (±0.33) a	2.28 (±0.27) a
Shoot nutrient content				
N (mg/plant)	1.13 (±0.04) c	2.17 (±0.14) b	3.25 (±0.18) a	3.36 (±0.16) a
P (mg/plant)	0.38 (±0.04) c	0.13 (±0.01) d	0.60 (±0.03) a	0.50 (±0.03) b
K (mg/plant)	2.43 (±0.1) b	2.71 (±0.11) b	5.49 (±0.18) a	5.83 (±0.25) a
Ca (mg/plant)	0.91 (±0.02) c	0.80 (±0.01) d	1.25 (±0.01) b	1.32 (±0.03) a
Mg (mg/plant)	0.74 (±0.03) b	0.68 (±0.06) b	1 (±0.02) a	1.06 (±0.04) a
Fe (mg/g)	0.06 (±0.02) a	0.06 (±0.02) a	0.06 (±0.01) a	0.06 (±0.01) a
Root nutrient content				
N (mg/plant)	0.55 (±0.02) c	1.05 (±0.06) b	1.24 (±0.05) a	1.13 (±0.04) ab
P (mg/plant)	0.18 (±0.02) b	0.08 (±0.004) c	0.26 (±0.02) a	0.18 (±0.004) b
K (mg/plant)	1.05(±0.04) d	1.92 (±0.04) c	2.19 (±0.03) a	2.02 (±0.02) b
Ca (mg/plant)	0.66 (±0.01) b	0.95 (±0.03) a	1.02 (±0.04) a	1.05 (±0.06) a
Mg (mg/plant)	0.48 (±0.04) b	1.01 (±0.14) a	1.23 (±0.1) a	1.07 (±0.11) a
Fe (mg/g)	0.12 (±0.01) b	0.38 (±0.08) a	0.46 (±0.05) a	0.39 (±0.05) a
Root growth potential				
Length[b] (cm)	10.97 (±2.44) a	9.82 (±2.40) a	8.92 (±2.46) a	13.01 (±2.33) a

Values in parentheses represent the standard error. N=12 (water relations, growth and root growth potential parameters); N=8 (mycorrhizal colonisation); N=4 (nutrient parameters). Values in the same row with different letters differ significantly ($p<0.05$) according to the Duncan test

$\Psi\pi_{full}$ osmotic potential at full turgor, $\Psi\pi_0$ osmotic potential at zero turgor, E_{max}: modulus of elasticity near full turgor

[a]Total number of total root tips per plant

[b]Total length of new roots per plant; covariate using the height parameter

because of the unfertilised peat that was used. Under these culture conditions, and by different mechanisms, *T. melanosporum* could facilitate the access of phosphorus to plants.

Rincón et al. (2008) observed increases in K concentrations in response to the inoculation with *P. fluorescens* Aur6 in *P. halepensis* seedlings. This positive effect was also observed in our study (*P. fluorescens* CECT 844) because all of the inoculations improved the content and concentration of K in the root, although its effects were not as positive as those of (*Tm*). The (*Pf*) inoculation did not

improve the absorption of K for aboveground growth, but inoculations with *T. melanosporum* [(*Tm*) and (*Tm*×*Pf*)] did, which is in agreement with previous studies (Domínguez et al. 2008).

All inoculations improved the Ca, Mg and Fe content of the roots, but only the inoculations with *T. melanosporum* [(*Tm*) and (*Tm*×*Pf*)] improved the Ca and Mg content in shoot. The only positive synergistic effect observed for both inocula was in the Ca content in the aboveground plant parts.

Mycorrhization can alter the bacterial community in the rhizosphere (Barea 1997) by modifying root exudates, which can later reduce nutrient availability by the action of such microorganisms (Simard et al. 2002) in plants. This may explain some of the reduced availabilities of certain nutrients such as N, P and Mn. However, in the present study, seedlings were grown on substrates with low nutrient availabilities, but mycorrhization with *T. melanosporum* improved the absorption of all nutrients.

Rincón et al. (2008) found that (*Pf*) inoculation, in the presence or absence of local bacterial colonies, caused differing concentrations of nutrients to be absorbed by the seedlings of *P. halepensis*. This suggests that alterations in rhizosphere microbial populations can lead to quantitative and qualitative changes in the absorption of nutrients in associated plants. In our work, we suggest that the addition of *P. fluorescens* inoculum may adversely affect the amounts of P and K that are taken up by plants inoculated with *T. melanosporum*. This may be the result of an imbalance in the populations in the mycorrhizosphere, some of which can be incorporated from within the same black truffle inoculum (Rivera et al. 2010).

The adhesion and colonisation of MHB (which are metabolically active) at the mycorrhizal surface can affect and improve the symbiotic relationship and the pre-symbiotic stages (Frey-Klett et al. 2007; Deveau et al. 2007); Several authors have reported the stimulatory effects of *P. fluorescens* and ectomycorrhizal fungi that are grown together (Rincón et al. 2005; Deveau et al. 2007). In the present study, the strain *P. fluorescens* CECT 844 was able to raise the average percentage of *T. melanosporum* mycorrhization from 15% to 28% (Table 1), which is a low to medium percentage. The relatively low percentages of truffle mycorrhizae that were obtained may have resulted from the use of a slightly alkaline substrate that was poorly suited for the truffle but was chosen in an attempt to more clearly elucidate the effects of the strain; *P. fluorescens* CECT 844 could facilitate the formation and establishment of *T. melanosporum* ectomycorrhizae, and therefore, this strain could be an MHB. Anyway, some authors have suggested that MHB may be beneficial to certain fungi but adversely affect others (Tarka and Frey-Klett 2008).

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. 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). 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 (Abrams and Kubiscke 1994). Other authors have reported changes in cell wall elasticity but not osmotic adjustment (Stewart and Lieffers 1993).

Authors have reported that during a drought, *T. melanosporum* can reduce the water deficit of the host plant (Domínguez et al. 2006). Dominguez et al. (2008) also observed that under conditions of abundant water availability, mycorrhization with black truffles can cause an elastic adjustment in seedlings of *P. halepensis*, which is a possible mechanism related to resistance to water stress. Hormonal effects may also be involved in the water stress tolerance of plants that have been inoculated with PGPR because some bacteria can produce abscisic acid, a plant hormone that is produced in response to drought (Boiero et al. 2007). Rincón et al. (2008) found that *P. fluorescens* Aur6 can enhance the water efficiency of associated forest species but only when they are subjected to a period of water stress. In the same way, in the present study and under well-watered conditions, none of the inoculations changed the water parameters; it will be important to study the effects of (*Tm*×*Pf*) inoculations under drought-like conditions in the future.

Potassium is an important solute associated with the regulation of cell turgor and stomatal opening (Benlloch-González et al. 2008). Thus, according to the results of this study, there was no clear relationship between the regulation of cellular osmotic potential and the amount of K absorbed by the plants.

P. halepensis does not normally produce *T. melanosporum* ascocarps; however, if it forms the ectomycorrhizae, we suggest that black truffle mycorrhizal seedlings be introduced and used as "carriers" of inocula of wild truffles in natural media for application in forestry truffle culture. This technique shows promise both in the development of truffle farming and the revival of natural truffle production for use in mountainous truffle cultures. An improved rate of *T. melanosporum* mycorrhization as a result of *P. fluorescens*, which was observed in the present study, indicates a high potential for truffle culture improvement. It is necessary to continue to study the effects of the co-inoculation of (*Tm*×*Pf*) in seedlings of species that produce the prized black truffle and to search for ways of improving the water stress tolerance of forest plants through the bioinoculation of soil microorganisms.

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