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Advantages of inoculation with immobilized rhizobacteria versus amendment with olive-mill waste in the afforestation of a semiarid area with *Pinus halepensis* Mill

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ABSTRACT

We performed a field assay to assess the influence of the inoculation with a mixture of two immobilized strains of rhizobacteria (*Azospirillum brasilense* and *Pantoea dispersa*) and the addition of organic olive residue (alperujo) on the growth of *Pinus halepensis* Mill. and plant stress parameters, as well as on soil physico-chemical and microbiological properties. Twenty-eight months after planting, the microbial inoculation was the most-effective treatment regarding stimulation of seedling growth (by 48% with respect to the control) and nutrient uptake. The inoculated plants had the lowest proline accumulation, less oxidative damage to lipids and higher shoot water potential. The microbial inoculation and combined treatment enhanced enzyme activities, total carbohydrates and microbial biomass C and nutrients in the soil. The effectiveness of the microbial inoculant with respect to promotion of plant growth and the lower cost of implementation of this restoration biotechnology support its preferential use in re-afforestation tasks with *P. halepensis* in semiarid environments.

1. Introduction

In semiarid Mediterranean areas, the establishment of plant cover is difficult under the severe climate, characterized by low precipitation and frequent drought periods, particularly in soils with low microbial activity. Therefore, it is necessary to use technological restoration methods that can improve both soil quality and the ability of the seedlings to resist semiarid environmental conditions (Caravaca et al., 2005). The establishment of native plant species is a practice widely used for reclaiming degraded lands and constitutes the most-effective strategy in semiarid areas (Alguacil et al., 2003; Mengual et al., 2014; Schoebitz et al., 2014). *Pinus halepensis* Mill. is the prevailing tree species in semiarid areas of central-southern Spain, and it has been used in afforestation programs for degraded soils as it is a pioneer species and one of the few tree species that can thrive in these conditions (Maestre and Cortina, 2004).

Recent studies about the reclamation of semiarid soils have shown the beneficial effects of the application of organic amendments on soil quality, with increase in the proliferation and development of natural populations of soil microflora, since the organic residues can be used by soil microorganisms, as substrates and as carbon and energy sources (Medina and Azcón, 2010), and also improve soil properties. This effect could be extended to the enhancement of soil enzyme activities, which are key factors contributing to soil-borne microorganism activity and soil fertility (Caravaca et al., 2005). The use of organic waste materials not only increases the organic matter and fertility of soils, but also contributes to the palliation of environmental and economic inconveniences related with waste disposal (Rincón et al., 2006).

The Spanish olive-mill industry produces a huge amount of wastes that are difficult to reuse (four million tons per year). The main by-product is alperujo, which can be composted before its application to the soil in order to obtain a high-quality amendment, rich in K and partially-humified organic matter (Alburquerque et al., 2009, 2006). Such characteristics suggest that this residue could be useful for improving soil quality and in the development of afforestation programs in semiarid and degraded areas. The beneficial short-term effects of the addition of alperujo compost in horticultural and revegetation practices have been reported (Alburquerque et al., 2006; Schoebitz et al., 2014).

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However, their effect on the establishment of tree seedlings under semiarid field conditions remains unknown. Additionally, the application of olive-mill waste interacts positively with soil microorganisms (Schoebitz et al., 2014). Among the components of the soil microbiota, rhizobacteria are free-living bacteria, often labeled as plant growth-promoting rhizobacteria (PGPR), which can colonize the rhizosphere and improve root system establishment (Antoun and Kloepper, 2001). In this regard, PGPR have a potential role in the establishment of plant cover in arid environmental conditions (Puente et al., 2004), where they can promote plant growth and improve both water and nutrients uptake (Bashan et al., 2004). Nevertheless, colonization around plant roots following the direct inoculation of free PGPR cells into soil is not easy because this process is highly susceptible to environmental variations (Wu et al., 2012). This unpredictability of the success of PGPR inoculation of plants is due mainly to the quality of the inoculant formulations containing effective rhizobacterial strains, which determines the success or failure of plant growth promotion. Immobilization of microbial inoculants has been used to enhance their effectiveness, by providing nutrients and protection from desiccation (Kim et al., 2012). The success of microbial inoculants introduced into soil requires that an adequate number of bacteria reach suitable habitats where they can survive (Heijnen and Van-Veen, 1991). The aim of the immobilization of rhizobacteria is to protect the microorganisms (Schoebitz et al., 2013) and ensure a gradual and prolonged release into the soil (Wu et al., 2011). In spite of their potential viability, the use of immobilized bacteria has never been tested in the reafforestation with tree species like *P. halepensis* in Mediterranean semiarid conditions. The aim of this work was to study the medium-term effect of olive-mill waste compost and a microbial inoculum constituted by two immobilized strains of rhizobacteria on *P. halepensis* establishment under semiarid field conditions. We hypothesized that the revegetation treatments assayed would confer drought tolerance on the plants and/or enhance soil quality, leading to enhanced plant growth. In this respect, we measured soil physico-chemical, biochemical and microbiological variations as well as the changes in shoot nitrate reductase activity, proline accumulation, oxidative damage to lipids and plant water relations induced by these treatments.

2. Material and methods

2.1. Study site

The experimental area was situated in Vicente Blanes Ecological Park in Molina de Segura (Southeast Spain) (Lat. 38° 12′ N, Long. 1° 13′ W, Elev. 392 m). The climate is semiarid Mediterranean, with a mean annual temperature of 17.5 °C and no frost period. The annual rainfall is around 300 mm and the potential evapotranspiration reaches out to 1000 mm per year. The soil is a Typic Torriorthent (SSS, 2010), with low organic matter content and a silty loam texture (Schoebitz et al., 2014). The vegetation in the study site was dominated by the invasive *Piptatherum miliaceum* (L.) Cosson and some native shrubs of *Thymus vulgaris* L., *Pistacia lentiscus* L., *Cistus clusii* Dunal and *Rosmarinus officinalis* L.

2.2. Plants

The selected plant to carry out this study was *Pinus halepensis* Mill. This is a tree species, belonging to the family *Pinaceae*, which can reach a height of 10 m, widely distributed in the Mediterranean area. It is well adapted to water stress conditions and high temperatures and, therefore, it has been used in revegetation assays of degraded (Díaz and Roldán, 2000; Maestre and Cortina, 2004) and arid soils (Oliveras et al., 2003). Seedlings were grown in Muzalé nursery (Murcia, Spain) with peat as substrate for 1 year prior to experimental procedures. At planting, *P. halepensis* was 20 cm high, with a shoot dry weight of 2.77 g and root dry weight of 1.37 g.

2.3. Microbial inoculant and organic residue

The microbial inoculant was a mixture of two plant growth promoting rhizobacteria (PGPR) *Azospirillum brasilense* Tarrand, Krieg & Döbereiner, 1978 and *Pantoea dispersa* Gavini, Mergaert, Beij, Mielcarek, Izard, Kersters, De Ley 1989 immobilized on clay pellets, being the cells concentrations of both rhizobacteria 10^9 CFU g^{-1}. Bacteria of genus *Azospirillum* that fix nitrogen under microaerobic conditions have a positive effect on plant growth (Flores et al., 2010). *Azospirillum* has been shown to be more successful when it is co-inoculated with other microorganisms such as phosphate-solubilizing bacteria (Bashan et al., 2004). To this end, *A. brasilense* was co-applied with *P. dispersa* whose beneficial effect on plant development arises from its capacity to solubilize phosphorus compounds and help to control pathogenic organism (Son et al., 2006). This microbial inoculant was developed by Probelte, S.A., Murcia. These strains were deposited in the Spanish Type Culture Collection (CECT) with the numbers CECT-5801 (*P. dispersa*) and CECT-5802 (*A. brasilense*).

A composted olive-mill waste was used as organic amendment for soil. Fresh cow bedding was added to the olive-mill waste as bulking agent and composted by using a combination of the Rutgers system and mechanical turning. The analytical characteristics of the organic residue are described on Schoebitz et al., 2014.

2.4. Experimental design

A full-factorial design was established with two factors and five fold replications in a split plot design. The first factor was the inoculation or not of *P. halepensis* seedlings with microbial inoculant and the second was the addition or not of organic olive residue into the soil.

In early February 2011, the seedlings were transported to the experimental field, where planting holes 15 × 15 cm wide and 15 cm deep were dug manually. There, an amount of 30 g of microbial inoculant pellets was applied per plant. The same quantity of sterilized inoculant was applied to the non-inoculated plants. Olive residue was added at a rate of 2% by weight (186 g of organic olive residue per plant). Microbial inoculant and organic olive residue were manually mixed into 2 kg of soil in plastic bags an introduced in the plantation holes. The seedlings were planted at least 1 m apart between holes, with 3 m between treatment levels. At least 10 seedlings per treatment level were planted.

2.5. Sampling procedures

Twenty-eight months after planting, in early June 2013, samples were collected. Five plants per treatment including root systems and rhizosphere soil were harvested, and introduced in polyethylene bags for transport to the laboratory. Rhizosphere soil samples were separated into two subsamples before physico-chemical and biochemical analyses: one subsample sieved to <2 mm and other subsample sieved between 4 and 0.25 mm.

2.6. Plant analyses

The sampling day, before the harvest, leaf water potential was measured in two fully developed needles per tree of each replicate in a pressure chamber (model 3005, Soil Moisture Equipment Co., Santa Barbara, CA, USA) (Mellisho et al., 2012). Midday (12 h solar time) stem water potential was measured in a similar number and