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Co-inoculation of *Halomonas maura* and *Ensifer meliloti* to improve alfalfa yield in saline soils

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ABSTRACT

Salinity is the major environmental factor limiting crop production. Alfalfa is a legume with high nutritional value that establishes a symbiosis relation with *Ensifer meliloti*. Under saline conditions the alfalfa yield decreases and this symbiosis is affected. The aim of this work is to study the effect of the co-inoculation of alfalfa plants with *Halomonas maura* (a moderately halophile bacterium) and *E. meliloti* in saline soils to improve their productivity and growth under greenhouse and field conditions. Alfalfa plants were grown in Leonard jar under greenhouse conditions, using a N-free mineral solution to mimic the conditions of an Orthic Solonchak. Then alfalfa plants were grown in the field in the same soil type. Seeds were inoculated with *E. meliloti*,*H. maura*, co-inoculated with *E. meliloti* and *H. Maura*, or non-inoculated as a control in both experiments. In greenhouse experiments the co-inoculation of alfalfa plants increased significantly the shoot dry weight (0.64 ± 0.02 *vs.* 0.79 ± 0.02), the leghaemoglobin content (10.17 ± 0.03 *vs.* 11.25 ± 0.06) and water potential (-3.12 ± 0.02 *vs.* -2.79 ± 0.02) compared with the single inoculation with *E. meliloti*. In the field experiments, biomass of co-inoculated plants clearly outyielded those of plants inoculated with any inoculant. The co-inoculation of *H. maura* and *E. meliloti* enhances alfalfa productivity in saline soils, thus contributing to the agricultural exploitation of low productive areas. *H. maura* and *E. meliloti* could be considered in formulation of bioinoculants to contribute in the reduction of the overuse of chemical fertilizers and their environmental impacts.

1. Introduction

High salt levels in the soil limit its agroecological potential and represent a considerable ecological and socio-economic threat to sustainable development. It is estimated that 20% of the irrigated land in the world is affected by salinity (Yamaguchi and Blumwald, 2005), with around 3.8 million ha in Europe. Soil salinity can be due to natural causes, such as evaporation, seawater intrusion, release of soluble salts from watering, with deposition of oceanic salts carried in wind and rain. In addition, a significant proportion of recently cultivated agricultural land has become saline due to land clearing or irrigation with saline water, both of which cause water tables to rise and salts to concentrate in the root zone (Munns and Tester, 2008). Areas with a dry climate can experience high soil salinity due to the evaporation of underground saline water that brings salt up to the soil surface (Yu et al., 2012). Salinity stress is of great importance in arid and semi-arid areas of the world due to its impact in reducing crop yield (Jalili et al., 2009). There is a negative correlation between the degree of salt stress and crop growth rate. The most important effects are inefficient photosynthesis and inhibition of cell division and expansion (Yu et al., 2012). Increased salinity causes a reduction in rate and germination percentage and a reduction of leaf area and plant dry weight (Bauddh and Singh, 2012; Bhattarai and Midmore, 2009; Muhammad et al., 2006; Sadeghi et al., 2012; Silini et al., 2012; Wankhade and Sanz, 2013). Crops are sensitive to salinity in different ways depending on the developmental stage of the plant, species and genotype (Shelden and Roessner, 2013; Turner et al., 2013); in general, vegetable crops are more salt sensitive than grains and forages (Shannon, 1997).

Research has been carried out to improve the crop productivity in saline soils. The use of plant growth promoting rhizobacteria (PGPR) is a well-known strategy to increase the yield of such soils (Ahmad et al., 2013 Zhang et al., 2004). Rhizobia are widely used to

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enhance the growth of crop legumes due to the capacity of the former microorganisms to fix atmospheric nitrogen. However, the symbiosis between plants and rhizobia also is affected by salinity (Bouhmouch et al., 2005; Shamseldin and Werner, 2005). The effect of the single inoculation with rhizobia can be improved by co-inoculating bacteria with different capacities.

Medicago sativa L. (alfalfa) is one of the most important perennial forage crops in the world and exhibits great agronomic interest. It is sensitive to salinity (Munns and Tester, 2008) and grows well in alkaline and carbonated soils, where it can develop a deep root system. *Ensifer* (formerly *Sinorhizobium*) *meliloti* is the specific microsymbiont of alfalfa. Although *E. meliloti* is capable to grow in the presence of 500 mM NaCl, this symbiosis is affected by saline stress that decreases the nitrogen fixation efficiency per plant.

Halomonas maura is a moderately halophile bacterium capable of growing in salt concentrations ranging from 1 to 15% w/v. It was first isolated from soils surrounding a saltern at Asilah in Morocco (Bouchotroch et al., 2001). *H. maura* is capable of fixing nitrogen under saline concentrations and uses nitrate as a final electron acceptor (Argandoña et al., 2006). *H. maura* S-30 has the ability to excrete large quantities of an exopolysaccharide known as mauran (Arias et al., 2003). This latter capacity could well result in beneficial applications as a soil inoculate in moderately saline, arid soils that until now have defied agricultural use (Llamas et al., 2006).

The aim of this work is to study the effect of the co-inoculation of alfalfa plants with *H. maura* and *E. meliloti* to improve their productivity and growth under the following experimental conditions: (1) *in vitro* greenhouse experiments reproducing saline soil properties, and (2) *in situ* experiments in saline soils Orthic Solonchak, (FAO, 2006) and Xeric Calcigypsid (Soil Survey Staff, 2010) located in an arid area (Granada, SE Spain).

2. Materials and methods

2.1. Bacterial strains and culture conditions

Cells of *E. meliloti* strain 1021 were routinely grown in tryptone-yeast extract medium (TY) (Beringer, 1974). *H. maura* strain S-30 was cultured in malt extract-yeast extract medium (MY) (Moraine and Rogovin, 1966) supplemented with 7.5% w/v mineral salts as described earlier (Quesada et al., 1993). Cells were grown at 30 °C.

2.2. Greenhouse experiments

Seeds of alfalfa (*Medicago sativa* L. cv. Aragón) were surface-sterilized and germinated as previously described (Torres et al., 2013). Selected seedlings were planted in autoclaved Leonard jars (Leonard, 1943) filled with vermiculite and containing nitrogen-free mineral solution (Rigaud and Puppo, 1975) to ensure that nitrogen came from atmospheric fixation. In order to mimic the soil solution of an Orthic Solonchack, the mineral solution was supplemented with a mixture of $CaSO_4$ (2.5 g/L), NaCl (1.29 g/L), $MgCl_2{\cdot}6H_2O$ (1.5 g/L) and $NaHCO_3$ (1.22 g/L) to reach a final ionic strength of 100 mM. Electrical conductivity of the mineral solution was determined with a conductivity meter Crison 525 (Crison Instruments, S.A., Barcelona, Spain). Seeds (5/jar) were inoculated at sowing with 1 mL of either *E. meliloti* (inoculant E) suspended in TY broth, *H. maura* (inoculant H) suspended in MY broth, or a mixture (1:1 ratio, about 10^8 CFU/mL each) of *E. meliloti* 1021 and *H. maura* S-30 (inoculant EH) suspended in TY and MY broth 1:1 ratio. Non-inoculated plants were used as a control (CT). Plants were grown for 60 d under greenhouse conditions. A 16/8 h light/dark photoperiod and 25/18 °C were used. Supplementary light was provided by Sylvania incandescent and cool-white lamps (500 μmol m^{-2} s^{-1}, 400–700 nm) at the plant tops. Leaf water potential (Ψ_w) was measured in the first fully expanded leaf, as indicator of stress resistance, using a C52 sample chamber connected to a HR-33T psychrometer (Wescor, Logan UT, USA). The leghaemoglobin content was measured in fresh nodules as previously described (Talbi et al., 2012) since leghaemoglobin facilitates the diffusion of O_2 which prevents inactivation of nitrogenase (atmospheric nitrogen fixation enzyme).

2.3. Field experiments

2.3.1. Site location and description of the study area

The experimental plots were located in the province of Granada (SE Spain, 30S WG 248483). Soils were classified as Orthic Solonchak (FAO, 2006) and Xeric Calcigypsid (Soil Survey Staff, 2010). The relief was plain, with gypsiferous vegetation developed on the gypsiferous sediments. Soils were under anthropogenic influence and their productivity was very low due to its poor agricultural quality. The climate is arid, characterized by a mean annual precipitation of 250 mm (Xeric/Aridic regimen, Soil Survey Staff, 2010) and a mean annual temperature of 14 °C (Mesic Regimen, Soil Survey Staff, 2010), leading to a strong hydric stress.

2.3.2. Field trials

The field experiment was carried out during the 2012 spring–summer season. It was conducted in a randomized block design where the plot size (4/block) was 1.20 m length × 1.20 m width in 5 replicates. The distance between each plot and each block was 0.80 m.

Seeds of alfalfa were immersed in three different solutions containing each bacteria, *E. meliloti*, *H. Maura* and a mixture of *E. meliloti* + *H. Maura* ($\sim 10^8$ CFU/mL), with their respective broth medium TY, MY and TY-MY (1:1 ratio). Every solution also contained Arabic gum (25%) as an adhesive agent. The seeds were kept for 20 min in solution under continuous stirring, filtered through four layers of cheesecloth, and then air-dried for 24 h before planting. A set of seeds immersed in TY medium supplemented with 25% arabic gum was used as a control. The following treatments were used: (1) seeds without bacterial treatment (control, CT); (2) seeds treated with *H. maura* (inoculant H); (3) seeds treated with *E. meliloti* (inoculant E), and (4) seeds treated with *H. Maura* and *E. meliloti* (inoculant EH). The seeds (5 g/plot, about 20 kg/ha) were scattered over the block and covered by a thin layer of soil. The plants were watered once every two weeks with 30 L tap water/plot and grown for 3 months until harvest at about 10% flowering. In July 2012, plant shoots from each plot were harvested, dried at 60 °C and weighed.

2.3.3. Soil analysis

Soils in every block were sampled before seeding (April, 2012) and after harvesting (July, 2012) at two depths (0–30 and 30–60 cm). Soil samples were air-dried, ground and passed through a 2 mm sieve for analysis. To determine the organic carbon, samples were ground and sieved again (0.125 mm screen size). Soil organic carbon content was determined using the method of Walkley and Black (1934) modified by Tyurin (1951); the pH was determined potentiometrically in a 1:1 suspension with distilled water, and the $CaCO_3$ equivalent content was measured using the method of Barahona (1974). Finally, a soil saturation extract was prepared according to Allison (1973) to determine the electrical conductivity (EC) using a Solubridge Beckman conductivity meter with a G-0.5 × 2 conductivity cell (Beckman Instruments, Inc., USA).