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Mitigation of negative effects of progressive soil salinity gradients by application of humic acids and inoculation with *Pseudomonas stutzeri* in a salt-tolerant and a salt-susceptible pepper

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This study is dedicated for the memory of the German/Spanish mycorrhizae researcher Horst Vierheilig (1964–2011) of Consejo Superior de Investigaciones Cientificas, Granada, Spain.

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ABSTRACT

Humic acids and inoculation with the plant growth-promoting bacteria (PGPB) *Pseudomonas stutzeri* was used alone and combined to mitigate negative effects of progressive soil salinity gradients in a bell and a chili pepper. Plant height, length of root system, dry weight of stems, leaves and roots, number of leaves, leaf surface area, chlorophyll *a* and *b* content, total chlorophyll, and content of Na^+, K^+, Ca^{2+}, and Mg^{2+} were measured in a salt-tolerant and a salt-susceptible pepper. We showed that applications of PGPB and humic acids did not have a clear-cut effect. Some plant parameters, such as leaf and root parameters, were positively affected at certain salinity gradients and others, such as plant height and number of leaves, did not. However, it appears that more positive effects by either treatment were more apparent in the salt-resistant cultivar. No synergism on plant growth parameters and salt mitigation was detected when humic acids and PGPB were applied together. The K^+/Na^+ and Ca^{2+}/Na^+ ratios showed that single applications of humic acids and the PGPB enhanced these ratios in several salinity regimes. More increases in these ratios were detected in the susceptible cultivar. In several salinity regimes, metabolic synergism, leading to enhancement of these ratios, was obtained when humic acids and the PGPB were applied together. In summary, under increased salt gradient, application of the PGPB or humic acids improved some plant growth parameters. Central to those are some improvements in the K^+/Na^+ and Ca^{2+}/Na^+ ratios. Combined application of PGPB and humic acids indicate a potential to use this strategy to combat salinity.

1. Introduction

Salinization of soils leads to soil degradation and reduced crop productivity on a global scale. Salinization occurred in all climate zones, but is mainly prevalent in dry regions (Acosta et al., 2011). Worldwide, over 930 million hectares are affected by salinization; this problem continues to worsen (Rengasamy, 2006).

Maize, beans, squash and peppers are *the* staple diet of Mexicans since antiquity (Allen, 1992). In 2010, peppers of numerous varieties and cultivars were cultivated on 150,000 ha in Mexico (http://www.inforural.com.mx/spip.php?article7381). They are high-value crops that can be cultivated in low to moderately saline soils. In Mexico, 1.1 million hectares, 3.2% of arable land is affected by salinity (SEMARNAT, 2008) and the area is expanding, largely from progressive salinization of water sources used for irrigation, especially in arid and semi-arid regions, and excessive fertilization of agroindustrial farms. With population increase and increased export, cultivation of peppers will require additional marginal saline lands. Alleviating salt stress opens the possibility of using newer approaches, such as application of soil microorganisms, humic and fulvic acids, algae and plant extracts, and salt-reduction additives (Bashan et al., 2014; Calvo et al., 2014).

Plant growth-promoting bacteria (PGPB) are a diverse group of bacteria capable of promoting growth and yield of many crops and wild plants (for reviews: Bashan and de-Bashan, 2005; de-Bashan et al., 2012; Lugtenberg and Kamilova, 2009) including pepper (Bashan et al., 1989; del Amor et al., 2008; del Amor and Porras, 2009). Many species of PGPB can mitigate salt stress in plants (Dimkpa et al., 2009; Karlidag et al., 2011; Mayak et al., 2004; Rojas-Tapias et al., 2012; Shilev et al., 2012; Sziderics et al., 2007),

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but mainly species of the genus *Azospirillum* (Bacilio et al., 2004; Barassi et al., 2006; Creus et al., 1997; del Amor and Cuadra-Crespo, 2011; Fasciglione et al., 2015; Hamdia et al., 2004; Pereyra et al., 2012), *Pseudomonas* and *Serratia* (Bano and Fatima, 2009; Cheng et al., 2007; Jalili et al., 2009; Zahir et al., 2009), and arbuscular mycorrhizal fungi (Kaya et al., 2009).

Although the general positive effects of humic and fulvic acids on plant growth are common knowledge (Calvo et al., 2014), there are indications that application of humic and fulvic acids can reduce negative effects of salinity. These organic compounds may improve plant growth parameters and enhance mineral uptake in several plant species, including pepper (Canellas et al., 2009; Demir et al., 1999; García et al., 2013; Nardi et al., 2009).

Because general positive effects of PGPB and humic acids on mitigating soil salinity are documented separately, under variable, but constant levels of salinity, we hypothesized that an application of PGPB and humic acids together will further mitigate negative effects of salinity and enhance growth of pepper plants under progressive saline conditions commonly found in irrigated and fertilized fields (BSTID, 1990). Combining PGPB and humic acids, even without saline conditions, is rarely documented (Young et al., 2006). This has been done by: (1) measuring the effects of several treatments on pepper growth under greenhouse cultivation, as salinity is progressively increased over time, (2) measuring if the inherent susceptibility of peppers to salinity (relative resistance and relative susceptibility) is a factor affected with a single supplement or in combination, and (3) determine which of the supplements provide more consistent improvement of plant growth. All this was done in a series of similar experiments carried out over a period of two years.

2. Materials and methods

2.1. Organisms and growth conditions

2.1.1. Plants

All preparations of inoculant and inoculation procedure followed established guidelines (Bashan et al., 2016). The peppers (*Capsicum annuum* L.) cv. Jupiter (bell pepper, Syngenta Seeds, Boise, Idaho) and cv. Ancho San Luis (chili pepper, Syngenta Seeds) were used. Peppers are susceptible to salinity (glycophytic species), but show significant genotypic variation in tolerance (Aktas et al., 2006), Therefore, from preliminary germination assays, the Jupiter cultivar is relatively tolerant to salinity and the Ancho San Luis cultivar is relatively susceptible (Supplementing material, Table S1). Seeds were first treated with 2% Tween-20 (P2287, Sigma-Aldrich, St. Luis MO), washed in distilled water five times, then disinfected by soaking in 3% household bleach (NaClO) for 5 min, and finally rinsed several times in sterile tap water. This treatment provided 50% germination within 3 days for cv. Jupiter and 7 days for cv. Ancho San Luis and 100% germination within 6 days for cv. Jupiter and 10 days for cv. Ancho San Luis.

2.1.2. Bacteria, growth conditions, and preparation of inoculant

The strain of diazotroph PGPB was first isolated from the desert epiphyte *Tillandsia recurvata* L. (Bromeliaceae) in the southern Sonoran Desert in 1994 (Puente and Bashan, 1994). It was first identified as *Pseudomonas stutzeri* by gas chromatographic analysis of cellular fatty acids (Sasser, 1990) and later confirmed by biochemical tests for *Pseudomonas stutzeri* (Krotzky and Werner, 1987). In 2011, the entire 16S rRNA gene was sequenced by a commercial service (Genewiz, South Plainfield, NJ). Identification of the isolate were compared with sequences in the GenBank database, using the BLAST tool (www.ncbi.nlm.nih.gov/Blast.cgi). The sequence was deposited in the Genbank as: *Pseudomonas stutzeri* strain TREC (GenBank accession number JX014305). In addition to the wild type, a *gfp*-labeled *P. stutzeri* was generated, similar to *gfp*-labeled *Azospirillum brasilense* (Rodriguez et al., 2006). Both strains were stored in liquid nitrogen and re-activated on nutrient agar medium: (Fluka, St. Louis, MO) at $30 \pm 2\,^{\circ}C$ for 24 h. A single colony was cultivated in 250 mL Erlenmeyer flasks containing 150 mL of nutrient broth (#N7519, Fluka) and incubated at $30 \pm 2\,^{\circ}C$ with rotation at 120 rpm for 48 h. Bacteria were harvested by centrifugation at 2683*g* for 10 min a $4\,^{\circ}C$. Bacteria were washed three times with saline solution (0.85%, w/v, NaCl) to eliminate all residues of nutrient broth. The bacteria suspension was diluted in the saline solution to 10^6 CFU mL^{-1}. This type of suspension served as the inoculant in all experiments.

2.2. Substrates and initial plant growth conditions

Plants were initially grown in a mixture of vermiculite and silica sand (6:4 v/v, Sun Gro^R Horticulture, Agawam, MA). Both substrates were washed with large volumes of tap water, dried at $160\,^{\circ}C$ for 48 h, and then autoclaved for 15 min. Plastic planting trays ($28 \times 54.5 \times 4$ cm), each containing 200 square planting cells (3.5 deep $\times$ 2.1 $\times$ 2.1 cm) were filled with the substrate mixture and one seed was inserted in each cell at 1 cm below the surface, then watered to field capacity. Trays were incubated in a dark growth chamber (125L, Conviron, Winnipeg, Canada) for four weeks for the germination phase and then grown under continuous light (200 μmol photon cm^{-2} s^{-1} at $26 \pm 2\,^{\circ}C$ and 70% relative humidity) in the same growth chamber until seedlings reached 4–5 cm height. Seeds were irrigated with distilled water almost to saturation every third day.

2.3. Transplanting, inoculation and formation of progressive salinity gradient

One month old seedlings were transplanted to black plastic round planting bags (11×17 cm; capacity 1.4 L), containing vermiculite and sand (6:4 v/v). Before transplanting, the root plug in each cell was inoculated with one mL of bacterial suspension at a concentration of 10^6 CFU·mL^{-1}. Plants were immediately watered to field capacity with distilled water.

Plants treated with humic acids were watered once a week with alternate applications of 50% Hoagland's nutrient solution (Hoagland and Arnon, 1950) and humic acids solution containing 1 g L^{-1} of commercial humic acids (active ingredients: minimum 65% humic acids and minimum 85% potassium humate; 93–98% water-soluble; Supplementing material Table S2) (Enersol SC, American Colloid, Hoffman Estates, IL) suspended in 50% Hoagland's nutrient solution.

Progressive salt gradients in the growth substrate were created by irrigating with 0, 25, 50, or 75 mM NaCl solutions dissolved in 50% Hoagland's nutrient solution once a week. All other irrigations during the week, to maintain 60–70% water field capacity, used 50% Hoagland's nutrient solution. The level of increased salinity of the substrate over time was measured at the end of the trials by mixing 13–15 g substrate with 30 mL deionized water. The mixture was placed in an orbital shaker at 120 rpm for 30 min. Conductivity was determined in the supernatant by a conductivity meter (model sensION+ 5, Hach, Loveland, CO).

The plants were cultivated for 90 days in controlled greenhouse at $29 \pm 1\,^{\circ}C$ at $50 \pm 5\%$ RH and natural illumination ranging of 200–220 μmol photon m^{-2} s^{-1}.

2.4. Plant analyses and mineral analyses

Ninety day after transplanting, the plants were extracted, the substrate was carefully removed; roots, stem, and leaves were separated. Diameter at stem base was measured with a digital