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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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Salinity
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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

caliper, stem length with a ruler, and leaf area with leaf area meter (LI-3000, LI-COR, Lincoln, NE). Plant parts were dried in an oven at 70 °C to constant weight and weighted with an analytical balance. Accumulation of minerals (Na, Ca, Mg, and K) in roots, stems, and leaves, separately, were analyzed by first drying plant materials (0.2 g per sample) in an oven at 65 °C for 48–72 h and the pulverized. The powder was dissolved in 10 mL solution of nitric-perchloric-sulfuric acids (10:4:1 ratio at concentrations of 68, 60 and 96%, respectively).The solution was heated and distilled water was added to provide a final volume of 50 mL. Each sample was analyzed in an atomic absorption spectrophotometer (AA-660, Shimadzu, Kyoto, Japan).

Analysis of phosphorus used the molybdate method (Gomori, 1942) with a calibration curve from concentrations of 0–100 μg of phosphorus. Absorbance was measured spectrophotometrically at 660 nm (U-1100, Hitachi High Technologies, Tokyo, Japan).

Anions (Cl^-, NO_2^-, NO_3^-, SO_4^-) were quantified by placing 0.3 g dry plant material in 100 mL volumetric flasks, adding 30–40 mL distilled water and boiling for 1 min. The mixture was filtered, and distilled water was added to provide a final volume of 100 mL. It was then analyzed by ion chromatography (HIC-6A, Shimadzu).

To measure total nitrogen, 0.2 g dry, ground plant material were digested in 5 mL solution of salicylic and sulfuric acids (1:30, w/v) and heated in a partial Kjeldhal digester (ME-6 Electric Heater, Sibata, Soka Japan) for 4–5 h. Then, the samples were cooled and adjusted to 50 mL with Nessler reagent. Absorbance at 415 nm was measured in Hitachi U-1100 Spectrophotometer (Tokyo, Japan).

K^+/Na^+ and Ca^{+2}/Na^+ ratios were separately calculated for leaves and roots. The results were recorded from the final experiment, using 5 pots per treatment, where 10 seedlings were grown in each pot. After drying the plants and performing mineral analysis, the ratios were calculated.

2.5. Presence of inoculum of PGPB on roots

Persistence of the inoculated bacteria on the roots in the greenhouse experiments was measured weekly in most trials. This was done by using a *gfp*-labeled strain of *P. stutzeri* (strain Ngfp, CIBNOR, Mexico). Young segments, from the root tips to the root hair zone, were excised, and tested for the presence of bacteria, as described in a previous study (Bacilio et al., 2004). No attempt was made to quantify root colonization.

2.6. Experimental design and statistical analysis

All greenhouse experiments were identical and had a factorial design, where four progressive gradient of salinity, plus a control treatment without NaCl, were tested. The treatments were: (1) supplementation of soil with humic acids, (2) inoculation with the PGPB, application of humic acid and the PGPB, and (3) plants growing without any treatment (the control). Each experiment had 5 or 6 replicates per treatment, where one replicate was a pot containing one plant. Each experiment was repeated two to five times. Because data from the wild-type strain and the *gfp*-modified strain had the same patterns and trends, the results of both strains were combined and analyzed together. Data were first analyzed by one-way ANOVA and then by Fisher LSD post-hoc analysis or Student's *t*-test at $P < 0.05$. All statistical analyses were done using the STATISTICA 6.0 software (Dell Statistica, Round Rock, TX).

3. Results

3.1. Soil salinity gradients

Four soil salinity gradients were tested during the 90-day trials. Even without any addition of salt, routine irrigation and fertilization significantly enhanced salinity ($P < 0.05$). When salt was added, salinization dramatically increased, creating extreme salinization when more salty water was used (Table 1, lower-case letter analysis). Increased salinity during cultivation of the two pepper cultivars was similar, even though statistically some values were different between the two cultivars (Table 1, capital-case letter analysis). Electrical conductivity of the soils was measured at the end of the individual experiments. In all treatments, concomitant with increases in periodic application of saline water, electrical conductivity linearly increased in the soil in the susceptible (Fig. S1, supplementary material) and resistant (Fig. S2, supplementary material) cultivars.

3.2. Effect of inoculation with PGPB and application of humic acids, separately or together, on plant parameters under progressively increasing salinity

Height of plant, length of root system, dry weight of stems, leaves and roots, number of leaves, leaf surface area, chlorophyll *a* and *b* content, and total chlorophyll were measured and compared in salt-resistant (cv. Jupiter) and salt-susceptible (cv. Ancho San Luis) pepper plants. Analyses of the results involved comparison of the application of humic acids, inoculation with the PGPB *P. stutzeri*, application of humic acids and inoculation with PGPB, and untreated controls at the four saline gradients. A second set of analyses compared the effect of each of these treatments at the four saline gradients.

Improvements in the growth of the salt-susceptible cultivar are presented in Fig. 1 and those of the salt-resistant cultivar in Fig. 2. Because the multiple parameters versus treatment generated large datasets, only part of the results is presented. General analyses of all the changes, negative, positive, and no effect, for each treatment, are summarized in Fig. 3. Additional quantitative data is presented in supplementing material Table S1 and S2.

Analyzing entire datasets, application of PGPB and humic acids did not have clear-cut effects. Some plant parameters were positively affected at some saline concentrations and some were not. It appears that more positive effects, indicated by blue markings in Fig. 3, by either treatment were more apparent in the salt-resistant cultivar than in the salt-susceptible cultivar. In the

Table 1
Electrical conductivity (EC) at the beginning and end of the experiments with continuous application of NaCl at three concentrations and one control.

NaCl application (mM)	Initial sampling EC; μS cm^{-1}	Final sampling. EC; μS cm^{-1}	
		Salt-tolerant cultivar	Salt-susceptible cultivar
0	208 ± 4.369^{a}	410 ± 19.502^{bA}	475 ± 25.003^{bB}
25	492 ± 9.703^{a}	1480 ± 85.391^{bA}	1700 ± 173.210^{bA}
50	618 ± 7.842^{a}	2470 ± 177.048^{bA}	2300 ± 204.124^{bA}
75	1181 ± 23.298^{a}	4800 ± 270.801^{bA}	3616 ± 33.334^{bB}

Different lowercase letters show significance between initial and final sampling time for each cultivar at $P < 0.05$ by Student's *t*-test. Different uppercase letters show significance between pepper cultivars at $P < 0.05$ by Student's *t*-test. Initial sampling of EC is similar for both cultivars. Data obtained from two independent experiments.

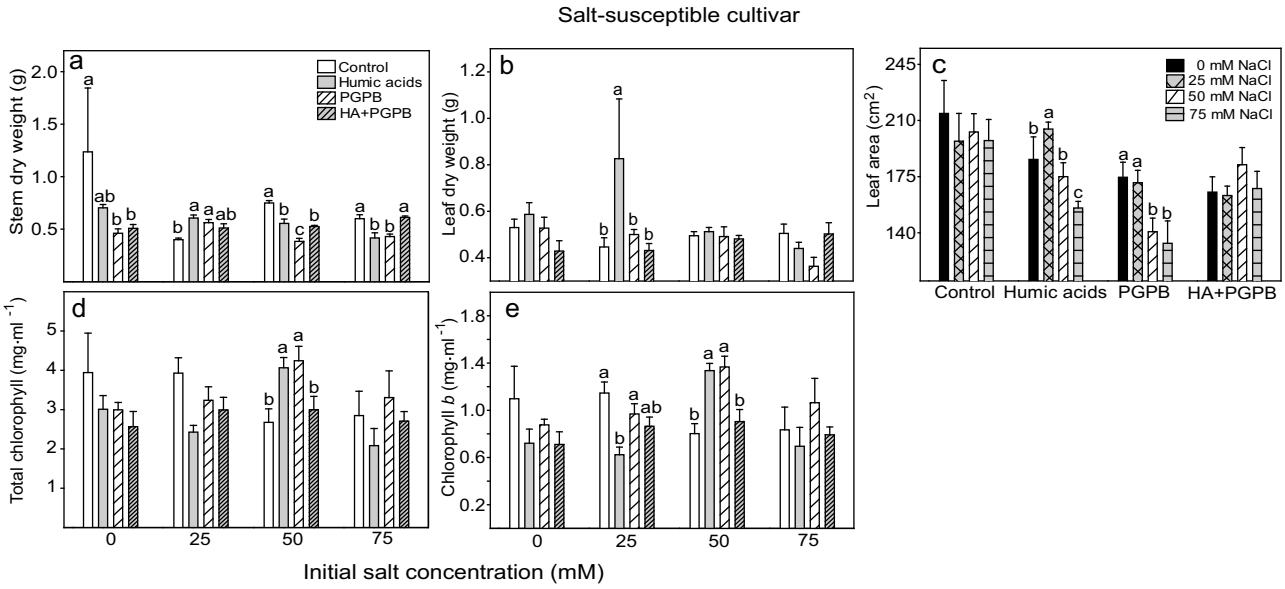

Fig. 1. Effect of (1) application of humic acids, (2) inoculation with the PGPB *Pseudomonas stutzeri*, and (3) combination of application of humid acids and inoculation with the PGPB on plant parameters in a salt-susceptible cultivar of pepper subjected to four progressing gradients of salinity. In each subfigure, columns followed by different letters differ significantly by one-way ANOVA and then by Fisher LSD post-hoc analysis at $P < 0.05$. Unlabeled columns are not significantly different. Whiskers represent standard error.

salt-susceptible cultivar, most of the negative effects, indicated by yellow markings in Fig. 3, prevailed in all treatments. No synergism on plant growth parameters or mitigation of salt stress was detected, where synergism is defined as an additional positive effect, compared with the effects of humic acid and PGPB used separately (analyses not shown).

3.3. Effect of inoculation with PGPB and application of humic acids, separately or together, on accumulation of Na^+, Ca^{2+}, Mg^{2+}, and K^+ and on K^+/Na^+ and Ca^{+2}/Na^+ ratios in plants growing under progressively increasing salinity

Accumulation or reduction of four elements (Na, Ca, Mg, and K), commonly analyzed in plants growing in saline soils, was measured in roots, shoots, and leaves (Fig. 4 for Na^+,Fig. 5 for K^+, Fig. 6 for Ca^{2+}, and Fig. S3 for Mg^{2+}). As is common for plants growing in saline soil, Na^+ and Ca^{2+} increased in the three plant parts, K^+ decreased, and no change occurred in Mg^{2+}. Calculation of K^+/Na^+ and Ca^{2+}/Na^+ ratios showed that either the application of humic acids or inoculation with the PGPB enhanced these ratios in several saline regimes, where larger increases in these ratios were detected in the susceptible cultivar (Tables 2 and 3, bold-face results). In several salinity regimes, synergism in enhancing these ratios were calculated when humic acids and PGPB were applied at the same time; a stronger effect than in the treatment with PGPB or humic acid (Tables 2 and 3, bold-face results with *).

4. Discussion

Soil salinization is a major agricultural problem that will only increase in magnitude and severity with time. Almost all crops are susceptible to various degrees to salinity. Innovative solutions to allow cultivation in these soils are always required. Our study assessed the effect of applying humic acids and PGPB, alone and combined, on performance of two pepper cultivars; one is relatively salt-resistant and the other is salt-susceptible under progressive gradients of increasing soil salinity.

Relatively little is known regarding mechanisms of salt tolerance in peppers, but in general, peppers are affected by salinity to various degrees of damage, depending on the cultivar and genotype (Aktas et al., 2006; Chartzoulakis and Klapaki, 2000; Lycoskoufis et al., 2005). The reduction in growth of peppers by salinity stress is caused by disruption of several physiological processes. This happened as a result of: (1) water deficit generated by increased osmotic potential in the growth medium, (2) ion toxicity related with high concentrations of Na^+ and Cl^-, (3) ionic imbalance caused by accumulation of salts within the plant, and/or (4) disturbance of nutrient absorption, especially nitrogen, and (5) interference with photosynthesis (del Amor and Cuadra-Crespo, 2011). In habanero peppers (*C. chinense*), a combination of several stress tolerance mechanisms, such as accumulation of proline, transport, control, and compartmentalization of Na^+ in vacuole-like structures, regulation of K^+, and increase in the H^+-ATPase activity mitigate salt-induced injury. Na^+ extrusion to apoplast was not an efficient strategy for salt tolerance (Bojórquez-Quintal et al., 2014). Application of several growth-promoting microorganisms may alleviate salt stress in peppers and other plant species (references in Introduction). Specifically, in bell peppers, application of the PGPB *Azospirillum brasilense* and *Pantoea dispersa* under constant saline conditions, mitigated many inhibited growth parameters. This is attributed to higher stomatal conductance that allows inoculated plants to increase photosynthesis, compared with non-inoculated plants and partly to improvement of nitrogen nutrition (del Amor and Cuadra-Crespo, 2011). Inoculation of peppers with the PGPB *Bacillus* sp. ameliorate osmotic stress (Sziderics et al., 2007) and inoculation of red peppers with several ACC deaminase-producing halo-tolerant PGPB (*Brevibacterium iodinum*, *Bacillus licheniformis*, and *Zhihengliuela alba*) mitigate salt stress by reducing induced ethylene production (Siddikee et al., 2011). In other plant species, other mechanisms prevail. Inoculation with *Azospirillum* sp. reduced the Cl^- concentration in leaves of salt-stressed strawberry plants (Karlidag et al., 2011) and suppression of photosynthesis in tomato plants was less severe in plants inoculated with the PGPB *Achromobacter piechaudii* (Mayak et al., 2004). As expected, in our study, application of PGPB or humic acids positively mitigated several plant growth parameters, such as leaf parameters, as shown in other plants species (for references, see Introduction). The effect was more pronounced in

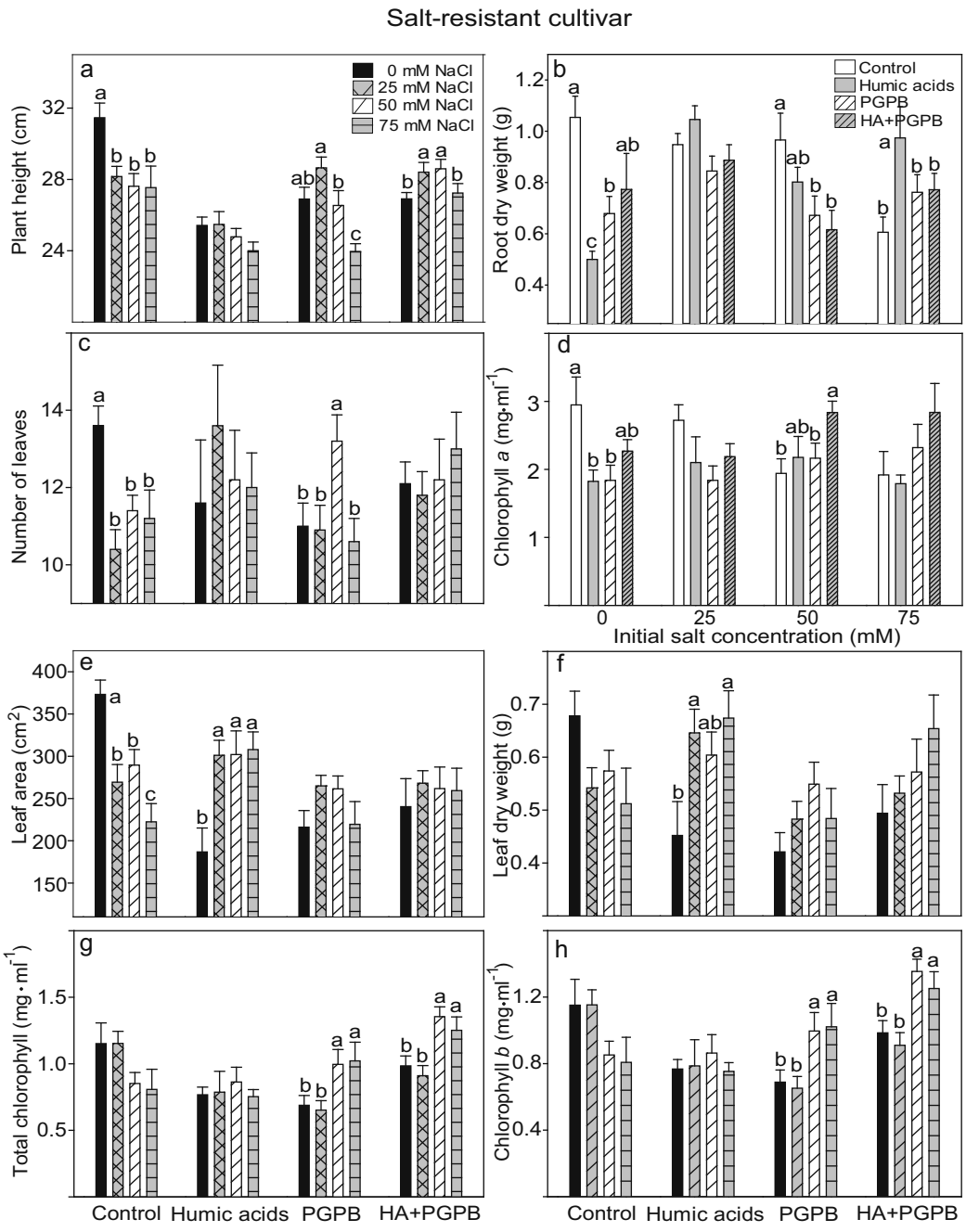

Fig. 2. Effect of (1) application of humic acids, (2) inoculation with the PGPB *Pseudomonas stutzeri*, and (3) combination of application of humid acids and inoculation with the PGPB on plant parameters in a salt-tolerant cultivar of pepper subjected to four concentrations progressing gradients of salinity. In each subfigure, columns followed by different letter differ significantly by one-way ANOVA and then by Fisher LSD post-hoc analysis at $P < 0.05$. Unlabeled columns are not significantly different. Whiskers represent standard error.

the salt-resistant cultivar ($P < 0.05$). No synergism on development of plant parameters was found when PGPB and humic acids were applied together. However, when K^+/Na^+ and Ca^{2+}/Na^+ ratios were calculated in tissues of relatively salt-susceptible and relatively salt-resistant pepper plants, synergism was found, as well as the improvements in these ratios when PGPB or humic acids were applied separately ($P < 0.05$). Interestingly, enhancement of the K^+/Na^+ and Ca^{2+}/Na^+ ratios was especially marked in the susceptible cultivar.

An explanation of these results is not simple. Plant response to salinity is complex. Even under constant salinity, it depends on interacting variables, including developmental stage, concentration of salt, duration of exposure to salt, and on the growth environment (Munns, 2002). The K^+/Na^+ and Ca^{2+}/Na^+ ratios are common markers for increased resistance to salinity. K^+/Na^+ is more prominent (Volkmar et al., 1998) and Ca^{2+}/Na^+ does not operate in all plant species (Cramer, 2002; Yeo and Flowers, 1985; Rengel, 1992). In our study, exposure of the plants to salt was more extreme compared to most studies because soil salinity was purposely increasing over time. The capacity of plants to maintain a high cytosolic K^+/Na^+ ratio, and to a lesser extent, a high Ca^{2+}/Na^+ ratio, are likely to be one of the key determinants of salt tolerance in peppers. This occurs because a large intrusion of Na^+ usually results in severe reduction of growth or even death in salt-sensitive varieties and leads to symptoms of mild toxicity in salt-tolerant varieties. Our results showed that the relative resistance of the

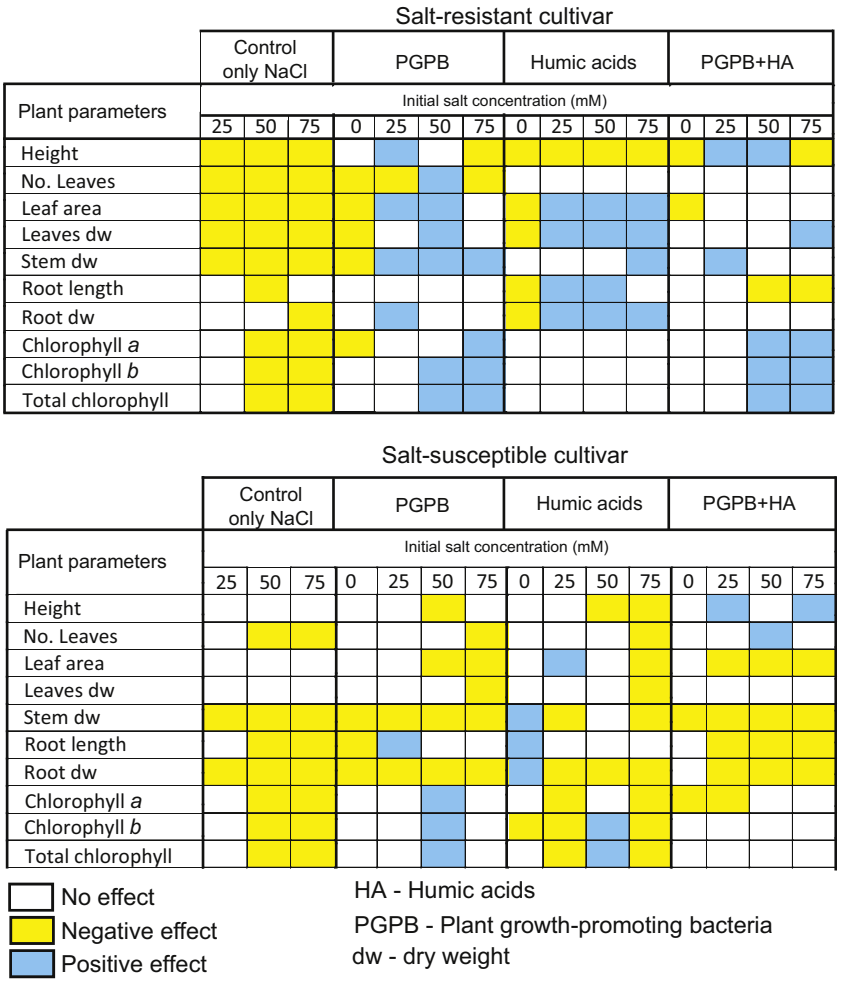

Fig. 3. General analysis of the effect of (1) application of humic acids, (2) inoculation with the PGPB *Pseudomonas stutzeri*, and (3) combination of application of humid acids and inoculation with PGPB on plant parameters in a salt-tolerant pepper and a salt-susceptible pepper subjected to increasing gradient of salt.

resistant cultivar did not depend on accumulating more K^+ or Ca^{+2} ions in the tissues. However, the susceptible cultivar did accumulate ions to avoid death. In many plants, the capacity of plants to counteract salinity stress strongly depends on the status of K^+ nutrition and on parameters that enhanced K^+ uptake under stress (Hamdia et al., 2004; Maathuis and Amtmann, 1999; Volkmar et al., 1998). Yet, this is not the leading mechanism for salt tolerance in other plants, such as barley (Kronzucker et al., 2006). Specifically in peppers, high K^+ concentration increased salt tolerance, as indicated by increased shoot and root dry weight (Rubio et al., 2010). In our study, even though K^+ content gradually decreased in all affected plants as salinity increased, a similar "relative increase" in K^+ content by the PGPB was demonstrated, especially in roots and leaves, but these differences were not significant by analysis of covariance (Supplementary material, Fig. S4). This slowed the decline of K^+, although this did not occur at all saline gradients and was variable among individual plants. This indicates that other salt-resistance mechanisms, not explored in this study, also play a role.

In saline soils, the amount of Na^+ is usually greater than K^+, especially in our study, where Na^+ was periodically added with irrigation water. In plants, Na^+ competes with K^+ because of the use of a common transport system. In this way, salt stress inhibits the uptake of K^+, yielding toxic accumulation of Na^+ and decreasing the K^+/Na^+ ratio. This occurred in many combinations of treatments in our study. In wheat, the PGPB *Pseudomonas putida* significantly increased the K^+/Na^+ ratio probably because it restricted uptake of Na^+ and enhanced uptake of K^+ when salinity was high, thus leading to enhanced salt resistance (Zahir et al., 2009). This did not happen in our study, where pepper plants were inoculated with the PGPB *P. stutzeri*. The increase in K^+/Na^+ and Ca^{2+}/Na^+ ratios we found in our study indicates that pepper plants are able to maintain relatively high K^+ content, and that this may act as the major monovalent cationic osmoticum in the presence of external salt, but not the single mechanism responsible for improvement in pepper plant functions and growth.

The common mechanism by which salinity disrupts the mineral ratios in plants is by reducing nutrient availability by competition with major ions in the substrate. These interactions often lead to Na^+-induced Ca^{2+} and/or K^+ deficiencies and Ca^{2+}-induced Mg^{2+} deficiencies (Grattan and Grieve, 1992). In our study, this did not happen. While some deficiencies in K^+ were detected, an increase in Ca^{2+} content with increase of salinity was found, but this elevated Ca^{2+} had no effect on the quantity of Mg^{2+} in peppers, which was stable at all concentrations of salt or in any treatment. Taken together, as occurred in many plant species, the mechanism of saline resistance in peppers is more complex than a simple effect on mineral acquisition in the plants.

Humic acids in general are beneficial for most plants, including pepper. They help break up clayey and compacted soils, assist in transferring micronutrients from the soil into plants, enhance water retention, increase seed germination, and stimulate development of microflora populations in soils. They also improve the performance of infertile soils, of soils with low native organic

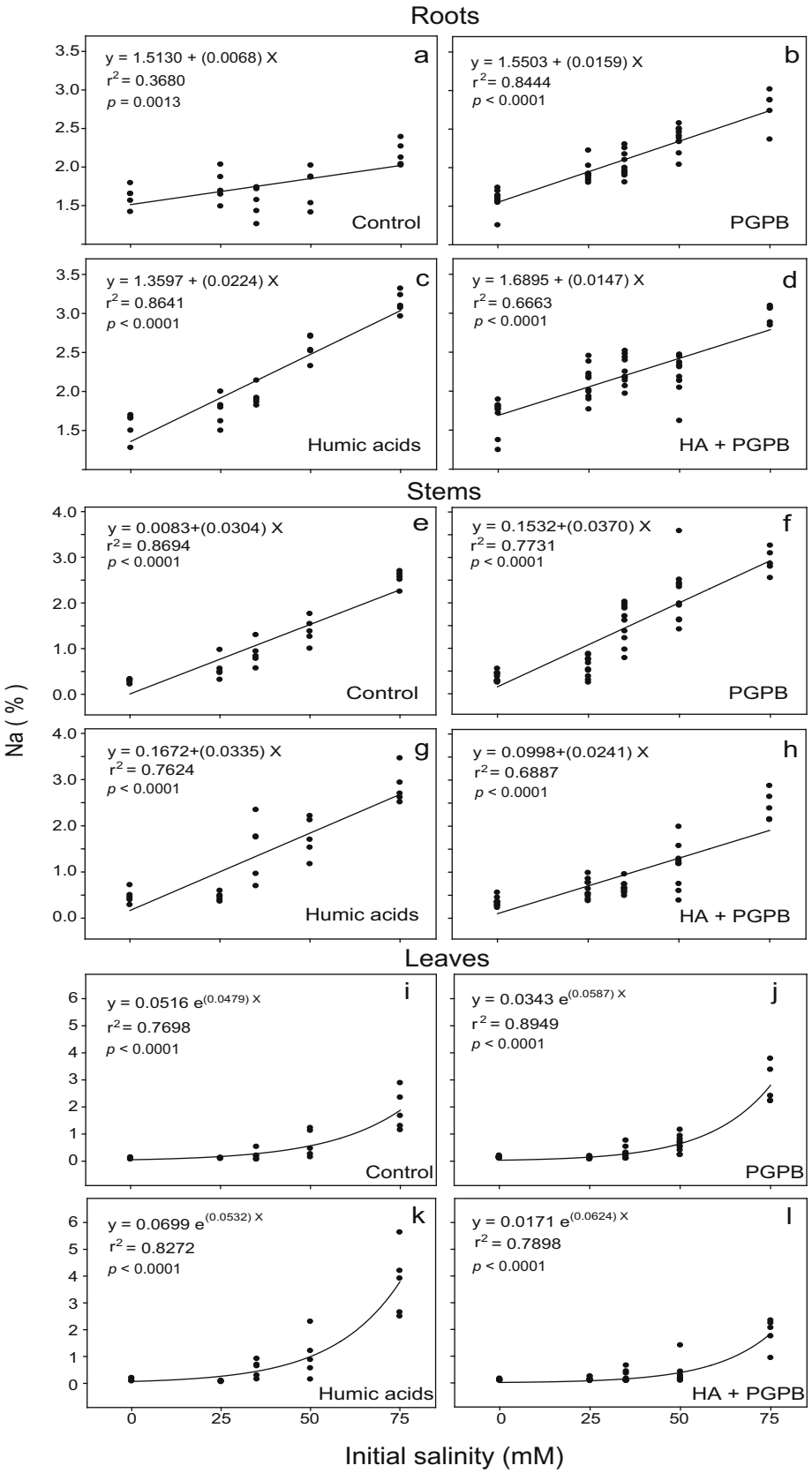

Fig. 4. Concentration of Na^+ at the end of experiments in plant parts after (1) application of humic acids, (2) inoculation with the PGPB *Pseudomonas stutzeri*, and (3) combination of application of humic acids and inoculation with the PGPB. Roots (a–d); stems (e–h); leaves (i–l).

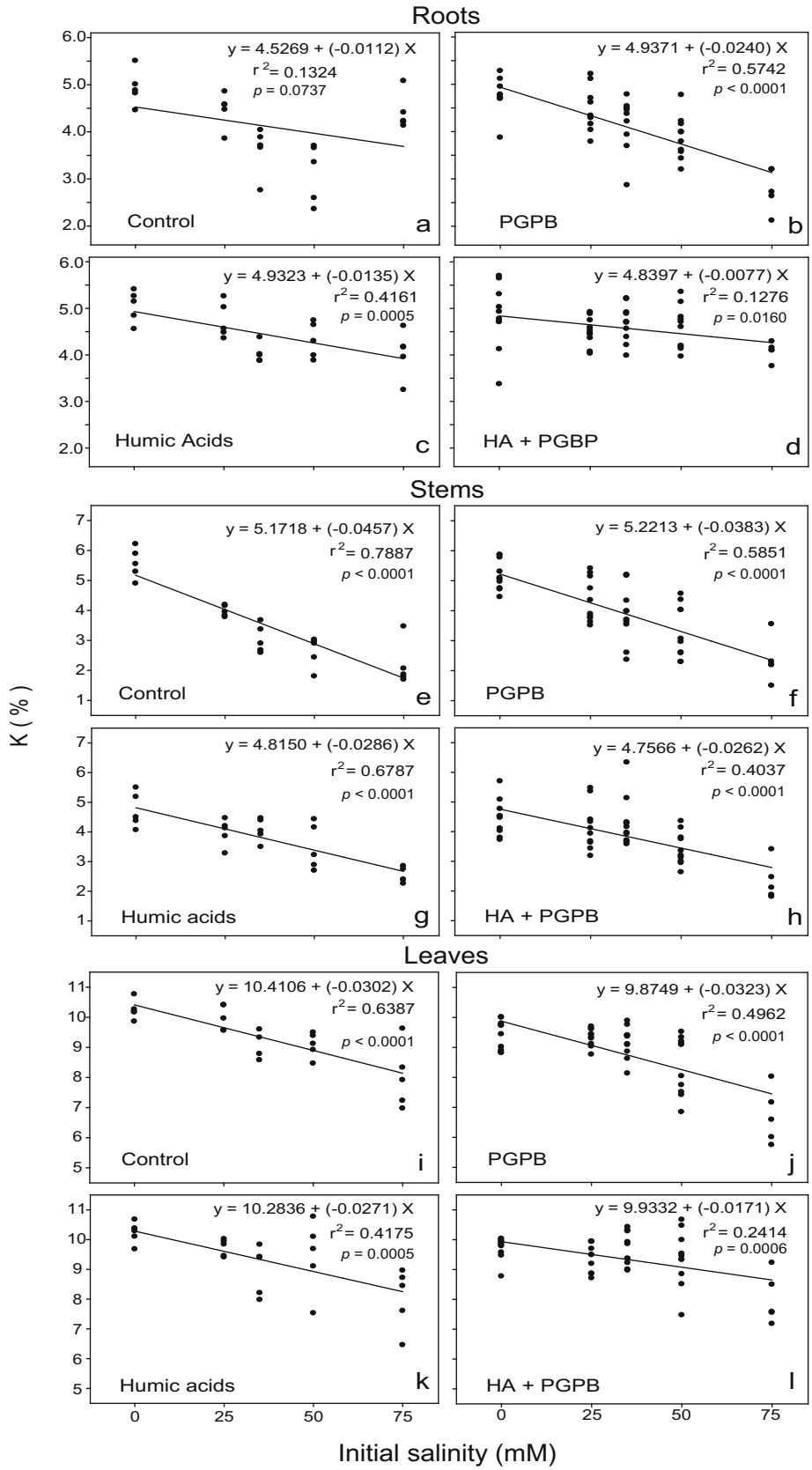

Fig. 5. Concentration of K^+ at the end of the experiments in plant parts after (1) application of humic acids, (2) inoculation with the PGPB *Pseudomonas stutzeri*, and (3) combination of application of humic acids and inoculation with the PGPB. Roots (a–d); stems (e–h); leaves (i–l).

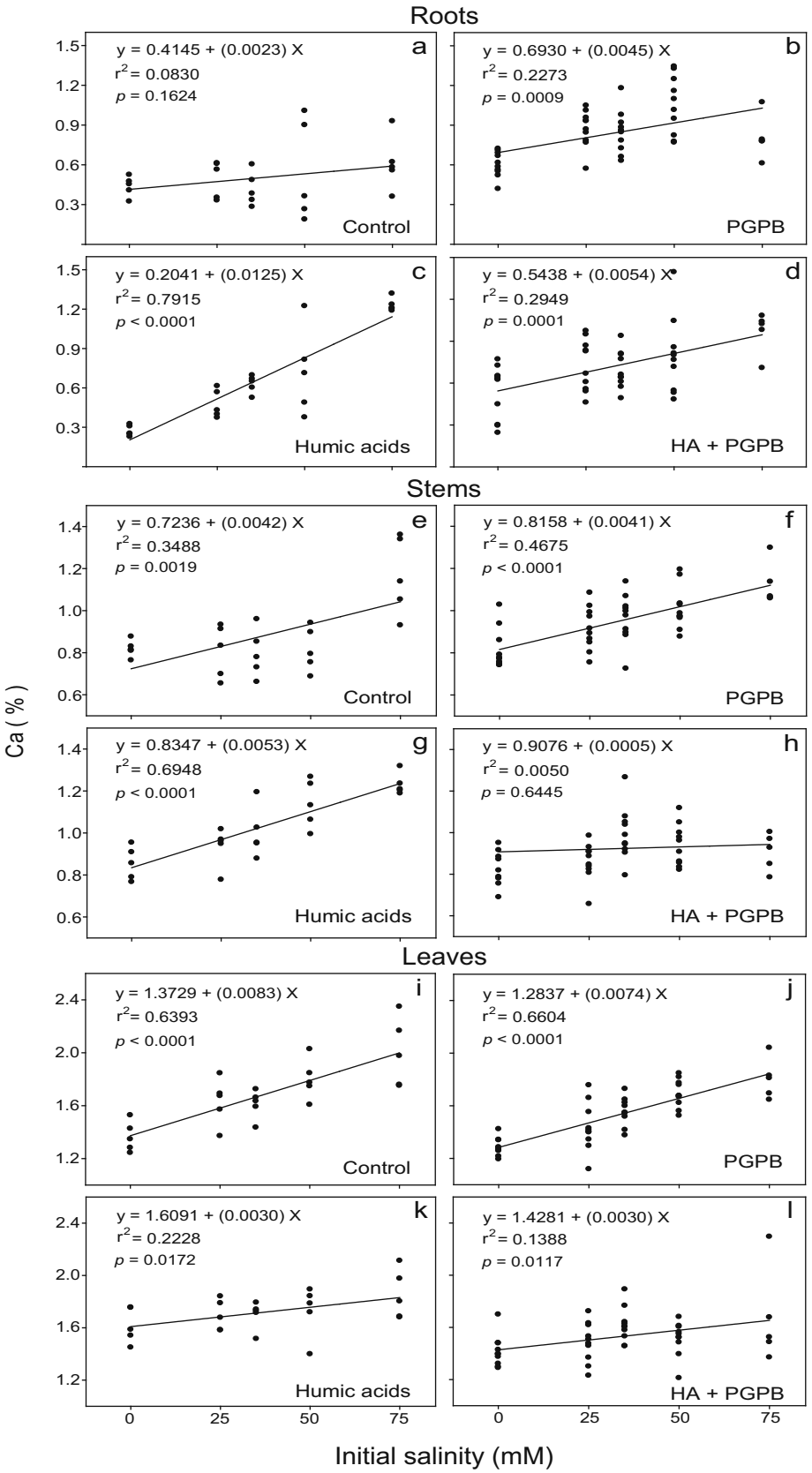

Fig. 6. Concentration of Ca^{2+} at the end of the experiments in plant parts after (1) application of humic acids, (2) inoculation with the PGPB *Pseudomonas stutzeri*, and (3) combination of application of humic acids and inoculation with the PGPB. Roots (a–d); stems (e–h); leaves (i–l).

Table 2
K/Na ratio in leaves and roots of the salt-resistant and salt-susceptible cultivars.

	K/Na ratio in leaves of the salt-resistant cultivar				
	Initial Salt Concentration (mM)				
	0	25	35	50	75
Untreated Control	105.6	93.2	39	13.9	4.2
Humic Acids	78.6	**131.4**	16.5	9.2	2.1
PGPB	66.1	80.65	33.8	15.1	
Humic Acids + PGPB	84.6	67.3	**47.6***	**44.53***	
	K/Na ratio in roots of salt-resistant cultivar				
	0	25	35	50	75
Untreated Control	3.08	2.6	2.4	1.84	2
Humic Acids	3.1	2.71	2.08	1.65	1.2
PGPB	2.965	2.31	2.03	1.65	
Humic Acids + PGPB	2.845	2.14	2.06	2.12	
	K/Na ratio in leaves of salt-susceptible cultivar				
	0	25	35	50	75
Untreated Control	66	31.7	19.6	31.7	6.6
Humic Acids	58	**68**	**31.7**	13.4	4.4
PGPB	**74.5**	19.05	18.65	7.05	
Humic Acids + PGPB	**80.75***	**66.8**	**49.2***	32.3	
	K/Na ratio in roots of salt-susceptible cultivar				
	0	25	35	50	75
Untreated Control	2.4	2.5	2.51	2.23	2.33
Humic Acids	**4.9**	**4.83**	**3.46**	**2.74**	2.2
PGPB	**7.23**	**4.16**	**3.85**	**3.9**	
Humic Acids + PGPB	**4.58**	**3.75**	**3.73**	**3.4**	

Bold face values indicate enhancement of K/Na ration over untreated controls.
Bold face values with additional *, a potential synergism.
Missing values indicate that most plants died.

Table 3
Ca/Na ratio in leaves and roots of salt-resistant and salt-susceptible cultivars.

	Ca/Na ratio in leaves of salt-resistant cultivar				
	Initial Salt Concentration (mM)				
	0	25	35	50	75
Untreated Control	13.6	16.3	8.05	2.8	1.07
Humic Acids	12.3	**24.1**	3.14	1.68	0.48
PGPB	9.85	12.5	5.75	2.86	
Humic Acids + PGPB	13.4	10.95	8.85	**7.3***	
	Ca/Na ratio in roots of the salt-resistant cultivar				
	0	25	35	50	75
Untreated Control	0.275	0.3	0.28	0.32	0.23
Humic Acids	0.16	0.27	**0.33**	**2.77**	**0.31**
PGPB	**0.385**	**0.455**	**0.405**	**0.435**	
Humic Acids + PGPB	**0.315**	**0.345**	**0.308**	**1.98**	
	Ca/Na ratio in leaves of salt-susceptible cultivar				
	0	25	35	50	75
Untreated Control	11.5	6.95	5	7.7	1.5
Humic Acids	10.6	**11.7**	**7.4**	3.25	1.25
PGPB	**15.5**	4.17	4.64	1.77	
Humic Acids + PGPB	**16.9***	**12***	**10.2***	6.8	
	Ca/Na ratio in roots of salt-susceptible cultivar				
	0	25	35	50	75
Untreated Control	0.23	0.24	0.43	0.37	0.32
Humic Acids	**0.52**	**0.34**	0.31	**0.406**	0.34
PGPB	**0.54**	**0.495**	0.45	0.39	
Humic Acids + PGPB	**0.475**	**0.44**	0.285	0.338	

Bold face values indicate enhancement of K/Na ration over untreated controls.
Bold face values with additional *, a potential synergism.
Missing values indicate that most plants died.

matter, and of crops grown in arid regions (Calvo et al., 2014). Adding humic acids to the PGPB *Bacillus subtilis* that was immobilized in alginate bead, as done in our study, increased the viability of the PGPB in the inoculant, but did not affect the dry weight of lettuce roots in sand over inoculation of the PGPB without humic acids. Yet, this occurred in the absence of saline conditions (Young et al., 2006). The combination of humic acid and the PGPB *P. stutzeri* had only a small improvement on plant performance, but was distinguished in improving K^+/Na^+ and Ca^{2+}/Na^+ ratios. Although this combination did not show sufficient potential for further development as an inoculant for peppers, the improvements that were detected demonstrated that the concept of PGPB–humic acids applications is correct and other combinations of PGPB with humic acids are worth testing.

This study used, as its basic strategy, a realistic scenario in fields irrigated with marginal water, where soil salinity increases over time. Most studies of salinity impact on plant growth use constant salinity rates. Our study indicates that continuous addition of Na^+ to the soil during growth, while mimicking irrigation with marginal water, is not a good strategy to study salinity effects on peppers. This happened because continuous increase in salt content over time increases salt stress. This creates an unwanted side effect that masked some of the positive effects that PGPB and humic acids have. A better strategy for studying mechanisms in reducing salt stress would be a constant concentration of salt, as happens in hydroponics, even if this does not represent reality in many fields.

This study has a broader impact than the specific case of alleviating salt stress in peppers. It shows that a combination of approaches (humic acid and PGPB) applied at the same time addresses the agronomic problem. Its potential for other crops is worth pursuing, even if the initial results presented in our study are not clear cut.

In summary, under increasing salt concentrations, application of either the PGPB *P. stutzeri* or humic acids improved some plant growth parameters. Specifically, some improvements in K^+/Na^+ and Ca^{2+}/Na^+ ratios occurred. Plant parameters measured in this study are not indicative of the synergisms, but salt mitigation ratios were more indicative. Combined application of the two treatments showed the potential to use this concept, but with other combination of PGPB and humic acids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2016.04.012.

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