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Research Paper

Microorganisms associated to tomato seedlings growing in saline culture act as osmoprotectant

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Abstract

Less than 0.5% of total water in the world is available for human consumption and agriculture. The major part of the world's water is saline and salinity in soils interferes in germination of seeds and the posterior development of the plant. In order to increase the osmotolerance of tomato, seedlings were associated with *Azospirillum brasilense* Cd, *Azospirillum brasilense* Cd transformed bacteria with a plasmid harboring a trehalose biosynthesis gene-fusion or *Chlorella vulgaris*. Two plant culture media: Hydroponic and Murashige and Skoog were tested. In the first set of studies seedlings were associated to single free cells meanwhile in a second set single and combined free cells were studied. A positive interaction between transformed *Azospirillum* and *Chlorella vulagris* and tomato plants was observed. Seedlings showed a salt concentration tolerance, as sodium chloride, up to 200 mM. According to our results, the association of plants with *A. brasilense* Cd-BIF and *C. vulgaris* is a viable approach to increase their salt tolerance and biomass, as consequence the possible use of sea water to irrigate horticultural plants.

Key words: hydroponic culture, microbial association, salt tolerance, Trehalose.

Introduction

The negative impact of salinity on plant growth in irrigated and non-irrigated areas of the world's arid regions is a major problem for agriculture (Nasr *et al.*, 2011; Meloni *et al.*, 2008; Ríos-Gómez *et al.*, 2010; Velarde *et al.*, 2003). Salinization is a process of soil enrichment with salts more soluble than calcium sulfate, usually chlorides and sulfates of sodium and magnesium. This causes osmotic stress and plant intoxication, thus interfering with the growth of most crops (Porta *et al.*, 1999). Plants develop a plethora of biochemical and molecular mechanisms to cope with salt stress. These processes include compartmentalization of compatible solutes, change in photosynthetic pathway, alteration in membrane structure, induction of antioxidative enzymes and plant hormones and as a consequence plants redirect their growth when exposed to stress (El-Baky *et al.*, 2008). It is hypothesized that such morphogenic responses are part of a general acclimation strategy that constitutes the 'flight' response of plants (Potters *et al.*, 2007). The initial effects of increasing soil salinity are very similar to those observed when plants are exposed to drought. Reductions in leaf water potential will reduce stomatal conductance and eventually inhibit photosynthetic metabolism (Zribi *et al.*, 2009).

Numerous studies have shown that in saline environments, adaptation to salinity during germination and early stages of seedling growth are crucial in determining the success of the establishment. Even in the late stages of development salinity may affect the distribution of plants in certain species (Tobe, 2000).

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Salinity tolerance and other adverse conditions in soils are currently overcome with an excess of irrigation water, thus given the increasingly shortage of fresh water at global level, it is necessary to develop strategies that include both salt-resistant crops and techniques to reduce stress injury (Bacilio *et al.*, 2004). A major effort in research has been dedicated to organic fertilization or use of biofertilizers to increase plant development. Biofertilizers are best described as microbial communities that add, preserve and mobilize soil nutrients. *Azospirillum* is considered one of the most studied plant growth promoting bacteria (PGPB) given its ability to significantly increase not only plant growth development, but also the yield of numerous agricultural crops (Givaudan and Bally, 1991; Strzelczyk *et al.*, 1994). *Azospirillum brasilense* promotes growth due to the accumulation and transport of indole-3-acetic acid to the plant (Umali-Garcia *et al.*, 1980; Hartmann *et al.*, 1983) and other plant growth regulation molecules such as absicic acid (ABA) and diamine cadaverine (CAD) (Canto Martín *et al.*, 2004; Perrig *et al.*, 2007).

Azospirillum spp. colonizes the roots of a wide range of crops and can efficiently colonize roots submerged in growth solutions, and thus increase plant growth. The ability of *Azospirillum spp*. to stimulate plant growth has been widely demonstrated in experiments both field and greenhouse. Several mechanisms have been suggested to be responsible for the stimulatory effect observed in the inoculated plants in numerous studies and inoculation with *Azospirillum spp*.

In the last decade it has been demonstrated that *Azospirillum brasilense* increases the growth parameters not only of superior plants but unicellular microalgae *Chlorella* spp. by changing its cytology and metabolic parameters (de Bashan *et al.*, 2004). This phenomena is also associated with the potential of *Azospirillium* spp. to produce plant growth regulators, because the microalga responds to the presence of *Azospirillum* similarly to higher plants, increasing their growth and changing its metabolism, due to the green microalgae meet the basic requirements of a higher plant (Mazur *et al.*, 2001; Stirk *et al.*, 2002).

Recently, it has been shown that a recombinant *Azospirillum brasilsence* Cd strain expressing the omoprotectant trehalose is able to grow in salt stress conditions (Rodríguez-Salazar *et al.*, 2009). The aim of this study is to find out if *Chlorella vulgaris*, *Azospirillum brasilsence* Cd and *Azospirillum brasilense* Cd-BIF, could increase the salt tolerance of tomato in hydroponic cultures.

Material and Methods

Microalgae and bacteria

Microalgae

Chlorella vulgaris (UTEX 2714) was grown in axenic cultures of Basal Bold Medium (Bischoff y Holtzer, 1969) at 2000 lux illumination with light/dark periods of 16/8 h for 14 days prior to its use. The cell concentration was adjusted to $1x10^6$ cells/mL prior to its use as inoculant.

Bacteria

Azospirillum brasilense Cd was grown in nutrient broth with ampicillin at 30 °C for 72 h prior to its use; *Azospirillum basilense* Cd-BIF which enables the accumulation of trehalose in excess (Rodríguez-Salazar *et al.*, 2009) was grown in nutrient broth supplied with ampicillin and gentamycin at 30 °C for 72 h prior to its use. The viability of the strains was tested on Congo Red agar plates. The cell concentration was adjusted to $1x10^9$ cells/mL prior to its use as inoculant.

In vitro hydroponic culture

Hydroponic media composition

Hydroponic media was modified from (Jensen y Malter, 1995). Composition in g/L: $MgSO4.7H_2O$, 0.5; K_2HPO_4, 0.27; KNO_3, 0.2; K_2SO_4, 0.1; $Ca(NO_3)_2$, 0.5; EDTA, 0.25; 250 μL of trace minerals. Trace minerals composition in g/L: H_3BO_3, 16.6; $MgCl_2$, 15.0; $CuCl_2$, 0.82; MoO_3, 0.33.

Tomato seeds germination

Seeds of *Solanum lycopersicum* var. cherry were germinated in sterile Petri dishes with hydroponic media for 10 days under light/dark periods of 16/8 h.

Plant growth and development

Tomato plants where placed axenically in plastic conical tubes in groups of five under different sodium chloride concentrations: 0, 50, 100, 150, 200 y 250 mM using hydroponic medium (Jensen y Malter, 1995) or Murashige and Skoog (MS) medium (Murashige y Skoog, 1962) as base. Each group of treatments was supplied with one of the following free cells cultures: AW, AB, CV, AWCV, ABCV, including a control treatment without microorganisms; note: combined microbial treatments were tested only in hydroponic culture in a second set of experiments. Tomato plants were placed in 13.5 mL of the corresponding media and 1.5 mL of the corresponding microbial culture (Table 1). Plant growth and development was monitored after 10, 20 and 30 days of culture; every period stem and root length were measured.

Experimental design and statistical analysis

In order to compare the effect of media composition, time of culture and salt concentration in stem and root elongation, a multifactorial design was used. Data was analyzed using the ANOVA and Least Significant Difference (LSD) analysis at level 0.05 of confidence.

Results

Two sets of experiments were planned. In a first group, two mediums (Hydroponic and Murashige and Skoog), three periods of time (10, 20 and 30 days), six NaCl concentrations (0 to 250 mM) and three single cells *A. brasilense* Cd (AW), *A. brasilense* Cd-BIF (AB) and *C.*

vulgaris (CV) were analyzed. In a second group, based on the results of group one, only hydroponic medium was used given its simplicity and microbial associations were tested with single or combined free cells.

Stem length

The ANOVA analysis of the first set of experiments showed that the associated microorganism, elapsed time and salinity were the only significant factors for plant growth, despite of the used medium (MS or hydroponic), (Table 1). It is remarkable that as long as NaCl increased in concentration form 0 to 250 mM the stem length diminished, in any case *A. brasilense* Cd-BIF promoted longer stems in the seedlings. *C. vulgaris* had the second better results in this measure (Figure 1a). Regarding salinity, two groups are clearly differentiated, Group I: NaCl concentrations 0, 50 and 100 mM with stem lengths average of 22.7 ± 0.6 mm and Group II: NaCl concentrations 150, 200 and 250 with stem lengths average of 17.8 ± 0.6 mm. When considering time as a second factor, it can be seen that as long as time passed the seedlings were less tolerant to salt, as stem lengths were shorter ~15 and 20 mm in comparison to the first ten days were stem lengths were in the range of 25 to 26 mm (Figure 1b). In general, those seedlings growing with any microorganism associated showed longer stems in comparison to the control, where no microbial association was used, which means that salt tolerance is favored by plant microbial associations.

If the average of stem elongation through the three periods of time is considered, *A. brasilense* Cd-BIF showed the major elongation in the stems (21.8 ± 0.07 mm) in comparison to the other microbial treatments. However, at the end of 30 days period the stems were shorter than those observed at 10 days, and this was true for almost all cases, except for *A. brasilense* Cd where the longitudes of the stems (22.3 ± 0.12 mm) were significantly larger ($p < 0.05$) than other treatments after 30 days (Figure 1b).

For the second set of experiments, where combined microorganisms were tested, according to the ANOVA analysis (Table 2) the combination of *A. brasilense* Cd and *C. vulgaris* (AWCV) had a better impact on stem growth (25.3 ± 0.7 mm) followed by the associated *A. brasilense* Cd-BIF and *C. vulgaris* (ABCV) (23.3 ± 0.8 mm) (Figure 2a). Longer stems were observed at 20 days in those seedlings associated to *A. brasilense* Cd and *C. vulgaris* (AWCV) (29.8 ± 1.3 mm), showing even higher results at NaCl concentrations below 100 mM (up to 30.6 ± 1.7 mm), but as before, in general the tendency of stems was to decrease in size as long as NaCl concentration increased; nonetheless, seedlings associated with ABCV showed longer stems than any other treatment at 250 mM of NaCl (25.7 ± 3.3 mM) (Figure 2b).

Root length

Conversely with stem length, the microbial association had no statistical significant differences ($p < 0.05$) in root length but growing medium (Table 3). Those seedlings grown in MS medium averaged longer roots (1.29 ± 0.03 mM) in contrast to those seedlings grown in hydroponic medium (1.13 ± 0.03 mM). Time and salinity also were significant factors for root length; though, salinity effects cannot be grouped in two as before (Figure 3a).

Regarding salinity, those seedlings grown in 50 and 100 mM NaCl showed the longer roots, 1.3 ± 0.05 and 1.4 ± 0.05 mm respectively, in comparison with the rest of the treatments. In the first 10 days, it can be observed that 50 and 100 mM of NaCl promoted longer roots and this

Table 1 - Analysis of Variance for Stem Length (mm) for two growing media and simple free cells.

Source	SS	df	MS	F-ratio	p-value
Main effects					
A: Microbial Association	919.315	3	306.438	10.69	0.0000
B:Time (d)	1075.01	2	537.505	18.75	0.0000
C:Salinity (mM)	3148.09	5	629.618	21.96	0.0000
D:Medium	39.7511	1	39.7511	1.39	0.2396
Interactions					
AB	1628.29	6	271.381	9.46	0.0000
AC	1199.53	15	79.9689	2.79	0.0004
AD	538.267	3	179.422	6.26	0.0004
BC	2292.38	10	229.238	7.99	0.0000
BD	570.949	2	285.475	9.96	0.0001
CD	35.2102	5	7.04204	0.25	0.9419
Residuals	13562.9	473	28.6743		
Total (Corrected)	25775.8	525			

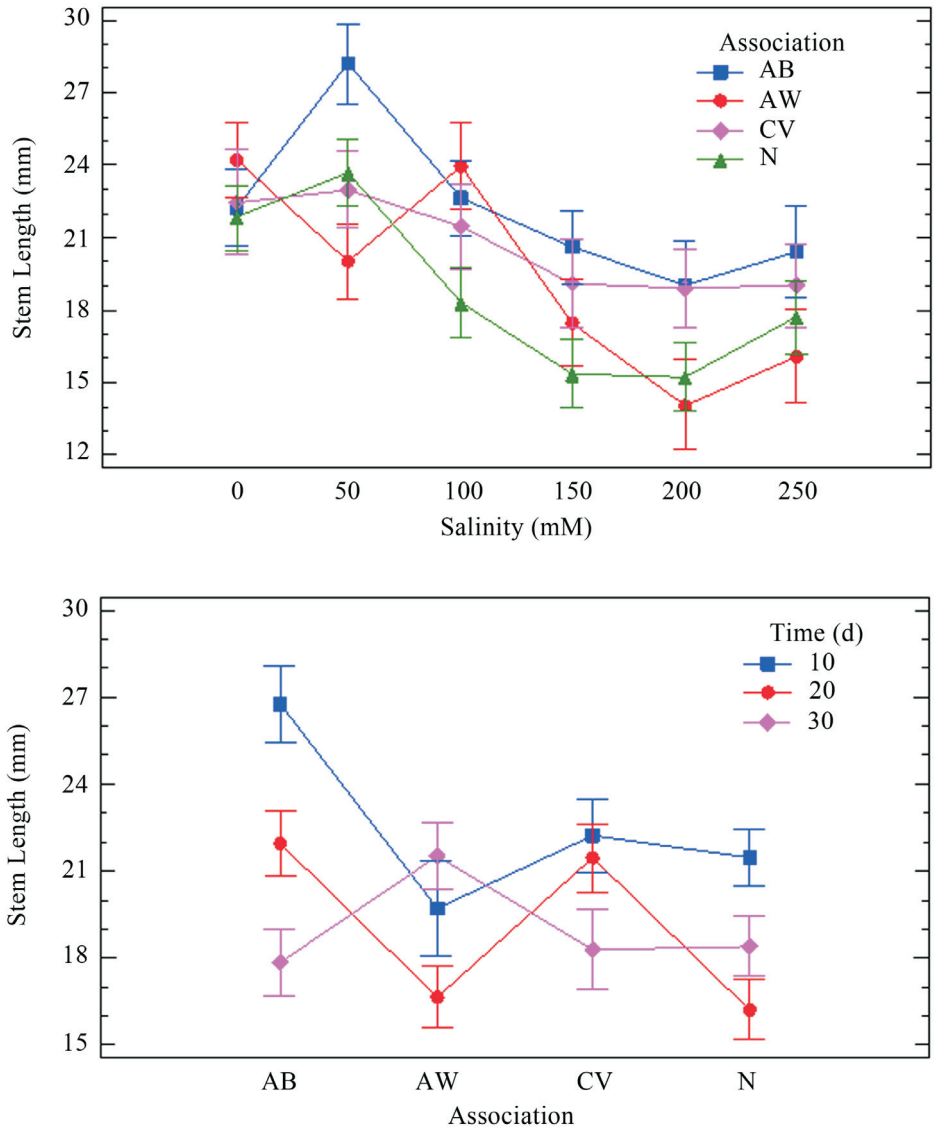

Figure 1 - a) Stem lengths for seedlings in relation to NaCl concentrations using different microbial associations for MS and hydroponic media. b) Stem lengths for seedlings growing in different microbial associations in three periods of time.

Table 2 - Analysis of Variance for Stem Length (mm) for hydroponic media using simple and combined cells.

Source	SS	df	MS	F-ratio	p-value
Main effects					
A: Microbial Association	1839.61	5	367.922	13.75	0.0000
B:Time (d)	73.1129	2	36.5565	1.37	0.2565
C:Salinity (mM)	1873.94	5	374.789	14.01	0.0000
Interactions					
AB	1878.47	10	187.847	7.02	0.0000
AC	2076.13	25	83.0454	3.1	0.0000
BC	602.975	10	60.2975	2.25	0.0147
Residuals	9419.46	352	26.7598		
Total (Corrected)	18329.9	409			

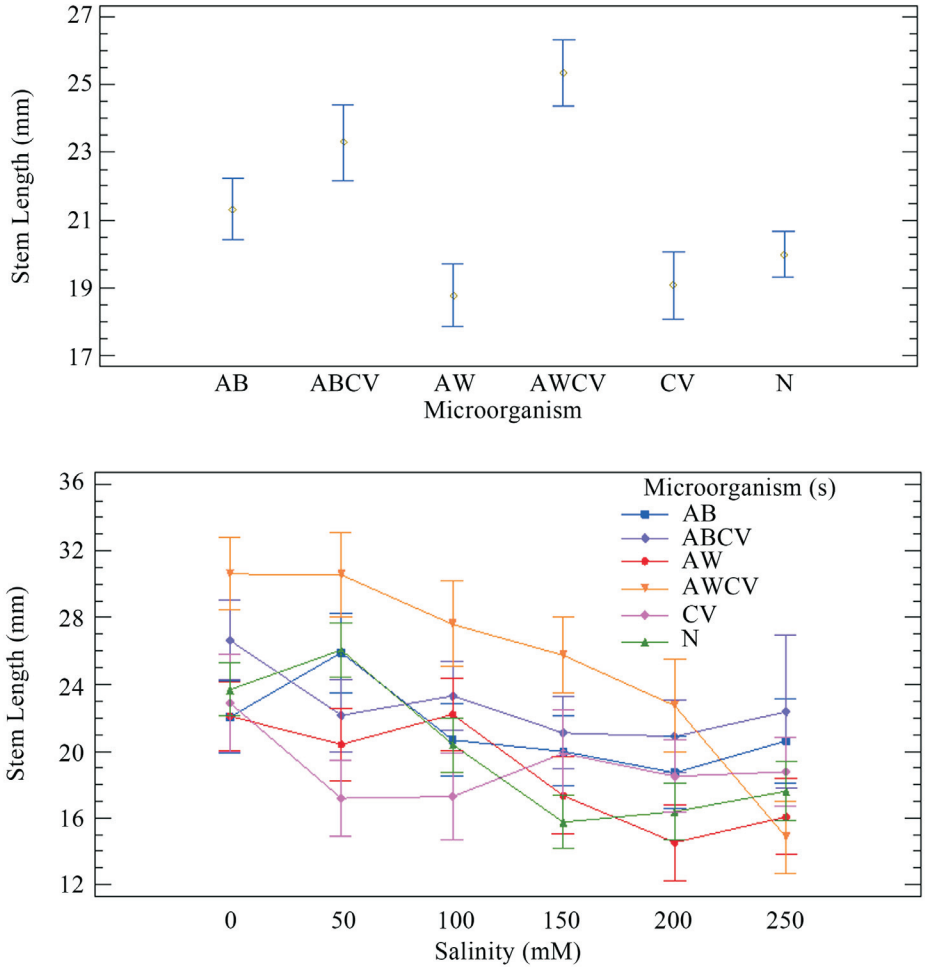

Figure 2 - a) Confidence intervals ($\alpha = 0.05$) for averaged stem lengths for seedlings growing in association with different microorganisms in hydroponic medium. b) Stem lengths for seedlings in relation to NaCl concentrations using different microbial associations for hydroponic media in the second set of experiments.

Table 3 - Analysis of Variance for Root Length (mm) for two growing media.

Source	SS	df	MS	F-ratio	p-value
Main effects					
A: Association	136.578	5	27.3155	2.48	0.0317
B:Time (d)	5.9548	2	2.9774	0.27	0.7633
C:Salinity (mM)	229.184	5	45.8369	4.16	0.0011
Interactions					
AB	737.614	10	73.7614	6.7	0
AC	531.986	25	21.2795	1.93	0.0053
BC	412.965	10	41.2965	3.75	0.0001
Residuals	3888.76	353	11.0163		
Total (Corrected)	5785.52	410			

growth pattern was consistent for the next 10 days but showing shorter roots and this is true until 30 days (Figure 3b).

In the second set of experiments where hydroponic medium was the only one used, time had no statistical effect on root length, but associated microorganisms and salinity (Figure 4). According to the analysis of variance, those

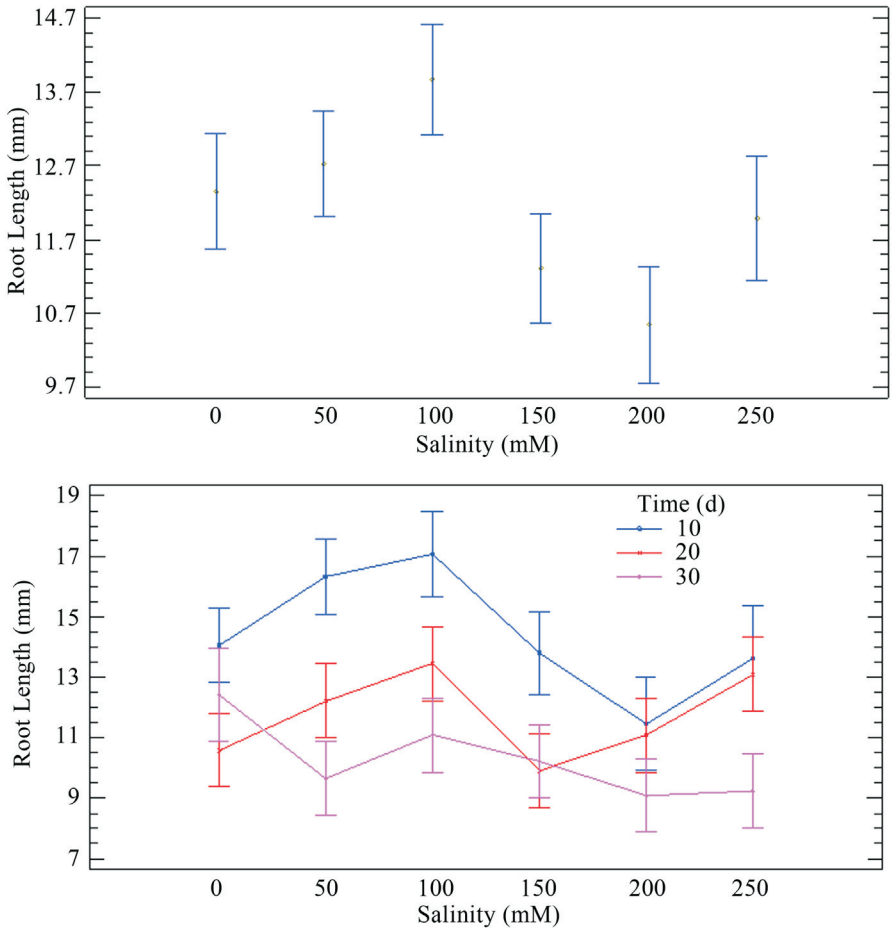

Figure 3 - a) Confidence intervals ($\alpha = 0.05$) for averaged stem lengths for seedlings growing at different NaCl concentrations in MS and hydroponic media. b) Effect of salinity concentration on root length through the time for MS and hydroponic media.

seedlings associated with *C. vulgaris* where different to control having longer roots (12 ± 0.4 mm) (Figure 4a). Even when the effects of NaCl concentration are not differentiated in groups as easily as with stems, in roots is noteworthy the influence of 100 mM of NaCl with longer roots (12.6 ± 0.4 mm).

Concomitantly, AW had a marked effect on root elongation after 30 days (13.2 ± 0.7 mm), similar to that observed for CV at the end of the first 10 days (13.2 ± 0.8 mm) with control showing the shorter roots after 20 and 30 days of treatment (Figure 4b).

Discussion

Hydroponic culture of plants with sea water or even salted water and the use of biofertilizers is a promising technology in order to mitigate the salinity effect on food crops. In the present work we planned to analyze the effects of *Azospirillum brasilense* Cd wild type, *A. brasilense* Cd-BIF able to over accumulate the osmoprotectant trehalose and *Chlorella vulgaris* in the growth of tomato seedlings using two different culture media and in microbial association.

According to the observed results, *Azospirillum basilense* Cd-BIF followed by *Chlorella vulgaris* showed the better influence in seedlings promoting longer stems, with good results even at 250 mM of NaCl. Given that microbial associations showed in general better results for stem and roots elongation in tomato seedlings, this could imply that these plants could be irrigated with sea water at least partially when associated to any of this microorganisms. Seemingly, if the concentration of NaCl is increased for those seedlings growing in hydroponic medium, the effect of *A. brasilense* Cd-BIF will be better; meanwhile the triple association of *A. brasilense* Cd, C. *vulgaris* plus seedlings appears to be sensitive to NaCl increments. These results are in agreement with those works reported previously about *Azospirillum brasilense* growing up to 200 mM of NaCl, where no drop in bacterial growth rate was observed, but when 300 mM of NaCl was used, the growth rate diminished in 66% (Rivarola *et al.*, 1998). In our results, *A. brasilense* Cd showed almost the same growing behavior,

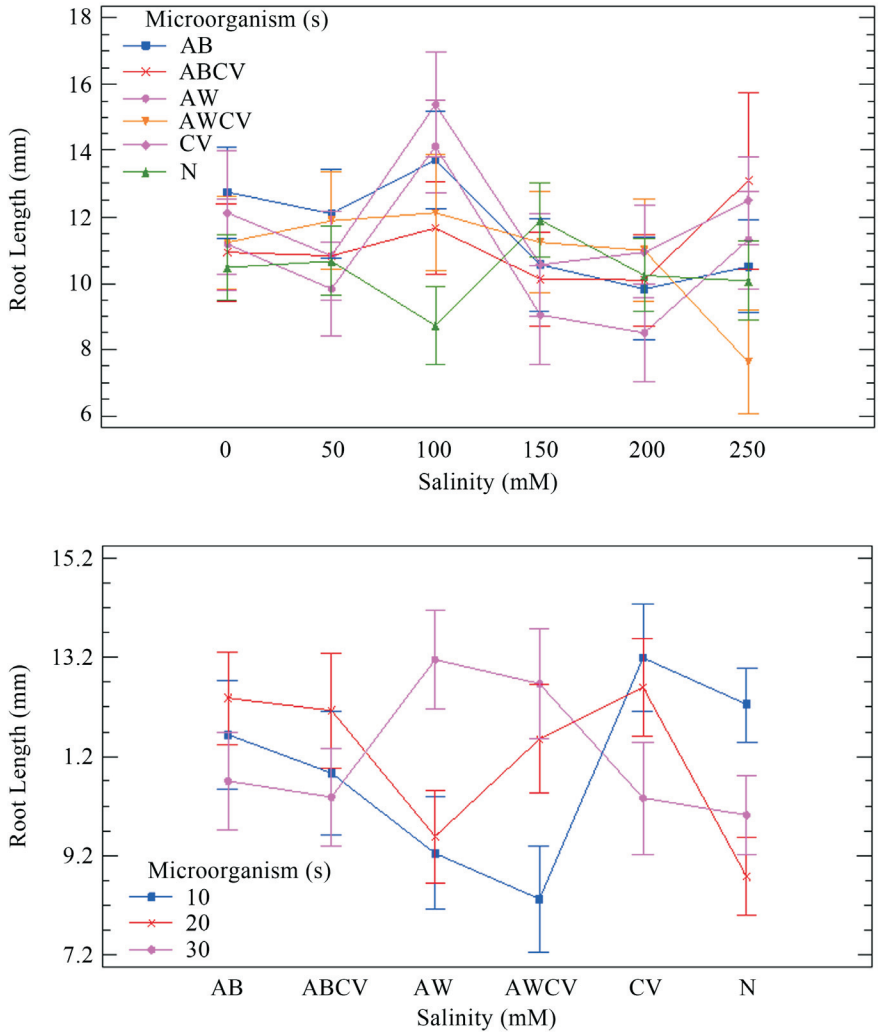

Figure 4 - a) Root lengths for seedlings in relation to NaCl concentrations using different microbial associations for hydroponic media in the second set of experiments. b) Root lengths for seedlings growing in different microbial associations for three periods of time in hydroponic media in the second set of experiments.

however we assume that *A. brasilense* Cd-BIF could grow with no problems even up to 300 mM.

In previous works with plants of *Zea mayz* under osmotic stress, Rodríguez-Salazar *et al.* (2009) observed an increased biomass, namely thicker roots, of those plants grown in association with *A. brasilense* Cd-BIF. Other works with maize and wheat report an osmoadaptative phenomena for those plant cells associated with *A. brasilense* (El-Baky *et al.*, 2008)(2, 16). Some other examples in wheat and barley suggest that the association plant-microorganism are beneficial for plant's salt tolerance due to an increase in its growing capacity (Caballero-Mellado, 2006; Zawoznik *et al.*, 2011).

On the other hand, Hiremath and Mathad *et al.* (2010) demonstrated that *Chlorella vulgaris* was positively stimulated by up to 200 mM of NaCl for chlorophyll and proline production, but when salinity concentration was increased up to 300 mM a reduction on chlorophyll was observed. Díaz *et al.* (1999)) reported that proline accumulates in plants in response to increased environmental salinity. These supports the evidence showed here regarding *C. vulgaris* and its effect on plant tolerance as mentioned above, where *C. vulgaris* had the second marked effect on salt tolerance over the seedlings of tomato.

In general, stems and roots lengths decreased with time, despite of the applied treatment and this could be a cause of the prolonged immersion time of the seedlings in the nutritive solutions, thus is advisable to add some kind of support for the plant. This is why for a future work we are planning to evaluate the effect of these microbial species in seedlings growing in alginate beads supports. As our results suggests, the use of *A. brasilense* Cd-BIF and *C. vulgaris* is a viable approach to increase the salt tolerance in plants and their biomass and the possible use of sea water to irrigate horticultural plants.

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Abbreviations

AW, *Azospirillum brasilense* Cd (wild type)
AB, *Azospirillum brasilense* Cd with plasmid pBBR1M:BIF
PGPB, Plant Growth Promoting Bacteria
CV, *Chlorella vulgaris*

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