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Full Length Research Paper

Flamingo is a new common bean (*Phaseolus vulgaris* L.) genotype with tolerance of symbiotic nitrogen fixation to moderate salinity

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Salinity is one of the major environmental threats to agriculture. In particular, soil salinization is a major limiting factor of symbiotic nitrogen fixation for legume growth. The adverse effects of salinity on the rhizobial symbiosis of common bean genotypes CocoT and Flamingo and the subsequent photosynthesis and plant-growth were investigated in glasshouse. Common bean plants were grown in sterilized sand and irrigated with salt concentrations of 2, 4, 6, 8 and 10 gl^{-1}, respectively and inoculated with rhizobial strains of *Rhizobium tropici* CIAT899 or *Rhizobium etli* 12a3. Nodule number and biomass, shoot content of nitrogen and chlorophyll were decreased by increasing salinity with all used symbiosis, whereas, the proline accumulation increased. The interaction between symbioses and salt levels for all parameters was highly significant. Overall, Flamingo inoculated with *R. etli* had the highest plant growth and nitrogen fixation under salinity. It is concluded that Flamingo has potential for application in field conditions exposed to soil salinization or irrigated with saline water, unless native rhizobial would interact negatively.

Key words: Legume, mediterranean area, rhizobia, salinity.

INTRODUCTION

Salinity is one of the major environmental threats to agriculture. It affects approximately 7% of the world's total land area (Ben-Salah et al., 2011). Nearly 40% of the cultivated world land surface can be categorized as suffering from potential salinity problem (Payakapong et al., 2006; Zahran, 1999). In plants, salinity drastically affects photosynthesis (Soussi et al., 1999), nitrogen metabolism (Santos et al., 2002), and carbon metabolism (Balibrea et al., 2003). It provokes disorders in plant nutrition that may lead to the deficiencies of several nutrients and accumulation of Na$^+$ (Mengel and Kirkby, 2001). Such physiological changes result in decreased plant growth and consequently decreased crop yield (Singla and Garg, 2005; Tejera et al., 2006). These effects limit the ability to generate further biomass or to maintain defense mechanisms (Zheng et al., 2009). They also cause a late development which promotes the accumulation of toxic ions that can lead to death of plants before the end of their development (Munns, 2002).

The negative effect of salinity on plant growth has also been attributed to physiological parameters, such as the inhibition of enzyme activities, particularly, those involved in the defense against oxidative stress (Turkan and Demiral, 2009). Also, several investigators have demonstrated that amino acid metabolism is strongly influenced by changes in the salinity concentrations (Li et al., 2010).

Munns (2002) found that the most legumes are salt sensitive, common bean, chickpea and pea were the most sensitive legumes (Soussi et al., 1999; Abdelly et al., 2005), whereas, soybean was the most tolerant

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(Delgado et al., 1994). Salinity is one of the most widespread problems for the limitation of symbiotic nitrogen fixation. It restricts the development of the extension of legume cultivation in arid and semi-arid areas, such as the Mediterranean basin. Indeed, high salinity in the soil affects the initiation, development and function of nodules (Saadallah et al., 2001). Symbiotic nitrogen fixation was found to be more affected by salt than plant growth (Rao and Tak, 2002). Generally, nodular activity is less affected by salt than nodulation, thus, the infection process seems to be the most sensitive to salt (Payakapong et al., 2006).

The most important strategies employed in the last few years to reduce the effect of salt stress on legume production have been focused on a selection of host genotypes that are tolerant to high salt conditions (Kucuk and Kivanc, 2008). Thus, an increase of tolerance to salinity of rhizobial bacteria might constitute another approach to improve plant productivity under symbiosis (Kenenil et al., 2010), or adaptation to various environmental limitations (Saleh Al-Garni, 2006).

The aim of the present study was to assess the effect of various salt concentrations on common bean-rhizobial symbiosis through growth, nodulation, N content, chlorophyll content and proline accumulation.

MATERIALS AND METHODS

Biological material

Two common-bean cultivars were used in this work: CocoT, selected as a pure line from the local cultivar Coco; Flamingo, selected on the basis of its adaptability to Mediterranean conditions (Trabelsi and Sifi, 1997) from a collection initially supplied by B. Voyesset from CIAT (Colombia). Inoculation was performed with *R. tropici* CIAT899 as a reference rhizobia initially isolated in an acid soil of Colombia kindly supplied by CIAT, or with the native rhizobia *R. etli* 12a3, that was previously isolated from the Cap Bon region in Tunisia and characterized at the phenotypic and molecular levels by Mhamdi et al. (1999).

Plant growth conditions

Experiment was carried out in a glasshouse in 1 L pots filled with sterilized sand. Seeds were surface-sterilized with 1.3% calcium hypochloride for 15 min with constant stirring and subsequently washed with sterile distilled water. They were germinated on 0.8% sterile agar plates for 3 days at 28°C in the dark, with a germination rate of 80%. Seeds were inoculated with 1 ml of liquid inoculants containing 10^8 bacteria ml^{-1} of *R. tropici* CIAT899 or *R. etli* 12a3. The seedlings were irrigated with the nutrient solution according to Saadallah et al. (2001): KH_2PO_4 (0.36 mM), K_2SO_4 (0.7 mM), $MgSO_4$ (1 mM), $CaCl_2$ (1.65 mM); Fe EDTA (40 µmol), H_3BO_3 (4 µmol), $MnSO_4$ (4 µmol), $ZnSO_4$ (1 µmol), $CuSO_4$ (1 µmol), $NaMoO_4$ (0.12 µmol), $CoCl_2$ (0.12 µmol). The solution was supplemented with 2 mM urea $plant^{-1}$ during the first two weeks before nodule emergence.

Trials were performed in a temperature-controlled glasshouse with night/day temperatures of 20 ± 2°C / 25 ± 5°C, and relative humidity of 75/55 ± 10% and natural illuminations. When the first trifoliate leaf appeared about the 21st day after sowing (DAS), plants were distributed into 6 treatments: the first one was irrigated with the aforementioned nutrient solution (control) and the others were watered with the same solution supplemented with 2, 4, 6, 8 and 10 gl^{-1} of NaCl. There were ten replicates for each line and each treatment with one plant only per pot. Plants were irrigated every two days with 100 ml distilled water per pot and the pots were distributed in a complete randomized block design.

Biomass determination and nitrogen content

The plants were harvested at the flowering stage, after 6 weeks of growth. Shoot, nodules and roots were separated and dried at 70°C for 2 days. Dry weight of each fraction was measured. The estimation of leaf area was carried out using a planimeter to measure directly the leaf area (cm^2) of detached leaves treated as screens to light rays. Total nitrogen (TN) was measured by Kjeldahl procedure on shoots.

Chlorophyll content

The accumulation of chlorophyll (Chl) was measured by the methodology of Moran and Porath (1980). For each treatment, three replicates of the third foliole of each leaf were excised and the fresh weight measured. Thereafter, the folioles of the plants were mixed with the N, N-dimethylformamide solvent (10w/w) in darkened and hermetic flask and transferred in a cold room. When the folioles became completely white after 48 h, the concentration of chlorophyll a and b in the solution was measured by spectrophotometer and calculated in mg chl g^{-1} fresh folioles, as (d E_{652} V) $(36\ W)^{-1}$ with d = 4, coefficient of dilution of chlorophyll in the solvent; E_{652}, absorbance of chlorophyll a and b at 652 nm; V, volume of the solution of chlorophyll; W, fresh weight of the sample of folioles immersed in the solvent; 36, coefficient of specific merger of the solvent.

Proline determination

The accumulation of proline was determined according to the method described by khedr et al. (2003) for each treatment at different concentrations of salt. Approximately, 0.5 g of fresh leaf material was homogenized in 10 ml of 3% aqueous sulfo-salicyclic acid, filtered through Whatman's No. 2 filter paper and 2 ml of solution was mixed with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4 ml toluene and cooled to room temperature and the absorbance measured at 520 nm.

Statistical analysis

The SAS software (1997) was used to perform the ANOVA of results and the comparison of means was achieved by the Duncan's multiple range test ($p \leq 0.05$). The regressions were performed using the general linear model procedure of the 2-D graphing analysis system package (File Version: 1.27).

RESULTS

Nodulation

Figure 1A presents the mean number of nodules under various salt concentrations in common bean genotypes.

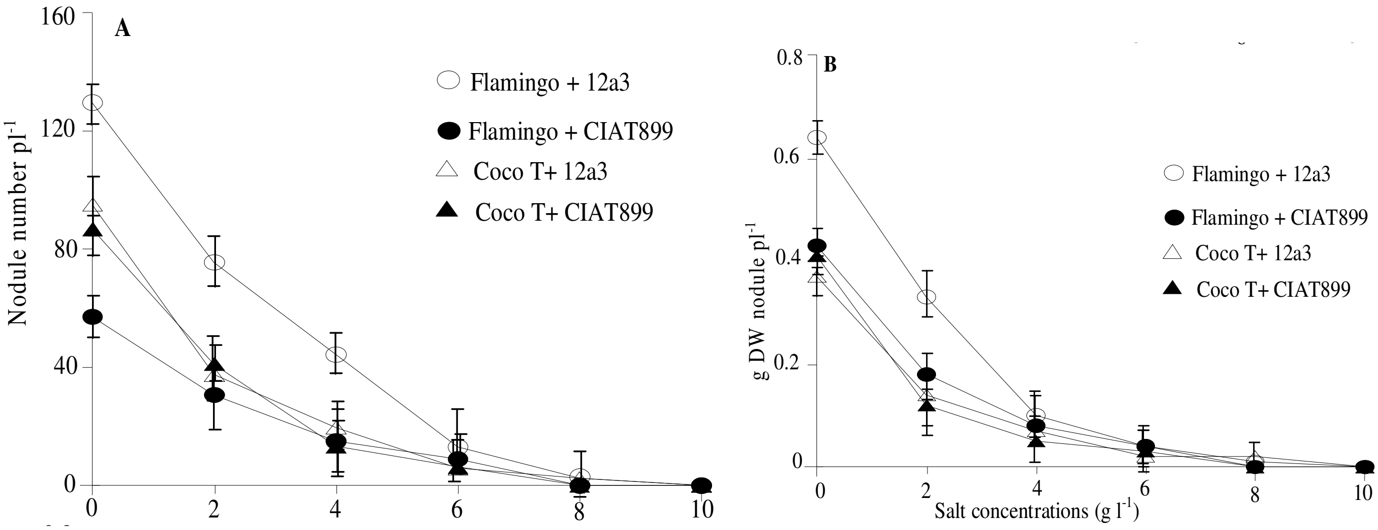

Figure 1. Salinity decreases number (A) and biomass (B) of nodules of common bean genotypes CocoT and Flamingo inoculated with *R. tropici* CIAT899 and *R. etli* 12a3. Data are means ± SD of ten replicates harvested at 50 days after sowing.

Thus, the nodule number varied significantly with rhizobia ($p \leq 0.05$), plant genotype ($p \leq 0.05$), and the interaction with salt concentrations ($p \leq 0.001$). The combination of Flamingo and 12a3 showed the highest values for number of nodules. By contrast, the lowest number of nodules was observed with CIAT899 for both genotypes in control plants and salt concentrations. $NaCl_2$ g l^{-1} induced a significant decrease in number of nodules of 53 and 47% with CIAT899 and 61 and 42% with 12a3 for CocoT and Flamingo respectively, in comparison with control plants (Figure 1A). Increasing salt concentration induced a significant decrease of nodules number above 60% for both genotypes and rhizobia. At concentration of 8 g l^{-1}, plants could not form nodules with CIAT899 by contrast with 12a3 that formed 3 nodules per plant for both genotypes. On the other hand, plants could not form any nodule at concentrations of 10 g l^{-1} regardless of the rhizobia.

Regarding nodule biomass, treatment with salt induced a significant decrease ($p \leq 0.01$) in the nodule mass per plant under all concentrations with both genotypes (Figure 1B). All salt concentrations induced more than 50% decrease in nodule biomass for both genotypes and rhizobia except 48% at 2 g l^{-1} for Flamingo with 12a3. On the other hand, above the concentration of 6 g l^{-1}, the decrease in nodule biomass was above 90% for all symbiosis (Figure 1B).

N accumulation and correlation with nodulation

Total nitrogen content accumulated in common bean shoots is presented in Figure 2. Generally, N_2 content decreased significantly ($p \leq 0.01$) with the increase of salt concentrations for all symbiosis. Nevertheless, Flamingo inoculated with 12a3 show the highest N content for all the salt concentrations. For CocoT, all concentrations of salt induced more than 50% decrease in N content, except with CIAT899 under 2 gl^{-1} (Figure 2). By contrast, for Flamingo, treatments of 2 and 4 gl^{-1} induced less than 50% decrease in N content with both rhizobia (Figure 2).

Consequently, a significant correlation between nodule biomass and nitrogen content was observed for all symbiosis under 2, 4 and 6 g l^{-1} (Figure 3). The detailed examination of the various regressions shows significant differences in the slope of the model between rhizobia, genotypes and salt concentrations, except between both genotypes with CIAT899 under 2 g l^{-1} by contrast with significantly higher values of 3.9 and 2.2% N g^{-1} NDW with 12a3 for Flamingo and CocoT, respectively (Figure 3 A and B).

Under 4 g l^{-1}, the model of adjustment was curvilinear indicating a maximal value of nodules biomass around 80 and 120 mg NDW pl^{-1} for CocoT and Flamingo with CIAT899, and of 130 mg NDW pl^{-1} with 12a3 for CocoT (Figures 3 C to D). Another difference between symbiosis was that the slope of the curve was significantly higher with CIAT899 for CocoT (29.6% N g^{-1} NDW) than for Flamingo (21.1% N g^{-1} NDW), and with 12a3 for CocoT (9.8% N g^{-1} NDW) than for Flamingo (5.0%N g^{-1} NDW) (Figures 3C to D).

Under 6 gl^{-1}, the correlation between nitrogen content and nodules biomass for CocoT was significant only with CIAT899 following a linear model of adjustment with a slope of 7.1%N g^{-1} NDW (Figure 3E). For Flamingo, the models of adjustment were curvilinear with both rhizobia; the slope was significantly higher with 12a3 (23.4% N g^{-1} NDW) than with CIAT899 (7.4% N g^{-1} NDW) (Figures 3E to F) and the maximal value of nodule biomass was 60 mg NDW pl^{-1} under this salt concentration whatever

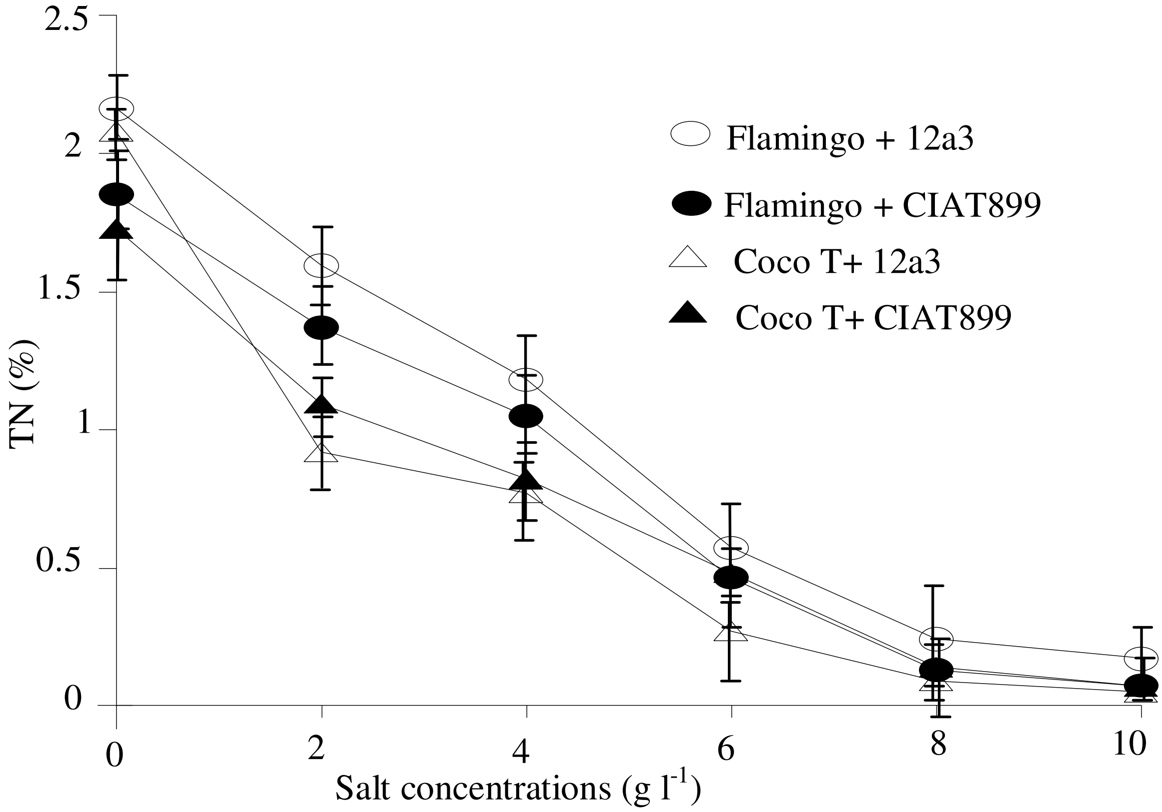

Figure 2. Salinity decreases shoot nitrogen content of common bean genotypes CocoT and Flamingo inoculated with *R. tropici* CIAT899 and *R. etli* 12a3. Data are means ± SD of ten replicates harvested at 50 days after sowing.

the rhizobia.

Shoot, root growth and leaf area

Figure 4 show that plant-growth was strongly decreased by salt treatment ($p \leq 0.05$). Thus, treatment with 2 g l^{-1} induced a significant decrease of shoot growth in the range of 30 to 33% in CocoT and 22 to 30% in Flamingo with 12a3 and CIAT899 respectively (Figure 4A). Treatment with 4 g l^{-1} induced less than 50% decrease in shoot with both genotypes inoculated with 12a3 and about 50% with CIAT899 in comparison to control plants. On the other hand, the highest decrease of shoot growth was observed in CocoT inoculated with CIAT899 which had a percentage of 82% under 10 g l^{-1} (Figure 4A). Root biomass was also affected by salt concentration ($p \leq 0.05$) (Figure 4B). In comparison to control plants, treatment of 2 g l^{-1}induced a significant decrease of root growth less than 20% in both genotypes inoculated with both rhizobia. The treatments of 4 and 6 g l^{-1} induced a significant decrease less than 50% for all symbioses. But, the highest decrease of root growth was observed in CocoT inoculated with 12a3 which had percentages of 71 and 82% under 8 and 10 g l^{-1} respectively (Figure 4B).

Regarding leaf area, generally, data in Figure 5 showed that leaf area decreased significantly ($p \leq 0.01$) with increase of salt concentrations with all symbioses. However, Flamingo showed the highest leaf area at all salt concentrations with both rhizobia in comparison to that in CocoT. Except for CocoT inoculated with 12a3, salt concentration of 2, 4 and 6 g l^{-1} induced a significant decrease of leaf area less than 50% with both rhizobia in both genotypes. However, the lowest leaf area was observed in CocoT inoculated with CIAT899 at 10 g l^{-1}of salt (Figure 5).

Leaf content of chlorophyll and proline

Figure 6A shows chlorophyll accumulation at the flowering stage of common bean genotypes inoculated with both rhizobia at different salt concentrations. Generally, the data showed that chlorophyll content decreased significantly ($p \leq 0.01$) with increase of salt concentrations with all symbioses. However, Flamingo inoculated with 12a3 showed the highest values for chlorophyll content at all studied concentrations. Thus, inoculation with 12a3 induced the highest quantity of chlorophyll in both genotypes at all salt concentrations. In comparison to control plants, treatments of 2 and 4 gl^{-1}induced a significant decrease less than 50% with both

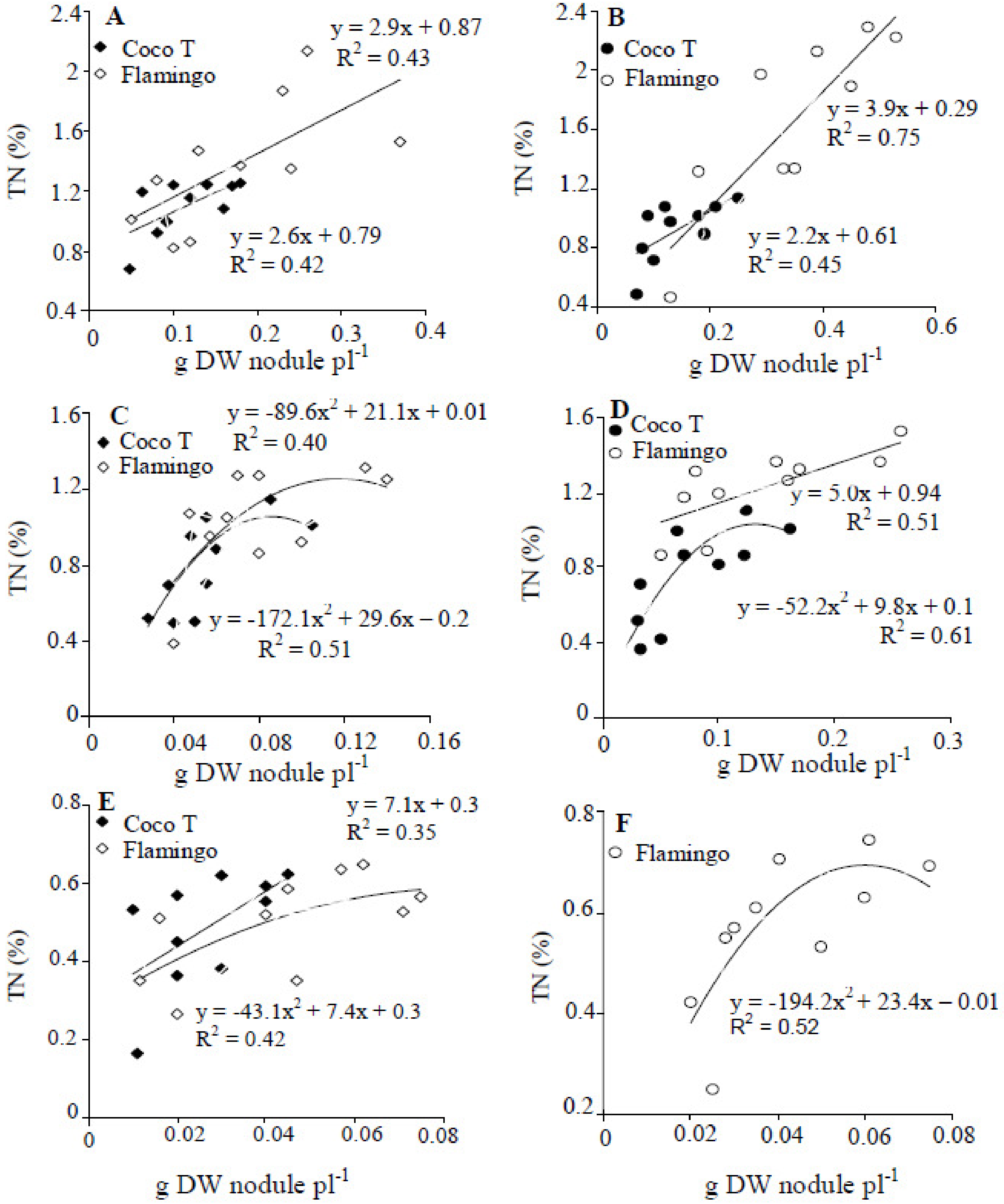

Figure 3. Relation between nitrogen content and nodule biomass (g plant^{-1}) at various salt concentrations of 2 (A and B), 4 (C and D) and 6 g l^{-1} (E and F) in common bean genotypes CocoT and Flamingo inoculated with *R. tropici* CIAT899 (A, C and E) and *R. etli* 12a3 (B, D and F). Data are means of ten replicates harvested at 50 days after sowing.

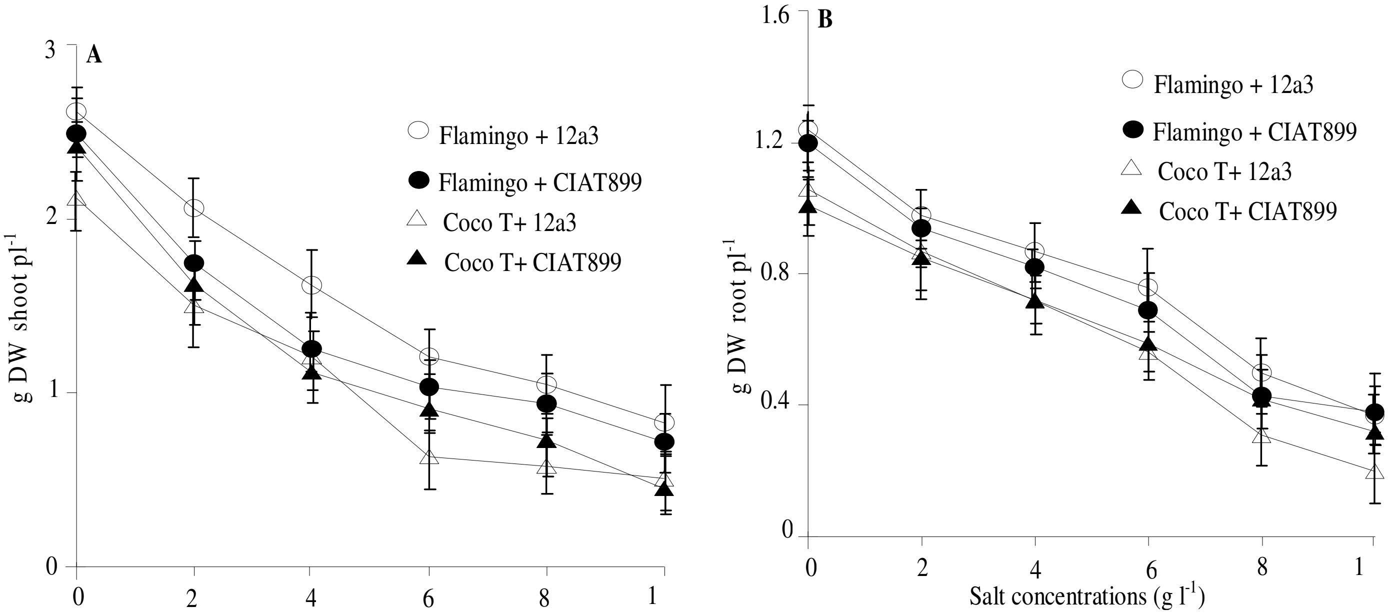

Figure 4. Salinity decreases shoot (A) and root growth (B) of common bean genotypes CocoT and Flamingo inoculated with *R. tropici* CIAT899 and *R. etli* 12a3. Data are means ± SD of ten replicates harvested at 50 days after sowing.

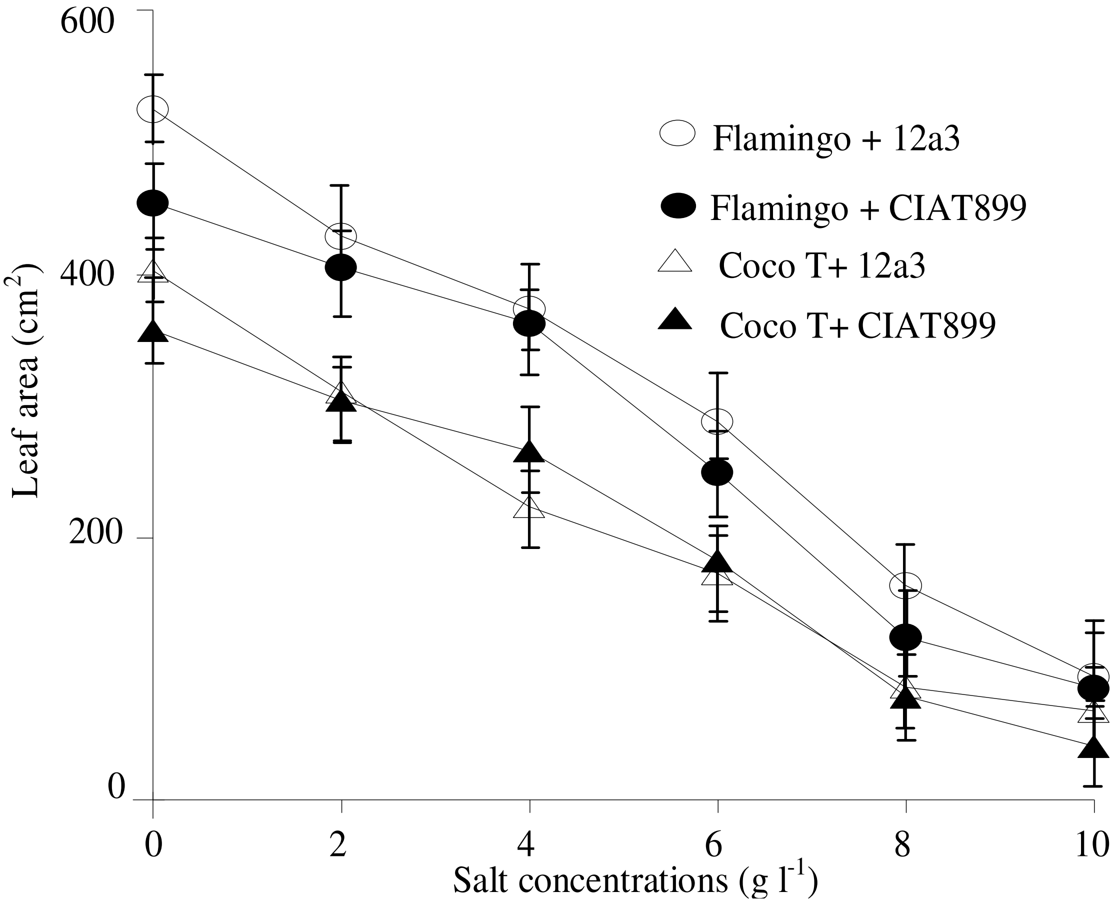

Figure 5. Salinity decreases leaf area of common bean genotypes CocoT and Flamingo inoculated with *R. tropici* CIAT899 and *R. etli* 12a3. Data are means ± SD of ten replicates harvested at 50 days after sowing.

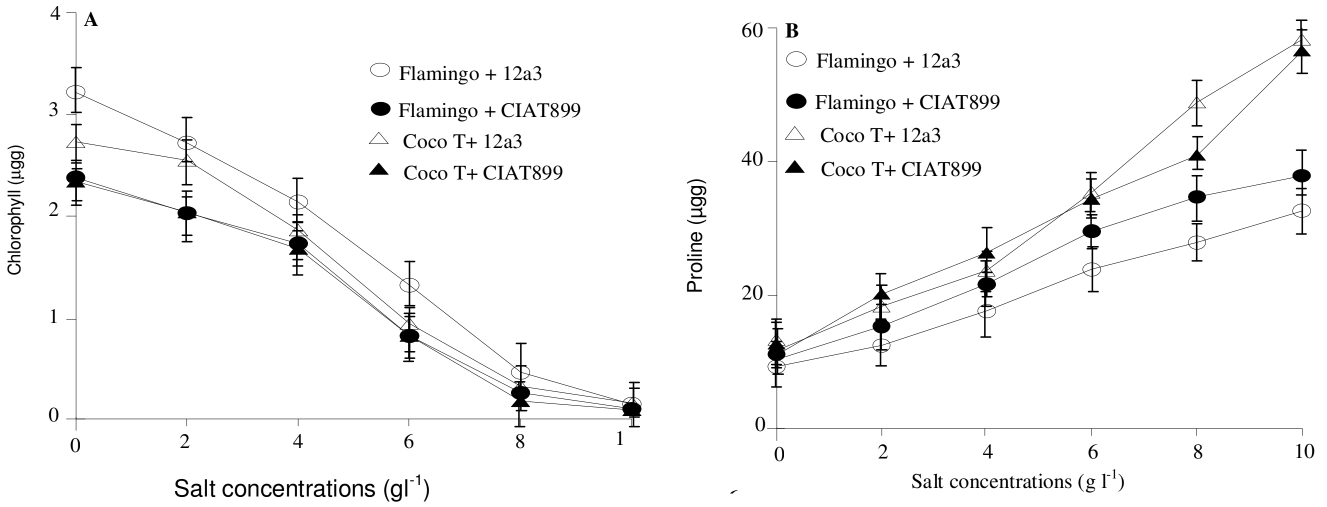

Figure 6. Effect of salt concentrations on chlorophyll content (A) and proline accumulation (B) of common bean genotypes CocoT and Flamingo inoculated with *R. tropici* CIAT899 and *R. etli* 12a3. Data are means ± SD of ten replicates harvested at 50 days after sowing.

rhizobia in both genotypes (Figure 6A). But other treatments of salt reduced the chlorophyll content more than 50% (Figure 6A).

Data in Figure 6B showed proline accumulation of common bean genotypes inoculated with rhizobia at different salt concentrations. Generally, proline accumulation increased in response to salt concentration increment ($p \leq 0.05$). The highest proline accumulation was found in CocoT plants inoculated with 12a3 while the lowest was in Flamingo with the same strain at 10 gl^{-1}. Relatively, symbiosis between Flamingo and 12a3 showed the lowest values for proline accumulation at all salt concentrations.

DISCUSSION

The present work shows the evaluation of the interaction between rhizobia strains and common bean genotypes on nitrogen fixation efficiency under salt concentrations stress. Many parameters were conducted which includes; nodulation, nitrogen content, plant growth, chlorophyll formation and proline accumulation.

The results showed that increasing salt concentrations decreased the mean number of nodules. Thus, nodule formation was sensitive to salt stress under this experiment condition which agreed with Tejera et al. (2004) and Fahmi et al. (2011) who found that the salt stress inhibits nodule formation by the inhibition of the initial steps of rhizobia-legume symbiosis. However, the combination of Flamingo and 12a3 was more tolerant to salt stress than the other symbioses (Figures 1 to 4), not only for the nodulation process but also for the nodule growth and the nodule function as previously described for other symbiosis (Drevon et al., 2001). Similarly, nitrogen content reduction increased with salt concentration increment for all symbioses. In symbiosis with 12a3, CocoT was more sensitive than Flamingo. Also, in symbiosis with CIAT899, Flamingo was more tolerant to salinity than CocoT, which agreed with Drevon et al. (2001). However, differences in this tolerance may also be due to other determinants such as the interaction with the plant genotype, as illustrated by the lower efficiency of 12a3 with CocoT than with Flamingo (Tajini et al., 2008). Reduction of N$_2$-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules (Kenenil et al., 2010). Also, the salt-induced distortions in nodule structure could be the reason for the decline in the N$_2$ fixation rate by legumes subjected to salt stress (Fahmi et al., 2011). The depressive effect of salt stress on N$_2$ fixation by legumes is directly related to the salt-induced decline in dry weight and N content in the shoot (Zahran, 2001) who found that the nitrogen content in soybean was more strongly reduced under increased irrigation with saline water. In the other hand, Saadallah et al. (2001) found that the plant growth was less affected by the salt treatment with mineral N than with N$_2$, and the amount of N fixed was the most limited.

The correlation of nitrogen content as a function of nodules biomass (Figure 3), establishes that the salt is a major threat of nitrogen fixation and bean growth; suggests a major factor that limit the nodulation and prevented the symbiosis contributing to plant-growth.

Interestingly, the curvilinear adjustment of nitrogen content as a function of nodulation with CIAT899 when nodule biomass ranges beyond 80 and 120 mg per plant for CocoT and Flamingo (Figure 3) respectively, and 130 mg per plant in CocoT with 12a3, under 4 gl^{-1}and 60 mg per plant with both rhizobia in Flamingo under 6 gl^{-1}, suggests that nodulation above this value does not bring any additional benefit to the nitrogen fixation of the plant and may even be slightly detrimental under these concentrations. However, differences in this efficiency parameter may also be due to other determinants such as the interaction with the plant genotype, as illustrated by the lower efficiency of 12a3 compared to CIAT899 with CocoT under 4 gl^{-1}.

The results presented in this study showed that salt reduce plant growth in both genotypes and shoot growth was more sensitive than root growth. This observation corroborates the findings of Rejili et al. (2007) and Khadri et al. (2007) that the root dry matter was not affected as severely as the aerial organs (shoots), but contradicts the report by Gama et al. (2007) that roots were more sensitive than shoots. This effect was more related to a reduction of leaf area and agrees with the previous observation of Saadallah et al. (2001) who reported that the reduction of growth of some *Medicago* subjected to salt was related to a decrease in leaf area rather than in assimilation efficiency. On the other hand, in *Phaseolus vulgaris*, the concentration of 50 mM of NaCl caused a decrease of growth due to the reduction in photosynthates caused by salt (Gama et al., 2007).This sensitivity of symbiotic plants was associated with an excessive accumulation of Cl^- in leaves, and over loading of nodules with toxic ions Na^+ and Cl^- (Drevon et al., 2001). Also, Gama et al. (2007) reported that in salt-sensitive plant, shoot, and to lesser extent, root growth is permanently reduced within hours of salt tress and this does not appear to depend on Na^+ concentrations in the growing tissues, but rather is a response to the osmolarity of the external solution. Although, reduction in biomass, photosynthetic capacity changes in leaf water potential and leaf turgor have been reported to have a cumulative effect attributed to salinity stress.

For Chlorophyll content, plants inoculated with both rhizobia showed reduction and no significant difference between all symbioses for the reduction. This result agreed with Li et al. (2010) who noticed reduction in photosynthetic activity under salt stress Chinese castor bean at increasing levels of salt stress. Also, Fahmi et al. (2011) found that chlorophyll a and b content were decreased by increasing sea water salinity in faba bean.

The decline in photosynthesis observed with increasing salinity could be attributed to stomata factors. During salt stress, the concentration of CO_2 in chloroplasts decreases because of a reduction in stomata conductance (Gama et al., 2007).

Proline content in plants inoculated with both rhizobia showed increment with increasing salt concentrations, while Flamingo inoculated with 12a3 showed the least proline levels. The concentrations of proline in the plant tissues were generally very low but the levels were significantly affected by salinity (Jampeetong and Hans, 2009). Also, they found that concentrations of proline were about three times higher in the plants grown at 150 mM salinity when compared to the control. Salt stress results in the formation of specific proteins in legumes (Parida et al., 2004) reported that the production of 41 proteins was increased at least 10-fold in salt-stressed.

Proline has been suggested to play multiple roles in plant stress tolerance. It acts as a mediator of osmotic adjustment, protects macromolecules during dehydration and serves as a hydroxyl radical scavenger.

In conclusion, this work showed the existence of variability among symbioses in the response to stress salinity: Flamingo inoculated with 12a3 was the most salt-tolerant symbiosis, which showed higher values in number of nodules, nitrogen content, chlorophyll content, plant growth and leaf area, but there were the least in proline accumulation. The compatibility of Flamingo and 12a3 might be considered as salt tolerant under our experimental conditions which might have potential for application in field conditions.

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