RESEARCH ARTICLE

Supplemental Ca^{2+} does not improve growth but it affects nutrient uptake in NaCl-stressed cowpea plants

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ABSTRACT

The growth and nutrient assimilation was evaluated in $CaCl_2$- and $CaSO_4$-supplemented cowpea plants subjected to salt stress (75 mM NaCl). The salinity significantly reduced the cowpea vegetative growth. The addition of $CaCl_2$ in the growth medium did not significantly affect plant growth, while for the $CaSO_4$, the beneficial effects of Ca^{2+} were moderate. Salinity increased the Na^+, K^+, Cl^-, N and P content in the plants, however it decreased the content of Ca^{2+} and Mg^{2+}. Increases in Ca^{2+} concentration in the nutrient solution caused decreases in the Na^+ and Mg^{2+} contents and increases in Ca^{2+}, K^+, P, and Cl^- contents. The supplemental Ca^{2+} may alleviate the Na^+ toxicity and may improve nutritional and ionic balance in cowpea, but it cannot overcome the osmotic effects associated with the increased total salt concentration.

Keywords: calcium, mineral nutrition, salinity, *Vigna unguiculata*.

INTRODUCTION

Soil salinity is one of the most important abiotic stresses limiting crop productivity worldwide (Hasegawa et al., 2000). The plant growth inhibition under saline conditions is due, in part, to both osmotic and ionic components, which may cause water deficit, ion toxicity, and nutritional unbalance (Hasegawa et al., 2000; Munns and Tester, 2008).

Salinity effects on plant mineral nutrition are associated with the salts predominating in the growth medium (Alam, 1999). Thus, excess Na^+ and Cl^- in the soil solution, and occasional excess SO_4^{2-} may lead to deficiencies in the essential nutrients (Sairam and Tyagi, 2004). In general, excess Na^+ may induce both K^+ and Ca^{2+} deficiencies, while NO_3^- uptake may be inhibited by Cl^- (Grattan and Grieve, 1999).

Calcium is an essential nutrient for the growth and development of plants and it plays a fundamental role as a second messenger in many signal transduction pathways within the cell (Marschner, 1995; Kim et al., 2009). In addition, Ca^{2+} helps maintaining the integrity and structure of the membranes and cell wall, and its displacement by Na^+ may occur in saline conditions, leading to altered plasma membrane integrity and to the leakage of intracellular solutes (Rengel, 1992).

According to Epstein (1998), the adverse effects of salinity on plants might be alleviated by the addition of supplemental Ca^{2+} in the growth medium. This is due, in part, to the ability of Ca^{2+} in decreasing the influx of Na^+ and the efflux of K^+ through the inhibition of non-selective cations and outward rectifying K^+ channels, respectively (Demidchick and Tester, 2002; Shabala et al., 2006). In addition, Ca^{2+} appears to alter root lipid composition (Cachorro et al., 1993) and to induce organic solute accumulation, such as proline and glycinebetaine (Girija et al., 2002) in salt-stressed plants.

Although an adequate supply of Ca^{2+} may reduce Na^+ content in tissues and improve plant growth under salt stress (Kaya et al., 2002; Dabuxilatu and Ikeda, 2005), there was no beneficial effect of Ca^{2+} when cowpea was grown in nutritive solutions supplemented with $CaCl_2$ (Silva et al., 2003). In order to clarify this issue, we have tested the hypothesis that the responses of cowpea to supplemental Ca^{2+} depend on the source of Ca^{2+} and/or the concentration in the growth medium. Furthermore, there is little information about the uptake and distribution of nutrients in cowpea under NaCl salinity (Sousa et al., 2007; Neves et al., 2009), and no studies conducted to date have examined the effects of supplemental Ca^{2+} on nutrient content in this species. The present study was undertaken to evaluate the effects of increasing $CaCl_2$ and $CaSO_4$ concentrations on growth and nutrient assimilation in NaCl-stressed cowpea plants.

MATERIAL AND METHODS

Plant material and growth conditions: Cowpea seeds [*Vigna unguiculata* (L.) Walp.], cultivar Pitiúba, were selected according to their size and shape, and their surface was sterilized with a 2% sodium hypochlorite solution for ten minutes. Afterwards, the seeds were rinsed in distilled water and sown in plastic cups containing vermiculite moistened with distilled water. On the fifth day after sowing, uniform-sized seedlings were transplanted into trays containing half-strength Hoagland's nutrient solution, and they were acclimated for four days.

On the ninth day after sowing, the seedlings were transplanted into pots (one plant per pot) containing 3 L of half-strength Hoagland's nutrient solution and were subjected to Ca^{2+} treatments: $CaCl_2$ or $CaSO_4$ were added to the nutrient solution at concentrations of 0.5, 1.25, 2.5, 5.0, 7.5, and 10.0 mM. Twenty-four hours after these calcium salts were added, the plants were subjected to salt stress, which was applied at a rate of 25 mmol L^{-1} every 48 hours, until the final concentration (75 mM) was reached.

NaCl was gradually added in an attempt to avoid osmotic shock. For the control treatment, a set of plants received only the half-strength Hoagland's nutrient solution containing 2 mM $Ca(NO_3)_2$. In the nutrient solutions supplemented with calcium salts, $NaNO_3$ was used as a source of nitrate to replace $Ca(NO_3)_2$. All nutrient solutions were kept aerated and were replaced every six days. Each day, the amount of water transpired was replaced with distilled water. The pH of the solution was daily measured and was adjusted to 5.5, as required, using HCl or NaOH. This was necessary to assure the solubility of $CaSO_4$, particularly at concentrations above 5.0 mM. The experiment was carried out in greenhouse conditions, with mean air temperatures of 32.5±4.5°C (day) and 25.5±4.5°C (night), a relative humidity of 40 to 80% and a mean maximum photosynthetic photon flux density of approximately 1,200 µmol m^{-2} s^{-1}.

Growth measurements and mineral analyses: Plants were harvested 24 days after the first addition of NaCl and they were separated into leaves, stems + petioles, and roots. Plant materials were dried in a forced-air oven at 60°C for 72 hours, and then the dry mass of the samples was recorded.

The extraction of Ca^{2+}, Mg^{2+}, Na^+, K^+, P, and S from the plant tissue material was performed by digestion of 0.5 g powder of the oven-dried samples in a mixture of concentrated nitric acid and perchloric acid (2:1; v/v; Malavolta et al., 1997). The Ca^{2+} and Mg^{2+} contents were determined by atomic absorption spectrophotometry, while the Na^+ and K^+ contents were determined by flame photometry. The P and S contents were estimated by colorimetric and turbidimetric methods, respectively (Malavolta et al., 1997). Total nitrogen extraction was based on the micro-Kjeldahl method, and the N content was determined according to Malavolta et al. (1997). Aqueous extracts were used for Cl^- determination by titration with $AgNO_3$ (Malavolta et al., 1997).

Statistics: The experimental design was entirely randomized and arranged as a factorial design (2 × 6 + 1), consisting of six saline treatments supplemented with $CaCl_2$ and six other ones supplemented with $CaSO_4$, plus a control treatment of half-strength Hoagland's nutrient solution. The experiment was conducted with five replications and a total of 65 pots (experimental units). All data were subjected to analysis of variance (ANOVA) using the *F* test, and the effects of Ca^{2+} concentration were assessed using the regression analysis. Dunnett's test was used to compare the differences between the control and the other treatments. Comparisons with p-values≤0.05 were considered to be significantly different. Statistical data analyses and graphics were made with SigmaPlot for Windows, version 11.0.

RESULTS

Growth: The cowpea plant growth was dramatically reduced ($p \leq 0.001$) by salt stress (NaCl at 75 mM) in both $CaCl_2$- and $CaSO_4$-supplemented plants (Figure 1). Increases in the concentration of $CaCl_2$ in the growth medium did not affect the leaf area (LA) and shoot dry mass (SDM), as seen in Figure 1. On the other hand, the plants supplemented with $CaSO_4$ showed a trend of increased LA and SDM (Figure 1). The root dry matter (RDM) was reduced by 68.3% after exposure to 75 mM NaCl, and there was no significant interaction ($p=0.280$) between the Ca^{2+} source and concentration, when the RDM was analyzed (Figure 1).

Na^+ and Cl^- accumulation: Salinity favored the accumulation of Na^+ in all cowpea tissues (Figure 2). The increasing $CaCl_2$ and $CaSO_4$ concentrations in the growth medium, however, significantly reduced the Na^+ content in cowpea plants (Figure 2). The Cl^- content in all analyzed plant parts increased with salinity, and this increase depended on the interaction between the Ca^{2+} source and concentration (Figure 2). It should be noted that regardless the plant part, the increasing $CaCl_2$ concentration in the growth medium gradually increased the Cl^- content of the plants. In the leaves and roots, the changes in the Cl^- content of $CaSO_4$-supplemented plants depended on the Ca^{2+} concentration in the growth medium, whereas in the stems + petioles, the Cl^- content did not change with increased Ca^{2+} concentration (Figure 2).

Macronutrient accumulation: The Ca^{2+} content of cowpea plants was significantly ($p \leq 0.001$) reduced by salinity, primarily in the leaves. As expected, the $CaCl_2$ and $CaSO_4$ supplementation increased the Ca^{2+} content in all plant tissues, resulting in values that were equal to or greater than those observed in control plants (Figure 3).

In general, the K^+ content was higher in salt-stressed plants in comparison to control plants (Figure 3). In leaves and stems + petioles, this increase occurred regardless the Ca^{2+} source, but only the K^+ content in the stems + petioles was influenced by the Ca^{2+} concentration (Figure 3). The root K^+ content in both plants supplemented with $CaCl_2$ and $CaSO_4$ increased linearly in response to increased Ca^{2+} concentration in the growth medium (Figure 3).

Salt stress increased ($p \leq 0.001$) the N content in leaves and stems + petioles of cowpea plants by 17.1 and 51.2%, respectively (Figure 4). Such increase occurred regardless the Ca^{2+} concentration and source in the growth medium. There was no significant change in the root N content of $CaSO_4$-supplemented plants (Figure 4). However, when $CaCl_2$ was used as the Ca^{2+} source, the root N content decreased linearly as the Ca^{2+} concentration in the growth medium increased (Figure 4).

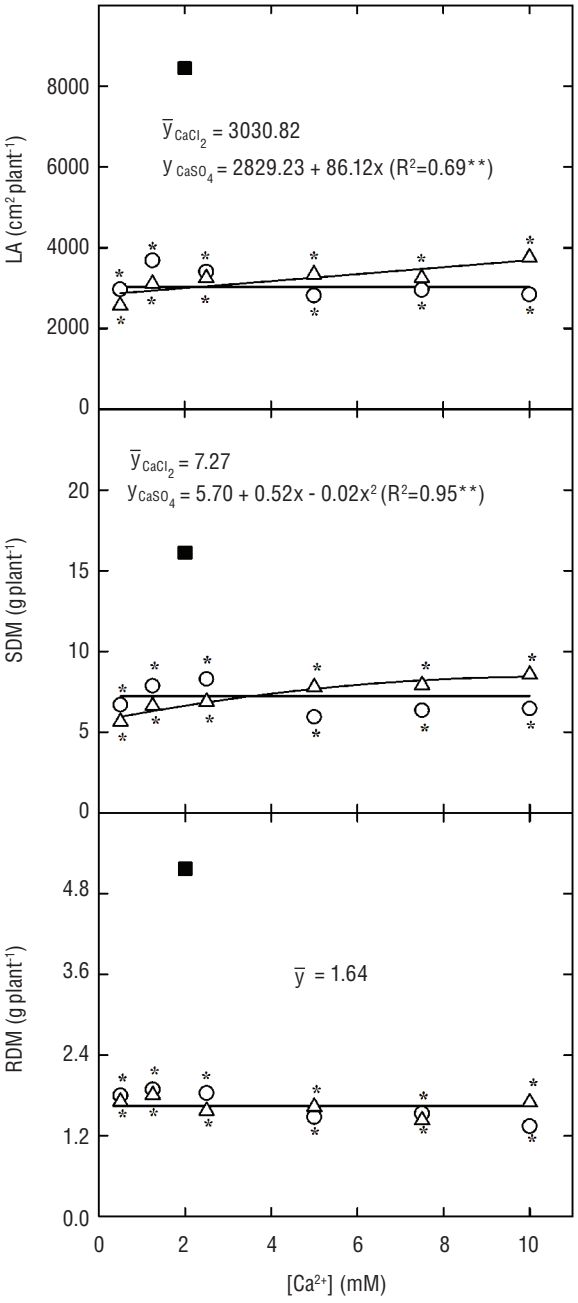

Figure 1. Leaf area (LA, A), shoot (SDM, B), and root (RDM, C) dry masses of cowpea plants grown under control (nutrient solution with 2.0 mM calcium – filled square) and saline conditions (nutrient solution containing 75 mM NaCl plus $CaCl_2$ – open circle – or $CaSO_4$ – open triangle – at 0.5, 1.25, 2.5, 5.0, 7.5 and 10.0 mM). The asterisk denotes statistical differences between the Ca^{2+} treatments and control (Dunnet's test; $p \leq 0.05$).

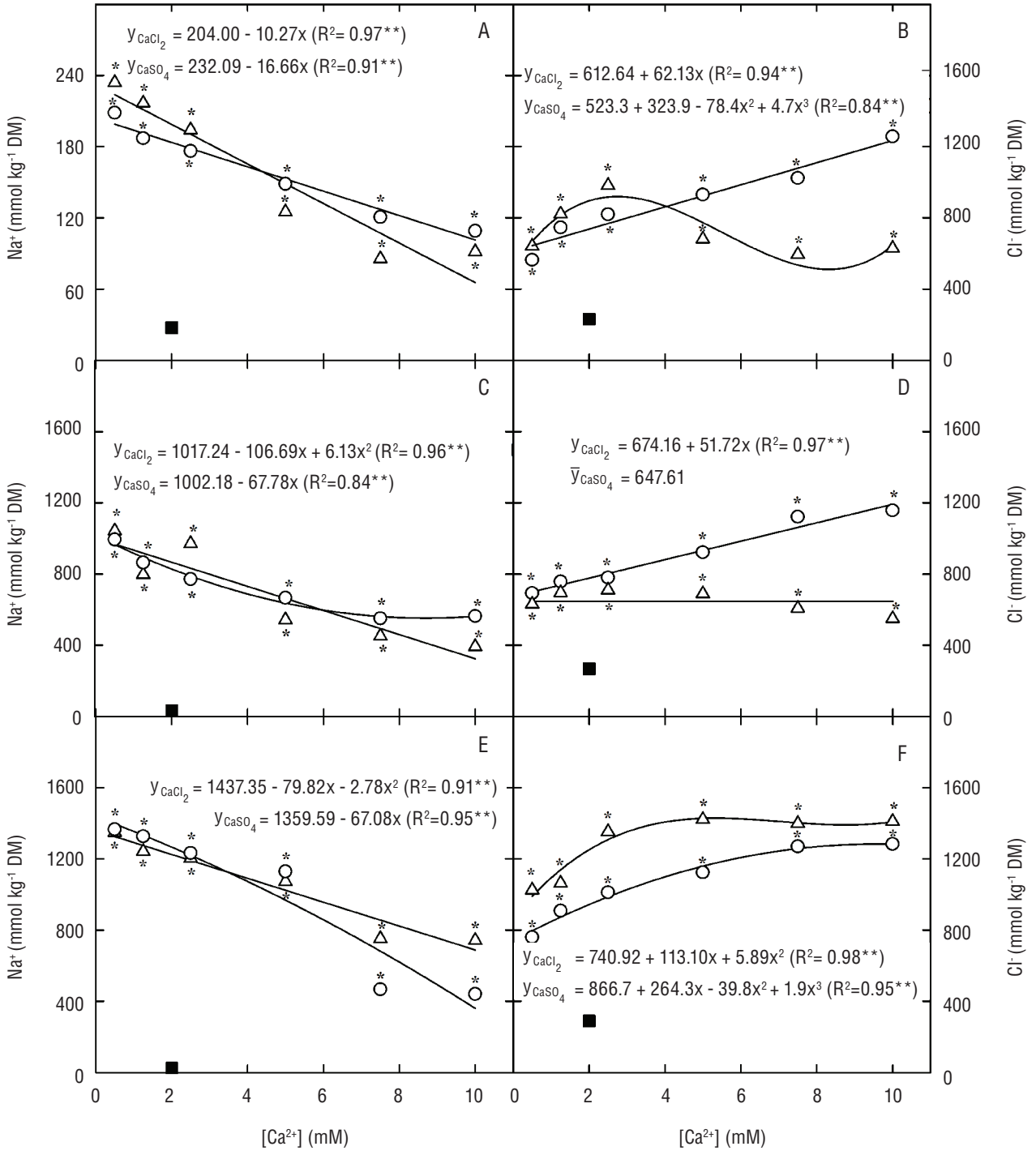

Figure 2. Sodium (Na^+) and chloride (Cl^-) contents in leaves (A and B), stems + petioles (C and D), and roots (E and F) of cowpea plants grown under control (nutrient solution with 2.0 mM calcium – filled square) and saline conditions (nutrient solution containing 75 mM NaCl plus $CaCl_2$ – open circle – or $CaSO_4$ – open triangle – at 0.5, 1.25, 2.5, 5.0, 7.5 and 10.0 mM). The asterisk denotes statistical difference between the Ca^{2+} treatments and control (Dunnet's test; $p \leq 0.05$).

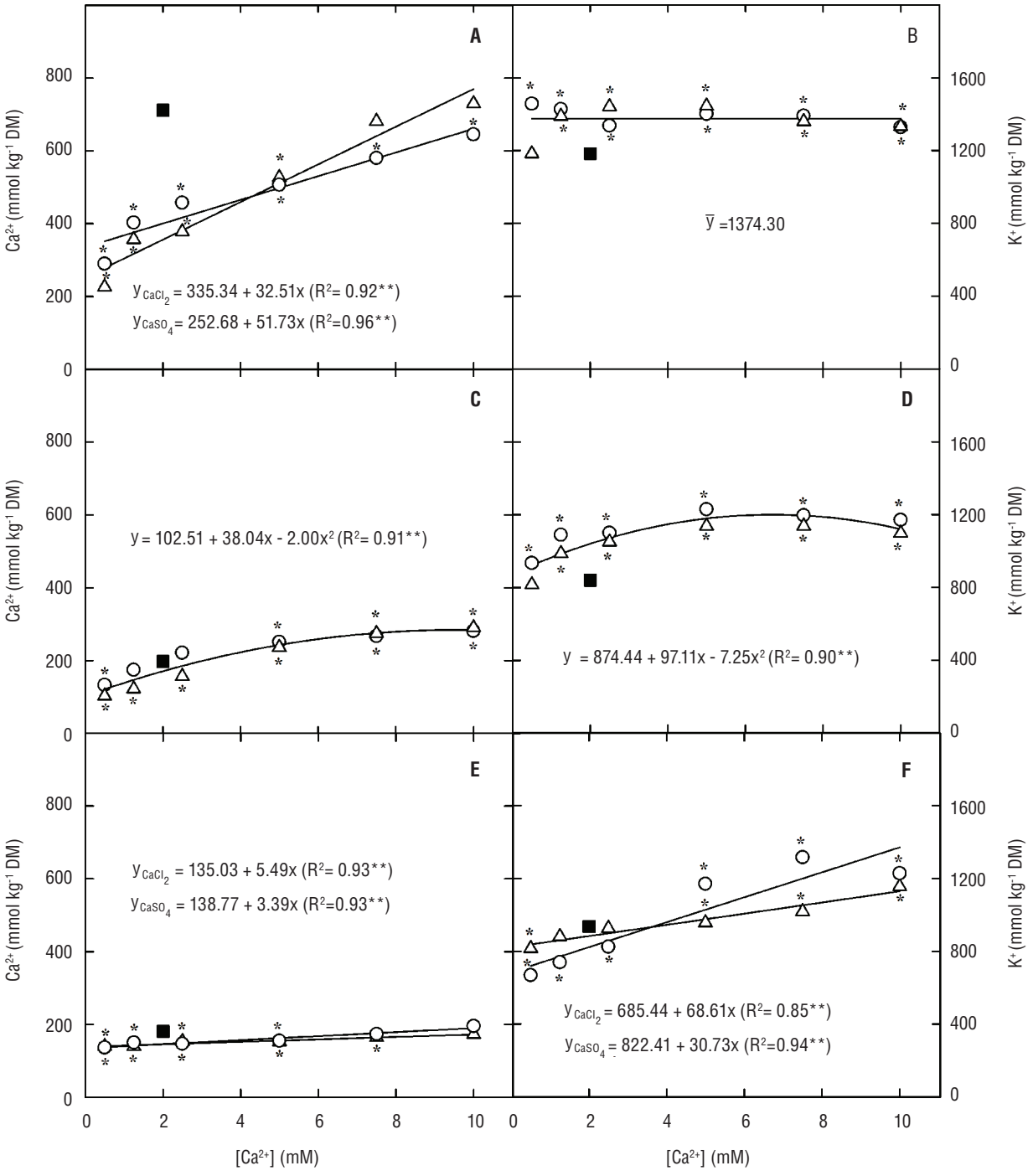

Figure 3. Calcium (Ca^{2+}) and potassium (K^{+}) contents in leaves (A and B), stems + petioles (C and D), and roots (E and F) of cowpea plants grown under control (nutrient solution with 2.0 mM calcium – filled square) and saline conditions (nutrient solution containing 75 mM NaCl plus $CaCl_2$ – open circle – or $CaSO_4$ – open triangle – at 0.5, 1.25, 2.5, 5.0, 7.5 and 10.0 mM). The asterisk denotes statistical difference between the Ca^{2+} treatments and control (Dunnet's test; $p \leq 0.05$).

The P content increased ($p \leq 0.001$) in different parts of the salt-stressed plants and was affected by both the Ca^{2+} source and concentration in the growth medium, except in the stem + petioles, in which no significant ($p=0.428$) interaction between these factors was found (Figure 4). Moreover, plant exposure to increasing Ca^{2+} concentration caused a linear increase in the P content in the leaves of $CaSO_4$-supplemented plants and in the roots of $CaCl_2$-supplemented ones, respectively (Figure 4).

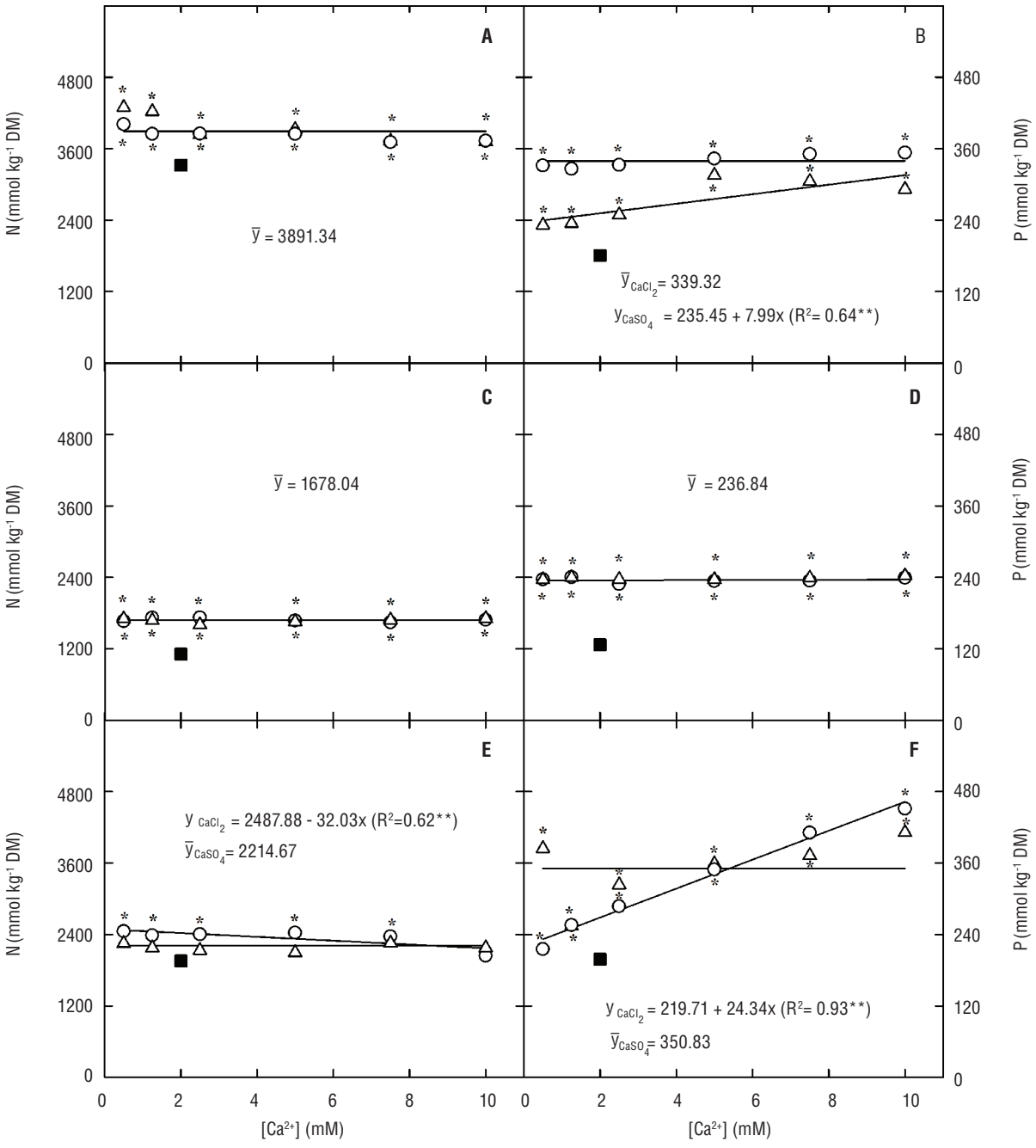

Figure 4. Nitrogen (N) and phosphorus (P) contents in leaves (A and B), stems + petioles (C and D), and roots (E and F) of cowpea plants grown under control (nutrient solution with 2.0 mM calcium – filled square) and saline conditions (nutrient solution containing 75 mM NaCl plus $CaCl_2$ – open circle – or $CaSO_4$ – open triangle – at 0.5, 1.25, 2.5, 5.0, 7.5 and 10.0 mM). The asterisk denotes statistical difference between the Ca^{2+} treatments and control (Dunnet's test; $p \leq 0.05$).

The Mg^{2+} content in leaves and roots was significantly ($p \leq 0.001$) reduced by salinity (Figure 5). However, unlike the trend observed for the Ca^{2+} content, the increasing $CaCl_2$ or $CaSO_4$ concentrations in the saline medium further reduced the Mg^{2+} content in these plant tissues. In the stems + petioles, the Mg^{2+} content was higher in the salt-stressed plants supplemented with a low Ca^{2+} concentration (especially when Ca^{2+} was provided as $CaCl_2$), while the Mg^{2+} content presented no significant differences between high Ca^{2+} treatments and control treatment (Figure 5).

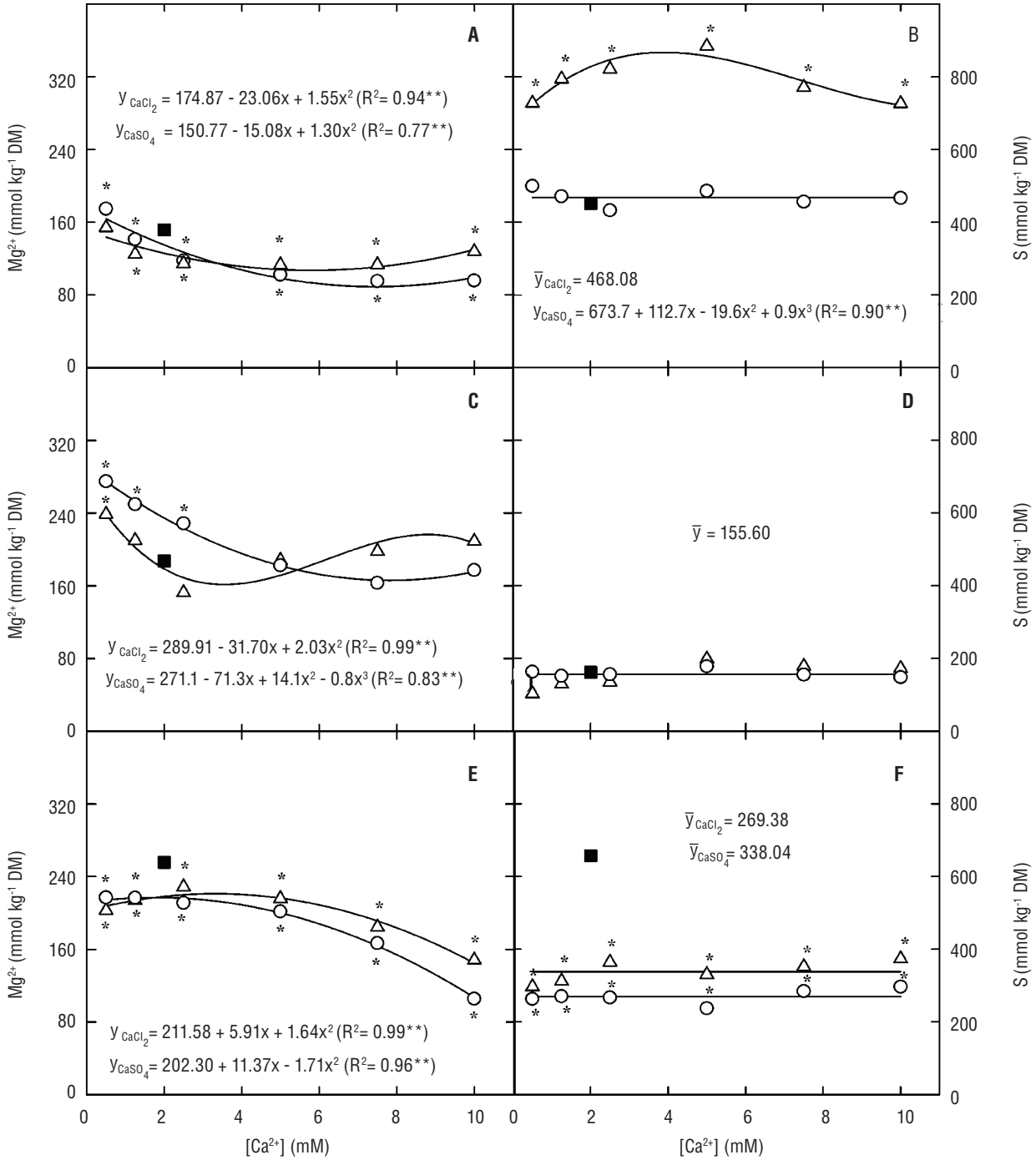

Figure 5. Magnesium (Mg^{2+}) and sulfur (S) contents in leaves (A and B), stems + petioles (C and D) and roots (E and F) of cowpea plants grown under control (nutrient solution with 2.0 mM calcium – filled square) and saline conditions (nutrient solution containing 75 mM NaCl plus $CaCl_2$ – open circle – or $CaSO_4$ – open triangle – at 0.5, 1.25, 2.5, 5.0, 7.5 and 10.0 mM). The asterisk denotes statistical difference between the Ca^{2+} treatments and control (Dunnet's test; $p \leq 0.05$).

Effects of salinity and Ca^{2+} on the S content depended on the plant part that was being analyzed (Figure 5). In leaves and roots, there was a significant ($p \leq 0.001$) interaction between the Ca^{2+} source and concentration, and the S content in the $CaSO_4$-supplemented plants was higher than that observed in the $CaCl_2$-supplemented ones (Figure 5). The root S content was reduced by salinity (Figure 5); however, neither salinity nor Ca^{2+} significantly affected the S content in the stems + petioles of cowpea plants (Figure 5).

DISCUSSION

Many interacting environmental variables contribute to salt tolerance/susceptibility in plants, including salt type and concentration, as well as developmental stage and cultivar. In previous papers, Pitiúba was considered a salt-tolerant cultivar (Costa et al., 2003; Maia et al., 2010). However, the results of the present study showed that a large proportion of the plant growth was inhibited by NaCl salinity, and only supplementation with $CaSO_4$ could counteract this inhibition (Figure 1). However, the magnitude of this beneficial effect was very moderate.

For most plant species, supplemental Ca^{2+} can reverse the adverse effects of salinity on growth and membrane permeability (Ebert et al., 2002; Kaya et al., 2002; Lacerda et al., 2004; Dabuxilatu and Ikeda, 2005; Onami and Hammes, 2006; Tuna et al., 2007). In general, the Ca^{2+} concentration that yields maximal growth under saline conditions varies between 5 and 10 mM, though important differences in the responses to supplemental Ca^{2+} between species and cultivars have been observed (Cramer, 2002). Our results confirmed that the growth of salt-stressed cowpea plants does not respond to supplemental Ca^{2+}, a finding which is supported by other studies in which supplemental Ca^{2+} did not alleviate (Schmidt et al., 1993; Cabot et al., 2009; Kwon et al., 2009), and, in some cases, even intensified (Silva et al., 2003), the effects of salinity on plants. According to Reid and Smith (2000), the restricted ability of Ca^{2+} to improve plant growth under saline conditions appears to be related to salinity-induced osmotic stress, which cannot be overcome by supplemental Ca^{2+}. Moreover, in the leaves and roots of salt-stressed cowpea plants, NaCl resulted in serious damage to membrane structures, and Ca^{2+} supplementation could not reduce this damage, therefore the limited response of cowpea plants to Ca^{2+} was also detectable at the cellular level (Guimarães et al., 2011).

Unlike plant growth, supplemental Ca^{2+} affected mineral nutrient uptake and distribution in salt-stressed cowpea plants. The addition of 75 mM NaCl to the growth medium significantly increased the Na^+ and Cl^- content in all cowpea tissues (Figure 2). As might be expected, supplemental Ca^{2+} reversed the increased Na^+ content in cowpea plants. In addition, Ca^{2+} reduced the Na^+ translocation to the shoot and retained this ion in the roots. Nevertheless, this was not enough to alleviate the effects of NaCl on plant growth (Figure 1). The reduced Na^+ content is considered the primary beneficial effect of Ca^{2+} on plants growing under NaCl stress (Rengel, 1992; Girija et al., 2002; Kaya et al., 2002). Apparently, in this species, the beneficial effects of Ca^{2+} on Na^+ toxicity reduction were surpassed by the osmotic effects, which were associated with the increase in total salt concentration (Silva et al., 2003). Surprisingly, in the roots, the $CaCl_2$ supplemented plants accumulated less Cl^- than the $CaSO_4$-supplemented ones (Figure 2). This was likely due to an increase in the transport rate of Cl^- from roots to shoot, which consequently led to higher Cl^- accumulation in the leaves and stems + petioles in these plants.

Supplemental Ca^{2+} provided as either $CaCl_2$ or $CaSO_4$ increased the Ca^{2+} content in cowpea plant tissues. In both Ca^{2+} supplementations, the Ca^{2+} content in the roots was much lower than that in the shoot (Figure 3). This phenomenon can be explained by the influx and translocation of Ca^{2+} to the shoot and its efflux through the root cells (Rengel, 1992). Maintaining an adequate supply of Ca^{2+} in saline soil solutions is an important factor to control the severity of specific ion toxicities (Grattan and Grieve, 1999).

Under saline conditions, high levels of external Na^+ not only interfere with K^+ acquisition by the roots, but may also disrupt the integrity of root membranes and alter their selectivity (Grattan and Grieve, 1999). In the present paper, there were increases in the K^+ content in salt-stressed cowpea regardless the Ca^{2+} source (Figure 3). This response, however, might simply be a consequence of reduced plant growth in saline conditions, and it is apparently unrelated to salt tolerance (Lacerda et al., 2004). Recently, Shabala et al. (2006) provided evidence for an additional mechanism of Ca^{2+} action on salt toxicity in plants: the inhibition of Na^+-induced K^+ efflux through outwardly directed, K^+- ermeable channels. In cowpea plants, it is likely that this alleviant effect of Ca^{2+} is responsible for increases in the K^+ content in the stems + petioles and roots, resulting from increased Ca^{2+} concentrations.

Frequently, when plants are exposed to NaCl, there is a decrease in the N content due to accumulation of Cl^- and competition of Cl^- with NO_3^- (Grattan and Grieve, 1998; Alam, 1999). In this experiment, the N content was higher in salt-stressed plants regardless the Ca^{2+} source in the growth medium (Figure 4). Increased N content in response to salinity was observed in other studies (Patel and Pandey, 2008; Keutgen and Pawelzik, 2009). However, as the nutrient contents were expressed in terms of dry mass, these increases in N content under saline stress could simply be a consequence of reduced plant growth (the concentration effect).

The influence of salinity on P accumulation in crop plants is variable and depends on the plant and on the experimental conditions (Grattan and Grieve, 1999; Alam, 1999). In cowpea plants, salinity favored P accumulation, which is in agreement with results previously obtained by Silva et al. (2003). In plants, there is an increase in the uptake and accumulation of P as its availability in the growth medium increases (Navarro et al., 2001), a condition that likely does not occur in plants growing in the field. Besides, little is known about the Ca^{2+} effects on P uptake and accumulation in salt-stressed plants. Regardless the concentration effect, the $CaCl_2$-supplemented plants had a higher P content in leaves and roots (over 5 mM Ca^{2+}) in comparison to $CaSO_4$-supplemented plants (Figure 4). Martinez and Läuchli (1991) observed an increase in the P translocation from roots to shoot, when cotton plants were subjected to 150 mM NaCl plus 10 mM $CaCl_2$, but it was not possible to determine whether this effect was due to increased P transport to leaves or to an indirect effect of Ca^{2+}. Similarly, no consistent relationship could be established between the supplemental Ca^{2+} and P accumulation in cowpea.

The reduction in the Mg^{2+} content of cowpea plants, mainly in the roots, occurred at high $CaCl_2$ or $CaSO_4$ concentrations (Figure 5). This effect was likely caused by competitive inhibition between Ca^{2+} and Mg^{2+}, since binding sites on the root plasma membrane appear to have a lower affinity for the highly hydrated Mg^{2+} than for Ca^{2+} (Marschner, 1995). In addition, a higher Mg^{2+} translocation from the roots to the shoot appeared to be detrimental to the Mg^{2+} accumulation in the root.

Little attention has been given to the salinity effects on the uptake and accumulation of S in plants (Grattan and Grieve, 1999). In the present paper, the S content was mainly dictated by the counter ion of Ca^{2+} salts used in this experiment (Figure 5). The highest S content in leaves and roots of $CaSO_4$-supplemented plants was likely due to an increased SO_4^{2-} concentration in the growth medium. On the other hand, the reduced leaf S content observed at Ca^{2+} concentrations of greater than 5.0 mM was likely due to S losses through the leaf stomata in the form of SO_2 (Marschner, 1995).

In conclusion, salinity significantly reduced plant growth and affected the uptake and distribution of nutrients in cowpea plants. Although the $CaSO_4$-supplemented plants have shown a trend towards increased LA and shoot, the addition of Ca^{2+} in the growth medium as either $CaCl_2$ or $CaSO_4$ did not improve plant growth. These findings confirm that cowpea growth is not responsive to supplemental Ca^{2+}.

In general, the NaCl-induced changes on nutrient content depended on the Ca^{2+} source and/or concentration. The increased Ca^{2+} concentration partially restored the Na^+/Ca^{2+} balance, reducing Na^+ concentrations and increasing Ca^{2+} ones in shoot and roots regardless of the Ca^{2+} source, but this effect was insufficient to alleviate the effects of NaCl on plant growth. Based on these observations and on the findings of previous studies, we concluded that supplemental Ca^{2+} can alleviate Na^+ toxicity and can influence the mineral nutrient content in cowpea plants, but this effect is insufficient to overcome the osmotic effects associated with increased total salt concentration.

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