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Growth, nutritional status and nitrogen metabolism in *Vigna unguiculata* (L.) Walp is affected by aluminum

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Abstract

Al toxicity is one of the most important abiotic stresses that reduce the growth and development of plants. From a physiological standpoint, Al affects the activity of several important enzymes such as nitrate reductase, which is the gateway of nitrogen in plants, and also triggers nutritional disorders and reduced plant growth. The content of nitrogen compounds, macronutrients and growth were assessed in order to better understand how cowpea plants respond to the toxicity of Al in a nutrient solution. The study was conducted in a greenhouse in a completely randomized design, consisting of the concentrations of 0 and 1 mM of Al. After ten days of applying the treatments we evaluated the activity of nitrate reductase, concentrations of protein and total soluble amino acids, proline, ammonia, macronutrients, and dry weight of the shoots and roots. Al reduced the nitrate reductase activity (-16%), concentration of nitrate (-22%), protein (12%) amino acids (-14%) and proline (-66%), however the concentration of ammonia increased (27%). Similarly, among concentrations of macronutrients that of calcium was most reduced (-21%), followed by magnesium (-16%) phosphorus (-19%) and potassium (-18%). The production of shoot and root dry matter was reduced by 18 and 27% when applying Al. The cultivar of cowpea Pretinho precoce 1showed to be sensitive to Al toxicity as a result of its inability to promote a biochemical response and satisfactory nutritional production when subjected to Al toxicity, which justifies the need for adoption of liming processes in the production of this cowpea crop which is appreciated by small agricultural producers in the North and Northeast regions of Brazil.

Keywords: Al, cowpea, nitrate reductase, nitrogen compounds, macronutrients.
Abbreviations: Al_Al; NR_nitrate reductase; Total soluble proteins_TSP; Total soluble amino acids_TSA; Adenosine triphosphate_ATP; Ca_Calcium; Mg_Magnesium; K_Potassium; Phosphorus; NH_4^+_Amonnia; NO_3^-_Nitrate; H^+_Hydrogen; $Al_2(SO_4)_36H_2O$ $_3$_Al sulfate; FM_Fresh matter; DM_Dry matter; SD_Standart deviation.

Introduction

Aluminum (Al) is one of the most abundant metals in the earth's crust. Most is in the form of non-phytotoxic aluminosilicate. However, hydrolysis of Al to the cation Al^{3+} under acidic conditions makes it soluble and toxic to plants. One of the abiotic factors that compromises the normal growth and development of plants is the presence of Al toxins associated with acidity and low base saturation, a fact that results in a variety of nutritional deficiency symptoms that contribute to lower crop yields. Al absorbed by the plant tends to preferentially accumulate in the root apex, inhibiting root elongation and cell division (Kochian et al., 2005). Studies show the negative role of Al in the metabolism of plants, especially with regards to nitrate reductase activity and levels of nitrogenous compounds such as amino acids and proteins (Justino et al., 2006; Ruiz et al., 2007; Cruz et al., 2011) given than NR is a key enzyme of the nitrogen metabolism and nitrogen is associated with crop productivity. However, in situations of stress such as that induced by Al, there is an increase in the concentration of proline, an amino acid which plays an important role in plants because of its participation as an attenuation agent of the effects of various abiotic stresses and by exerting important biochemical functions, for example, osmotic adjustment, stabilization of subcellular structures, detoxification of free radicals and source of nitrogen (Kavi Kishor et al., 2005; Ashraf and Foolad 2007). In plants, there is a close correlation between concentrations of proline and stress tolerance (Ashraf and Foolad, 2007; Giannakoula et al., 2010), including stress caused by Al (Giannakoula et al., 2008). Besides having a negative effect on physiology, mineral metabolism and plant growth, this metal induces premature senescence manifested by the increase of ammonia content in plant tissues due to protein degradation (Balestrasse et al., 2006), a fairly common process in stress conditions for plant growth. In general, plants present reduced leaf concentrations of macronutrients due to Al toxicity, as evidenced in rice cultivars with differential tolerance to Al (Mendonça et al., 2003). As an initial symptom of its toxicity, a reduction and thickening of roots is observed with consequent impact on the nutritional status of the plants, because Al competes with other nutrients for absorption sites in the root system, triggering a nutritional imbalance. Reduction in the concentration of macronutrients implies decreased growth of both the shoot and root systems and the appearance of nutritional disorders represented by nutritional deficiency in plants. However, maintenance of satisfactory levels of macronutrients in the tissues of plant roots and shoots has characterized genotypes as tolerant to Al (Giannakoula et al., 2008). *Vigna unguiculata* (L.) Walp. (*V. unguiculata*) is one of the important crop species and belong to the subfamily Papilionaceae of the family Leguminosae (Manivannan et al., 2007). *V. unguiculata* is one of the most important legumes for the human kind and has long been used as food particularly because its seeds provide vitamins, minerals, and good quality dietary proteins ranging from 23% to 32% of the seed dry weight, besides to be rich in lysine and tryptophan (Moura et al., 2014). Cowpea is grown to obtain seeds and pods for human consumption, as a source of green manure and organic material on unproductive soils, primarily in semi-arid regions (Murillo-Amador et al., 2006). Although the species *V. unguiculata* is a rustic plant tolerant to salt (Souza et al., 2011) and water stress (Nascimento et al., 2011), the impact of Al toxicity on nitrogen metabolism, macronutrient concentrations and growth in this species is poorly reported in literature. This justifies the need for a better understanding of its growth in toxic conditions induced by Al. In this study, Al toxicity was assessed in hydroponic growing conditions of *V. Unguiculata* plants. The combination of biochemical analysis of nitrogen metabolism components, leaf macronutrients and growth were conducted to better understand the effects of Al on *V. unguiculata* plants.

Results

Al contents

Imposition of Al treatments increase Al contents observed in the leaves of cowpea in the treatment of 1 mM were significantly high ($p < 0.05$), and not detected in leaves of the control plants (Fig. 1).

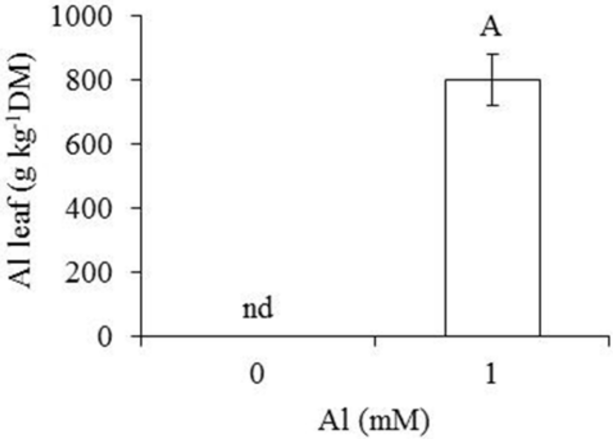

Fig 1. Al concentration in the leaves of cowpea submitted to Al toxicity. Values are means ± SD (n=15). nd, not detected.

Nitrate reductase activity and nitrate content

The activity of nitrate reductase (NR) was significantly affected ($p < 0.05$) by Al with reduction of 16% in relation to the control treatment (Fig. 2A). Along with the lower NR activity found in plants treated with 1 mM of Al, there was a significant reduction ($p < 0.05$) in the concentration of nitrate in the leaves of plants of the same treatment, with observed reduction of 22% compared to control plants (Fig. 2B).

Nitrogen compounds contents

Nitrogen compounds in cowpea leaf tissues were affected by toxic levels of Al, where the concentration TSP was significantly reduced ($p < 0.05$) by the imposition of Al toxicity (Fig. 3A) which was 12% lower compared to plants of the control treatment. The same response pattern was observed in the concentration of TSA (Figure 3B) and proline (Fig. 3C) for which significant reductions were observed ($p < 0.05$) of 14 and 66% in relation to the control plants, respectively. However, despite the negative impact of Al on the levels of TSP, TSA and proline, a significant increase ($p < 0.05$) of 27% was observed in the foliar concentration of free ammonia in plants grown in the presence of 1 mM of Al (Fig.3D).

Leaf macronutrients contents

Leaf concentrations of macronutrients in cowpea plants were significantly reduced by the toxic action of Al ($p < 0.05$), verifying decreases of 21, 16, 19 and 18% in the concentrations of calcium (Fig. 4A), magnesium (Fig. 4B), phosphorus (Fig. 4C) and potassium (Fig. 4D), respectively, relative to plants of the control treatment.

Dry mass shoot and root

The dry mass of the shoots was significantly reduced ($p < 0.05$) by the toxic level of Al (Fig. 5A), where the plants subjected to 1 mM of Al showed an 18% decrease compared to the control treatment plants. Similar to the shoots, root dry mass of the plants submitted to 1 mM of Al was significantly reduced, noting a 30% decrease in relation to roots of the control plants (Fig. 5B).

Discussion

In this study Al treatments increase Al contents in leaf cowpea (Fig. 1). Similarly, Maejima et al. (2014) observed higher Al content in shoots in the presence and absence of phosphorus in a nutrient solution for rice subjected to 0.1 mM Al. In conditions of low soil pH, a considerable number of exchange sites of the mineral constituents able to establish cation exchange are occupied by Al, replacing cations such as Ca and Mg , where Al is in its soluble form and is readily absorbed by plants. However, high levels of Al in the plant shoots may reflect its inefficiency to neutralize the Al phytotoxicity in the rhizosphere. For example, synthesis and exudation of organic chelating agents such as citric, malic and acid oxalic are strategies adopted by plant genotypes expressing some level of tolerance to Al in growth conditions of acidic substrate, as was observed in the cowpea cv. Lulutong 1, which synthesizes and exudes greater quantities of malate in growth conditions presenting toxic levels of Al (Yan et al., 2012). However, the cowpea cultivar adopted in this study appears to possess little ability to restrict the absorption and translocation of Al to the shoots (Fig. 1), suggesting that the strategy of synthesis and exudation of organic chelates is quantitatively insufficient or that the dose of 1 mM is highly detrimental to the point of triggering physiological disorders and damages to plants. In this study the close relationship between NR activity and nitrate concentration was observed, since the availability of this substrate is one of the factors that compete for activation of this enzyme. In parallel with the reduced leaf NR activity, the nitrate concentration also decreased in cowpea plants submitted a 1 mM. Due to the fact that there was interaction

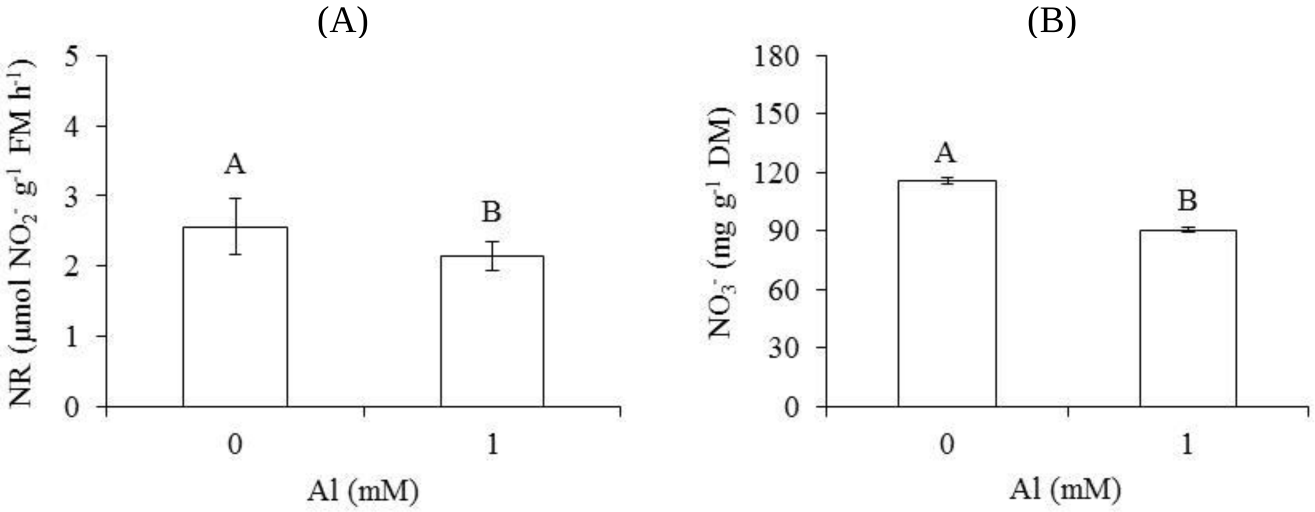

Fig 2. Activity of nitrate reductase (A) and concentration of nitrate (B) in leaves of cowpea submitted to Al toxicity. Values are means ± SD (n=15).

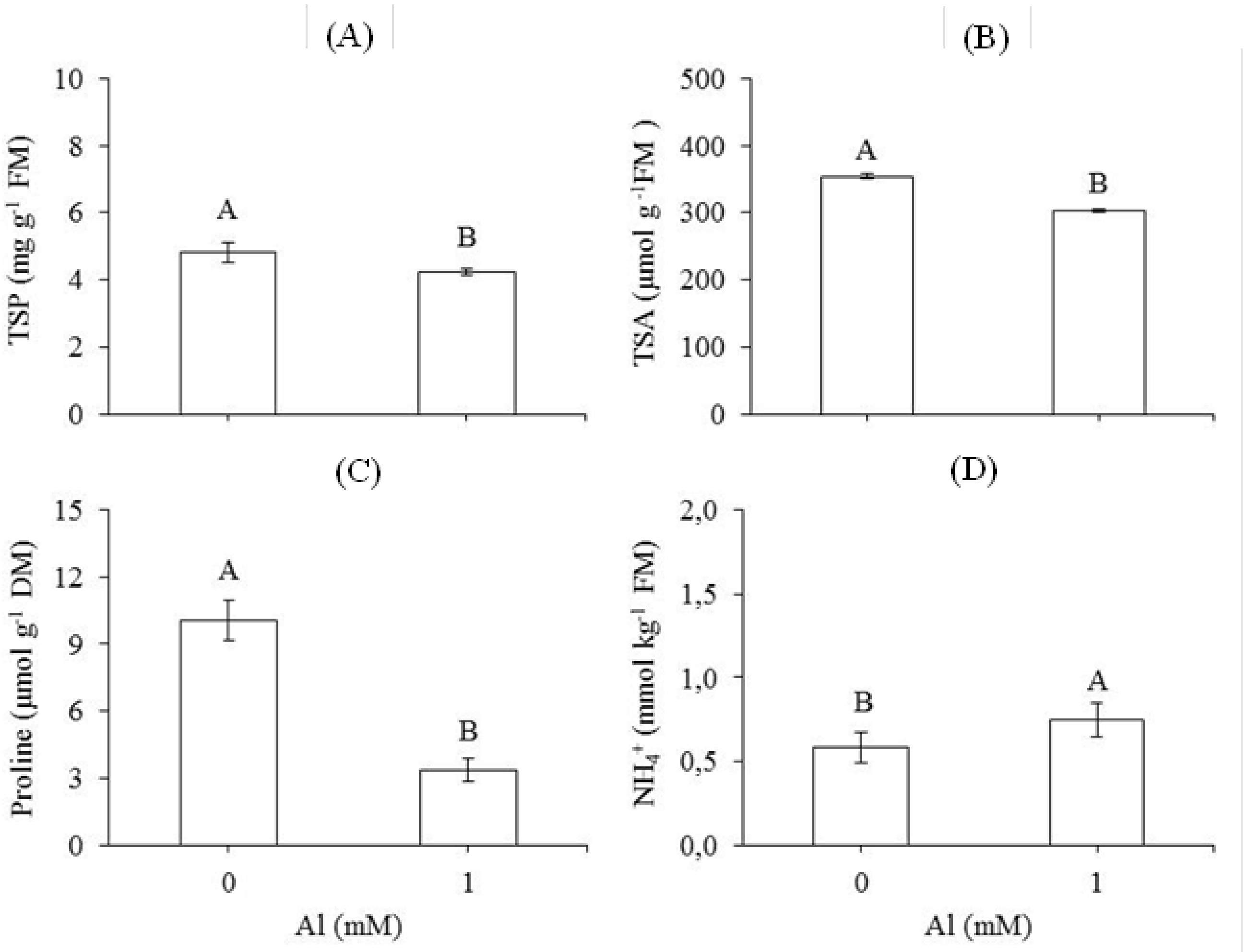

Fig 3. Concentration of total soluble proteins (A), total soluble amino acids (B), proline (C) and free ammonia (D) in leaves of cowpea submitted to Al toxicity. Values are means ± SD (n=15).

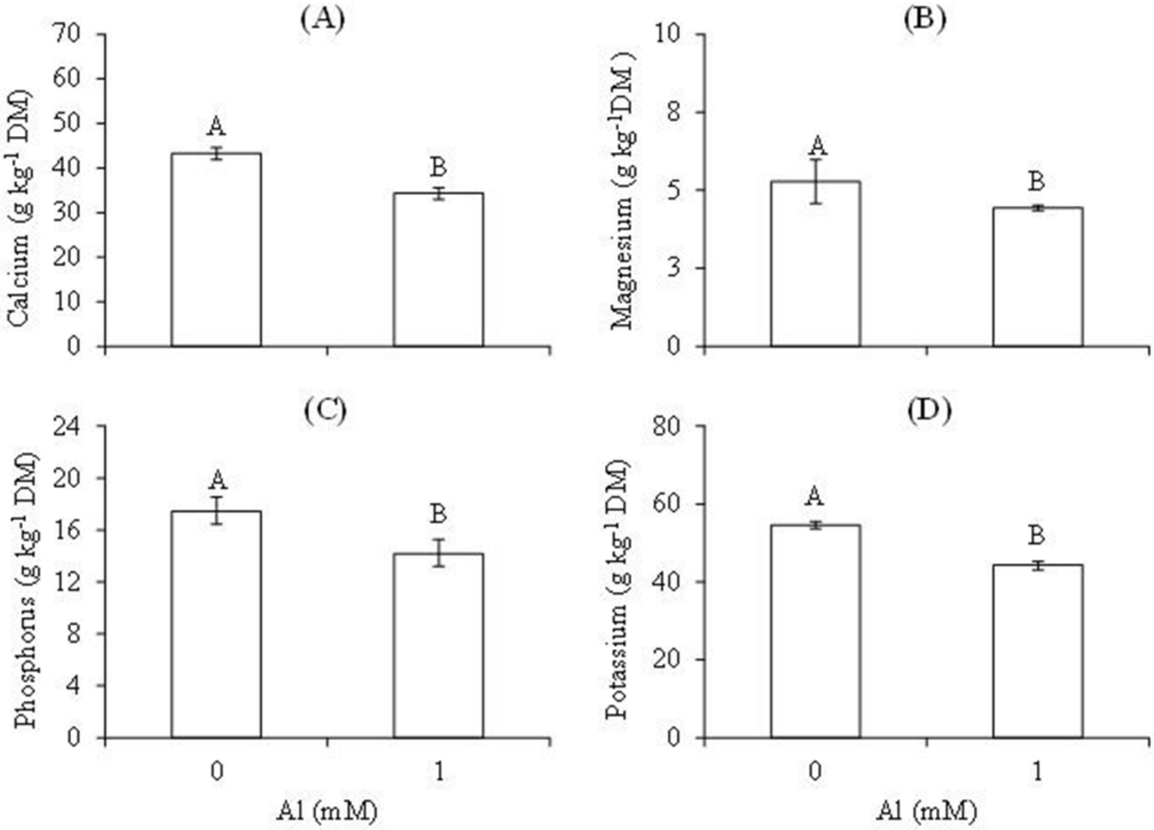

Fig 4. Concentration of the macronutrients calcium (A), magnesium (B), phosphorus (C) and potassium (D) in leaves of cowpea submitted to Al toxicity. Values are means ± SD (n=15).

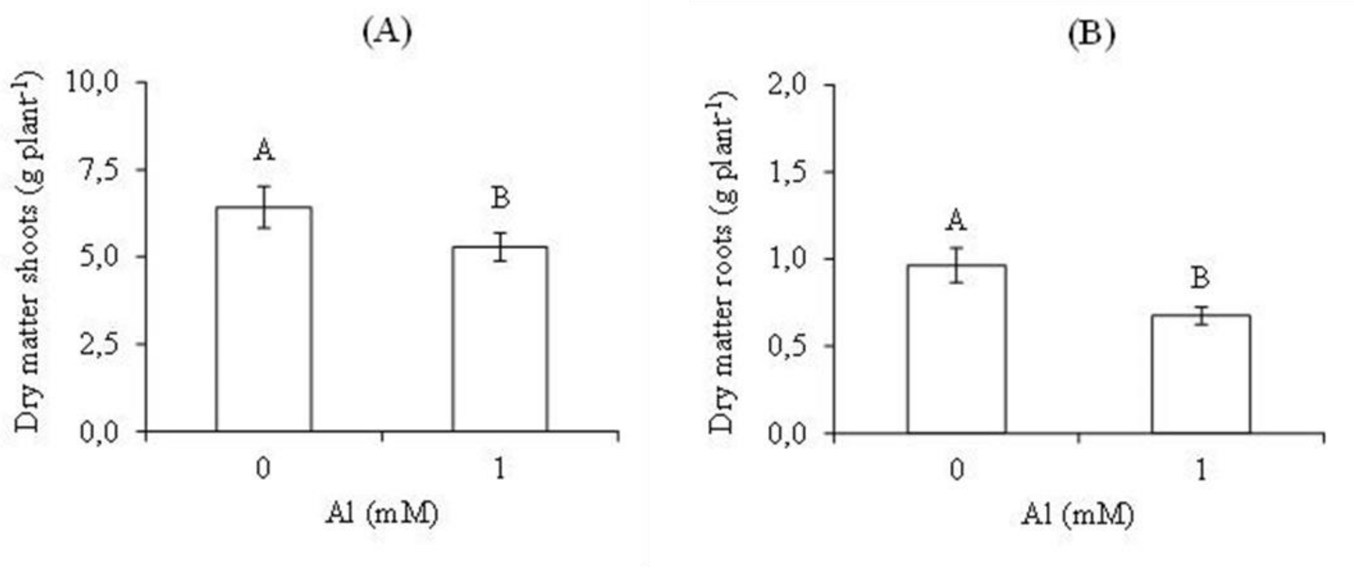

Fig 5. Dry matter in the shoots (A) and roots (B) of cowpea submitted to Al toxicity. Values are mean ± SD (n=15).

of Al with nitrate transporters in cortical and epidermal root cells in acidic medium (Durieux et al., 1993), the conformational structure of these specific membrane carriers may be altered, so that its functionality is compromised. This causes a reduction in the availability of nitrate as a substrate required for transcription of genes associated with the synthesis of this enzyme (Sharma and Dubey, 2005), since one of the primary factors that regulate *de novo* synthesis and NR activity is nitrate availability (Ruiz et al., 2007). Moreover, NR activity may be affected by the ability of Al to interact with sulfhydryl groups (-SH) of its amino acid cysteine residues, as was observed in leaves and nodules of *Vicia faba* (Shalaby and Al-Walkell, 1995), causing changes in the structure and function of this enzyme. In this sense, there are several pathways that compete for reduction of NR activity due to the ability of Al to interact with the enzyme structure and substrate, as in the operation of nitrate transporters. In studies of sorghum (Cruz et al., 2011) and rice plants (Sharma and Dubey, 2005; Justino et al., 2006) cultivated in nutrient solution, the activity of nitrate reductase was reduced by Al, indicating decreased activity of this key enzyme in nitrogen metabolism by Al, as observed in this study. In general, Al promotes negative changes in the nitrogen metabolism of plants from the process of absorption and reduction of nitrate (Justino et al., 2006) to its assimilation in organic compounds. In this study, the negative impact of this metal on the concentrations of TSP, TSA and proline is evident (Fig. 3A, B and C, respectively). Some authors have reported changes in the concentration of proteins with the energy demand required for protein synthesis represented by ATP, which in the presence of Al, suffers depletion of cell levels with possible impairment of mitochondrial activity (Yamamoto et al., 2002). Accordingly, protein synthesis in a final stage of Al toxicity would be reduced by the significant interaction of Al with binding sites of phosphoric groups of the ATP molecule, which would imply the reduction of cellular levels of this energy compound, sufficient for protein synthesis (Lorenc-Plucinska and Ziegler, 1996). This fact implies a reduction in the concentration of amino acids resulting from the degradation of proteins, including proline which is associated with the tolerance of plants to abiotic stresses (Verbruggen and Hermans, 2008). These results are consistent with those reported by Shalaby and Al-Walkell (1995) in *Vicia faba*, Ruiz et al. (2007) in *Heliantus annuus* and Cruz et al. (2011) in *Sorghum bicolor*. Regarding the amino acid proline, several studies have reported that tolerance to abiotic and biotic stress (Ashraf and Foolad, 2007; Verbruggen and Hermans, 2008) and its accumulation in plant tissues, among other functions, is attributed to maintenance of the water equilibrium status which can be disturbed by Al (Barceló and Poschenrieder, 1990). In this sense, proline would contribute to relieve water disturbances resulting from decreased hydraulic conductivity and extensibility of root cells (Gunsé et al., 1997) resulting from the toxic action of Al, acting in the acclimation of plants to stress caused by this metal, since osmotic homeostasis requires an increase in the absorption of solutes from the soil or synthesis of compounds with osmotic properties (Tester and Davenport, 2003). However, in this study an insignificant contribution of this amino acid to induce tolerance to Al is evident because of its low concentration in the leaves of plants subjected to 1 mM of Al (Figure 3C), indicating that Al affects a possible strategy of plants to use proline as a mitigating agent of stress induced by this metal, as observed in *Sorghum bicolor* (Cruz et al., 2011). Some authors have reported increased ammonia levels in the normal process of senescence in plants (Loulokakis et al., 1994). However, Al causes oxidative stress and indirectly causes premature senescence (Balestrasse et al., 2006), as was observed in nodules of soybean plants grown in the presence of 0.5 mM of Al (Balestrasse et al., 2006) as well as in nodules and roots of soybean plants cultivated under Al toxicity (Sandalios et al., 2001). Considering growth of cowpea plants under conditions of acidity and Al toxicity, the results encountered for leaf contents of ammonia suggest that the cultivar used in this study was not able to assimilate and/or reassimilate NH_4^+ to glutamate via the enzyme glutamate synthetase as evidenced by the high concentration of this compound in the leaves. One of the factors that may contribute to this response is the prevalence of NH_4^+ in detriment of NO_3^- in the conditions of acidity and Al toxicity experienced by cowpea plants (Kerbauy, 2008). Additionally, assimilation of NH_4^+ is affected by Al, since this metal compromises the process by altering the activity of enzymes able to incorporate it into amino acids (Purcino et al., 2003).

The results of this study clearly suggest that mineral metabolism of cowpea plants was affected by the toxic level of Al tested. The ability of Al to interact with membrane carriers is an important factor for reducing the concentration of macronutrients in leaf tissues and this interaction involves the decreased absorption of calcium by direct blocking of the absorption channels by Al (Rengel and Eliot, 1992). Another mechanism which contributes to reduce the calcium concentration in plant tissues is the ability of Al to compete for binding sites of Ca on the cell wall since this cation establishes ionic bonds between unesterified carboxyl groups of adjacent polygalacturonic chains, contributing to spatial conformation of pectin walls, because Ca is functionally involved in extensibility, stiffness and permeability of the cell wall (Rengel and Zhang, 2003). With respect to Mg, reduced concentration of this metal in plants occurs due to direct inhibition of absorption of this metal by Al (Giannakoula et al., 2008) or by stimulating the efflux of Mg and influx of H^+ induced by Al as observed in soybean, a strategy for promoting alkalization of the root surface to greater than pH 5.0 (Silva et al., 2001). Under low pH conditions, the interaction between Al and P results in the formation of complexes, and especially low solubility compounds such as $Al_4(PO_4)_3$ which reduces the absorption of Al and its translocation to the shoots. However, the emergence of P deficiency in plants has also been reported resulting from the interaction of these two metals which occurs in acidic medium and remains on the surface and inside root cells (Gaume et al., 2001), a fact that impedes the translocation of P to the shoots. Similar to calcium, reduction in the concentration of potassium appears to be linked to blocking of the channels of this metal by Al (Giannakoula et al., 2008). Therefore, plants considered tolerant to Al are able to maintain satisfactory nutrient concentrations while submitted to toxic levels of Al, as a result of physiological mechanisms involved in internal detoxification of Al (Piñeros et al., 2002) or by root exudation of organic acids (Piñeros et al., 2008) that promote chelating, and thus neutralizing of the toxic effect of Al. This fact allows for increases in nutrient uptake and translocation from the root to shoots, despite toxic levels of Al near the root system. Al causes extensive injury to the root system, leading to reduced water and nutrient uptake, constituting the root apex, meristem zone and elongation structures highly sensitive to Al and that easily accumulate it (Panda et al., 2009). It is known that root growth consists of cell elongation and division; however, a rapid inhibition of root elongation induced by Al suggests that the initial effect of the metal is probably caused more by inhibiting of elongation than cell division (Ma, 2007). According to this author, although cell division alone does not increase root growth, both the rate of cell division and the time period that mitotic cells remain active determine the supply of cells to the elongation zone, and therefore the rate of elongation. Accordingly, the inhibitory action of cell division caused by Al can affect root elongation in later stages (Ma, 2007) which depends on the intensity and duration of stress caused by Al, and especially the plant genotype studied. Thus, biometric alterations observed in the roots may have considerably contributed to trigger nutritional and biochemical disturbances in the shoots of plants subjected to treatment with 1 mM of Al, since the initial site of Al action resides in the root system and later the toxicity of this metal is manifested in the shoot. This was indicated by the variables analyzed, particularly the concentration of macronutrients, given the direct or indirect functional importance of these nutrients on plant growth and total soluble protein, by virtue of essential functions that these biomolecules exert on plants. Therefore, this study showed that the cultivar of cowpea Pretinho precoce 1was affected by Al toxicity indicated by its sensitivity to this metal in a nutritional, physiological and biometric plan, although Fery (1990) characterized this species as adapted to acid soils. The results encountered here suggest the need for adoption of liming in soils with high Al saturation or even the planting of this legume in soil with low saturation of Al, since it is a species highly valued in the North and Northeast regions of Brazil and contributes as an important source of protein in the diet of small farmers in these regions.

Material and Methods

Plant material and experimental site

The experiment was conducted in a greenhouse located at the Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias (UNESP - FCAV), Jaboticabal – São Paulo. Seeds of the cowpea cultivar Pretinho precoce 1 were initially sterilized with sodium hypochlorite 1% and then washed with deionized water for 10 minutes to remove excess NaClO. They were then sown in polystyrene plastic trays containing sterilized sand moistened with deionized water. Three days after sowing, seedlings showing uniform vigor were selected and transplanted in pots with 1.5 L of capacity containing the nutrient solution of Hoagland and Arnon (1950). The plants were maintained in a greenhouse with average irradiance of 1800 µmol m^{-2} s^{-1}, average relative air humidity of 60% and minimum/maximum temperatures of 27°C ± 1 and 34°C ± 1, respectively. At 26 days after sowing the treatments of Al toxicity were applied which consisted of the addition of $Al_2(SO_4)_3{\cdot}6H_2O$ at concentrations of 0 and 1 mM in pots containing nutrient solutions, finalizing the study 10 days after applying the Al treatments. The nutrient solution was aerated by its substitution at two day intervals. The pH of the solution of the control plants was maintained at 5.6 ± 0.1 and the plants grown under Al toxicity were maintained at 4.5 ± 0.1. Maintaining of the solution pH, when necessary, was performed by the addition of HCl or NaOH 0.1 N.

Experimental design

Experimental design was completely randomized with 2 treatments (0 and 1 mM of Al). Experiment was composed by 15 replicates and 30 experimental units, where each experimental was constituted by 1 plants.

In vivo nitrate reductase activity determination

The determination of NR (EC 1.6.6.1) was conducted based on a 0.2 g sample of fresh leaf tissue which was incubated in 5 mL of extraction buffer (0.1 M of KH_2PO_4; 50 mM of KNO_3; isopropanol 1% (v/v), pH 7.5) for 30 minutes at 30 ° C, where the entire procedure was conducted in test tubes protected from the light. Quantitation of enzyme activity *in vivo* was performed at 540 nm according to the method proposed by Hageman and Hucklesby (1971).

Nitrate determination

Nitrate extraction was performed using 1 g of dry matter incubated in 5 mL of deionized water and maintained in a waterbath at 100°C for 30 minutes. Next, the samples were centrifuged twice at 19,000 g for 30 minutes at room temperature, followed by collection of the supernatant. Nitrate quantification was performed at 410 nm according to Cataldo et al. (1975).

Total soluble proteins determination

The extraction of TSP was conducted using 0.1 g of fresh matter incubated in 5 mL of extraction buffer (Tris-HCl at 25 mM, pH 7.6). The homogenate was maintained under stirring for 2 hours, followed by centrifugation at 2000 g for 10 minutes at 20°C. Quantification of total soluble proteins was performed at 595 nm according to Bradford (1976) and the protein dosage was obtained according to a standard curve constructed with bovine serum albumin.

Total soluble amino acids

The total soluble TSA extract was obtained from 0.05 g of fresh matter incubated in 5 mL of deionized water maintained in a water bath at 100°C for 30 minutes. Next, the homogenate was collected and centrifuged at 2000 g for 5 minutes at 20°C and the supernatant collected. Quantification of total soluble amino acids was performed at 570 nm according to Peoples et al. (1989), using a standard curve made with L-glutamine.

Ammonia determination

Extraction of ammonia was conducted from 0.05 g of fresh matter incubated in 5 mL of deionized water at 100°C for 30 minutes. Next, the homogenate was centrifuged at 2000 g for 5 minutes at 28°C and the supernatant collected. Quantification of ammonia was performed at 625 nm according to Weatherburn (1967), using a standard curve made with ammonium sulfate.

Proline determination

Extraction of proline was performed using 0.05 g of dry matter which was incubated in 5 mL of deionized water at 100°C for 30 minutes. Next, the homogenate was centrifuged at 2000 g for 5 minutes at 20°C and the supernatant collected. Proline quantification was conducted at 520 nm according to Bates et al. (1973), using a standard curve made with L-proline.

Dry mass, macronutrients and Al determination

To determine the dry mass of the plants, they were collected, separated into shoots and roots, and placed in a forced ventilation oven at 65°C until reaching constant mass, then weighed on an analytical balance to determine the mass of dry matter. Concentrations of calcium, magnesium, potassium and phosphorus were determined according to Malavolta et al. (1997). Levels of Al, P and K were determined according to the methods of alumina, molybdate colorimetry and flame photometry, respectively. Levels of Ca and Mg, in turn, were determined by the atomic absorption spectrometry method.

Statistical analyses

All data were subjected to analysis of one-way variance (ANOVA) and mean comparisons were made using Tukey's multiple range test at 5% level of probability.

Conclusions

Al toxicity affects plant growth of cowpea where the structure most affected is the root system. Al has a negative impact on the concentration of nitrogenous components, nitrate reductase activity and levels of the macronutrients Ca, P, K and Mg which are important for growth of cowpea plants. This study highlights the sensitivity of the cultivar Pretinho precoce 1 to Al toxicity suggesting the need for adoption of liming in soils with high Al saturation or even the planting of this legume in soil with low saturation of Al, since it is a species highly valued in the North and Northeast regions of Brazil and contributes as an important source of protein in the diet of small farmers in these regions.

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