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Nitrogen compounds and enzyme activity in young muruçi (*Byrsonima crassifólia* L.) plant subjected to water stress

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Abstract

The objective of this study was to verify the nitrogen compounds and enzyme activity in muruçi plants subjected to water stress. The experiment was conducted in a greenhouse on 4-month-old seedlings. The experimental design was completely randomized with two water conditions (control and water stress), with 14 repetitions, totaling 28 experimental units. The parameters analyzed were relative water content, nitrate concentrations, ammonia, total soluble amino acids, total soluble proteins, proline and glycine betaine and the activities of the enzymes nitrate reductase (ARN) and glutamine synthetase (GS). The suspension of irrigation (water stress) for 25 days provided significant changes in all parameters. The suspension was sufficient to alter the metabolic and enzymatic pathways of young muruçi plants, reducing the relative water content, nitrate concentration and total soluble protein, nitrate reductase and glutamine synthetase activity. However, the suspension raised the free ammonium, total soluble amino acids, proline and glycine betaine concentrations in the plant parts. Therefore, these changes indicate that this species is susceptible and can change its metabolic pathways. However, some important enzymes in nitrogen metabolism cannot be activated, compromising the entire process of assimilation of this element in soils with low water availability, contributing to the knowledge about abiotic stresses in murucizeiro plants.

Keywords: nitrogen compounds; water stress; muruçi; osmoregulation; metabolic parameters.
Abbreviations: DM_ dry matter; FM_ fresh matter; GS_glutamine synthetase; GB_glycine betaine; RWC_ relative water content.

Introduction

The *Byrsonima* genus comprised of several fruit species popularly known in the Brazilian Amazon as muruçi and in other regions of Brazil as muricizeiro. *Byrsonima crassifolia* (L.) H.B.K., is a Brazilian Amazon plant and its center of origin, genetic diversity and spread is located in tropical America. Furthermore, it is a very important species, not only for being the most cultivated, but for presenting better quality fruits for consumption (Cavalcante, 2010). It is considered a food with good nutritional quality and has a variety of volatiles such as ethanol, butyl hexanoate, butyric acid, hexanoic acid and methyl butyrate in its composition, responsible for the distinctive aroma of the fruit (Alves et al., 2003). Moreover, it is rich in polyphenols and flavonoids, which gives it high antioxidant capacity and may be framed in the group of functional foods (Silva et al., 2007; Souza et al., 2008). Despite of known economic, social and cultural importance this species for small communities who use it for consumption and trade, little is known about its physiological and biochemical aspects, especially when the species is subjected to environmental conditions that influence their growth and development in periods and conditions of water stress in the soil (Pollardy, 2008; Almeida et al., 2011).

Stressful conditions is caused by lack of water leading to induce many changes in the metabolic pathways of carbon and nitrogen, where the nitrogen is the most demanded element for the cultures and its deficiency affects the growth and productivity of plants (Martinez et al., 2005; Debouba et al., 2007; Carvalho et al., 2012). The drought induces many physiological and biochemical processes. It is considered as a limiting factor for the development and growth of plants. Only plant species with drought resistance mechanisms can grow in environments with a minimum amount of water (Gonzales et al., 2012). Low water availability is currently the main environmental factor that restricts metabolic reactions and their disability affects plant growth and productivity. Water stress induces many physiological and biochemical processes such as amino acid synthesis. Other metabolisms, such as those of carbohydrates, proteins and other organic compounds, are also modified by water stress (Alves, 2010). It can cause changes in guard cell turgor during the opening and closing of stomata, controlling the transport of water with the variation of the stomatal opening (Mendes et al., 2013). Furthermore, water stress results in nitrate reductions and consequently in its enzyme from small decreases in water potential (Oliveira et al., 2011).

Among the various limiting factors for crop production, water stress occupies a prominent position due to affecting the water relationships in plants by altering their metabolism, a phenomenon occurring in large areas of farmland. The level of water stress of a plant is evaluated through variables related to leaves and roots (Pereira et al., 2012). The aim of this study was to evaluate the metabolic nitrogen answers depending on water availability in plants of *Byrsonima crassifolia* L., where the lack of knowledge on this biochemical process is evident.

Results

Relative water content, nitrate concentration, nitrate reductase activity and free ammonium concentration

The relative water content was significantly affected in muruçi plants after 25 days of water stress (Fig. 1A), with a reduction of 26.92% with water content values of 57%, when compared to control plants that obtained relative water content values of 78%.

A decrease in nitrate concentration was observed in both the root and in the leaves of plants subjected to water stress (Fig. 1B). The values found in the leaves were 0.38 and 0.05 µmoles of NO_3^-/Kg MS in control and water stress plants, respectively, a decrease of 86.8%. For the roots the values were 0.61 and 0.11 µmoles of NO_3^-/Kg MS in the control plants and water stress plants respectively, indicating a decrease of 81.9%.

In the nitrate reductase activity (Fig. 2C), a decrease in plants subjected to water stress was observed. The values found in the leaves were 0.18 and 0.06µmoles $NO_2^-.g.MF^{-1}.h^{-1}$ in control and water stress plants, respectively, presenting a decrease of 66.6%. For the roots the values were 0.32 and 0.04 µmoles $NO_2^-.g.MF^{-1}.h^{-1}$ in control plants and water stress plants, respectively, indicating a 87.5 % decrease.

An increase was observed in the free ammonium concentrations (Fig. 1D) in plants subjected to water stress. The values found in the leaves ranged from 5.83 to 9.24 mmol of NH_4^+/Kg of DM in control and water stress plants, respectively, an increase of 36.9%. In the roots it ranged from 10.1 to 17.87 mmol of NH_4^+/Kg of DM in control plants and water stress plants, respectively, presenting a 43.4% increase.

Glutamine synthetase (GS) activity, amino acid concentration and total soluble proteins

The glutamine synthetase (GS) activity decreased in plants under water stress presenting statistical differences, when compared to control plants (Fig. 2A). The values found in the leaves were 17 and 4.2 mmol de GGH/Kg DM in the control plant and water stress plant, respectively, a 75.29% decrease. The roots presented 12 and 5.94 mmol of GGH/Kg DM in control and water stress plants, respectively, indicating a 50.5% decrease.

An increase in total soluble amino acid concentrations was observed in both the root and in the leaves of plants subjected to water stress (Fig. 2B). The values found in the leaves ranged from 14 to 44.21 µmol of AA/g DM in control and water stress plants, respectively, showing an increase of 68.33%. The values found in roots ranged from 11.1 to 28.12 µmol of AA/g DM in control and water stress plants, respectively, a 60.52% increase.

A decrease in the concentrations of total soluble protein in both the root and in the leaves of plants subjected to water stress was observed (Fig. 2C). The values found in the leaves ranged from 2.4 to 1.2 mg protein/g DM in control and water stress plants, respectively, which is 50% reduction. The values found in the roots ranged from 1.6 to 0.47 mg protein/g DM in control and water stress plants, respectively, a reduction of 70.62%.

Concentration of proline and glycine Betaine

The proline concentrations increased in plants under water stress, presenting statistical differences, when compared to control plants (Fig. 3A). The values found in the leaves ranged from 5.18 to 12.91 µmol Pro/g DM in control and water stress plants, respectively, presenting an increase of 59.87%. The roots presented 2.23 and 8.43 µmol Pro/g DM in control plants and water stress plants, respectively, an increase of 73.54%. The glycine betaine concentrations increased in plants under water stress, presenting statistical differences when compared to control plants (Fig. 3B). The values found in the leaves ranged from 21.21 to 29.21 mg of glycine betaine/g DM in control and water stress plants, respectively, an increase of 27.38%. The values found in the roots ranged from 14.3 to 18.34 mg of glycine betaine/g DM in control and water stress plants, respectively, an increase of 22.02%.

Discussion

The decrease in the relative water content (Fig. 1A) can be explained by the retention of water molecules in the colloids, since the soil contains a low amount of water and release of this water to the plant takes place with greater difficulty. Chaum et al. (2010) point out that even the soil has low water availability the plant can still absorb water molecules in its colloids, but the amount is not enough for the growth. The root system is the first to detect water stress signals and sends the message to other plant cells and tissues (Geronimo, 2014). According to Silva et al. (2014), osmotic potential decreases due to soluble solute accumulation in cells, which is another response to water stress. The osmotic adjustment produces a more negative leaf water potential; thereby, helping to maintain the water movement towards the leaves, and consequently favoring their turgor, causing a decrease in the relative water content.

In a study by Sant'anna, (2009) the 'Sour' orange CRA reduced 31.72% and 'Rangpur' lime 32.45%, when compared to control plants. By lowering the relative water content (Fig. 1A), absorption and transport of NO_3^- by the roots tends to decrease due to low transpiration stream and may affect other biochemical factors (Souza et al., 2014). A recent research suggests that the nitrogen can regulate transpiration and NO_3^- concentrations modulate the hydraulic conductance of the roots, through the control of aquaporins (Matimati et al., 2014). Oliveira et al. (2011) observed similar results in the treatment of *Campomanesia* plants sp. under water stress.

The low level of this substrate directly influenced the enzyme activity responsible for its incorporation within the transpiration stream, the RN as is seen in Fig. 1C. The low water flow in the transpiration current decreased the nitrate reductase activity (Fig. 1C) in plants under water stress, probably due to the low energy level arising from photosynthesis. It brings a decreased transpiration with the nitrate flow (Fig. 1B) to the leaf since this enzyme is highly dependent on its substrate (Horchani et al., 2010).

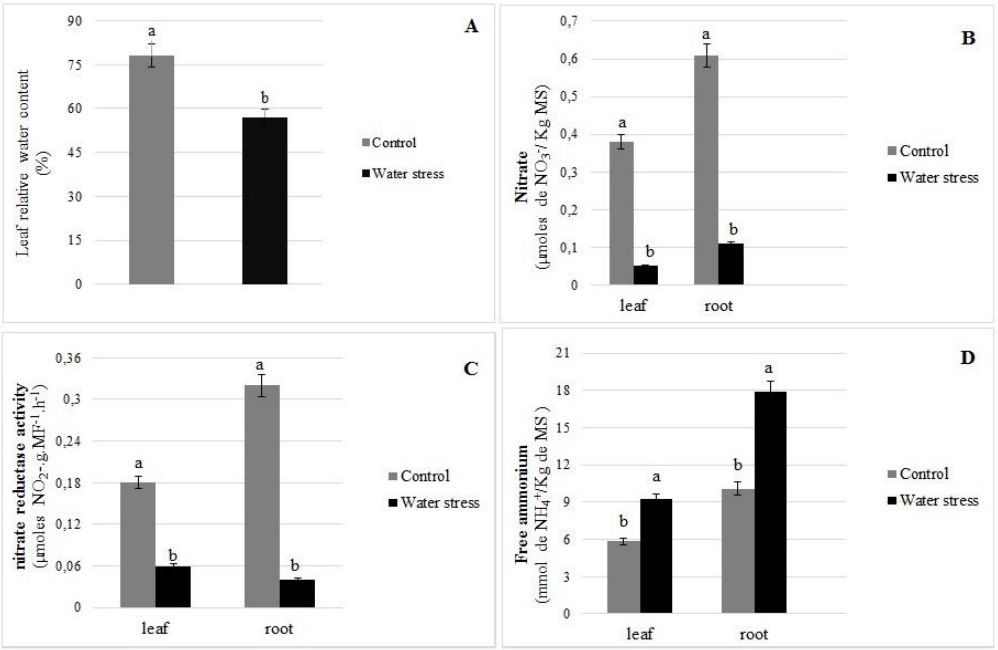

Fig 1. Leaf relative water content (A), Nitrate (B), nitrate redutase activity (C) and free ammonium (D) in leaves of young plants of murucizeiro subjected to 25 days of water stress. The letters a and b show statistically significant differences between treatments, compared by Tukey test at 5% probability. The bars represent the standard deviations of the means.

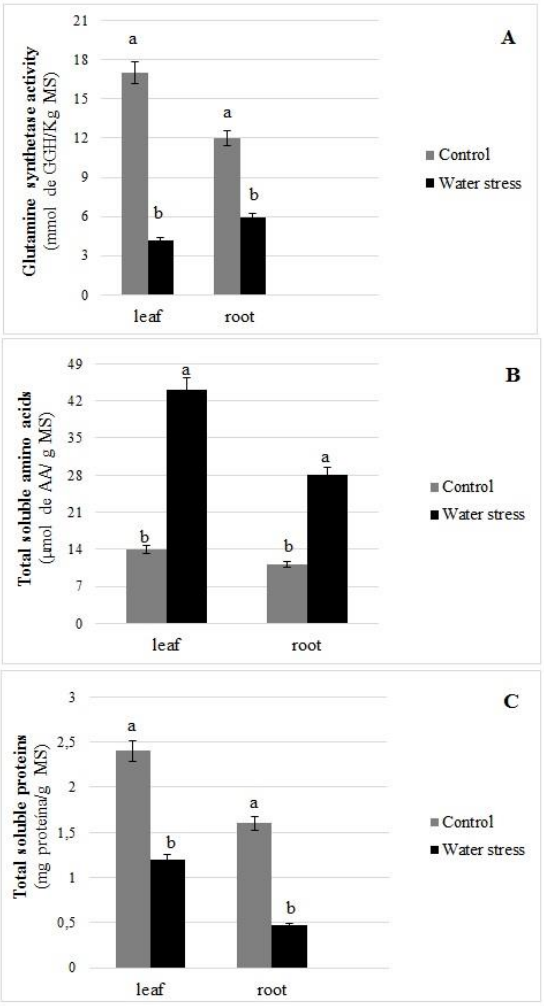

Fig 2. Glutamine synthetase activity (A), total soluble amino acids (B) and total soluble proteins (C) in leaves of young plants of murucizeiro after 25 days of water stress. The letters a and b show statistically significant differences between treatments, compared by Tukey test at 5% probability. The bars represent the standard deviations of the means.

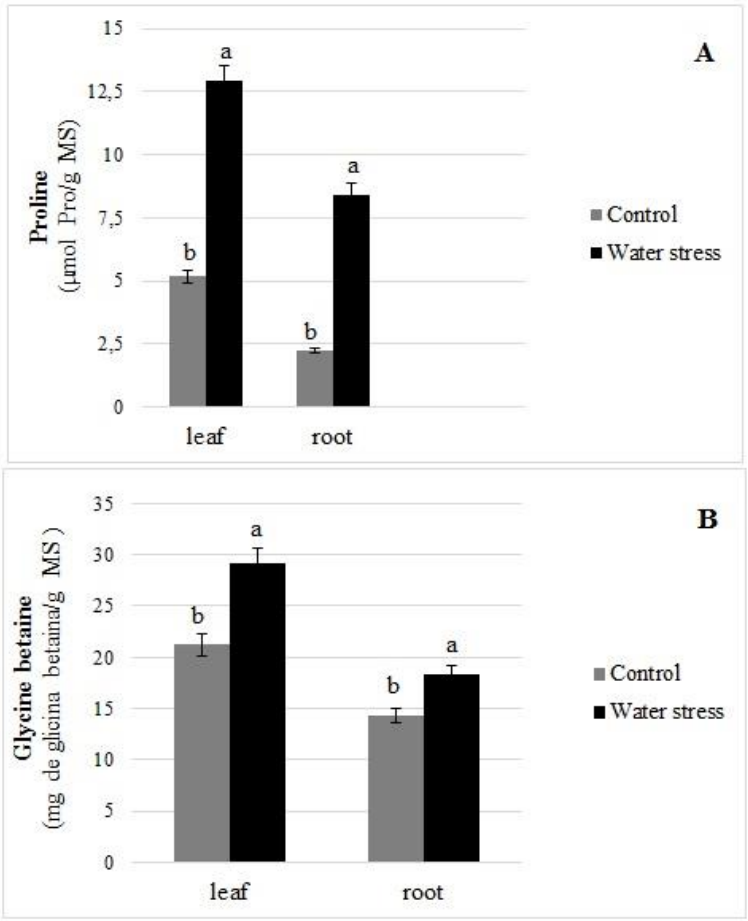

Fig 3. Proline (A) and glycine betaine (B) in leaves of young plants of murucizeiro after 25 days of water stress. The letters a and b show statistically significant differences between treatments, compared by Tukey test at 5% probability. The bars represent the standard deviations of the means.

Lima (2015), evaluated young andiroba plants subjected to 30 days of water stress and obtained a marked decrease in nitrate and nitrate reductase content. The increase in free ammonium concentrations (Fig. 1D) in plants under water stress can be related to other free ammonia formation pathways, like the pathways of glutamate dehydrogenase (GDH), which operates dependent on NADH, deaminating glutamate generating ammonium (NH_4^+) and 2-oxoglutarate; thereby, promoting ammonium accumulation due to its increased production and the reduction of the glutamine synthetase enzyme activity (Fig. 2A) (Lea and Miflin, 2011; Terce-Laforgue et al., 2013). Another possible explanation for the high ammonium concentrations is the low energy level for its assimilation, given that the assimilation of ammonium into amino acids is more feasible in terms of energy (Li et al., 2013). A study by Carvalho (2012) in the yellow ipê plant showed that ammonium concentrations increased in both leaves and roots when subjected to water stress conditions for 9 days.

Possibly, the reduction in glutamine synthetase activity (Fig. 2B), could be related to decreased photosynthesis according to Freitas (2014), showing that these conditions occur the low ATP concentrations in the cellular medium, where the enzyme is highly ATP dependent, arising from the photosynthetic activity in the leaves and the root respiratory process (Tercé-Laforgue et al., 2013). This result also indicates that the NH_4^+ used by GS may be due to photorespiration occurring in different isoforms and locations in the plant, since this enzyme needs ammonium as a substrate (Carneiro, 2014). Carneiro (2014) working with rubber tree seedlings noted that during 21 days of water stress the glutamine synthetase (GS) enzyme activity declined compared to activity in plants kept under field capacity.

The amino acid accumulation (Fig. 2B) may be due to decreased protein synthesis (Fig. 2C) due to proteolytic enzymes, reducing the translocation of amino acids to other organs (Maraghni et al., 2011), where this accumulation can be considered as a sign of plant tolerance to water stress leading the plant to metabolically adjust by decreasing osmotic potential (Carneiro, 2014). These compounds promote the turgor maintenance, and function as a nitrogen reserve for the possible resumption of growth, when environmental stress ceases or lessens (Oliveira Neto, 2010). Oliveira et al. (2013), working with 40 days of water stress in *Annona muricata* L. plants also reported an increase in amino acid concentrations in plants under water suspension. In the study developed by Lobato et al. (2009) in *Capsicum annuum* plants (cv. Red Giant) under water stress, a reduction was observed in the concentration of total soluble proteins.

Low water content in the plant promotes proline (Fig. 3A) as a means of adaptation, allowing the maintenance of vital processes as well as preserving protein, enzyme and

membrane integrity (Monteiro et al., 2014). This increase in the proline concentration was probably a result of its production by the metabolic pathway, which uses glutamic acid as a precursor, involving the coordinated action of the pyrroline 5-carboxylate synthase and reductase enzymes (Szabados and Savoure, 2010). Its increase can ensure better soil water absorption and transport to the areial part when acting as compatible osmolyte (Sousa et al., 2012). Similar results were found by Nogueira (2015) who submitted *Ochroma pyramidale* to water stress and found a large increase of proline compared to the control plants.

The increase in glycine betaine (Fig. 3B) in plants under water stress was probably due to formation of soluble amino acids (Fig. 2B) through the degradation of soluble proteins (Fig. 2C) and also to the high ammonium concentration (Fig. 1D), possibly derived from photorespiration (Mota et al., 2015). This increase contributes to obtaining a better soil water assimilation and transport to the aerial part through osmotic adjustment, also influencing cell membrane protection against oxidative stress in plants (Alves, 2010). Similar results were found by Lima et al. (2015) who exposed *Carapa guianensis* Aubl. Plants to the water stress and obtained an increase in the concentration of this metabolite in plants.

Materials and Methods

Plant materials

The seedlings obtained from the association of exporting industries of wood in the state of Pará (AIMEX). The seedlings age was 4-month-old (after germination). The seedlings were acclimatized in a greenhouse for a period of 03 months for ambiance.

Experimental conditions

The study was conducted at the Federal Rural University of Amazonia (UFRA), state of Pará, campus Capitão Poço, Brazil (Latitude 01º 44 '47' 'and longitude 47 03'34' '). This experiment was conducted in a greenhouse, with temperature of air minimum-maximum with values of 24.5 / 39.1 and 53.3% / 91%, respectively.

Substrate, pots and plant nutrition

The substrate used was a mixture in the proportion of 3: 1: 1 (v/v/v), black earth, chicken manure and earthworm humus, respectively. The polyethylene vessels were used in the dimensions 0.30 m × 0.30 m (height × diameter), and capacity of 20 kg. Corrections were made in the concentrations of macro and micronutrients from the soil and the pH soil, through the results of the soil chemical analysis in the laboratory of soils in Embrapa Eastern Amazon, applying 600 mL of complete nutrient solution (Hoagland and Arnon, 1950), divided in three months, for every month 200 mL of complete nutrient solution before the start of the experiment.

Experimental design and treatments

The experimental design was completely randomized with two water conditions (control and drought stress), with 14 repetitions, totaling 28 experimental units, where each experimental unit consisted of one plant per pot. The experiment was conducted from April to July 2013, in which the water suspension occurred in the 25 days period and the control plants were irrigated daily in an average of 400 mL of water to compensate the losses by evapotranspiration.

Leaf relative water content

The leaf relative water content was evaluated using leaf disks with 10 mm of diameter and it was carried out in each plant, in which 40 disks were removed and the calculation was done in agreement with the formula proposed by Slavick (1979):
$LRWC = [(FM_1 – DM)/(FM_2 – DM)] \times 100$
Where; FM_1 is fresh matter, FM_2 is turgid matter evaluated after 24 h and saturation in deionized water at 4°C in dark, and DM is the dry matter determined after 48 h in oven with forced air circulation at 80°C.

Concentrations of nitrate

For determination of nitrate, 100 mg of leaf dry matter powder was incubated with 5 ml of sterile distilled water at 100°C for 30 min, and the homogenized mixture was centrifuged at 3.000 g for 15 min at 25°C, and the supernatant was removed. The quantification of the nitrate was carried out at 410 nm in accordance to Cataldo et al. (1975), with KNO_3 (Sigma Chemical) as standard.

In vivo nitrate reductase activity

Nitrate reductase enzyme (E.C. 1.6.6.1) was extracted from 200 mg of leaf and root samples. and incubated in 5 mL of extraction buffer (KH_2PO_4 at 0.1 M, KNO_3 at 50 mM, isopropanol at 1% (v/v) and pH 7.5) for 30 minutes at 30ºC, and all the procedures were carried out in the dark. The quantification of the enzyme activity was in accordance to the method of Hageman and Hucklesby (1971) with absorbance at 540 nm using spectrophotometer (Quimis, model Q798DP).

Concentrations of free ammonium

Free ammonium was determined with 50 mg of leaf dry matter powder incubated with 5 ml of sterile distilled water at 100°C for 30 min, after the homogenized mixture was centrifuged at 2.000 *g* for 5 min at 20°C and the supernatant was removed. The quantification of free ammonium was carried out at 625 nm in accordance with Weatherburn (1967), with $(NH_4)_2SO_4$ (Sigma Chemical) as standard.

Glutamine synthetase activity

Extraction of the glutamine synthetase enzyme (E.C. 6.3.1.2) was carried out with 200 mg of leaf tissue ground in liquid nitrogen. The samples were then incubated in 5 mL of extraction mix (Tris-HCl buffer pH 7.6 containing 10 mM $MgCl_2$, 10 mM _-mercaptoethanol, 5% (w/v) PVP, and 5 mM EDTA), homogenized, centrifuged at 3.000 *g* for 10 min, and the supernatant was removed. All the procedures were carried out in the interval of 0-4°C. The quantification of the enzyme activity was carried out using the method of Kamachi et al. (1991) with absorbance at 540 nm, and gglutamyl hydroxamate (Sigma Chemicals) was used as a standard.

Concentrations of total soluble amino acids

Determination of amino acids was performed using 50 mg of leaf dry matter powder and incubated in 5 mL of sterile distilled water at 100ºC for 30 minutes. After incubation, the homogenized was centrifuged at 2.000 *g* for 5 minutes at

20ºC and supernatant was removed. Quantification of the total soluble amino acids was carried out at 570 nm according to Peoples et al. (1989), and L-asparagine + L-glutamine (Sigma Chemicals) was used as standard.

Concentrations of total soluble proteins

Determination of the total soluble proteins was carried out with 100 mg of powder, incubated with 5 mL of extraction buffer. This was homogenized and kept in agitation for 2 h, and centrifuged to 2.000 *g* for 10 minutes at 20ºC. Quantification of the total soluble proteins was carried out at 595 nm in accordance with Bradford (1976) with albumin bovine (Sigma Chemicals) as standard.

Concentrations of proline

Proline level was determined with 50 mg of leaf dry matter powder, which was incubated with 5 mL of sterile distilled water at 100 ºC by 30 minutes, after the homogenized was centrifuged to 2.000 g for 5 minutes at 20 ºC. Quantification of proline was carried out at 520 nm according to Bates et al. (1973), in which L-proline (Sigma Chemicals) was utilized as standard.

Concentrations of glycine betaine

The glycine betaine was determined with 25 mg of leaf dry matter powder, which it was incubated with 2 mL of sterile distilled water at 25°C by 4 h and under agitation, after the homogenized was centrifuged to 10.000 g by 10 min at 25°C and the supernatant was removed. The glycinebetaine quantification was carried out at 365 nm according to Grieve and Grattan (1983), in which was utilized glycinebetaine (Sigma Chemicals) as standard.

Data analysis

Data were subjected to variance analysis and when significant differences occurred, the Tukey's test at 5% level of error probability was applied. Standard errors were calculated for all means (Steel et al., 2006). The statistical analysis was carried out with the SAS software (SAS Institute, 2008).

Conclusion

Young muruçi plants subjected to 25 days of water stress showed significant changes in all parameters, reducing the relative water content, nitrate levels, nitrate reductase activity, glutamine synthetase and total soluble proteins, but increased the free ammonium levels, amino acids, glycine betaine and proline in the evaluated parts. Therefore, these changes indicate that these plants are susceptible and they can change their metabolic pathways; however, some important enzymes in nitrogen metabolism cannot be activated, compromising the entire process of assimilation of this element in soils with low water availability, contributing to the knowledge about abiotic stresses in murucizeiro plants.

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