Nitrogen compounds and enzyme activities in sorghum induced to water deficit during three stages

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ABSTRACT

The aim of this study was to evaluate and explicate the changes in the nitrogen compounds and enzyme activities in *Sorghum bicolor* plants (cv. BR-700) submitted to water deficit during vegetative, reproductive and maturation stages. The experimental design used was entirely randomized in factorial scheme, with two conditions (control and stress) combined with three stages (vegetative, reproductive and maturation). The nitrate level was increased at 180.6, 72.9 and 78.9% during the vegetative, reproductive and maturation stages, respectively. The total soluble amino acids and glycinebetaine increased only during vegetative and reproductive stages, if compared with control plants. However, besides protein levels during all stages, significant reductions were reported in nitrate reductase and glutamine synthetase activities; free ammonium presented decreases at 37.3 and 77.6% in vegetative and reproductive stages, respectively, when compared with control plants.

Keywords: *Sorghum bicolor* L.; nitrogen; nitrate; ammonium; water deficit

The sorghum crop (*Sorghum bicolor* L.) presents a great potential when used to forage yield, as it has a great adaptation capacity, as well as tolerance to high temperature, which are important characteristics for cultivation in environments under influence of abiotic stress as water deficit, salinity stress, heat stress and mineral stress (Oliveira et al. 2002, Lobato et al. 2008b). This species presents two important mechanisms of adaptation to water deficit; one is the water tolerance linked to biochemical metabolism with efficient osmotic adjustment, lower metabolic activity and fast recuperation after rehydration, whereas the other one is the escape due the deep and intense root formation (Silva et al. 2001).

Water deficiency in higher plants promotes different behaviors depending on plant stage, exposition time, and intensity of factors that can attenuate or intensify the consequences (Pimentel 2004). Water deficit normally presents changes in compounds and enzyme activities linked to nitrogen metabolism; moreover, lower protein formations, amino acids accumulation as glycinebetaine and praline are reported, besides reduction in nitrate reductase and glutamine synthetase activities (Lobato et al. 2008c, 2009).

The aim of this study was to evaluate and explicate the changes in the nitrogen compounds and enzyme activities in *Sorghum bicolor* plants (cv. BR-700) submitted to water deficit during vegetative, reproductive and maturation stages.

MATERIAL AND METHODS

Experimental conditions. The study was carried out in the Instituto de Ciências Agrárias (ICA) of the Universidade Federal Rural da Amazônia (UFRA), Pará State, Belém City, Brazil (01°27'S, 48°26'W), during the period between May and August of 2007. The experiment was conducted in greenhouse under natural day/night conditions (the air temperature minimum/maximum and relative humidity during the experimental period

were 24.5/39.7°C and 40/89%, respectively). The photoperiod medium was of 12 h of light and the photosynthetic active radiation (PAR) was 968 μmol/m^2s (at 12:00).

Substrate, pots and plant nutrition. The substrate used for plant growth and development was a mix in proportion 3:2 (v/v), soil and organic matter, respectively. The pots had the dimensions of 0.3 × 0.3 m (height × diameter) and capacity of 21.1 l or 18 kg of the substrate used. The soil is classified as yellow latosol and presented medium texture, in which it was previously dried and filtered (10 mm) to remove all impurities. The soil chemical analysis was carried out, the pH was adjusted to 6.0 and the macro and micro nutrients were applied using nutritive solution of Hoagland and Arnon (1950) modified to crop, in which the nitrogen amount was 10 mg/kg of substrate. The supplementations were applied three times: 15 days before the experiment implantation, 30 days and 60 days after the experiment implantation.

Plant material. The seeds of the sorghum cultivar BR-700 were used in this study, which were developed and obtained from the Empresa Brasileira de Pesquisa Agropecuária – Milho e Sorgo (Embrapa/Brazil), harvested in the 2006 season.

Experimental design. The experimental design used was entirely randomized in factorial scheme, with two conditions (control and stress) combined with three stages (vegetative, reproductive and maturation; 30, 60 and 90 days after the experimental implementation, respectively); the experiment comprised five repetitions and 30 experimental units (each plant being one experimental unit).

Plant conduction. The seeds were placed into substrate and the seedlings were thinned in the 6^{th} day after the experimental implantation to have only one plant/pot. The plants were submitted to two conditions (control and stress), in which the water deficit was simulated by the period of 15 days. The control plants were normally irrigated during all the experimental period; however the stress plants were induced to water suspension during the periods 15^{th} to 30^{th} day, 45^{th} to 60^{th} day and 75^{th} to 90^{th} day after the experimental implementation; these periods were denominated as vegetative, reproductive and maturation stages, respectively. The plants were harvested in the 30^{th}, 60^{th} and 90^{th} day and the physiological and biochemical parameters were evaluated.

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):

$$LRWC = [(FM - DM)/(TM - DM)] \times 100$$

where: FM is fresh matter, TM 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.

Nitrate reductase activity. The extraction of the nitrate reductase enzyme (E.C. 1.6.6.1) was carried out with leaf disks until the weight of 200 mg was reached, the samples were incubated in 5 ml of extraction mix [0.1M KH_2PO_4, 50mM KNO_3, isopropanol at 1% (v/v) and pH 7.5] by 30 min at 30°C, and all the procedures were carried out in dark. The quantification of the enzyme activity was made by the method of Hageman and Hucklesby (1971) with absorbance at 540 nm and using spectrophotometer (Quimis, model Q798DP), nitrite (Sigma Chemicals) was used as standard.

Glutamine synthetase activity. The extraction of the glutamine synthetase enzyme (E.C. 6.3.1.2) was carried out with 200 mg leaf tissue ground in liquid nitrogen, the samples were incubated in 5 ml of extraction mix [Tris-HCl buffer pH 7.6 containing 10mM $MgCl_2$, 10Mm β-mercaptoethanol, 5% (w/v) PVP, and 5mM EDTA]; after the homogenized mixture was centrifuged at 30 000 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 made by the method of Kamachi et al. (1991) with absorbance at 540 nm; γ-glutamylhydroxamate (Sigma Chemicals) was used as standard.

Leaf powder. The leaves were harvested and placed in oven with forced air circulation at 70°C for 96 h. The leaf dry matter was triturated and the powder was kept in glass containers, where it remained in the dark and under the temperature of 15°C until the moment to carry out the biochemical analysis.

Nitrate. Nitrate was determined with 100 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 10 000 g for 15 min at 25°C and the supernatant was removed. The quantification of the nitrate was carried out at 410 nm in agreement with Cataldo et al. (1975), KNO_3 (Sigma Chemical) was used as standard.

Glycinebetaine. The determination of glycinebetaine was carried out with 25 mg of powder

incubated with 2 ml of distilled water. The homogenized mixture was kept in agitation for 4 h at 25°C, after this period centrifuged at 10 000 g for 10 min at 25°C and subsequently the supernatant was removed. The quantification of glycinebetaine was carried out at 365 nm in agreement with Grieve and Grattan (1983), glycinebetaine (Sigma Chemicals) was used as standard.

Free ammonium and amino acids. The free ammonium and amino acids were 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 2000 g for 5 min at 20°C and the supernatant was removed. The quantification of the free ammonium was carried out at 625 nm in agreement with Weatherburn (1967), $(NH_4)_2SO_4$ (Sigma Chemical) was used as standard. The 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) were used as standard.

Total soluble proteins. The determination of the total soluble proteins was carried out with 100 mg of powder incubated with 5 ml of extraction buffer (Tris-HCl at 25mM and pH 7.6). The homogenized was kept in agitation for 2 h, after this period centrifuged at 2000 g for 10 min at 20°C and subsequently the supernatant was removed. The quantification of the total soluble proteins was carried out at 595 nm in agreement with Bradford (1976), albumin bovine (Sigma Chemicals) was used as standard.

Data analysis. The data were submitted to variance analysis and when significant differences occurred the Tukey test at 5% level of error probability was applied; the standard errors were calculated in all evaluated points. The statistical analysis was carried out with the SAS software.

RESULTS AND DISCUSSION

Leaf relative water content. The results revealed significant decrease of 23, 35 and 42% in vegetative, reproductive and maturation stages, respectively, in relation with control plants and within each stage (Figure 1A). In addition, the vegetative stage presented higher values in control and stress plants.

The decrease obtained in all the stages result from the lower water absorption from substrate and consequent reduction in cell turgor. In this study, these reductions showed to promote lower growth and development, flower aborting and reduction in yield during vegetative, reproductive and maturation stages, respectively. The control and stress treatments in maturation stage presented lower leaf relative water content, if compared with others stages. In control plants this fact is probably linked to senescence process, which causes a decrease of water and nutrient absorptions promoting thus cell death in the root tissue, mainly the root hairs. Stress plants during maturation stage were also more sensitive, probably due to the transpiration rate higher than the water absorption carried out by the roots.

Nitrate. The results in nitrate reveal a significant difference among the treatments; we obtained an increase by 180.6, 72.9 and 78.9% during the vegetative, reproductive and maturation stages, respectively (Figure 1B).

The values obtained in nitrate level were probably promoted by lower nitrate reductase activity, in which this enzyme is responsible for nitrate reduction (NO_3^-) to nitrite (NO_2^-). Similar results were reported by Costa (1999) working with *Vigna unguiculata* under water deficit.

Nitrate reductase activity. The results of nitrate reductase present a significant decrease by 98.2, 84.2 and 98% in the vegetative, reproductive and maturation periods, respectively (Figure 1C). In addition, the maximum activity was obtained during the reproductive stage in control and stress plants.

The reduction in nitrate reductase activity is directly linked to degradation and consequent inactivation of this enzyme under limited water supplement (Lobato et al. 2008a). Moreover, lower water absorption of the soil/substrate promotes a decrease in the transpiration rate and indirectly reduces nitrate influx, since this enzyme depends on nitrate coming from soil/substrate (Plhák 2003). The maximum activity in reproductive stage was probably promoted by grain filling, as this period increases the exigency and translocation of nitrogen compounds to seed and panicle.

Free ammonium. The free ammonium levels were decreased by 37.3 and 77.6% in the vegetative and reproductive stages, respectively. However, in the maturation stage a significant increase by 43.6% was reported compared to control treatment (Figure 2A). In addition, the plants submitted to water deficit presented lower ammonium level during the reproductive stage.

The decrease in free ammonium is a direct consequence of reduction in nitrate reductase activity, in which it is the first enzyme for nitrogen

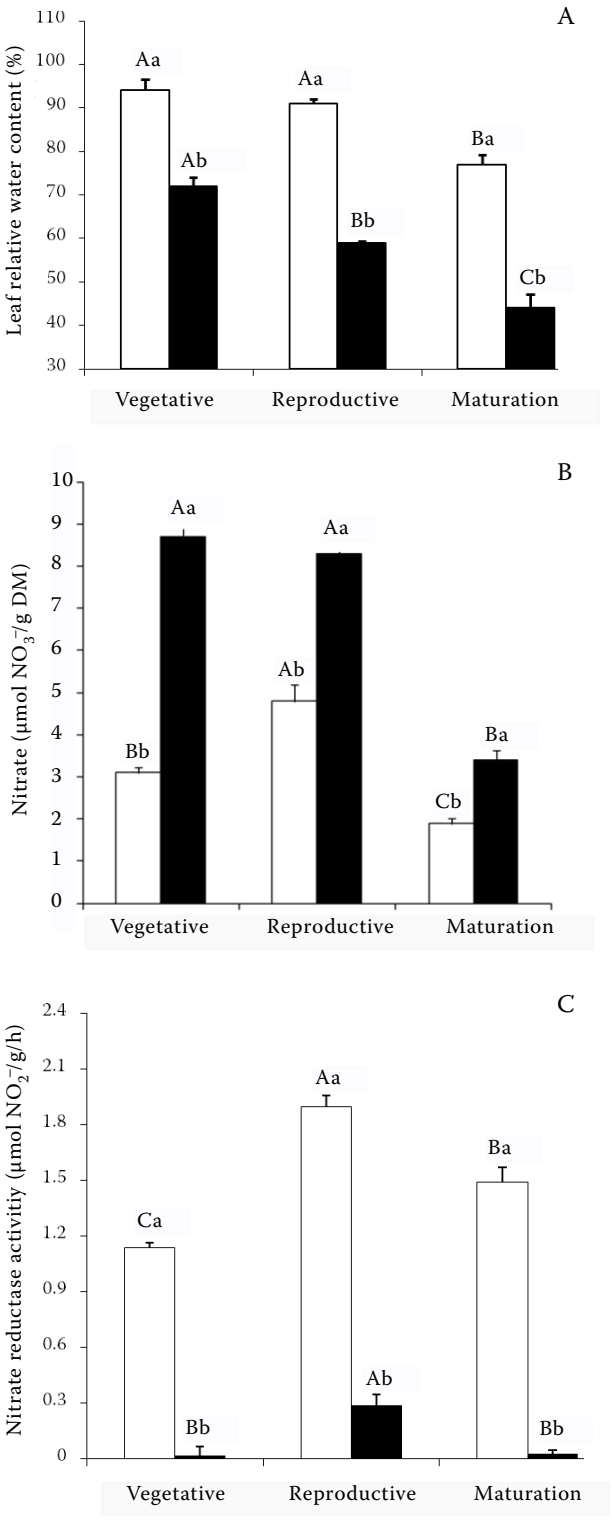

Figure 1. Leaf relative water content (A), nitrate (B) and nitrate reductase activity (C) in *Sorghum bicolor* plants cv. BR-700 submitted to 15 days of water restriction. Averages followed by the same lowercase letter within of the stages (vegetative, reproductive and maturation) and uppercase letter among the conditions (control and stress), do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

metabolism. The reduction also showed that it can be attributed indirectly to the probable photosynthesis reduction, as the assimilation and translocation of nitrogen compounds depend on the photosynthetic rate. However, the increase during the maturation stage is linked to lower glutamine synthetase activity (Kerbauy 2004). The lower level in stress plants during reproductive stage occurred as a result of reduction of the nitrate reductase activity, nitrite level and consequent ammonium formation. Similar results on increase in ammonium were reported by Debouba et al. (2006) investigating *Lycopersicon esculentum* plants induced to salt stress; similarly, Patel and Pandey (2008) reported an increase of nitrogen contents in *Holoptelea integrifolia* tissues growing under soil salinity.

Glutamine synthetase. The glutamine synthetase activity was decreased in all the stages, and the reductions were of 47.8, 51.9 and 73.5% during vegetative, reproductive and maturation stages, respectively. The higher activity was obtained during reproductive stage in control and stress plants (Figure 2B).

The decrease shown in glutamine synthetase activity is a result of lower ammonium supplement, as it uses ammonium as substrate for formation of amino acids and other compounds. The higher activity in control and stress treatments during the reproductive stage, when compared to other stages, is linked to high nitrate reductase activity and consequent protein catabolism.

Total soluble amino acids. The amino acids of the stress plants increased by 37.4 and 26.2% in the vegetative and reproductive stages, respectively, when compared with control plants. The maturation stage presented a decrease of 42.8% (Figure 2C). The maximum values in control and stress treatments were found in reproductive stage, and the minimum values in both treatments were obtained in maturation stage.

The increase in total soluble amino acids was promoted by the proteases activities, because these enzymes can breakdown reserve proteins in stress situations as water deficit (Costa 1999). Through this mechanism, the amino acids carry out the osmotic adjustment and cause a consequent increase of the plant tolerance. The maximum value during the reproductive stage in control treatment resulted from a high activity in nitrogen metabolism. The reduction in amino acids in the control and stress treatments during the maturation was probably related to leaf senescence and mobilization of photo-assimilates to grain and

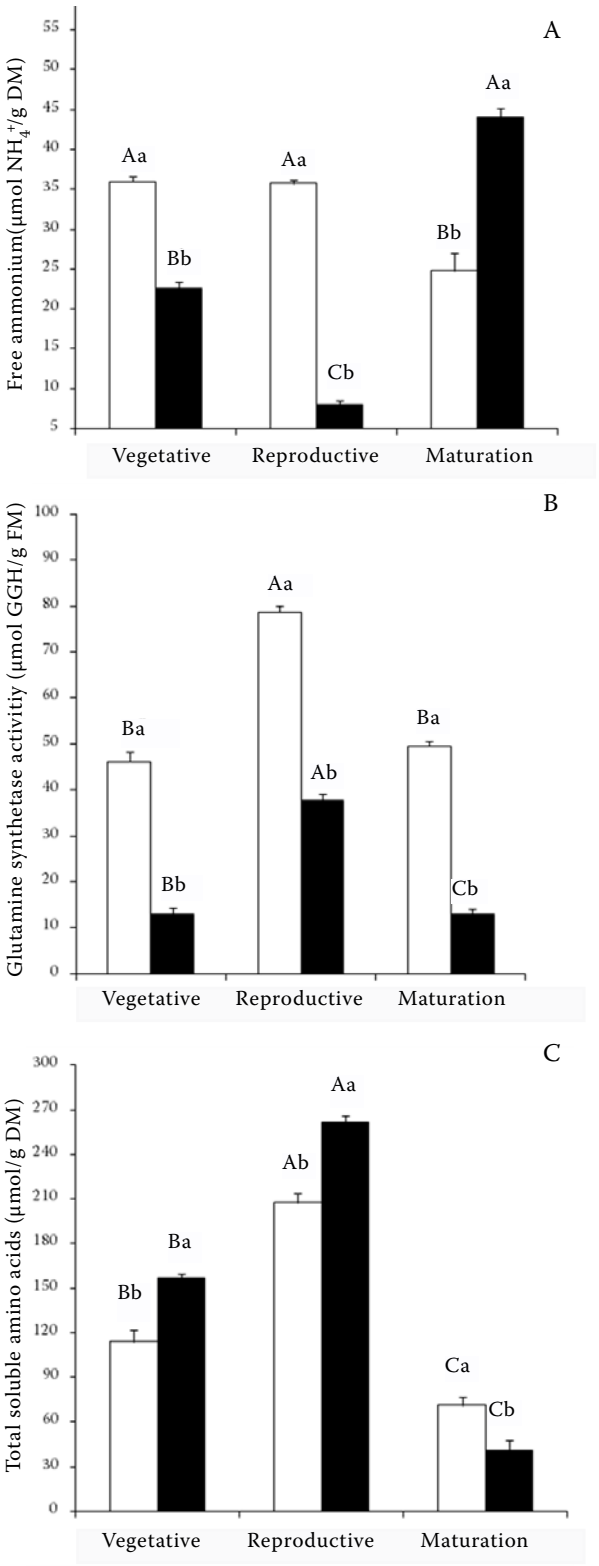

Figure 2. Free ammonium (A) and glutamine synthetase activity (B) and total soluble amino acids (C) in *Sorghum bicolor* plants cv. BR-700 submitted to 15 days of water restriction. Averages followed by the same lowercase letter within of the stages (vegetative, reproductive and maturation) and uppercase letter among the conditions (control and stress), do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

panicle (Pimentel 2004). Similar results on increase in amino acids were reported by Yadav et al. (2005) with *Sorghum bicolor* investigating the effects of water deficit.

Glycinebetaine. The glycinebetaine levels in stress plants were increased by 31.1 and 68% during the vegetative and reproductive stages, respectively. However, in the maturation stage a decrease of 18.5 to 9 mg/g DM was recorded after the stress simulation. The maximum values in control and stress plants were found during the reproductive stage (Figure 3A).

The accumulation in glycinebetaine level during the stress is a result of this nitrogen compound being used in osmotic adjustment and consequently enhancing the plant tolerance under inadequate situations. The glycinebetaine is synthesized through two reactions of choline oxidation, in which the first reaction is catalyzed by ferredoxin monooxygenase choline and the second is catalyzed by betaine-aldehyde dehydrogenase NAD^+ (Chen and Murata 2002). Therefore, the main factor that can limit the glycinebetaine production is the choline availability. The reduction during the maturation stage is linked to leaf senescence and consequent chloroplast degradation. The maximum value in reproductive stage is probably due to the amino acids formation through the nitrogen metabolism in control treatment and protein breakdown in stress treatment. The results described in this study are similar with those found by Agboma et al. (1997).

Proteins. The results on total soluble proteins obtained in vegetative, reproductive and maturation stages in control were 3.76, 4.7 and 2.69 mg/g DM and in stress plants they were 1.5, 2.49 and 0.7 mg/g DM, respectively (Figure 3B). The reproductive stage presented higher levels in control and stress plants.

These results on proteins were promoted by two factors, i.e. the increase in proteolysis, because these enzymes breakdown the proteins, and a decrease of the protein synthesis due the water deficit. The maximum values obtained during the reproductive stage prove the necessity of proteins for plant development and grain filling, as well as the strong metabolic activity and consequent protein formation through nitrogen metabolism. This type of stress affects the plant biochemical processes and provokes changes in metabolic behavior as protein degradation to form amino acids as proline and glycinebetaine, which is linked to maintenance of the cell turgor promoted by plant osmotic adjustment during abiotic stress.

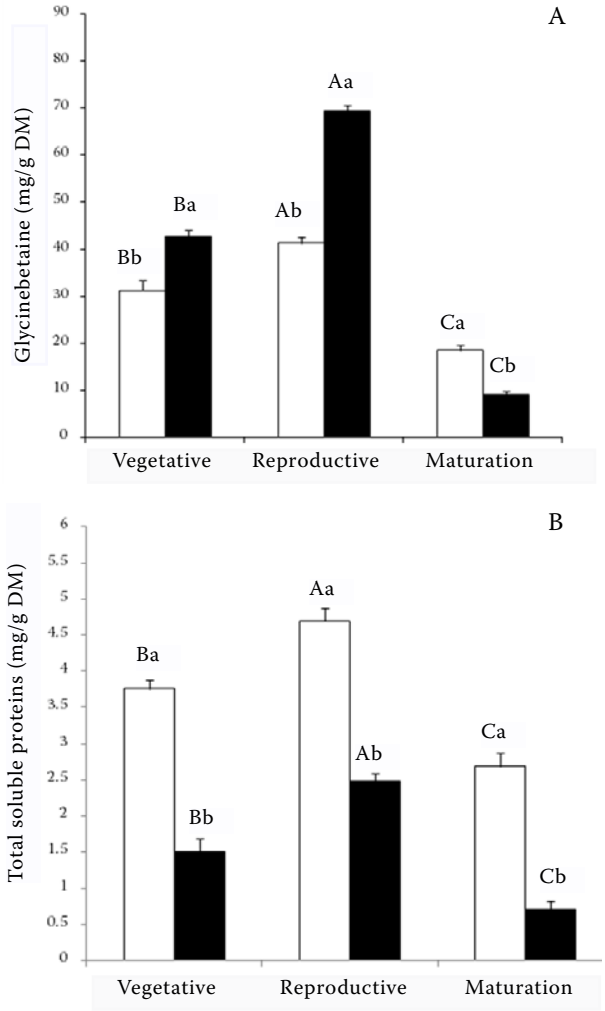

Figure 3. Glycinebetaine (A) and total soluble proteins (B) in *Sorghum bicolor* plants cv. BR-700 submitted to 15 days of water restriction. Averages followed by the same lowercase letter within of the stages (vegetative, reproductive and maturation) and uppercase letter among the conditions (control and stress), do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

Besides, several proteins can be produced with the objective to establish the metabolism and to act in recuperation of the damage promoted by water stress (Xiong et al. 2002). In addition, the proteins have several functions as ions request, membrane protection, protein saturation and indirectly water retention (Zhu et al. 1997).

The study revealed that the period under water deficit was sufficient to provoke significant changes in nitrogen compounds and enzyme activities, and to identify the stages when sorghum plants were more sensitive to simulated stress. In addition, an increase was showed in nitrate level during all stages, as well as accumulation in total soluble amino acids and glycinebetaine during vegetative and reproductive stages, if compared with control plants. However, significant reductions were reported in nitrate reductase and glutamine synthetase activities; besides protein levels, they were obtained in all stages, while free ammonium decreased only in vegetative and reproductive stages, when compared with control plants.

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