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Original Article

Osmotic regulators in cowpea beans plants under water deficiency

Reguladores osmóticos em plantas de feijão-caupi sob deficiência hídrica

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Abstract

Cowpea is a leguminous plant belonging to the fabaceae family cultivated in the North and Northeast regions of Brazil, with productive potential. Among the abiotic factors, water deficiency is one of the main environmental limitations that influence agricultural production in the world. The objective of this work was to study the relative water content and osmoregulators of cowpea plants subjected to water stress. The experiment was carried out in a greenhouse at the Universidade Federal Rural da Amazônia (UFRA, Belém, PA), cowpea plants BR-17 Gurguéia *Vigna unguiculata* (L.) Walp were used. The experimental design was completely randomized (DIC) in a 2 × 2 factorial scheme, two water conditions (control and water deficit) and two times of stress (four and six days of water suspension), with 7 replications, totaling 28 experimental units. The water deficit affected plants, causing a reduction in relative water content (69.98%), starch (12.84% in leaves and 23.48% in roots) and carbohydrates (84.34%), and an increase in glycine-betaine, sucrose (114.11% in leaves and 18.71% in roots) and proline (358.86%) at time 2. The relative water content was negatively affected by water conditions, with a decrease in relation to the interaction of the aerial part and the root system. Therefore, greater metabolic responses were noted in plants that were subjected to stress treatment at time 2 (6 days).

Keywords: water stress, abiotic factors, *Vigna unguiculata* (L.) Walp.

Resumo

O feijão-caupi é uma planta leguminosa pertencente à família das Fabaceae cultivada nas regiões Norte e Nordeste do Brasil, com potencial produtivo. Entre os fatores abióticos, a deficiência hídrica é uma das principais limitações ambientais que influenciam a produção agrícola no mundo. O objetivo do trabalho foi estudar o conteúdo relativo de água e os osmorreguladores de plantas de feijão-caupi submetidas ao estresse hídrico. O experimento foi conduzido em casa de vegetação da Universidade Federal Rural da Amazônia (UFRA, Belém, PA), foram utilizadas plantas de feijão-caupi BR-17 Gurguéia *Vigna unguiculata* (L.) Walp. O delineamento experimental foi inteiramente casualizado (DIC) em esquema fatorial 2 × 2, duas condições hídricas (controle e déficit hídrico) e dois tempos de estresse (quatro e seis dias de suspensão hídrica), com 7 repetições, totalizando 28 unidades experimentais. O estresse hídrico afetou as plantas, provocando redução no conteúdo relativos de água (69,98%), amido (12,84% nas folhas e 23,48% nas raízes) e carboidratos (84,34%), e aumento na glicina-betaína, sacarose (114,11% nas folhas e 18,71% nas raízes) e prolina (358,86%) no tempo 2. O conteúdo relativo de água foi afetado negativamente pelas condições hídricas, com diminuição em relação à interação da parte aérea e do sistema radicular. Diante disso, notou-se maiores respostas metabólicas nas plantas que foram submetidas ao tratamento de estresse no tempo 2 (6 dias).

Palavras-chave: estresse hídrico, fatores abióticos, *Vigna unguiculata* (L) Walp.

1. Introduction

Cowpea is a leguminous plant belonging to the fabaceae family cultivated in the North and Northeast regions of Brazil, with potential productive representation in the place (Benevides et al., 2013). This culture has its origins in Africa and was introduced in Brazil by Portuguese colonizers who arrived in the State of Bahia during the second half of the

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16th century, thus its cultivation was being disseminated throughout the country (Freire Filho, 2011). This legume constitutes an excellent source of proteins and carbohydrates (Oliveira et al., 2009). The production of cowpea in the state of Pará is equivalent to 20,922 tons (IBGE, 2022), however, despite all its benefits, cowpea has low productivity of grains at the national level (491 kg ha^{-1}) (Brasil, 2022), due to the use of traditional cultivars with low agronomic aptitude.

Despite this scenario, agricultural production can be limited by biotic and abiotic factors, causing losses of plant biomass, reductions in plant growth and development and, consequently, reduced production. Among the abiotic factors that most affect production, water deficiency stands out, this stress caused by the lack of water is one of the main environmental limitations harmful to agriculture (Chaves and Oliveira, 2004). However, studying the ability of plants to withstand drought becomes important for the development of agribusiness around the world (Shao et al., 2008).

Water is a fundamental resource for plant growth, responsible for stimulating cell elongation, synthesis and hydration of proteins, in addition to the entry of essential nutrients from the soil solution. Water limitation in the soil-plant-atmosphere continuum inhibits germination and plant establishment, exposing it to a series of changes in its metabolism (Tavares et al., 2021), such as inhibition of photosynthesis, less root expansion, stomatal closure, wilting of leaves and subsequent death of plants (Campos et al., 2021). Therefore, aiming to maintain both the growth and reproduction of plants in dry conditions, that is, lack of water. Thus, the plant responds both physiologically and molecularly to the water restriction condition, and one of the most pronounced responses is the ability of some species to carry out the osmotic adjustment of their cells and maintain cell turgor (Maia et al., 2007; Pintó-Marijuan and Munné-Bosch, 2013).

Osmotic adjustment is essential for protecting plants that are in water deficit (Monteiro et al., 2014), acting in the vacuole or citisol through the storage of solutes such as proline, glycine betaine, sucrose, among other solutes (Ashraf et al., 2011; Pintó-Marijuan and Munné-Bosch, 2013). For these same authors, these solutes favor the maintenance of water balance and the protection of the integrity of proteins, enzymes and cell membranes. Furthermore, according to Monteiro et al. (2014) the accumulation of these solutes favors the increase in osmostic pressure inside the cells, maintaining water absorption and turgor, which contributes to the continuity of physiological processes.

Water deficiency is one of the abiotic stresses responsible for restricting food production on the planet (Filippou et al., 2014). That way, studies that address this theme are necessary to better understand the mechanisms of adaptation of plants to drier environments (Fariduddin et al., 2013).

Therefore, with the hypothesis that water deficiency generates changes in the metabolism of cowpea plants, the objective of this study was to examine whether the relative water content in the leaves and the behavior of osmotic are affected regulators in cowpea plants exposed to water deficiency.

2. Material and Methods

2.1. Plant material and growth conditions

The plant material used in the study was the cowpea cultivar BR-17 Gurguéia *Vigna unguiculata* (L.) Walp., with seeds from the Germplasm Bank of Embrapa Amazônia Oriental. The experiment was carried out in a greenhouse at the Laboratory of Plant Physiology at the Federal Rural University of Amazonia (UFRA) in Belém, State of Pará, Brazil located in the geographic coordinates 01° 27' 21" S and 48° 30' 16" W.

The cowpea seeds were selected based on their uniformity, thus, those that did not present deformities and obtained similar sizes. After selection, they were sown in pots with a capacity of 3L, with washed and sterilized sand. Three cowpea seeds were placed per pot, moistened with distilled water.

Germination occurred on the 3rd day after sowing (DAS), then thinning was performed, leaving only one plant per pot. On the 8th DAS, the plants began to be fed with nutrient solution (¼ of ionic strength), according to the methodology of Hoagland and Arnon (1950) (Table 1), modified in the Laboratory of Plant Physiology at UFRA, with the pH maintained in 5.5 ± 0.5, using 1N NaOH or HCl solutions, when necessary. With the release of the third pair of leaves, at 18° DAS, the strength of the nutrient solution was increased, thus passing to 1/2 ionic strength of its original concentration. At 25° DAS, when the plants were in the V5 vegetative stage, the nutrient solution was suspended, and the plants were exposed to water deficit until the material was collected for analysis.

Table 1. Composition of Hoagland and Arnon (1950) nutrient solution.

COMPOSITION	CONTENT	mL/L
Macronutrients		
KNO_3	1 M	5
NH_4NO_3	1M	2
K_2SO_4	0.5 M	2
KH_2PO_4	1M	0.5
$CaCl_2.2H_2O$	1M	2
$MgSO_4.7H_2O$	1M	1
Fe (EDTA)	-	1
a) $FeSO_4.7H_2O$	0.1 M	
b) Na_2 (EDTA)	0.08 M	
Micronutrients	-	1
a) H_3BO_3	0.04 M	
b) $MnCl_2.4H_2O$	0.009 M	
c) $CuSO_4.5H_2O$	0.003 M	
d) $ZnSO_4.7H_2O$	0.007 M	
e) $Na_2MoO_4.H_2O$	0.001 M	
$CoCl_2.6H_2O$	0.004 M	1
$Ca(NO_3)_2.4H_2O$	1M	2.5

2.2. Material collection and storage

The plants were collected in two moments, at 29° and 31° DAS when they were exposed to water deficit for 4 and 6 days, respectively, in the early hours of the morning. The determination of the relative water content (WRC) was carried out *in vivo*, selecting in a greenhouse, completely expanded primary leaves in each repetition.

Subsequently, the plants were separated into aerial and root parts, wrapped in aluminum foil and stored in a -80 °C freezer. For the determination of the biochemical analyses, the material was placed in a forced air ventilation oven at 65 °C for 48 h. After drying, the leaf and root dry mass was determined. The dry material was crushed in a mill until obtaining a fine powder, stored in a falcon tube until use in the tests.

2.3. Analysis of variables

To determine the relative water content (RWC), fresh leaf discs were removed from each plant per treatment, at random, through a stainless steel pourer, following the methodology proposed by Slavick (1979), expressing the results as a percentage, as per described in Equation 1:

$$RWC = (FM1 - DM) / (FM2 - DM) x100 \quad (1)$$

on what: FM1 is the fresh mass 1; FM2 is the fresh mass 2 and DM is dry mass.

Free proline content was obtained from 2 mg of dry mass (DM) of leaves and roots, lyophilized in test tubes by adding 2 mL of distilled water, kept in a water bath for 30 minutes at 100 °C. After extraction, the samples were submitted to a bench centrifuge (2500 rpm for 5 minutes), collecting and supernatant to obtain the total extract (Bates et al., 1973).

To determine the glycine-betaine concentration of the plants, the methodology of Grieve and Grattan (1983) was followed, where 25 mg of DM, leaves and roots, were transferred to 2 mL eppendorf tubes, adding 2 mL of distilled water. The solution was shaken for 4 hours in a shacker at 25 °C (cold extraction), then centrifuged at 10,000 rpm for 10 minutes at 25 °C. After this process, the supernatant was collected to obtain the aqueous extract and the precipitate was discarded.

Starch concentrations were obtained using the method described by Dubois et al. (1956), in which an ethanolic extraction (20 mg of dry mass of roots and leaves) was performed in 2.5 mL of 80% ethanol for 30 min at 80 °C. Afterwards, a new extraction was performed with 2.0 mL of 30% perchloric acid (HClO4) for 20 minutes at 25 °C. After the first and second extractions, they were taken to a centrifuge (2,000 rpm for 10 m minutes) and the supernatants were collected. The supernatants of each extraction were united and calibrated to the volume of 10 mL with distilled water to obtain the total extract.

For the extraction of total soluble carbohydrates, 20 mg of dry matter from leaves and roots were weighed and placed in test tubes, followed by addition of 2.0 mL of 80% ethanol. Immediately the samples were placed in a water bath for 1 hour at 75 °C, with stirring every 15 minutes. The material was centrifuged at 3000 rpm and the supernatant collected, following the same author's methodology above.

Sucrose was determined according to the van Handel (1968) method, in which samples of 50 mg of dry mass of roots and leaves were used. These were homogenized in Eppendorf tubes with a volume of 2.0 mL, containing 1.5 mL of MWC solution (methanol, chloroform and water; 12:5:3 v/v/v), and shaken in a "shacker" for 30 minutes at room temperature. The homogenate was centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected. The residues were again extracted with an equal volume of MCW, followed by a new centrifugation and another collection of supernatants, in which they were pooled to obtain the total extract.

2.4. Experiental design and statistical analysis

The experimental design used was completely randomized (DIC) in a 2 × 2 factorial scheme, totaling 4 treatments, being analyzed as factor A the two water conditions (control and water deficiency) and as factor B (two times: four and six days of suspension water). Each treatment consisted of 7 repetitions, totaling 28 experimental units.

To evaluate the effect of comparing the water condition between the times of water suspension, analysis of variance was performed, in which the mean values were compared by Tukey's test at 5% probability, using the AgroEstat (2017) program.

3. Results

Water deficit affected the relative water content (RWC) of cowpea leaves (Figure 1). There was a difference between treatments (control and stress) at the same time of water suspension. This difference was observed at time 2 (6 days of stress), where the reduction in water content of leaf

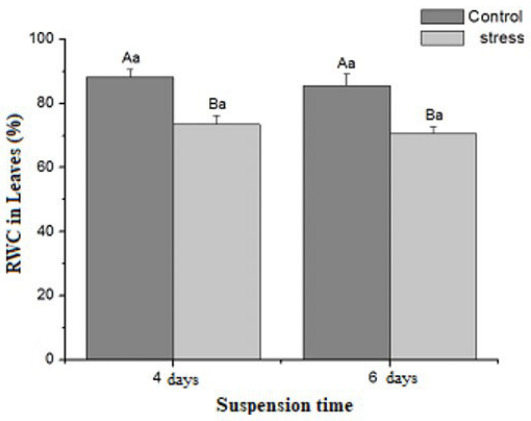

Figure 1. Relative water content in cowpea [*Vigna unguiculata* (L.) Walp.] leaf submitted to water deficit. Different lowercase letters indicate statistical differences ($p<0.05$) between the water suspension time in the same water condition, and different capital letters represent statistical differences between the water conditions in the same water suspension time. Bars indicate standard errors of means.

tissues was from 85.35% and 69.98% of control and water deficit plants, respectively.

The results demonstrate that there was a reduction of this starch concentration (GLU) in roots and leaves in all treatments when compared to control plants (Figure 2), there was in starch concentration in the leaf and in the root between the treatments (control and stress) in the same water suspension time. In the leaves, this difference was at time 2 (6 days), with reductions in the order of 0.257 to 0.224 µmol g^{-1} DM of GLU in the control and water deficit treatments, respectively, representing a reduction of 12.84% in the water deficit treatment. For the roots (Figure 2B) this difference was evidenced on the fourth day, where there were reductions from 0.247 to 0.189 µmol g^{-1} DM of GLU in the control and water deficit treatments, respectively, equivalent to a reduction of 23.48% in the stressed plants.

For sucrose concentrations there were differences in the leaf and root (Figure 3). Being observed that for the leaves between the treatments (control and stress) in the same time of water suspension the most expressive result occurred in the sixth day, where the plants presented values of 9.06 and 19.40 mg of sucrose/g of DM for the control and stress treatments, respectively, showing an increase of 114.11% in sucrose levels in the stress treatment. For time 1 (4 days) the plants expressed values of 10.57 and 8.33 mg of sucrose/g of DM for the control and stress treatments, respectively.

The same happened to the roots (Figure 3B) where the plants presented values of 16.08 and 19.09 mg of sucrose/g of DM for the control and stress treatments, respectively, equivalent to an increase of 18.71% in the levels of sucrose in the stress treatment at time 2, while for the first time the values were 13.06 and 10.96 mg of sucrose/g DM.

A difference was also observed between the times in the same water condition, for the leaves the sucrose concentration of the stressed plants in time 2 increased significantly (132.86%) compared to those in time 1, and for roots there was also a significant difference between the times in same condition, both for control plants and for plants subjected to stress. The control plants in time

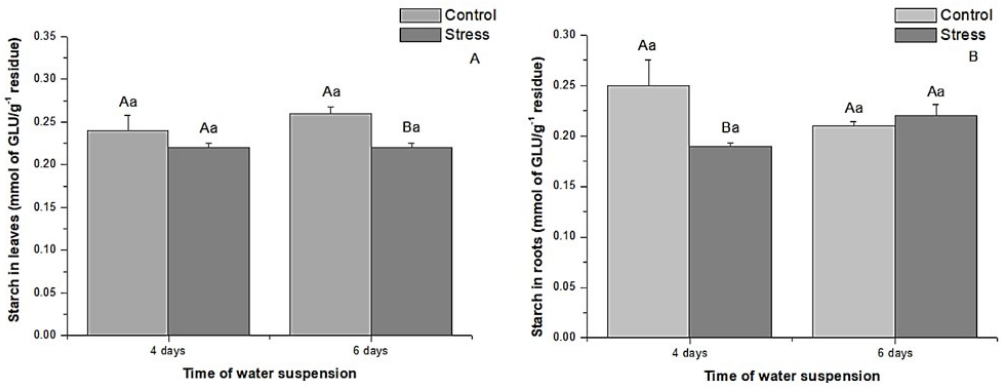

Figure 2. Starch concentration in leaves (A) and roots (B) of cowpea [*Vigna unguiculata* (L.) Walp.] submitted to water deficit. Different lowercase letters indicate statistical differences ($p<0.05$) between the water suspension time in the same water condition, and different capital letters represent statistical differences between the water conditions in the same water suspension time. Bars indicate standard errors of means.

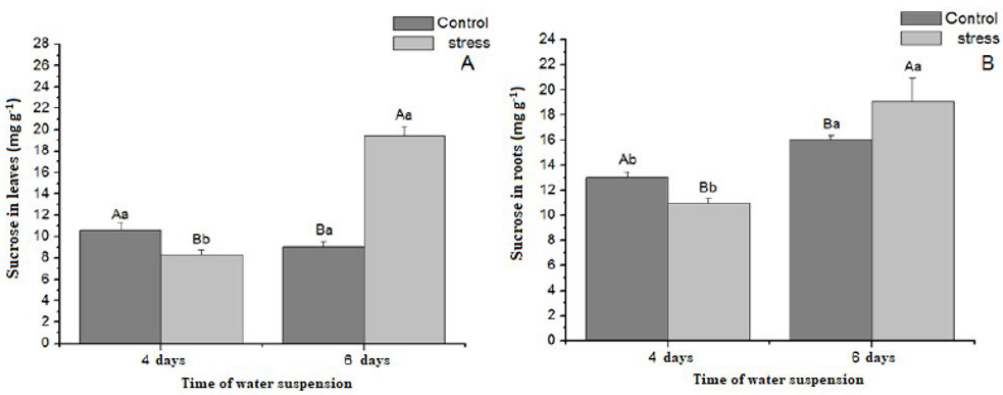

Figure 3. Sucrose in leaf (A) and root (B) of cowpea [*Vigna unguiculata* (L.) Walp.] submitted to water deficit. Different lowercase letters indicate statistical differences ($p<0.05$) between the water suspension time in the same water condition, and different capital letters represent statistical differences between the water conditions in the same water suspension time. Bars indicate standard errors of means.

2 increased 23.11%, while for the roots of plants subjected to water deficit the increase was 74.18%, in the second time, showing that as the time of exposure of the plants to drought increases, the concentration of sucrose in their tissues.

In the concentrations of total soluble carbohydrates (TSC) there were differences only for the leaves (Figure 4), both for the isolated factors and for their interaction. This difference in TSC in leaves was observed between treatments (control and stress) at the same time of water suspension. Where in time 1 (4 days) the plants showed values of 3.24 and 4.24 µmol/g^{-1} DM of TSC for the control and stress treatments, respectively, representing an increment of 31.22% in the carbohydrate contents of the plants under water stress. At time 2 (6 days) the treatment under stress showed a marked reduction, with values of 0.67 µmol/g^{-1} DM of TSC, while the control plants showed an average of 5.50 mg/g^{-1} of TSC. A difference was observed between the times in the same water condition, with greater expression in stressed plants, where the TSC contents of time 2 reduced by 84.34% compared to time 1.

An increase in glycine-betaine concentrations was observed in plants under water stress (Figure 5).

The concentration of proline in the roots increased (Figure 6), this difference in the roots was observed between the treatments (control and stress) at the same time of water suspension. At time 2 (6 days) the plants showed values of 0.902 and 4.139 µmol pro/g of DM for the control and stress treatments, respectively, representing an increase of 358.86% in the proline contents of the plants under water stress.

4. Discussion

The determination of relative water content (RWC) is a way of knowing the water status of plants, which reflects changes in the metabolic activity of plant tissues. Thus, the

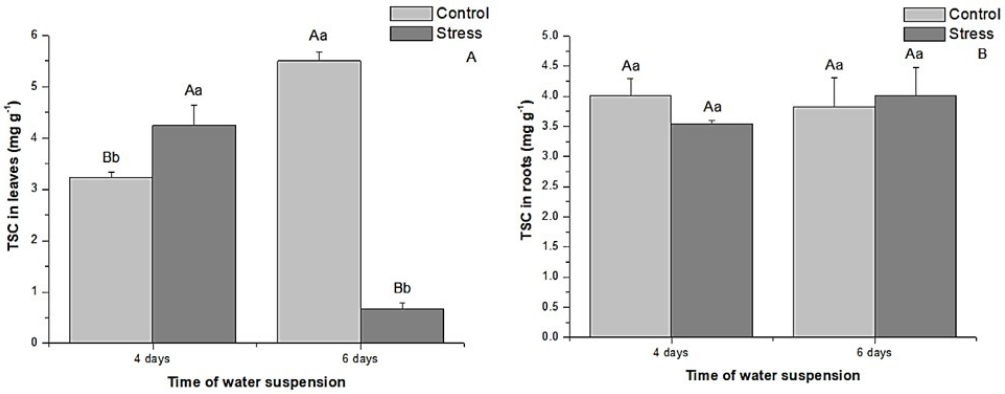

Figure 4. Concentration of carbohydrates in leaves (A) and roots (B) of cowpea [Vigna unguiculata (L.) Walp.], submitted to water deficit. Different lowercase letters indicate statistical differences ($p<0.05$) between the water suspension time in the same water condition, and different capital letters represent statistical differences between the water conditions in the same water suspension time. Bars indicate standard errors of means.

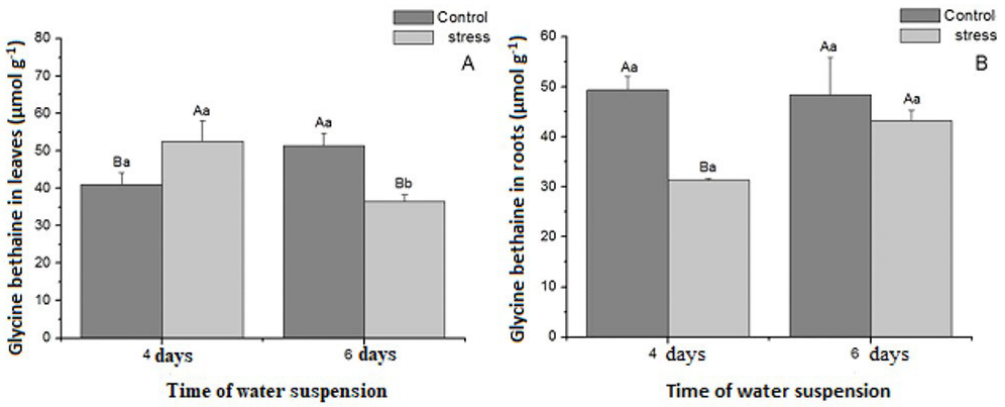

Figure 5. Concentration of glycine betaine in leaves (A) and roots (B) of cowpea [*Vigna unguiculata* (L.) Walp.] submitted to water deficit. Different lowercase letters indicate statistical differences ($p<0.05$) between the water suspension time in the same water condition, and different capital letters represent statistical differences between the water conditions in the same water suspension time. Bars indicate standard errors of means.

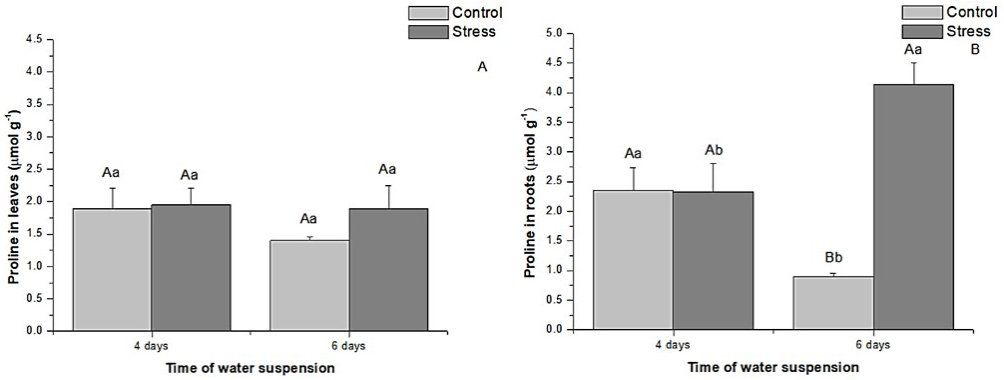

Figure 6. Proline concentration in the leaf (A) and root (B) of cowpea [*Vigna unguiculata* (L.) Walp.] submitted to water deficit. Different lowercase letters indicate statistical differences ($p<0.05$) between the water suspension time in the same water condition, and different capital letters represent statistical differences between the water conditions in the same water suspension time. Bars indicate standard errors of means.

decrease in RWC in the leaves promotes stomatal closure and, consequently, a reduction in stomatal conductance (Palheta, 2017). It is important to maintain the turgor of plant cells in order to maintain the physiological processes that promote plant growth, such as: expansion, cell division and photosynthesi (Petry, 1991). Smit and Singels (2006) state that RWC values below 75% already promote limitations in the physiological activities of plants, which was observed in this study. Fioreze et al. (2011) also observed a decrease in RWC in soybean plants (Glycine max) kept in conditions of water deficit, when compared with plants in treatment under irrigation.

The reduction of starch contents in plant tissues in plants under water stress occurs due to the reduction of photosynthetic activity, with an increase in the degradation of starch by the α and β amylase enzymes, forming new sugars (mainly sucrose), with the purpose of carrying out the osmotic adjustment in cells (Belo et al., 2015).

This reduction in starch concentrations in root tissues is due to a decrease in the flow of photoassimilates translocated from leaves to roots, due to water deficiency promoting a reduction in the positive pressure potential of the phloem, in addition to causing an increase in energy consumption through of cellular respiration driven by nutrient absorption, metabolic activities and root growth, preventing the possibility of storage of reserve sugars in the root system (Pimentel, 2004).

This increase in sucrose concentration in leaf and root tissues of plants under water deficiency is related to reduced plant growth, and consequently, less distribution of assimilates to other tissues (Palheta, 2017). In addition, starch hydrolysis is another process that is involved in the accumulation of sugar in plants subjected to water stress conditions (Lee et al., 2008).

Ataide (2015) in his experiment with a tree legume obtained results similar to those of this study, where sucrose levels rose as the exposure times of the plants to water deficiency increased. When reserve polysaccharides are mobilized, the hydrolysis product is sucrose, the main transport sugar in plants which acts in the photosynthetic cell, with the function of protecting the integrity of membranes and proteins under stress conditions (Hoekstra et al., 2001). For growing organs (drains) to be able to metabolize this sucrose, its degradation becomes necessary (Martins et al., 2003).

The concentration of total soluble carbohydrates (TSC) tends to reduce under conditions of water stress, as the consumption of these sugars becomes essential for maintaining plant survival, in addition to contributing to osmotic adjustment. Thus, the osmotic adjustment assists in the stomatal opening and in the functioning of the photosynthetic apparatus, allowing it to operate even in conditions of low water potential (Hayat et al., 2012).

Water deficit alters the concentration of total soluble carbohydrates (sucrose, fructose and glucose mainly) and insoluble carbohydrates (starch) in the tissues by decreasing the efficiency of the translocation of photoassimilates in plants, thus affecting their development process and respiration (Moura et al., 2016).

The reduction of carbohydrate contents in the leaf tissues of the plants with 6 days under stress is due to the loss of the photosynthetic activity, causing a lower production of TSC, however there was an increase of sucrose in the plants, a non-reducing sugar, which is the main sugar exported from the synthesis sites for the consuming regions. Melo et al. (2007) report that a decrease in starch content not accompanied by an increase in total soluble sugars indicates an immediate consumption of sugars to maintain plant survival. Pereira et al. (2012) found similar results, in their experiment there was no increase in carbohydrate content after reducing the concentration of starch in 2 peanut genotypes considered sensitive and moderately sensitive to water deficit.

The accumulation of glycine betaine observed in the leaves of plants subjected to water deficit in the first period of the experimente (Figure 5A) is associated with better absorption and transport of water from the soil to the shoot via osmotic adjustment, in addition to greater protection of the cell membrane of the plants (Ashraf and Harris, 2004).

This result was also observed by Palheta (2017), where in his experiment there was an increase in the

concentrations of glycine betaine in the leaves of plants under water deficiency. Biochemically, when plants are in adverse conditions for their development, they alter their metabolism and produce osmoregulatory compounds, such as glycine betaine (Graciano et al., 2016).

However, in this study it was verified that for the leaves in the second time and for the roots there was no accumulation of glycine betaine in the plants under water deficit, according to Sakamoto and Murata (2002), some plants accumulate significant amounts of glycine betaine in response to high salinity, cold and drought. When plants are subjected to water stress conditions, they need to reduce their intracellular osmotic potential to tolerate such conditions. This osmolyte acts as an osmoprotector, stabilizing the structure of proteins and the cell membrane. The main role of glycine-betaine is to protect plant cells by preserving the osmotic balance, stabilizing the protein structure and protecting the photosynthetic apparatus (Cha-Um et al., 2013). Although the occurrence of a possible osmotic adjustment through the accumulation in the glycine betaine concentration of the stressed plants was not observed, the increase in the proline content observed in the roots of the stressed plants of the second time is a strong indication that stressed plants of this species can perform it.

Some nitrogenous metabolites, such as the amino acid proline, tend to accumulate in plant tissues under water stress, in order to act in the osmotic adjustment of cells (Ferreira et al., 2002). Santos et al. (2010) report that proline accumulation can be considered a biochemical osmoregulator of water stress for mid-cycle cowpea genotypes.

In response to dehydration, plants tend to activate some mechanisms, such as the activation of the enzyme P5CS (Pyrroline-5-carboxylate synthetase) that converts glutamate to proline, simultaneously the enzyme PDH (proline dehydrogenase) responsible for the degradation of proline is inactivated, causing the increase in the level of this metabolite in the cell (Szabados and Savoure, 2010).

The accumulated proline has the functionality of providing energy and redistributing nitrogen and carbon, for the recovery of physiological activities in the plant (Hemaprabha et al., 2013). Playing an essential role in stabilizing proteins and cell membranes in plant cells in the presence of high levels of osmolytes (Farooq et al., 2009).

The synthesis of osmoregulators, such as proline, is abundantly used by plants to balance membranes and maintain protein disposition under low water potential (Efeoğlu et al., 2008). In studies carried out by Vantini et al. (2016) in a sugarcane culture, found that proline synthesis was stimulated when the plants were subjected to biotic and abiotic stresses, so that the plants maintained cell turgor.

5. Conclusion

The relative water content was negatively affected by water conditions, with a decrease in relation to the interaction of the aerial part and the root system. Therefore, greater metabolic responses were noted in plants that were subjected to stress treatment at time 2 (6 days).

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