Metabolic alterations in cowpea submitted to water stress

Alterações metabólicas em feijão caupi submetido ao estresse hídrico

Cambios metabólicos en frijol sometidos a estrés hídrico

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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 was to evaluate changes in carbon and nitrogen metabolism in cowpea plants exposed to water deficit. The experiment was conducted 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 x 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. Changes in the metabolism of cowpea plants were evident, with alterations in the activity of the nitrate reductase enzyme, concentrations of total soluble proteins, soluble amino acids, free ammonium and nitrate in the leaf tissue and roots.

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

RESUMO
O feijão-caupi é uma leguminosa pertencente à família fabaceae cultivada nas regiões Norte e Nordeste do Brasil, com potencial produtivo. Dentre os fatores abióticos, a deficiência hídrica é uma das principais limitações ambientais que influenciam a produção agrícola no mundo. O objetivo foi avaliar as alterações no metabolismo do carbono e do nitrogênio em plantas de feijão-caupi expostas ao déficit hídrico. O experimento foi conduzido em casa de vegetação na 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 x 2, sendo 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. Foram evidenciadas mudanças no metabolismo das plantas de feijão-caupi, com alterações na atividade da enzima nitrato redutase, nas concentrações de proteínas solúveis totais, aminoácidos solúveis, amônio livre e nitrato no tecido foliar e nas raízes.

RESUMEN
El caupí es una leguminosa de la familia de las fabáceas cultivada en las regiones Norte y Nordeste de Brasil, con potencial productivo. Entre los factores abióticos, la deficiencia de agua es una de las principales limitaciones ambientales que influyen en la producción agrícola en todo el mundo. El objetivo fue evaluar los cambios en el metabolismo del carbono y del nitrógeno en plantas de caupí expuestas a déficit hídrico. El experimento se realizó en un invernadero de la Universidad Federal Rural de Amazonia (UFRA, Belém, PA), utilizando plantas de caupí BR-17 Gurguéia Vigna unguiculata (L.) Walp. El diseño experimental fue totalmente aleatorizado (DIC) en un esquema factorial 2 x 2, con dos condiciones hídricas (control y déficit hídrico) y dos momentos de estrés (cuatro y seis días de suspensión hídrica), con 7 repeticiones, totalizando 28 unidades experimentales. Se observaron cambios en el metabolismo de las plantas de caupí, con alteraciones en la actividad de la enzima nitrato reductasa, en las concentraciones de proteínas solubles totales, aminoácidos solubles, amonio libre y nitrato en tejido foliar y raíces.

Palabras clave: deficiencia hídrica, factores abióticos, Vigna unguiculata (L.) Walp.

1 INTRODUCTION
Currently, the agricultural sector is facing problems due to biotic and abiotic factors, mainly in regions with prolonged drought. What can cause losses in production due to water deficiency, and thus, the lack of this resource has become a limiting factor for the good growth and development of crops, negatively impacting agricultural production systems.

Cowpea is a leguminous plant that belongs to the Fabaceae family, grown in the North and Northeast regions of Brazil, with potential productive representation in the area (BENEVIDES et al., 2013). Its production in Pará is equivalent to 28,751 tons, where the northeast of the state alone represents 45% of the total produced (12,901 tons) (IBGE, 2016).

During the first harvest of 2016/2017 there was a production of 201.5 thousand tons, increasing to 404 thousand tons of cowpea in the second harvest. The increase in production is related to the incorporation of new areas for cultivation, in addition to the good average productivity of the current harvest, which was not greatly influenced by the climatic conditions of the 2016 agricultural year (CONAB, 2017).

Despite the culture studied presenting a positive scenario and with prospects of increasing the amount produced, in the two regions mentioned above, concerns regarding the impacts of drought have been the object of study, in order to assess the capacity of the culture to resist water stress, because it is one of the problems that most harms world agriculture (SHAO et al., 2008).
These researches become necessary to observe the drought tolerance level of the cowpea, and thus, to bring better results during the vegetative and productive phase of the culture to obtain good levels of productivity in regions where the low water availability has been a reality. Therefore, the aim of this study was to evaluate metabolic changes in cowpea plants exposed to water deficit.

Given the above, the objective of the work is to evaluate the biochemical responses in cowpea plants subjected 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 of the Federal Rural University of Amazonia (UFRA) in Belém, State of Pará, Brazil. (01º 27' 21" S and 48º 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 18º DAS, the strength of the nutrient solution was increased, thus passing to 1⁄2 ionic strength of its original concentration.

<table>
<thead>
<tr>
<th>COMPOSITION</th>
<th>CONTENT</th>
<th>mL/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>1 M</td>
<td>5</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1 M</td>
<td>2</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>0.5 M</td>
<td>2</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1 M</td>
<td>0.5</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>1 M</td>
<td>2</td>
</tr>
</tbody>
</table>
### Table: Micronutrients

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) H3BO3</td>
<td>0.04 M</td>
</tr>
<tr>
<td>b) MnCl2.4H2O</td>
<td>0.009 M</td>
</tr>
<tr>
<td>c) CuSO4.5H2O</td>
<td>0.003 M</td>
</tr>
<tr>
<td>d) ZnSO4.7H2O</td>
<td>0.007 M</td>
</tr>
<tr>
<td>e) Na2MoO4.2H2O</td>
<td>0.001 M</td>
</tr>
<tr>
<td>f) CoCl2.6H2O</td>
<td>0.004 M</td>
</tr>
<tr>
<td>g) Ca(NO3)2.4H2O</td>
<td>1M</td>
</tr>
</tbody>
</table>

Source: authors.

At 25º DAS, the nutrient solution was suspended, and the plants were exposed to water deficit until the material was collected for analysis.

### 2.2 COLLECTION AND MATERIAL STORAGE

The plants were collected in two moments, at 29º and 31º DAS when they were exposed to water deficit for 4 and 6 days, respectively, in the early hours of the morning. Subsequently, the plants were separated into aerial and root parts, wrapped in aluminum foil and stored in a -80ºC freezer. To determine the biochemical analyses, the material was taken to a forced air ventilation oven at 65ºC 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 ANALYZED VARIABLES

Nitrate reductase activity was obtained using the method described by Hageman & Hucklesb (1971). With the aid of a cork borer, leaf discs (0.5 cm² in diameter) were removed and then approximately 200 mg of leaf discs were weighed. To obtain the extract, the leaf discs were transferred to test tubes to be subjected to vacuum, containing 5.0 ml of phosphate buffer (reaction medium) for 2 min. Then, the test tubes were placed in a “water bath” at 300 ºC for 30 min and protected from light (dark).

To obtain total soluble proteins, the method described by Bradford (1976) was used. Using 15 ml test tubes, 100 mg lyophilized DM / 5.0 ml of extraction buffer (25 mM Tris-HCl pH 7.6) were added and then they were shaken for 2 h in the shacker (with tubes duly sealed) to obtain the extract.
In the extraction of total soluble amino acids, 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, the process was repeated twice more, thus obtaining a total extract (Yemm & Cocking, 1955).

The determination of free ammonium followed the methodology proposed by Weatherburn (1967), 50 mg of dry mass (DM) of powdered leaves and roots were weighed, and placed in 15 ml test tubes, where 5 ml of distilled water were added. and placed in a water bath for 30 minutes at 100°C. After extraction, the samples were centrifuged in a benchtop centrifuge (1000 rpm) and the supernatants collected to obtain the total extract.

The nitrate concentration was obtained by the method described by Cataldo et al. (1975), in which 50 mg samples of previously lyophilized leaves and roots were added to test tubes containing 5.0 ml of distilled water and incubated in a “water bath” for 30 minutes at 100 ºC. Then, it was centrifuged at 3,000 rpm for 10 min to obtain the total extract.

2.4 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

The experimental design used was completely randomized (DIC) in a 2 x 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, the analysis of variance was performed, in which the mean values were compared by the Tukey test at 5% probability, using the AgroEstat program (2017).

3 RESULTS AND DISCUSSION

The activity of the nitrate reductase (NR) enzyme showed a statistically significant difference (p<0.05) in leaves when subjected to water stress and compared with control plants (Figure 1). Plants subjected to water deficit in leaf tissues showed 0.158 μmoles of NO₂⁻g⁻¹ DM h⁻¹ when compared to control plants (0.716 μmoles of NO₂⁻g⁻¹ FM h⁻¹) at time 1 (4 days) (Figure 1A).
For time 2 (6 days) the plants showed values of 2.921 and 0.175 μmoles of NO₂⁻ g⁻¹ DM h⁻¹ for the control and stress treatments, respectively, which represented a 94% reduction in the activity of the enzyme. A significant difference was also observed between the times in the irrigation condition (control), and the RN concentration in the plants at time 2 increased by 75.50% compared to time 1.

For root tissue (Figure 1B) there were differences on the fourth day of water suspension (p<0.05), reducing from 0.063 in the control to 0.027 μmoles of NO₂⁻ g⁻¹ FM h⁻¹ when exposed to water deficit. On the sixth day of water suspension, the control plants changed from 0.081 to 0.016 μmoles of NO₂⁻ g⁻¹ FM h⁻¹ in the water deficit treatment, representing an 80.25% reduction in enzymatic activity. There was also a significant difference between the times in the irrigation condition (control), with an increment of 22.20%.

It is normal for the nitrate reductase enzyme to show a reduction in its activity with the advancement of stress due to water deficit. According to Freitas et al. (2007), when going through a situation of moderate water stress, even with nitrate levels, the plant can reduce its enzymatic activity to 20% due to other factors. For example, high amounts of glutamine can negatively affect the expression of the nitrate reductase enzyme, decreasing its activity. (ANDRADE NETTO, 2005).

The close relationship between nitrate reductase regulation and photosynthesis may be important to prevent the accumulation of nitrite which is a highly reactive and potentially toxic ion in the plant, as the reduction of nitrite to ammonium uses reduced ferredoxin as an electron source, a product obtained through the photosynthetic process, as water deficiency can cause a decrease in photosynthesis, this change in this process can generate nitrite accumulation if the activity of nitrate reductase was not regulated (LILLO et al., 2003).

As expected, there was a reduction in leaf and root nitrate activity with increasing stress. For Serraj and Sinclair (1996), several species of tropical legumes, such as Vigna unguiculata (L.) Walp. are partially affected by water restriction. In addition, similar results were found in two maize genotypes, where the lowest enzyme activity occurred when there was water deficiency (FERREIRA et al. 2002).
Figure 1. Nitrate reductase activity 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.

Source: authors.

It was possible to observe an effect on leaves and roots of cowpea (p<0.05), for the interaction of factors between treatments regarding the concentration of total soluble proteins (TSP) (Figure 2). In leaf tissue (Figure 2A), for factor 1 interaction, plants showed averages of 9.118 and 9.933 mg protein/g DM in control and under stress, respectively, with an increase of 8.93% in TSP levels in plants subjected to deficiency water for 4 days, and when under stress for 6 days this difference was not evident, with difference between the times for the control treatment of the plants.

For concentration of proteins in the root system of the culture (Figure 2B), time 1 (4 days) presented an average of 10.546 mg protein/g DM to control plants, while those submitted to water deficit the value was 9.908 mg protein/g DM, representing a 6.04% reduction in the TSP content of stressed plants. However, at time 2 (6 days) there was an increase in TSP levels of 3.80% in stressed plants. There was a difference in the roots between times in the same water condition, being more expressive in plants under deficit, where the concentration of TSP in time 2 increased by 6.22% compared to time 1.

The increase in protein content in cowpea plants subjected to water deficit is associated with non-degradation of proteins, justified by osmotic adjustment. This change promotes an accumulation of metabolites, in addition to modifying enzymatic activities and protein synthesis (SANTOS et al. 2010).
Furthermore, the accumulation of protein in the plant cell may come from the conservation of a nitrogen stock to maintain plant metabolism at the end of water stress (MANSOUR, 2000). In this way, proteins can synthesize or have a high expression in response to water stress. (BELO et al. 2015).

Figure 2. Protein 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.

Source: authors.

The results obtained in the leaves and roots of Vigna unguiculata (L.) Walp were significant (p<0.05) both in the interaction of factors and in the concentration of amino acids (AA) (Figure 3). Leaf tissue from control plants showed averages of 9.363 and 7.018 μmol of AA g\(^{-1}\) DM at time 1 and 2, respectively. In plants under water stress, the values were 5,600 and 2,408 μmol of AA g\(^{-1}\) DM, at time 1 and 2, respectively, corresponding to a reduction of 40.2% and 65.7% (Figure 3A).

In root tissue (Figure 3B) there was no difference between treatments (control and stress) at time 1 (4 days), but at time 2 (6 days) there was an increase in the concentration of AA in stressed plants, where they showed values of 3.404 and 7.473 μmol AA g\(^{-1}\) DM in control and under stress, respectively.

For the same water condition there were also differences, being more expressive in stressed plants, where the water deficit promoted an increase in the order of 4.742 to 7.473 μmol of AA g\(^{-1}\) of DM in time 1 and 2, respectively. Aminoacids in roots (μmol g\(^{-1}\))
Decreases in the levels of free amino acids, resulting from stress, are associated, among other factors, with their use in the synthesis of new proteins (ROCHA, 2003). In the roots, the concentrations of these solutes varied according to the time of exposure to the deficit, with a reduction in AA contents followed by an increase, attributed to the longer time under water stress. Similar results were found by Pimentel (1999) when working with two corn hybrids exposed to water deficit for two cycles.

The increase in AA in the roots of cowpea plants exposed to water deficit can be attributed to changes in nitrogen metabolism (BRITO et al., 2008), thus maintaining cell turgor, in addition to serving as a nitrogen reserve for the resumption of plant growth at the end of the water deficit.

Figure 3. Amino acid concentration in the leaf (A) and root (B) of cowpea [Vigna unguiculata (L.) Walp.] submitted to water deficit.

![Amino acid concentration in the leaf (A) and root (B) of cowpea](image)

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.

Source: authors.

For the concentrations of free ammonium, there was a difference (p<0.05) for the interaction of factor 1, in the concentration of ammonium (NH$_4^+$) in leaves of Vigna unguiculata (L.) Walp (Figure 4). In leaf tissues (Figure 7A), water deficit promoted an increase in the NH$_4^+$ content in the order of 2.180 (control) to 2.673 (stress) μmol of NH$_4^+$/g of DM in the first time (4 days), showing an increase of 22.61% in ammonium concentration.

There was no difference between treatments (control and stress) only in time 2 (6 days), between times in the same water condition the differences were evident, being more expressive in stressed plants, where the ammonium levels of plants in time 2 (6 days) reduced by 52.30%
compared to time 1 (4 days). There was no significant difference for root tissue, either for isolated factors or for their interaction (Figure 4B).

The ammonium absorbed by the root, produced by nitrate assimilation, or still originating from photorespiration, is converted into the amino acids glutamine and glutamate by sequential action (BECKER et al., 1993). It is used in transamination reactions for the production of all other amino acids necessary for protein synthesis (TAIZ; ZEIGER, 2013).

Due to being subject to adverse reactions such as water deficiency, plants can induce the formation of ammonium through proteolysis, or through the induction of other routes of formation of this compound (TAIZ; ZEIGER, 2013). The accumulation of ammonium in plants under water stress is also related to the photorespiration process of the catabolism of nitrogenous compounds, such as amino acids and by deamination (DEBOUBA et al., 2007).

The results obtained in this work were also observed by Ferreira et al. (2002) stating that another response to this concentration of free ammonium in tissues would be the increase in photorespiratory activity. Teixeira et al. (2015), when working with seedlings of *Morinda citrifolia* L., also observed an increase in the concentration of ammonium in the leaf tissue.

![Figure 4](image)

**Figure 4.** Ammonium 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 error of means.

Source: authors.

For the levels of nitrate (NO$_3^-$) there was a statistically significant difference (p<0.05) for the leaf tissue between the treatments (control and stress) at the same time of water suspension (Figure 5). At time 1 (4 days) the plants showed values of 0.028 and 0.044μmoles of NO$_3^-$/mg
DM for the control and stress treatments, respectively, representing an increase of 57.14% in the NO$_3^-$ contents of the plants under water stress.

At time 2 (6 days) there was the highest accumulation of 0.029 and 0.049 μmoles of NO$_3^-$/mg DM for control and stress plants, respectively. No statistically significant difference was observed between water suspension times in the same water condition. The observations obtained for the roots were not significant, both for isolated factors and for their interaction (Figure 5B).

The increase in nitrate concentration in the leaves is related to the low activity of the nitrate reductase enzyme. This increase in the concentration of organic compounds, such as nitrate, in plants under water deficit, is a response induced by the decrease in the activity of the RN enzyme, since nitrate acts as a substrate in the reaction catalyzed by this enzyme, which is, therefore, responsible for the reduction of nitrate (NO$_3^-$) into nitrite (NO$_2^-$) (FERREIRA et al., 2002), promoting accumulation of organic nitrate.

The plant essentially assimilates nitrogen through ion-root contact, preferably carried out by mass flow, where nitrate and ammonium are absorbed and distributed to all parts of the plant (PALHETA, 2017). These results demonstrate that, in general, the nitrate absorbed by the roots can be assimilated in these organs or in the air, depending on availability and plant species (OLIVEIRA, 2010).

Most species are intermediates referring to the ability to assimilate NO$_3^-$ in the roots. In this case, the leaf becomes fundamental only when the NO$_3^-$ in the medium is in sufficient concentration to overcome the reduction capacity of the root. However, despite prevailing a greater capacity for assimilation of NO$_3^-$ in the root, some legumes carry a significant part of the NO$_3^-$ to the leaf even when the capacity of the root is not exceeded (DELFINI et al. 2010).
Figure 5. Nitrate concentration in the leaf (A) and root (B) of cowpea \([Vigna unguiculata \text{ (L.) Walp.}]\) submitted to water deficit.

Distinct lowercase letters indicate statistical differences (p<0.05) between the time of water suspension in the same water condition, and distinct capital letters represent statistical differences between water conditions in the same water suspension time. The bars indicate the standard error of the means.

Source: authors.

4 CONCLUSION

Biochemical processes in cowpea plants were negatively affected when exposed to water deficit, promoting disturbances in metabolism and carbon pathways, in leaf and root tissues of plants.

ACKNOWLEDGEMENTS

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