

## WATER STRESS RESPONSE ON THE ENZYMATIC ACTIVITY IN COWPEA NODULES

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### ABSTRACT

A greenhouse experiment was carried out aiming to study the effect of water stress on metabolic activity of cowpea nodules at different plant development stages. Cowpea plants were grown in pots with yellow latosol soil under three different matric potentials treatments: -7.0 (control-S<sub>1</sub>), -70.0 (S<sub>2</sub>) and <-85.0 KPa (S<sub>3</sub>). The experimental design was randomized blocks with sub-divided plots, each plot containing a different degree of water stress, divided in sub-plots for the four different developmental stages: E<sub>1</sub> (0-15), E<sub>2</sub> (15-30), E<sub>3</sub> (20-35) and E<sub>4</sub> (30-45) days after emergence. Water stress treatments were applied by monitoring soil water potential using a set of porous cups. The effect of water stress was most harmful to cowpea when it was applied at E<sub>2</sub> than at other symbiotic process stages. Shoot/root ratio decreased from 2.61 to 2.14 when matric potential treatment was <-85.0 and -70.0 KPa respectively. There was a reduction in the glutamine synthetase activity and phosphoenolpyruvate carboxylase activity with increased stress, while glutamine synthase activity was the enzyme most sensitive to water stress. Glutamate dehydrogenase activity increased in more negative matric potential, indicating that this enzyme is sufficiently active under water stress.

**Key words:** drought stress, *Vigna unguiculata*, nodulins

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### INTRODUCTION

The effects of water stress on plants physiology vary with species and degrees of tolerance, as well as with the magnitude of the water deficit and how fast the plants experience this water deficit. Generally, drought affects the processes related to cell turgidity and particularly meristematic growth quickly. If drought persists other physiological processes is affected (21,26). For example, changes in stomatal opening may lead to a decrease in photosynthesis rate, and water transport through the xylem, which in turn may decrease transport flux of absorbed nutrients by roots and in the whole plant (13). Regardless the physiological mechanism of N<sub>2</sub> fixation inhibition by drought stress, there is evidence that legume species have significant genetic variation in their ability to fix N<sub>2</sub> under drought conditions.

Drought is one of a range of environmental stresses, which can cause considerable reductions in N<sub>2</sub> fixation (7,17,27).

However, it is not obvious which particular physiological process of the stressed plant actually affect nodule growth and metabolism (9,30). The relationship between plant water status, photosynthesis and N<sub>2</sub> fixation, under water stress and the changes in nodule morphology have been studied for some temperate legumes (32). However, tropical legumes grown in arid regions, have not received such adequate attention. Even where available information, exists the degree of water stress in the plants was not clearly defined and making difficult comparisons between cultivars. The structural basis for the difference in sensitivity of N<sub>2</sub> fixation in tropical legumes, under water stress, is not clearly understood (32). A number of studies have indicated that a nitrogenous signal(s), associated with N accumulation in the shoot and nodule, exists in legume plants so that N<sub>2</sub> fixation is inhibited early in drying soil (22). Some data indicate that water stress directly separates interactions between the bacteria and the host plant by alteration of nodule

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structure and enzymatic activity (4,8). Others suggest that the water stress limits many processes of nodule activity (18). Adaptive response in the metabolism of any organism during any environmental stress must reflect changes either in activities of enzymes or in gene expression (22). Although the GS-GOGAT pathway is considered to be the major route for ammonia assimilation under normal growth conditions, the role GDH under some environmental and nutritional conditions cannot be excluded and, therefore, the possible factors under which GDH may play a significant aminating or deaminating role in cell metabolism (28). The PEPC magnitude on  $N_2$  fixation was presented by Coker and Schubert (2). The objective of the present investigation was to evaluate the response to water stress on some key enzyme of cowpea nodule metabolism at different developmental stages of  $N_2$  fixation.

## MATERIALS AND METHODS

The experiment was conducted in a greenhouse at a temperature range from 27 to 35°C and air relative humidity range from 50 to 80%. Pots were filled with soil samples (0-20 cm) of a sandy loam Yellow Latosol (16). The soil was air-dried, sieved (5.0 mm), corrected to pH 6.3 by the addition of calcium and magnesium oxides a 3:1 ratio. It was autoclaved for 30 min at 121°C and 101 KPa, once a day for three consecutive days and 14 kg of soil was used in each 15 L pot. Chemical and physical analyses of the soil were conducted at the Pernambuco Agricultural and Livestock Research Corporation (Empresa Pernambucana de Pesquisa Agropecuária-IPA) and showed the following results: pH (water<sup>1:2.5</sup>) 4.8;  $Ca^{2+}$  7.0 mmol<sub>c</sub> kg<sup>-1</sup>;  $Mg^{2+}$  4.0 mmol<sub>c</sub> kg<sup>-1</sup>;  $K^+$  0.7 mmol<sub>c</sub> kg<sup>-1</sup>;  $Na^+$  0.4 mmol<sub>c</sub> kg<sup>-1</sup>;  $Al^{3+}$  3.0 mmol<sub>c</sub> kg<sup>-1</sup>; P 6.1 mg kg<sup>-1</sup>; N 0.6 g kg<sup>-1</sup>; clay 190 g kg<sup>-1</sup>; silt 50 g kg<sup>-1</sup>; fine sand 90 g kg<sup>-1</sup>; coarse sand 670 g kg<sup>-1</sup>; porosity 493 m<sup>3</sup> m<sup>-3</sup>; particle density 2650 kg m<sup>-3</sup>; and bulk density 1420 kg m<sup>-3</sup>.

The plant species used was cowpea (*Vigna unguiculata* (L.) Walp.) cv IPA 204 (L. 1429). The seeds were inoculated with a strain of *Bradyrhizobium* spp., from Embrapa Agrobiologia (National Center for Research in Agrobiologia), Embrapa, Rio de Janeiro, Brazil. Five seeds of cowpea were surface sterilized (15) and sown in each pot and immediately inoculated with 5 ml pot<sup>-1</sup> of liquid culture of *Bradyrhizobium* spp. (10<sup>9</sup> cfu ml<sup>-1</sup>). After emergence three plants were left per pot. Hoagland and Arnon (12) solution without N was applied weekly at a rate of 2 ml kg<sup>-1</sup> of soil.

### Water stress application and enzyme activities

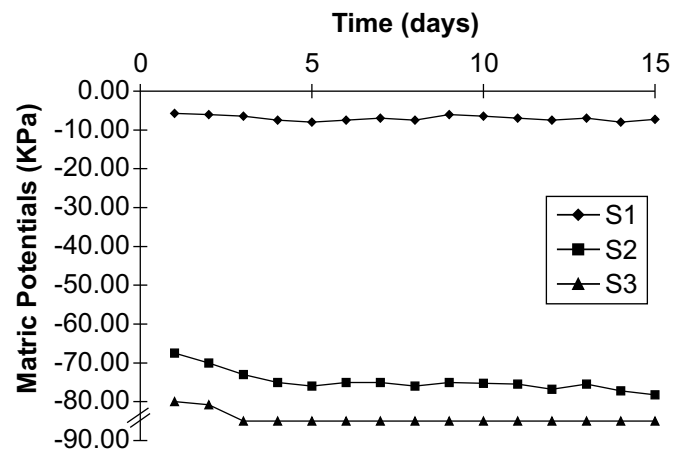
Water stress was applied by means of a porous cup arrangement similar to that described by Bataglia (1). The different soil water content were obtained by setting the vertical distances between the middle of the cups and the reservoir with a constant level of  $\Psi_m$  at 15, 40 and 100 cm equivalents, respectively, to  $S_1 = -1.5$  KPa,  $S_2 = -4.0$  KPa, and  $S_3 = -10.0$  KPa

representing  $\Psi_m$  values at the porous cup walls and consequently of the soil water when in equilibrium. As the plant roots absorbed water a potential gradient developed, inducing water flow from cup to soil. For this reason the  $\Psi_m$  at the treatment  $S_1$  represents a soil  $\Psi_m$  of -7.0 KPa; at the treatment  $S_2$  the soil  $\Psi_m$  reached -70 KPa, and at the treatment  $S_3$  the soil  $\Psi_m$  exceeded -85 KPa (the exact value not being measured due to the limited range of the tensiometer) (Fig. 1).

Water stress treatment started 5 days after seedling emergence and during a period of 15 days at each plant growth stage:  $E_1 = 0-15$  days, corresponding to the initial period of nodule formation and  $N_2$  fixation;  $E_2 = 15-30$  days, corresponding to the nodule growth period and establishment of  $N_2$  fixation;  $E_3 = 20-35$  days, corresponding to the peak of dinitrogen fixation by the nodules; and  $E_4 = 30-45$  days, corresponding to the final stage of the bacteriological cycle and the beginning of nodule senescence. At the end of each stage, the plants were harvested for analysis and comparison with non-stressed plant (control  $S_1$ ).

The parameters analysed regarding water stress were: leaf water potential (19) (readings taken from 09:00 to 10:00 h), soil water matric potential, using the tensiometer Soil Moisture, mod. 2725 (the readings taken daily at 10:00 h, throughout the entire drought period).

To determine enzyme activities, nodules were kept in liquid  $N_2$  until assay. Nodule cytosol was prepared in extraction solution (6) and the extraction method according to Hungria *et al.*, (14). Supernatant was desalted at 4°C on a Sephadex G-25 column equilibrated with a buffer suitable (6) to GS and GOGAT and (15) to GDH enzymes assays. The glutamine synthetase (GS) activity was measured spectrophotometrically (540 nm) by the biosynthetic hydroxamate assay (6). Glutamate synthase (NADH-GOGAT) activity was assayed spectrophotometrically (340 nm) according



**Figure 1.** Soil matric potentials during 15 d periods, at the different degrees of water stress (-70.0 and <-85KPa) and control (-7.0).

to Farnden and Robertson (6) and glutamate dehydrogenase (GDH) activity was assayed spectrophotometrically (340 nm) according Hungria and Araujo (15). Phosphoenolpyruvate carboxylase (PEPC) activity was assayed spectrophotometrically (340 nm) according Schweizer and Erismann (20).

### Statistical design and analysis

The experimental design was randomized blocks with sub-divided plots, each plot containing a different degree of water stress, one without stress (control  $S_1$ ) and two with stress ( $S_2$  and  $S_3$ ) divided in sub-plots for the four different developmental stages ( $E_1$ ,  $E_2$ ,  $E_3$ , and  $E_4$ ). An analysis of variance was done for each variable studied according to the mathematical model of the experimental layout adopted (29).

## RESULTS AND DISCUSSION

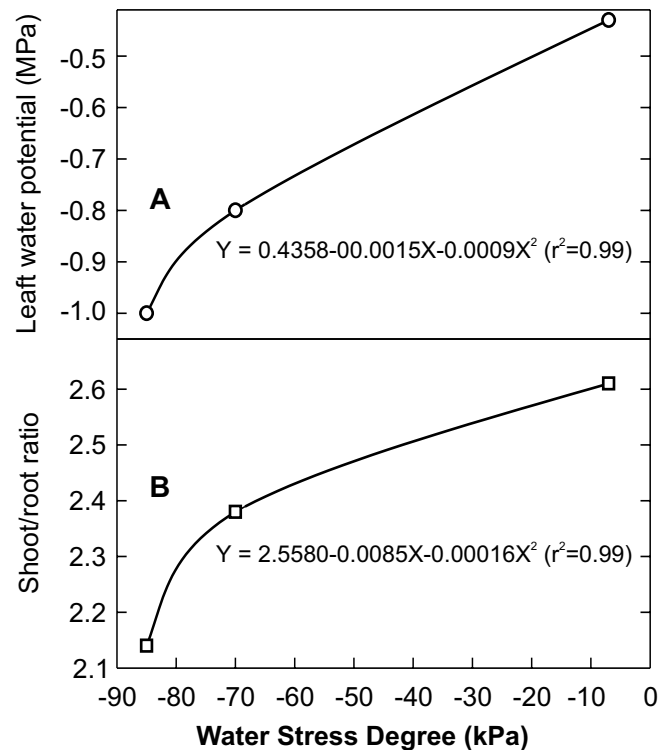
Plants were not exposed to  $\Psi_m$  values extremely low through the experiment, though the period of stress had been relatively long, reaching values of -1.0 MPa (Fig. 2A). Studies conducted by Turk *et al.*, (31) with cowpea and by González *et al.* (8) with soybean, presented  $\Psi_w$  -1.2 MPa with magnitude similar to the data found in this experiment. Practically, the negative effects on  $N_2$  assimilation is observed when the  $\Psi_w$  is reduced below -0.75 MPa as happened in stressed treatments. The sensitivity of cowpea nodules under water deficit conditions inclosed with the plant development and with the  $\Psi_w$ .

The results indicate that the water stress controlled through a porous cap resulted in a decline in the enzymes activity in metabolic paths concerning the  $N_2$  fixation, NADH-dependent glutamate synthase (NADH-GOGAT), glutamine synthetase (GS), phosphoenolpyruvate carboxylase (PEPEC) and an increase in glutamate dehydrogenase (GDH) (Fig. 3 A, B, C and D). However, it can be clearly noted that NADH-GOGAT was the most sensitive enzyme under water stress and its activity in the nodule decreased rapidly with an increase of water stress.

GS and NADH-GOGAT activities in the nodules are not strongly coupled, and the GS/NADH-GOGAT coupling became weaker as drought progressed. According to Groat and Vance (10), a strong GS/NADH-GOGAT coupling would not be required once glutamate may be supplied as a substrate to GS by some other mechanism, that is, through NADH-GDH.

Nodules PEPC exhibited a bell shape tendency with maximal activity around flowering. These results are similar to those reported by Silveira *et al.* (25). There is a synergism between  $CO_2$  dark fixation and N fixation. PEPC has an important role in energy production and carbon skeleton supply necessary for  $N_2$  reduction and  $NH_3$  assimilation, and is a key enzyme for anaplerotic reactions that occur in plants (24).

In this study, a positive correlation ( $r=0.899$ ) between PEPC and GS activities for cowpea nodules was also observed. This positive correlation and the  $N_2$  fixation suggest a potential use



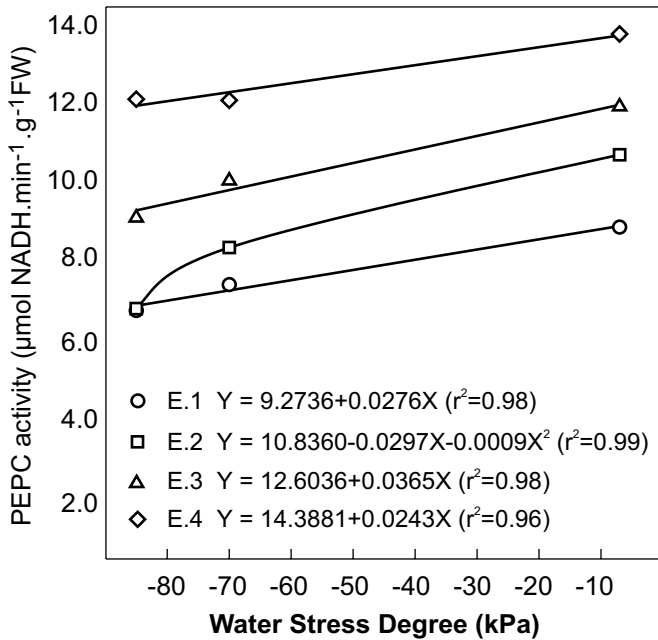
**Figure 2.** Leaf water potential (A) and shoot/root ratio (B) in cowpea at different degrees of water stress (-70.0 and <-85KPa) and control (-7.0KPa).

of these enzymes and their specific isoforms in nodule as molecular markers for genetic breeding program aiming maximization of  $N_2$  fixation.

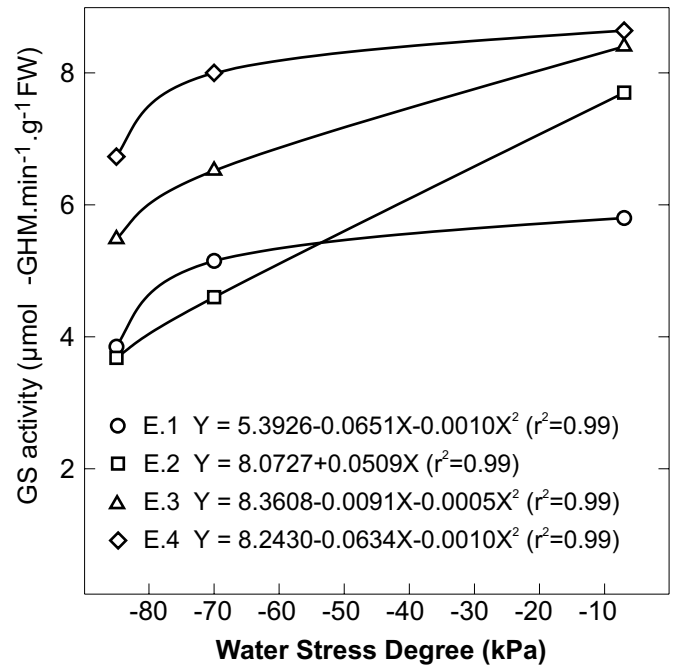
GDH activity of nodules (Fig. 3D) increased with a more negative  $\Psi_m$ . It is believed that increased GDH activity might occur as a measure of ammonia detoxication resultings from protein and aminoacid degradation due to during water stress (28). The higher increase at the  $E_4$  stage may be due to the beginning of nodules senescence. Groat and Vance (10) report that the GDH can function in assimilation of ammonia during the nodules senescence in alfalfa.

The proportion between NADH-GOGAT and GDH activities varied greatly and presented a low correlation ( $r=0.323$ ) indicating that the affinity of the GDH or the NADH-GOGAT can control the synthesis, activation and inhibition of one another.

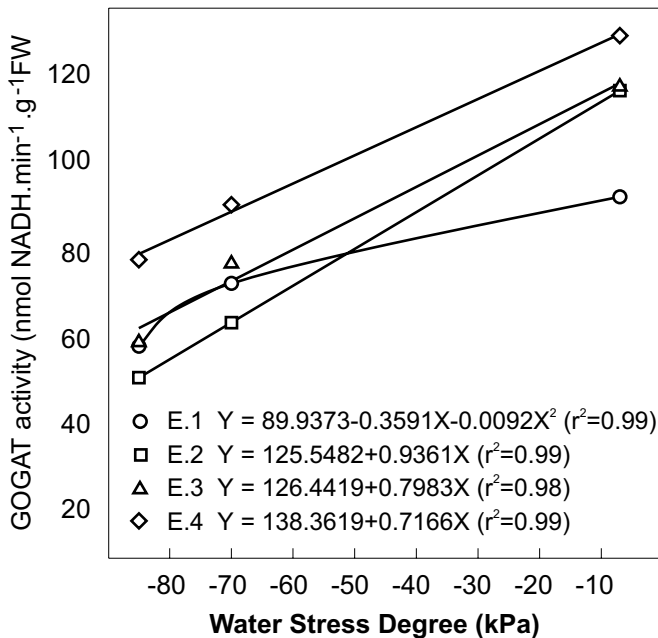
The inhibition of  $N_2$  fixation by water stress was established, corroborating the observations of Guerin *et al.* (11) and Diaz del Castillo *et al.*, (4), but information about the effects of water stress on enzymes of ammonia assimilation are insufficient and difficult to evaluate. Sheoran *et al.* (23) found that water stress caused a drastic reduction in the GS activity in roots and nodules of pigeon-pea that was not reflected in the leaves, whereas González *et al.* (8) didn't find any significant difference



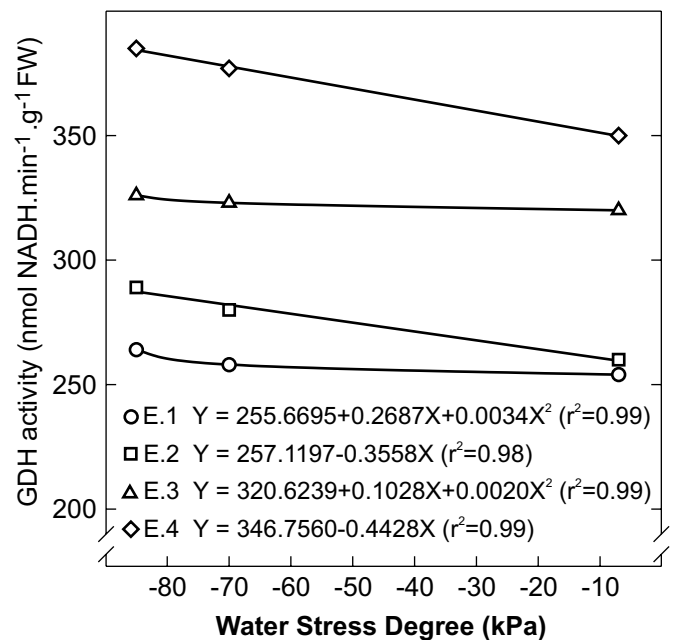
**Figure 3A.** Phosphoenolpyruvate carboxilase (PEPEC) activity in cowpea nodule, measured at four developmental stages of  $\text{N}_2$  fixation (E<sub>1</sub> 0-15; E<sub>2</sub> 15-30; E<sub>3</sub> 20-35 and E<sub>4</sub> 30-45 days from emergence at different degrees of water stress (-70.0 and <-85.0KPa) and control (-7.0KPa).



**Figure 3C.** Glutamine synthetase (GS) activity in cowpea nodule, measured at four developmental stages of  $\text{N}_2$  fixation (E<sub>1</sub> 0-15; E<sub>2</sub> 15-30; E<sub>3</sub> 20-35 and E<sub>4</sub> 30-45 days from emergence at different degrees of water stress(-70.0 and <-85.0KPa) and control (-7.0KPa).



**Figure 3B.** Glutamina synthase (NADH-GOGAT) activity in cowpea nodule, measured at four developmental stages of  $\text{N}_2$  fixation (E<sub>1</sub> 0-15; E<sub>2</sub> 15-30; E<sub>3</sub> 20-35 and E<sub>4</sub> 30-45 days from emergence at different degrees of water stress (-70.0 and <-85.0) and control (-7.0KPa).



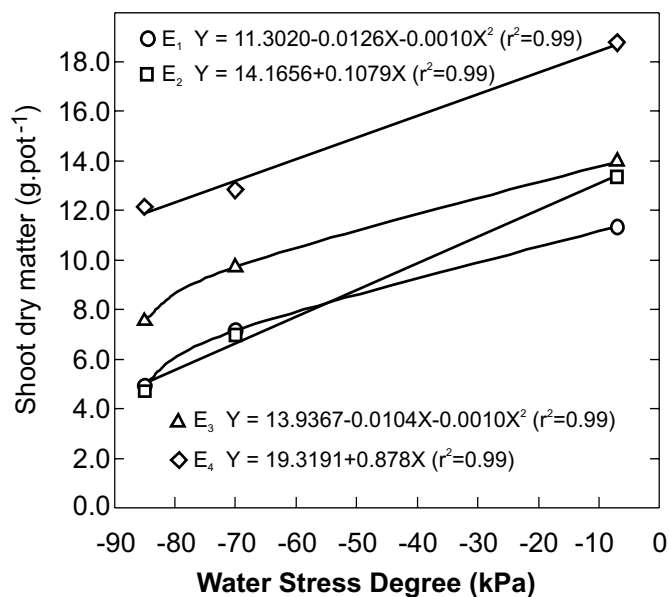
**Figure 3D.** Glutamate dehydrogenase (GDH) activity in cowpea nodule, measured at four developmental stages of  $\text{N}_2$  fixation (E<sub>1</sub> 0-15; E<sub>2</sub> 15-30; E<sub>3</sub> 20-35 and E<sub>4</sub> 30-45 days from emergence at different degrees of water stress(-70.0 and <-85.0KPa) and control (-7.0KPa).

in the reduction of the GS activity in soybean nodules submitted to -1.2 MPa water stress.

The plants submitted to water deficit presented a reduction in leaf area (due to a reduction in the expansion of the leaf), lower production of leaves and greater senescence. This generally causes a quite significant effect in the total dry matter produced. The leaf area presented a highly significant correlation with the dry matter ( $r = 0.905$ ), coinciding with the study by Costa *et al.* (3). It was also seen that the shoot dry matter in relation to the control at the most negative  $\Psi_m$  ( $S_3$ ) was more affected at the  $E_2$  stage, indicating that stressed plants in this period suffer reduction in leaf area expansion as much as in dry matter accumulation. Fig. 2 presents reductions in the shoot/root due to water stress, in accordance with the study of Costa *et al.* (3).

It was verified that the shoot dry matter (Fig.4) plants at the most negative  $\Psi_m$  ( $S_3$ ) was more affected in  $E_2$  stage, indicating that stressed plants in this period suffer reduction in the leaf area expansion as much as in the dry matter accumulation. It was also observed that water stress determined reductions in the shoot/root ratio (Fig. 2B), in accordance with the study of Costa *et al.*, (3).

Nitrogen content in the shoot of most stressed plants ( $25.70 \text{ mg g}^{-1} \text{ DW}$ ) was greater than the control ( $24.50 \text{ mg g}^{-1} \text{ DW}$ ), but did not present any statistical significance in relation to the stress. Even with the dry matter production of stressed plants being low, the nitrogen content was greater than in control plants. Some studies have shown the effect of water stress on the availability of nutrients (33).



**Figure 4.** Shoot dry matter in cowpea nodule, measured at four developmental stages of  $N_2$  fixation ( $E_1$  0-15;  $E_2$  15-30;  $E_3$  20-35 and  $E_4$  30-45 days from emergence at different degrees of water stress (-70.0 and <-85.0KPa) and control (-7.0KPa).

Growth and dry matter accumulation of legumes reduction due to low water stress degrees has been reported in *Phaseolus vulgaris*, *Glycine max* and *G. wightii*. The existence of variations among cultivars within legume species in  $N_2$  fixation sensitivity to water deficit (21) indicates that the tolerance trait found in some genotypes may be useful in breeding programmes for  $N_2$  fixation drought tolerance in legumes.

The results presented herein indicate that water stress applied in the  $E_2$  stage was the most harmful, suggesting this way be a critical period for cowpea nodules. The GS and NADH-GOGAT coupling was lost as the drought conditions progressed and there was a slight reduction in the PEPC activity with increased stress. The GDH activity increased in more negative  $\Psi_m$ , indicating that the cowpea nodules maintain sufficient enzyme activity under stress.

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## RESUMO

### Resposta ao estresse hídrico na atividade enzimática dos nódulos de caupi

Experimento em casa de vegetação foi conduzido com objetivo de estudar os efeitos do estresse hídrico nas atividades metabólicas dos nódulos de caupi nos diferentes estádios de desenvolvimento da fixação de  $N_2$ . As plantas de caupi foram crescidas em vasos com solo Latossolo amarelo sob diferentes potenciais matriciais: -7,0 (control- $S_1$ ), -70,0 ( $S_2$ ) e <-85,0 KPa ( $S_3$ ). O desenho experimental foi em blocos ao acaso com parcela sub-dividida, na parcela principal contendo os diferentes níveis de potencial matricial e a sub-parcela contendo os diferentes estádios de desenvolvimento:  $E_1$  (0-15),  $E_2$  (15-30),  $E_3$  (20-35) e  $E_4$  (30-45) dias após a emergência. Os tratamentos de estresse foram aplicados gradualmente através do sistema de cápsula porosa. O efeito do estresse de água foi mais prejudicial ao caupi quando aplicado no estádio  $E_2$  do que nos demais estádios. A relação parte aérea/raiz decresceu de 2,61 para 2,14 no potencial matricial <-85,0 e -70,0 KPa respectivamente. Foi observado pequeno decréscimo nas atividades da glutamina sintetase e fosfoenolpiruvato carboxilase com o aumento do estresse, enquanto que a glutamina sintase foi a enzima mais sensível ao estresse. A atividade da glutamato desidrogenase aumentou no potencial matricial mais negativo indicando que esta enzima é suficientemente ativa sob estresse hídrico.

**Palavras-chave:** estresse hídrico, *Vigna unguiculata*, nodulinas

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