

## N-compound accumulation and carbohydrate shortage on N<sub>2</sub> fixation in drought-stressed and rewatered cowpea plants

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### Abstract

In this study nodulated cowpea plants were exposed to a progressive drought-stress followed by rewatering in order to establish mechanisms related to the nodule sensitivity to N<sub>2</sub> fixation. Water stress caused a prominent decrease in the dry matter of leaf and nodule in contrast to the root growth that was stimulated. The drought-stressed plants showed a great decline in the ureide concentration of xylem sap whilst the concentration of the nodule leghaemoglobin decreased slightly. The stressed-nodules had significant accumulation of soluble carbohydrates in parallel to intense increase in the concentration of ureide, total amino acid and proline. After two days rewatering, the leaf growth showed a significant enhancement whereas in nodules a poor recovery was observed. In addition, the nodule N<sub>2</sub> fixation capacity did not improve after rehydration whereas the sap ureide experienced only a slight recovery and surprisingly the content of nodule soluble protein continued to drop. In contrast, a significant recovery in the nodule leghaemoglobin content was observed. Rewatering greatly induced depletion of both sucrose and total soluble sugar contents of nodules that were associated to a great decrease in the concentration of ureide, proline and amino acids, especially in leaves. Conversely the poor recovery of cowpea nodules after rehydration was apparently related to a carbohydrate shortage.

**Key words:** leghaemoglobin, ureide, symbiose, water stress, *Vigna unguiculata*.

### Resumen

#### Acumulación de componentes nitrogenados y reducción de carbohidratos en la fijación de nitrógeno de plantas de *Vigna unguiculata* (L.) Walp sometidas a estrés hídrico y rehidratadas

A fin de establecer los mecanismos relacionados con la capacidad de los nódulos para fijar nitrógeno atmosférico, se expusieron plantas de *Vigna unguiculata* (L.) Walp noduladas a un estrés progresivo de sequía seguido de rehidratación. La sequía produjo una importante disminución en la materia seca de las hojas y de los nódulos, y en cambio estimuló el crecimiento de las raíces; por otro lado, hubo una gran disminución en la concentración de ureidos de la savia del xilema. Los nódulos sometidos a estrés presentaron una acumulación significativa de carbohidratos solubles, junto con un intenso aumento de la concentración de ureidos, aminoácidos totales y prolina, disminuyendo levemente la concentración de leghemoglobina. Tras dos días de rehidratación, el crecimiento de las hojas aumentó significativamente, pero tanto los ureidos de la savia como los nódulos sólo se recuperaron de forma leve, y no aumentó su capacidad de fijación de N<sub>2</sub>. Además, sorprendentemente, el contenido de proteínas solubles de los nódulos siguió disminuyendo, aunque el contenido de leghemoglobina se recuperó significativamente. Una rehidratación intensa indujo reducción tanto en el contenido de sacarosa como de azúcares solubles totales de los nódulos, junto con una gran disminución en la concentración de ureidos, prolina y aminoácidos, especialmente en las hojas. La pobre recuperación de los nódulos de *Vigna unguiculata* (L.) Walp tras la rehidratación estuvo aparentemente relacionada con la escasez de carbohidratos.

**Palabras clave:** leghemoglobina, ureidos, simbiosis, estrés hídrico, *Vigna unguiculata*.

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## Introduction

Legume N<sub>2</sub> symbiotic fixation is greatly sensitive to drought stress, particularly in tropical species like cowpea [*Vigna unguiculata* (L.) Walp.] (Sinclair and Serraj, 1995; Figueiredo *et al.*, 1999a). It has been reported that N<sub>2</sub> fixation is more sensitive to water stress than CO<sub>2</sub>-photosynthetic assimilation (Dekoun and Planchon, 1991). However, the carbohydrate supply from leaves intensively limits the nodule activity (Schubert *et al.*, 1995). Nevertheless, the inhibition mechanisms of nitrogenase and N<sub>2</sub> fixation caused by water restriction are still controversial. The effects could be related to: (a) the reduced photosynthesis and therefore, reduced availability of carbohydrate, (b) less water for the transport of N-products away from nodule, (c) some direct effect on nodule gas permeability, or (d) the alteration of nodule metabolic activity (González *et al.*, 1998). In addition, the drought-induced accumulation of N-compounds in shoot might also inhibit the N<sub>2</sub> fixation through a negative feedback mechanism (Serraj *et al.*, 1999a).

There is a direct correlation between decreased nodule water potential and decline of nitrogenase activity (Guerin *et al.*, 1990). The nodule of alfalfa plants required a higher  $\Psi_w$  (leaf water potential) than leaf tissues to maintain its fixation process at maximum rates, suggesting that leaf tissue was more tolerant to dehydration (Irigoyen *et al.*, 1992). On the other hand, unlike the root tissue, the transport of water to the nodules is almost exclusively through the phloem (Serraj *et al.*, 1999a). Therefore, the great sensitivity of N<sub>2</sub> fixation to water deficit could be a consequence of phloem flow rate from leaves, which, in turn, is sensitive to variations in leaf water potential (Walsh *et al.*, 1998). Recently, some researchers (Guerin *et al.*, 1990; González *et al.*, 1998) suggested that N<sub>2</sub> fixation during drought decreases by a feedback mechanism caused by N-compound accumulation. They concluded that ureide accumulation in the shoot might indirectly inhibit the nitrogenase activity through a derivative compound, being asparagine a potential candidate.

In some temperate legumes (*Vicia faba* L. and *Medicago sativa* L.) the drought stress acted directly on the nodule metabolic potential, defined as the sum of the nodule biochemical machinery and its capacity to support nitrogen fixation at maximum rates, since they were unable to overcome the inhibition by raising the concentration of oxygen (Guerin *et al.*, 1990; Irigoyen *et al.*, 1992; Guerin *et al.*, 1991). Thus the early res-

ponses to water stress in these indeterminate nodules might be related to a decrease in leghaemoglobin (legh) content rather than to the carbohydrate shortage found in nodules of soybean (Gordon *et al.*, 1999) and pea (González *et al.*, 1998). Nevertheless several works cited by Serraj and collaborators in a recent review (Serraj *et al.*, 1999a) have pointed out that there are conflicting results in the literature and that different hypotheses to explain the inhibition mechanisms of nodule activity by water stress were put forward.

The coordinated balance that occurs among nodule and leaf C and N metabolism under water stress conditions is still unknown (González *et al.*, 1998). Thus, it is very important the comprehension of the mechanisms involved in the recovery of the plant associated with the nodule activity for N<sub>2</sub> fixation under short periods of drought-stress followed by rehydration, an environmental condition that is recurrent in the semi-arid tropical regions, where cowpea is largely cultivated (Figueiredo *et al.*, 1999a). Recently we demonstrated that cowpea was more sensitive to drought-stress in terms of the nodule growth and nitrogenase activity than in relation to activities of nitrogen assimilation enzymes (Figueiredo *et al.*, 1999b; Silveira *et al.*, 2001). In this study nodulated cowpea plants were exposed to a progressive drought-stress followed by rewatering in order to establish mechanisms related to the nodule sensitivity to N<sub>2</sub> fixation.

## Material and methods

Seeds of *Vigna unguiculata* (L.) Walp. cv. Vita 7 (EMBRAPA/CNPMN, Piauí, Brazil), surface sterilized with 2.5% sodium hypochlorite solution, were planted in jars (diameter 9 cm and height 20 cm) containing 1.25 dm<sup>3</sup> of vermiculite. This substrate presents a high water holding capacity (0.70 cm<sup>3</sup> water cm<sup>-3</sup> substrate). Two days after planting the seeds were inoculated (10<sup>9</sup> cells/plant) with *Bradyrhizobium* spp. BR-3256 (CB-756) strain host legume *Vigna unguiculata* L. Walp. origin Zimbabwe supplied by EMBRAPA/CNPAB (National Agrobiology Research Center), Rio de Janeiro, Brazil. The seedlings were reinoculated four days later. The plants were irrigated daily by a capillary sub-irrigation system (Leonard jars modified) with 800 cm<sup>3</sup> of a N-free nutrient solution composed of (mol m<sup>-3</sup>): K<sub>2</sub>SO<sub>4</sub>=2.0; CaCl<sub>2</sub>=3.0; K<sub>2</sub>HPO<sub>4</sub>=1.0; MgSO<sub>4</sub>=1.0 and micronutrients as previously described (Silveira *et al.*, 2001). The nutrient solution was changed each four days and

the pH maintained at  $6.0 \pm 0.5$  with addition of  $0.1 \text{ mol m}^{-3}$  HCl or NaOH. Once a week pots were rinsed with tap water to avoid salt accumulation. The experiments were carried out in a greenhouse under natural conditions with mean temperature varying from 24°C (night) to 27°C (day). The average of relative humidity was 55% (day) and 87% (night), a 12 h photoperiod with a means of photon maximum flux of approximately  $1,100 \mu\text{mol m}^{-2} \text{ s}^{-1}$  measured at plant level (190SA quantum sensor, LI-COR).

Inoculated cowpea plants were grown until 28 days after emergence as described above. Next the plants were transferred to one of the following conditions of water content in the substrate: during 4 consecutive days, half of the plants received no watering (drought-stressed plants) and the remaining ones received nutrient solution to jar capacity on a daily basis (well-watered control). Because of the high water holding capacity of vermiculite, the cessation of nutrient application resulted in a gradual water stress. The jars were covered to avoid evaporation and next the control and the water-stressed plants were weighed every day to calculate transpiration rates. After cessation of the short-term drought-stress, water stressed plants were resupplied with nutrient solution for 2 subsequent days. A completely randomized design with two treatments (drought-stressed and well-watered control) and seven harvesting period (0, 1, 2, 3, and 4 days of water withdrawal and 1 and 2 days after rewatering) was employed. Three independent replicates (one individual jar containing two plants represented a replicate) per treatment were performed. Fresh nodules, roots and leaves were excised, washed with distilled water, dried with filter paper, weighed, instantaneously frozen in liquid nitrogen, kept at  $-20^\circ\text{C}$  and immediately lyophilized for later analyses. The experiment was repeated twice and the data trend was similar in both experiments. Statistical analyses were performed by the SAS statistical analysis system and means were compared by the least significant difference (LSD) test at the 0.05 level of confidence.

The leaf and nodule relative water content were measured according to Irigoyen *et al.* (1992) with minor modifications. Thirty leaf discs (diameter 1.3 cm) of the fully expanded leaf and nodule samples were collected at 09:00 am under laboratory conditions (24°C and 52% relative humidity) and weighed (fresh weight, FW) immediately. These tissues were then plunged in Petri dishes containing water for 7 h at 25°C under a photon flux density of  $38 \mu\text{mol m}^{-2} \text{ s}^{-1}$  (leaves) or in

the dark (nodules), and their turgid weights (TW) determined. These samples were dried in an oven at 80°C for 48 h, their dry weights (DW) obtained. Relative water content (RWC) was calculated using the formula:  $(\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$ . The leaf water potential ( $\psi_w$ ) was measured by the pressure chamber method (Soilmoisture Equipment Corp., USA). In order to obtain standardized measures, the following precautions were taken: (a) the determination was made on the leaf with the same physiological age for which the RWC was determined, (b) the readings were taken from 09:00 to 10:00 am, (c) the apparatus was installed as close as possible to the plants to speed up the measurements.

Xylem exudate was collected from bleeding rootstocks after shoots had been decapitated through the hypocotyl region about 3 cm above the surface of the rooting medium. The sap was collected after pressurization in the stem using a vacuum pump (Figueiredo *et al.*, 1999b). The xylem sap collected was transferred immediately into ice-cooled glass vials and stored at  $-20^\circ\text{C}$ . The ureide-N was determined by a colorimetric method (Vogels *et al.*, 1970) using alantoin as standard and it was expressed as  $\text{mmol alantoin dm}^{-3}$  xylem exudate. The total N was determined by a colorimetric method (Baethgen and Alley, 1989) after sample digestion; soluble proteins were measured by the protein dye-binding method (Bradford, 1973) using bovine serum albumin as standard; total free amino acids (L-glutamine as standard) and ureides (alantoin as standard) were determined according to Peoples *et al.* (1989); proline according to the Bates's method (Bates *et al.*, 1973) and nodule leghaemoglobin determined by the cyanmethemoglobin method (Wilson and Reisenauer, 1963) using human hemoglobin as standard. The contents of amino acid, ureide and proline were expressed as  $\text{mmol kg}^{-1}$  dry matter (DM). The nodule efficiency was expressed as  $\text{mol N-fixed kg}^{-1}$  nodule and the N<sub>2</sub> fixation rate was estimated from the direct measurement of total N accumulated in the whole plant ( $\text{mol N plant}^{-1}$ ) and indirectly through ureide concentration in xylem sap. Previously we demonstrated that these parameters presented a significant correlation with both the current nitrogenase activity (acetylene reduction method) and the total N<sub>2</sub> fixation (measured as the N accumulated in the whole plant) in cowpea plants exposed to a progressive water stress (Figueiredo *et al.*, 1999a).

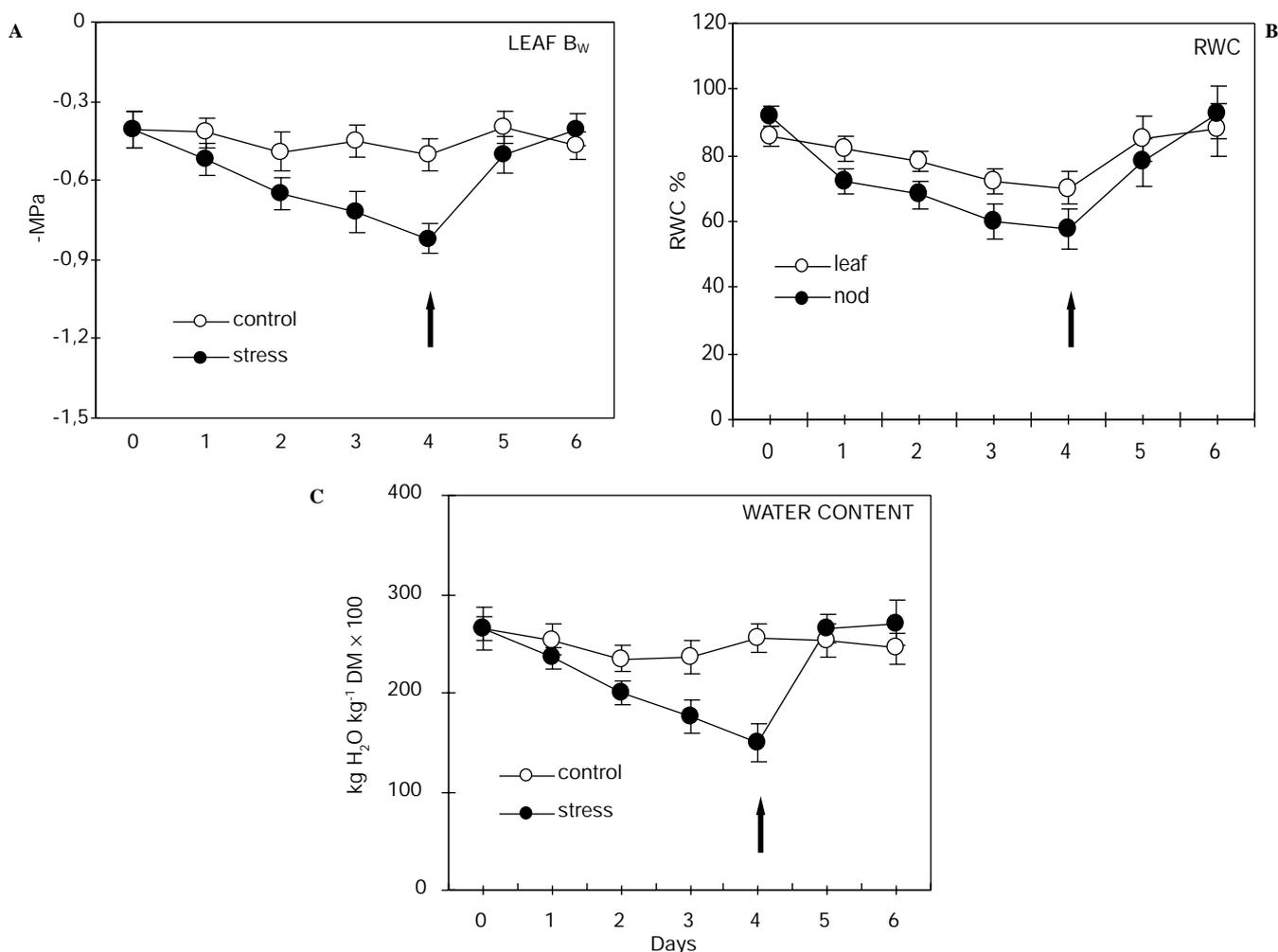
Lyophilized samples of leaf, nodule and root tissues were extracted with 80% (v/v) ethanol at boiling tem-

perature. The ethanol-soluble extract was dried under vacuum, and the soluble compounds were redissolved in distilled water for determination of sucrose and total soluble sugars. The sucrose concentration in water-extract was estimated after acid-hydrolysis in presence of  $0.5 \text{ mol dm}^{-3} \text{ HCl}$ ,  $70^\circ\text{C}$ , 30 min. Both total reducing sugars (TRS) and free reducing sugars (FRS) in water-extract were determined according to Somogyi-Nelson's colorimetric method (Somogyi, 1952). The nodule sucrose content was estimated through difference between TRS (determined after acid hydrolysis) minus FRS (determined before acid-hydrolysis). Total soluble sugars were determined by phenol-sulfuric method (Dubois *et al.*, 1956). D-glucose was utilized as standard for both determinations. The contents

of sucrose and total soluble sugars (TSS) were both expressed as  $\text{mmol glucose kg}^{-1} \text{ DM}$ .

## Results

In this study, a group of 28-d-old nodulated cowpea plants, having an initial mean of nodule dry weight of 0.60 g per plant exemplifying an excellent level of nodulation, was submitted to a short-term drought stress (4 d) followed by 2 consecutive days of rewatering. The high water holding capacity of vermiculite associated to the cessation of nutrient solution application during the drought period allowed for a progressive depletion in the vermiculite-water content (Fig. 1) resulting in a

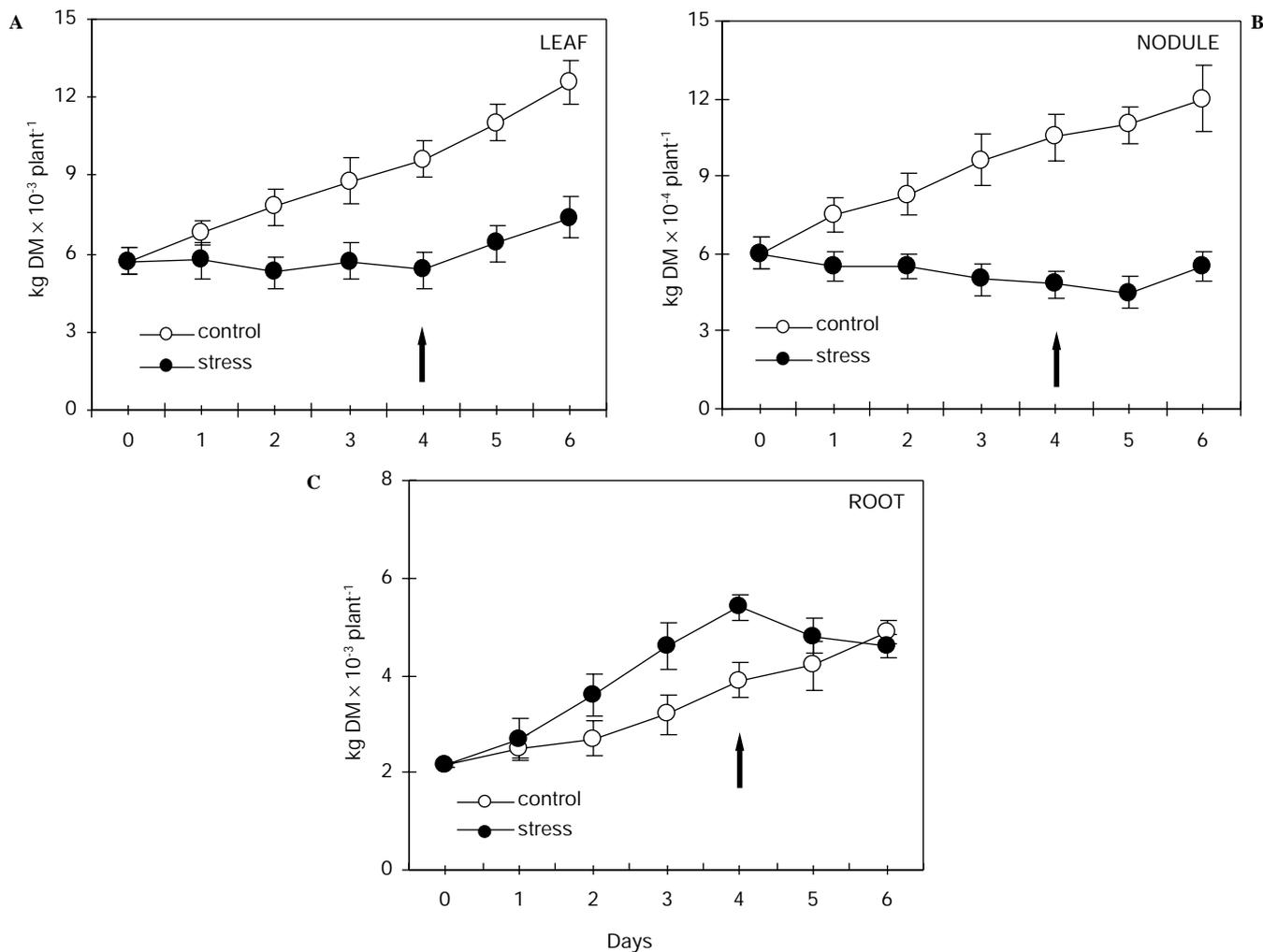


**Figure 1.** A) Leaf water potential of the water-stressed plants (●-●) and in well-watered control (○-○), B) nodule- (●-●) and leaf (○-○) relative water content of cowpea plants subjected to water withdrawal followed by recovery, and C) water content of the vermiculite substrate of the water-stressed plants (●-●) and in well-watered control (○-○). The arrows indicate the rewatering day and each point represents the mean of three replicates  $\pm$  SE.

progressive plant-water deficit. The water-stress at the end of the drought period was intensified by the hot and dry environmental conditions over the experimental period. The transpiration rates (expressed as kg water kg<sup>-1</sup> shoot DM day<sup>-1</sup>) of water-stressed plants declined drastically during the water withdrawal period. Indeed, at day 4 it was represented by 20% of the control (data not shown). Therefore, the design utilized in the current study was able to induce a progressive and severe water stress as well as contrasting responses among leaf and nodule.

The leaf  $\Psi_w$  of water-stressed plants presented a steady decrease during the drought period, which was in parallel with the actual water content of vermiculite (Fig. 1A, C). During the 4 d of water stress, the leaf

$\Psi_w$  decreased from  $-0.41$  to  $-0.83$  MPa and the vermiculite water content from 2.78 to 1.49 kg water kg<sup>-1</sup> DM (Fig. 1A, C). These changes represented approximately 50% decrease in both parameters in relation to control. The relatively high values of leaf  $\Psi_w$  reached after 4-d of drought ( $-0.83$  MPa) did not adequately reflect the severity of the water status in stressed plants. Actually, the leaf- $\Psi_w$  is not a good index to reflect the plant-water status in this species exposed to water stress according to the pertinent literature. During water withdrawal period, leaf RWC was less sensitive to desiccation than leaf  $\Psi_w$ . In addition, the leaf RWC was less affected by water stress than was the nodule RWC (Fig. 1A, B). Rewatering of the stressed cowpea plants led to a rapid recuperation in both leaf- $\Psi_w$  and

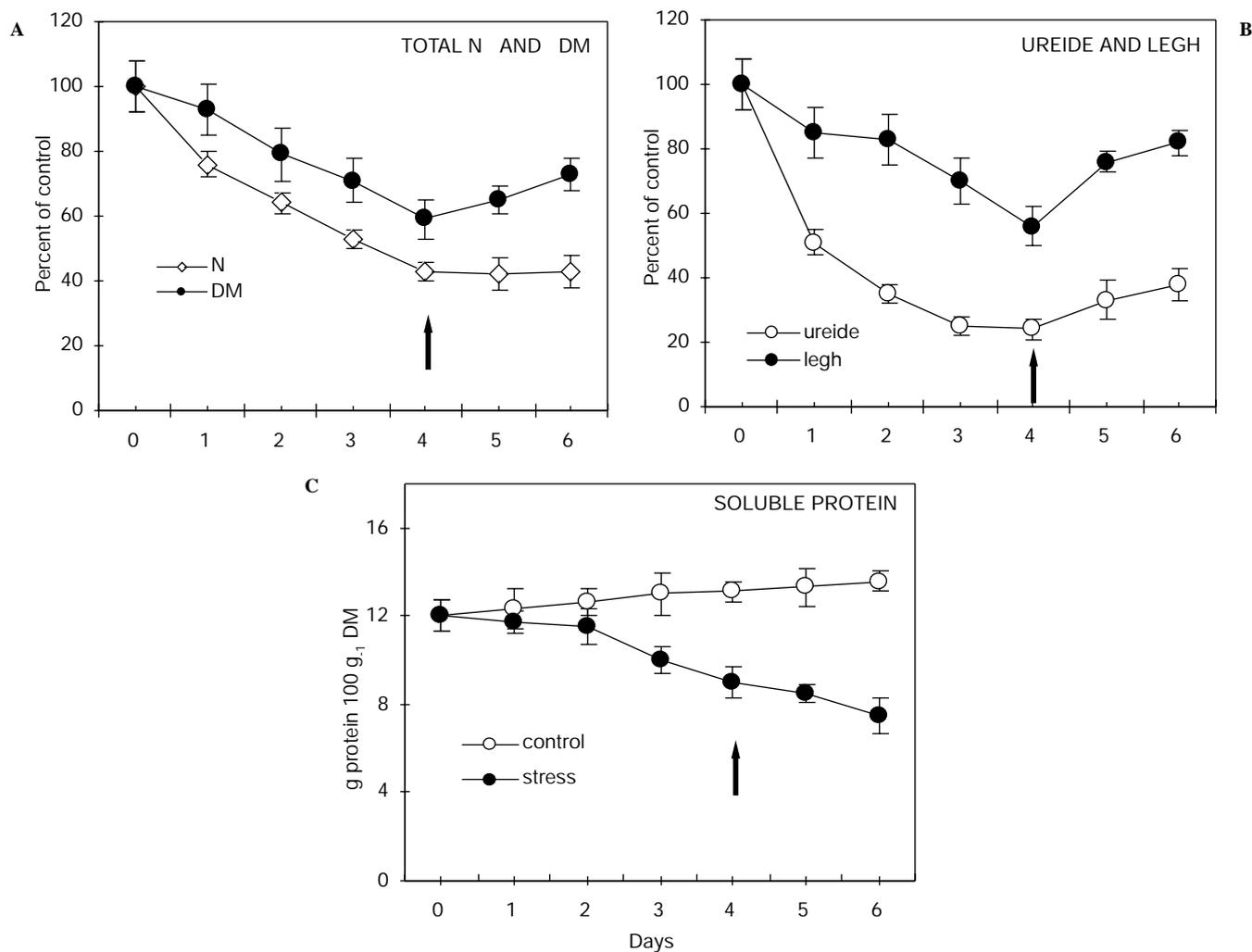


**Figure 2.** A) Dry matter accumulation of leaf, B) nodule and C) root of the water-stressed (●-●) and well-watered control (○-○) cowpea plants subjected to water withdrawal followed by recovery. The arrows indicate the rewatering day and each point represents the mean of three replicates ± SE.

leaf and nodule RWC. Indeed, after 1-d of the rewatering these plants reached values similar to those of the well-watered control (Fig. 1A, B). Drought stress caused a pronounced and proportional effect on both the leaf and nodule growth. After 4 d of the water withdrawal the leaf and nodule dry mass represented 56% and 47%, respectively, of that of the well-watered plants. Moreover a cessation in dry matter accumulation in these plant parts after the second day of the drought was observed. Interestingly, the root growth rate was stimulated by the water stress during the drought period in comparison with the well-watered plants (Fig. 2A, B, C). On the other hand, after 2-d rewatering the leaves showed a 33% recovery in DM when compared to the pre-stressed plants whereas in

the nodule mass only a 10% recovery was observed. Curiously the root dry mass of pre-stressed plants decreased 20% after 2-d rewatering.

The progressive water stress caused a continuous decrease in total N content ( $\text{g plant}^{-1}$ ) derivative from  $\text{N}_2$  fixation paralleled with the whole plant dry matter ( $\text{g plant}^{-1}$ ) both expressed as percent of control (Fig. 3A). Nevertheless, the relative decrease of the total-N content was higher than that of dry matter production. After 4 d of drought the N content of water stressed plants represented 40% of that of well-watered plants whereas the plant dry mass was equivalent to 60% of control (Fig. 3A). After rewatering the stressed plants presented 30% recovery in total dry matter production whereas the total N content remained un-

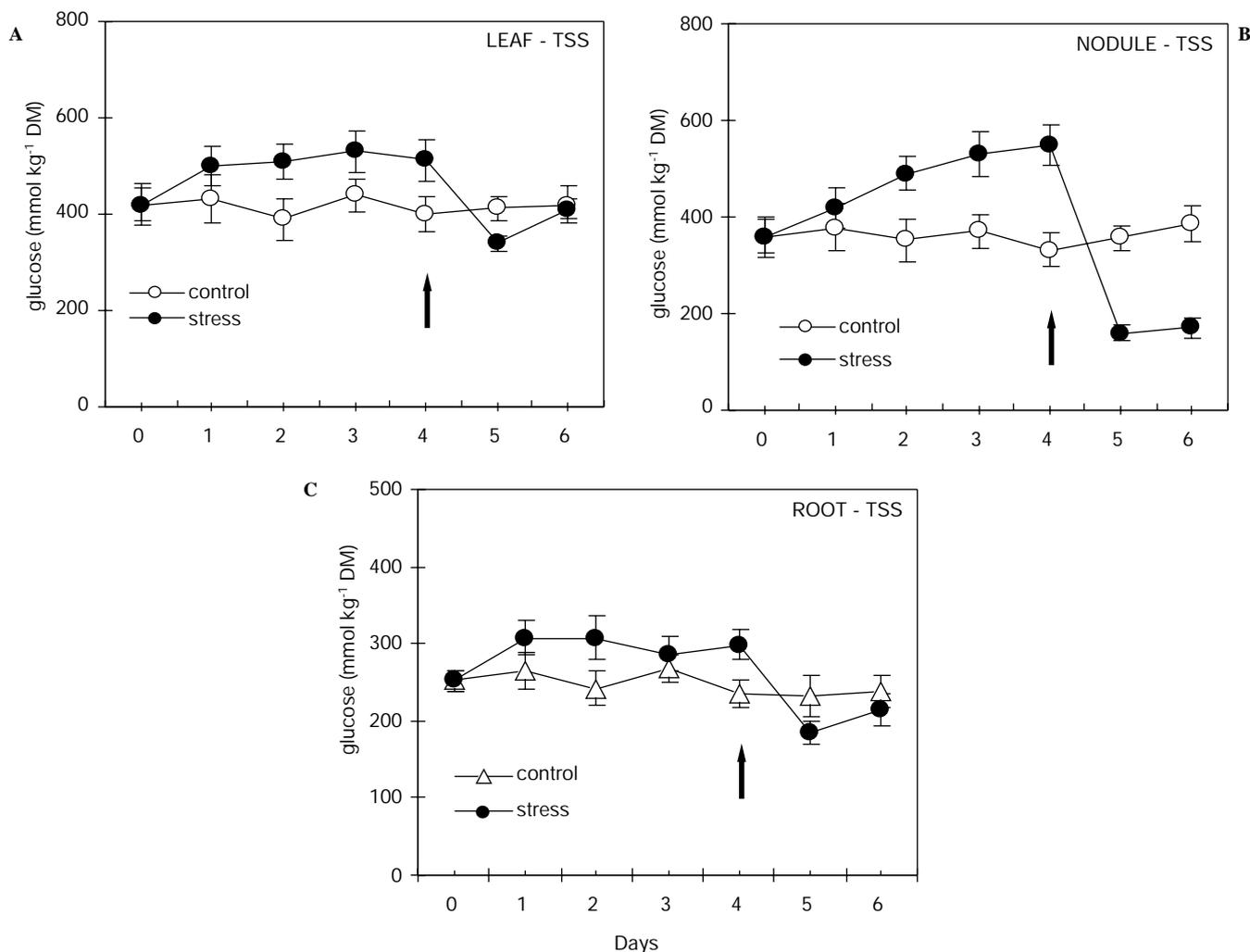


**Figure 3.** A) Relative accumulation of dry matter (●—●) and total N (○—○) in the whole plant, B) leghaemoglobin (●—●) and sap ureide concentration (○—○) and C) nodule soluble protein of water-stressed (●—●) and control (○—○) cowpea plants subjected to water withdrawal followed by recovery. The arrows indicate the rewatering day and each point represents the mean of three replicates  $\pm$  SE.

changed. The nodule efficiency, expressed as mol N-fixed kg<sup>-1</sup> nodule, was not improved after rewatering (data not shown). The sap ureide concentration, which indirectly estimates the N<sub>2</sub> fixation, drastically declined during the water withdrawal period. Indeed, after 1-d of water withdrawal its concentration experienced a 50% reduction and 4 d later it represented only 25% of the well-watered plants (Fig. 3B). In contrast, the nodule leghaemoglobin concentration showed a slight and lower decrease induced by the water stress as compared to those presented by ureide. At day 4 the leghaemoglobin concentration represented 56% of that of control (Fig. 3B). Curiously, whereas the leghaemoglobin concentration showed a faster and significant recovery after rewatering, reaching around 80% relative to control, the sap ureide concentration showed

just a slight recuperation. The nodule and leaf soluble protein content showed a similar response to the drought and rewatering. Both presented a significant decrease only after 3 d of water deficit and curiously they continued to decrease even after rewatering (Fig. 3C).

The water stress induced a slight but consistent increase in the leaf-TSS concentration during the drought period. The nodule presented a similar response except that the increase in TSS was higher than that of the leaf. Furthermore, the nodule-TSS content continued to increase throughout the water withdrawal period. The root-TSS content exhibited a response similar to those of the leaf (Fig. 4A, B, C). Interestingly rewatering caused a significant decrease in TSS concentration in the root, nodule and leaf. However, the



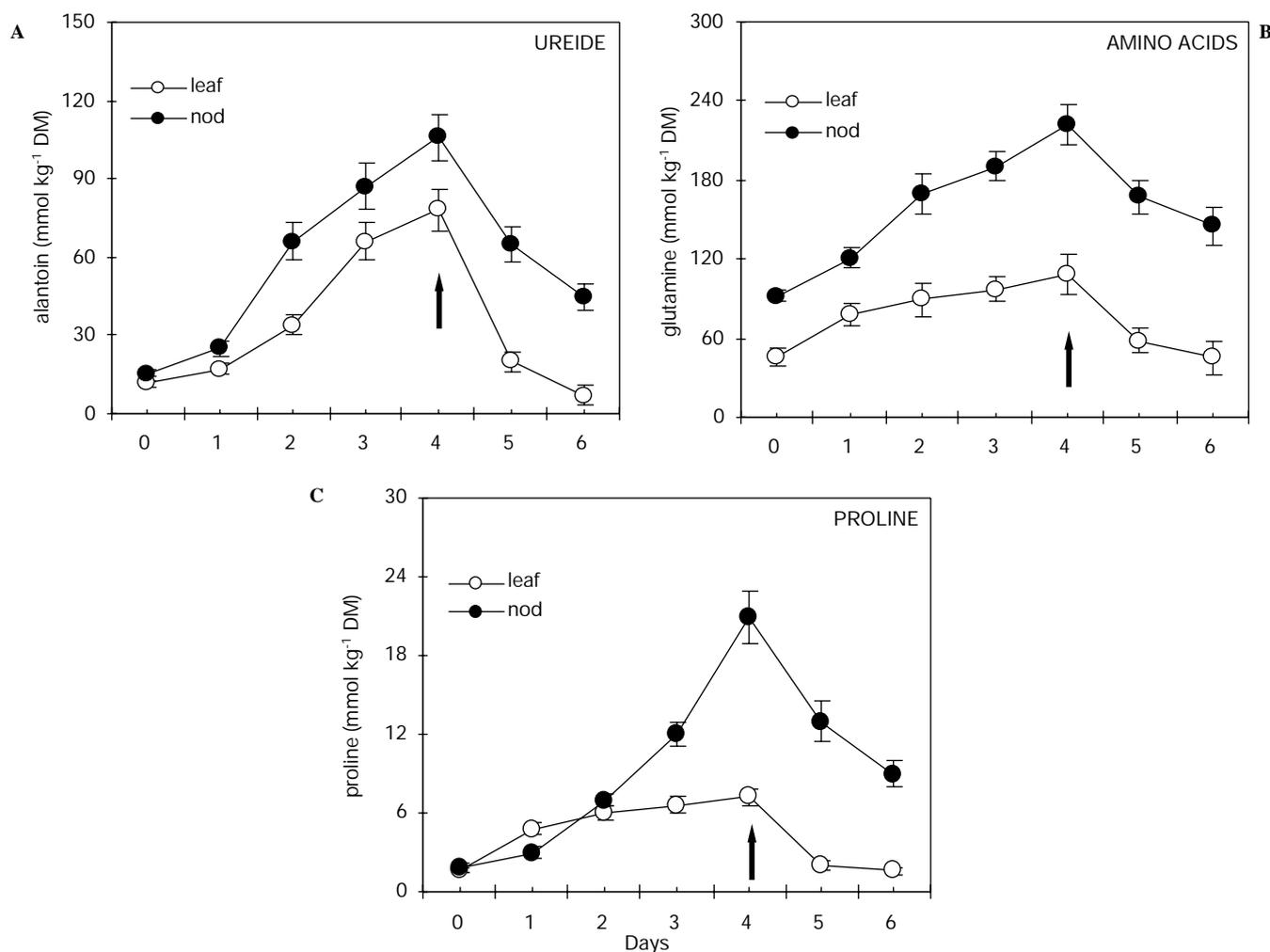
**Figure 4.** A) Total soluble sugar concentration of leaf, B) nodule and C) root of well-watered (○-○) and water-stressed (●-●) cowpea plants subjected to water withdrawal followed by recovery. The arrows indicate the rewatering day and each point represents the mean of three replicates ± SE.

nodule tissue showed a more drastic decline in the sugar content (3.5-fold) when compared to the leaf (1.5-fold) and the root (1.6-fold) after 1-d rewatering. The nodule sucrose content represented approximately 85% of that of total soluble sugars and they varied similarly during the experimental period (data not shown). An intense and rapid leaf and nodule ureide accumulation in response to the drought stress was observed. Indeed, their concentrations increased 6- and 7-fold, respectively, after 4 days of water withdrawal (Fig. 5A). Rewatering drastically reduced the leaf ureide concentration to levels near to those of the well-watered plants, whereas in the nodule the decrease was less pronounced. Similar responses of the leaf and nodule free amino acid contents were observed as compared to

their ureide content (Fig. 5B). Regarding to the leaf and nodule proline concentrations drastic increases (5- and 12-fold, respectively) were observed after 4 days of the water deficit (Fig. 5C). One day after rewatering a rapid and intense decrease in the leaf proline concentration to values near to that of the control plants was observed, whereas the decline of the nodule proline concentration was less pronounced.

## Discussion

In this study water withdrawal was able to induce a progressive and severe water stress in the nodulated cowpea plants even in a short-term exposure to



**Figure 5.** A) Changes in the concentrations of ureides, B) total free amino acids and C) proline in nodule (●—●) and leaf (○—○) of cowpea plants subjected to water withdrawal followed by recovery. The value plotted at day zero for each parameter represents the means from seven points of control obtained along of the experimental period. The arrows indicate the rewatering day and each point represents the mean of three replicates  $\pm$  SE.

drought. Recently in maize and soybean plants it was concluded that regardless of the pot or plant size the overriding factor determining water deficit plant response to drought stress was the soil water content (Ray and Sinclair, 1998). We have previously demonstrated that when nodulated cowpea plants were exposed to a progressive drought-stress and cultivated in large pots (15 dm<sup>3</sup>) containing soil at different controlled water potentials (-0.15 to -7.00 MPa) they presented similar behavior, in terms of plant-water relations and decline in N<sub>2</sub> fixation (Figueiredo *et al.*, 1999a), as compared to the current results. Moreover, under field conditions of the semi-arid tropical regions of Brazil, cowpea plants are frequently challenged by short and severe periods of drought-stress.

The data of this present study clearly showed that during the drought period the cowpea nodules were more sensitive to desiccation than the leaves, as previously observed in nodulated alfalfa plants (Irigoyen *et al.*, 1992). However, after rewatering these tissues presented a contrasting recovery performance. As previously observed (Silveira *et al.*, 2001), the leaf growth and nitrate reductase activity of drought-recovered cowpea plants showed a significant recovery whereas the nodules had a poor growth and a low capacity of N<sub>2</sub> fixation. Moreover the comparative data of total-N and dry matter accumulation during the drought period suggest that N<sub>2</sub> fixation was more sensitive to drought than photosynthesis. In addition the recovery of photosynthesis was apparently greater than that of N<sub>2</sub> fixation. These results are in agreement with those obtained for several other legume species that showed a similar higher sensitivity of N<sub>2</sub> fixation as compared to the photosynthesis rate (Huang *et al.*, 1974; Dekoun and Planchon, 1991; Sinclair and Serraj, 1995; Serraj *et al.*, 1999a).

It is noteworthy that, in contrast to what was observed for leaf and nodule, the root of the drought-stressed plants presented a growth rate higher than those of the well-watered plants during the whole drought period (Fig. 2). These data together with the higher content of soluble sugars in the roots and nodules of the stressed plants (Fig. 4) suggest that the reduction in nodule growth was not caused by a soluble sugar shortage neither due to restriction in the supply of sucrose from leaves as had previously been proposed (Guerin *et al.*, 1990). The supply of carbohydrates to the root and nodule from the leaf through the phloem by similar unloading kinetics (González *et al.*, 1998) suggests that the reduced nodule growth of the water-stressed

plants was not caused by restriction in the supply of carbohydrates, since root growth was stimulated under the same stress conditions. The increase in the content of nodule soluble sugar induced by the drought-stress was similar to those observed in other legumes exposed to drought, such as pea (González *et al.*, 1998), soybean (Gordon *et al.*, 1997), alfalfa (Irigoyen *et al.*, 1992) and many others (Serraj *et al.*, 1999a). This increase in the soluble sugar was probably caused by a decline in the hydrolytic sucrose synthase activity associated with starch degradation, as has recently been proposed (González *et al.*, 1998; Gordon *et al.*, 1999).

After rewatering the intense and rapid decrease in the contents of soluble sugars, amino acids and ureides in the cowpea nodules (Figs. 4 and 5) might indicate a great catabolic activity on these substrates. Nevertheless, it can be suggested that this intense metabolism was not enough to recover the soluble protein content (Fig. 3C) as well as to promote a significant export of ureides into the xylem sap (Fig. 3B). These data suggest that the soluble sugars, amino acids and ureides accumulated during the drought period, might have been used as substrates for nodule maintenance respiration (Serraj *et al.*, 1999a) rather than for N<sub>2</sub> fixation and protein synthesis. The progressive decrease in the content of the nodule soluble protein even after 2-d of rewatering was previously confirmed by the nodule protein pattern after SDS-PAGE in which several protein bands decreased in intensity specially those with apparent molecular mass of 24 and 35 kD. These band molecular weights are close to those of the peribacteroid membrane proteins (nodulin 23 and 24) and uricase, respectively (Vance and Griffith, 1990).

Nodulated alfalfa plants when subjected to a progressive water withdrawal period also showed similar results (Irigoyen *et al.*, 1992) to those found here, particularly in relation to the increase in proline and soluble sugar concentrations in parallel with the decrease of the soluble protein. For nodulated alfalfa plants proline was suggested as a possible involvement in the osmotic adjustment and also in the protection of nodule proteins against denaturation. Although cowpea plants have a low capacity of osmotic adjustment in response to water stress (McCree and Richardson, 1987) it was verified a prominent accumulation of proline particularly in the nodules and roots, when compared to the leaves in response to drought (Silveira *et al.*, 2001). Nodule proline turnover has been proposed to be involved in providing energy for the nodule

metabolism under low oxygen pressure conditions occurring in water stress (Khol *et al.*, 1994). Thus, the intense accumulation of ureide and free amino acids, particularly proline, in cowpea nodule in response to drought stress might have exerted favorable effects in relation to C and N storage, osmotic adjustment and some protection against the deleterious effects of dehydration.

Contrary to soybean nodule, the hypothesis that water stress causes initially a reduction in the nodule leghaemoglobin concentration and then a decrease in O<sub>2</sub> diffusion to infected region (Guerin *et al.*, 1990, 1991; Irigoyen *et al.*, 1992) seems to be relatively less important to explain the decrease in N<sub>2</sub> fixation in cowpea plants (Figueiredo *et al.*, 1999a, 1999b). Indeed, the leghaemoglobin concentration in this study was less affected by water stress and had an excellent recovery after rewatering in contrast with the slow recovery in the sap ureide concentration and in the nodule capacity for N<sub>2</sub> fixation. Recently, it was shown that when cowpea plants were subjected to a long-term drought-stress the leghaemoglobin concentration was less affected than was both the sap ureide concentration and nitrogenase activity (Figueiredo *et al.*, 1999b). The question of decrease in the nodule permeability to O<sub>2</sub> under water stress is very important, but it is still not clear whether the nitrogenase activity inhibition is a cause or a consequence of increasing resistance in O<sub>2</sub> diffusion toward the infected region (González *et al.*, 1998; Serraj *et al.*, 1999a).

Recently Sinclair's group (Serraj *et al.*, 1999b; Valdez *et al.*, 2000) reinforced the general hypothesis of feedback regulation by N compound when they observed that ureide accumulation in the shoot of nodulated soybean submitted to a progressive water stress condition inhibited indirectly nitrogenase activity through a derivative compound, possibly asparagine. Although the hypothesis of a negative feedback control caused by accumulation of amino acids and ureides in shoot has been partially reinforced by our present data during the period of drought in cowpea plants, it did not yield a clear cut explanation for the results obtained during the recovery period. In this study, even when the content of leaf amino acids and ureides diminished to the status of the well-watered plants after 2-d of rewatering, the nodule growth and its capacity for N<sub>2</sub> fixation remained very low. On the other hand, the intense depletion in levels of total soluble sugars in the nodule after rewatering suggests that the supply of photosynthates from the leaves might have limited

nodule activity during the earlier metabolic events involved with N<sub>2</sub> fixation.

In conclusion, our data suggest that the high sensitivity of N<sub>2</sub> fixation observed in cowpea nodules during the drought-stress period was not associated with either soluble carbohydrate shortage or restriction in the leghaemoglobin content. However, after rewatering the poor recovery of cowpea nodules was apparently related to carbohydrate shortage.

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