ORIGINAL PAPER

Drought stress response on some key enzymes of cowpea (*Vigna unguiculata* L. Walp.) nodule metabolism

M. V. B. Figueiredo · H. A. Burity · C. R. Martínez · C. P. Chanway

Abstract A greenhouse experiment was carried out aiming to evaluate the response to drought stress of cowpea nodule enzymatic activities during different plant developmental stages leading to biological N_2 fixation. Stress was applied by controlling soil's water-potential through a porous cup. Cowpea plants cv IPA 205 were grown in pots with yellow latosol soil under three different matric potential (ψ_m) treatments. Even with high evaporative demand and limited soil water availability, cowpea could not induce an extremely low leaf water potential (ψ_w). Sap ureides concentration in cowpea declined during the drought stress period. There was a decline in enzyme activity in the metabolic pathways concerned with N_2 fixation: NADH-dependent glutamate synthase (EC 1.4.1.14), glutamine synthetase (EC 6.3.1.2) and phosphoenolpyruvate carboxylase (EC 4.1.1.31). In contrast, an increase in glutamate dehydrogenase (EC 1.4.1.4) was observed as the ψ_m declined. Metabolism associated with N_2 assimilation was impaired every time that the ψ_w was reduced below – 0.73 MPa as had happened in the stressed treatments. The stress applied by the porous cup was gradual and the plant recovered its turgor, avoiding permanent deleterious alterations in the cellular metabolism, even from a limited cowpea-growth ψ_m.

Keywords *Bradyrhizobium* sp. · N_2 fixation · Nodulins · Symbiosis · *Vigna unguiculata* · Water stress

M. V. B. Figueiredo (✉) · H. A. Burity
Laboratório de Biologia do Solo, Empresa Pernambucana de Pesquisa Agropecuária IPA/EMBRAPA, Rua Sá e Souza, 1020/1401, CEP 51030-065 Boa Viagem-Recife-PE, Recife, PE 51030-065, Brazil
e-mail: mbarreto@elogica.com.br

C. R. Martínez
Departamento de Biologia Molecular, Universidade Federal da Paraíba, João Pessoa, PB, Brazil

C. P. Chanway
Department of Forest Sciences, University of British Columbia, Vancouver, British Columbia, Canada

Introduction

Water is a major limiting factor in world agriculture. In general, most crop plants are highly sensitive to even mild dehydration stress (Mundree et al. 2002). One of the most important sites of biological N_2 fixation are nodules that form on the root systems as a result of symbiosis of the host plant with bacteria. Legumes and their symbiotic root nodule bacteria are extremely sensitive to drought stress (Serraj et al. 2001; Sinclair et al. 2001). The fact that N_2 fixation is more sensitive to decreasing soil water content relative to leaf gas exchange constitutes an important constraint on N accumulation and yield potential of legumes subjected to soil drying (Serraj et al. 1999).

Drought is one of a range of environmental stresses which can cause considerable reductions in N_2 fixation (Sprent 1981; Figueiredo et al. 1998a; Rengel 2002; Ramos et al. 2003). Although such stress is known to affect all steps of nodule formation and functioning, most previous work on mechanisms of drought stress effects on N_2 fixation has focused on nitrogenase activity, rather than nodulation (Streeter 1993). However, it is not obvious which particular physiological processes of the stressed plant actually affect nodule growth and metabolism (González et al. 1995).

The relationship between plant water status, photosynthesis and N_2 fixation, particularly under water stress, and the changes in nodule morphology have been studied for some temperate legumes. However, tropical legumes grown in arid regions, have not received adequate attention. Even where there was available information, the degree of water stress in the plants was not clearly defined, rendering it difficult to compare cultivar and/or different crops responses. The structural basis for the difference in sensitivity of N_2 fixation in tropical legumes under water stress is not clearly understood (Venkateswarlu et al. 1990). A number of studies have indicated that a nitrogenous signal (or possibly signals) associated with N accumulation in the shoot and nodule, exists in legumes so that N_2 fixation is inhibited early as soil dries out (Figueiredo et al. 1998b; Silveira et al. 2003). Some data indicate that water stress directly separates interactions between the bacteria and the host plant by alteration of nodule structure and enzymatic activity (Diaz del Castillo et al. 1994; González et al. 1995). Others suggest that the drought stress limits many processes of nodule activity (Parsons et al. 1993). Adaptive responses in the metabolism of any organism during environmental stress necessarily reflect changes in activities of enzymes, gene expression and in some cases, morphology (Serraj et al. 1999).

The objective of the present investigation was to evaluate the response to drought stress of cowpea nodule enzymatic activities during different developmental stages leading to biological N_2 fixation.

Materials and methods

Soil preparation, inoculation and planting

The experiment was conducted in a greenhouse at a temperature range of 26–33°C with 50–70% relative humidity. Pots were filled with yellow latosol, a sandy loam from the Araripina Experimental Station (0–20 cm), located in a semi-arid region of Pernambuco State at 7°29′00″ S, 40°36′00″ W and, 816 m of altitude. The soil was air dried, sieved (5.0 mm), and adjusted to pH 6.3 by the addition of a calcium and magnesium oxides mixture (3:1, w/w). Fourteen kilograms of soil contained in 15-l pots were autoclaved for 30 min at 121°C and 101 kPa, once a day for three consecutive days. Chemical and physical analyses of the soil were conducted at the Pernambuco Enterprise of Agricultural and Livestock Research (Empresa Pernambucana de Pesquisa Agropecuária—IPA) in accordance with the Empresa Brasileira de Pesquisa Agropecuária—EMBRAPA (Brasil 1997).

Seeds of cowpea (*Vigna unguiculata* L. Walp.) cv. IPA 205 (result of the crossing of ''Sendo x TVu-59'', IPA-Recife-Brazil) were sterilized superficially in 80% (v/v) ethanol for 30 s and then in 5% (w/v) sodium hypochlorite for 2 min before washing nine times in sterilized distilled water. The seeds were inoculated with a BR2001 strain of *Bradyrhizobium* sp. supplied by the Microbiological Resources Center (MIRCEN, Porto Alegre—Rio Grande do Sul, Brazil). The BR2001 strain was isolated from *Crotalaria juncea* L. host-legume by the National Agrobiology Research Center (EMBRAPA/CNPAB, Rio de Janeiro, Brazil) and catalogued under number 6145 SEMIA. Five seeds of cowpea were sown in each pot and then inoculated with 5 ml pot^{-1} of liquid culture of *Bradyrhizobium* sp. with 10^9 c.f.u. ml^{-1} inoculum size. The inoculum was evaluated by bacterial count in a Petroff-Hausser chamber, as well as by the count of colony forming units by dilution and counting in Petri dishes. After emergence was complete, three plants were left per pot. Hoagland solution (Hoagland and Arnon 1938) without N was applied weekly at a rate of 2 ml kg^{-1} of soil.

Water stress application and analysis

Water stress was applied by means of a porous cup arrangement similar to that described by Bataglia (1989). The autoirrigation system consisted of a porous ceramic filter cup (3.5 cm diameter and 14 cm height) placed in the centre of the pot (Fig. 1A). The porous cup and tubing were filled with distilled water. The different soil water contents were obtained by setting the vertical distances (15, 40 and 100 cm) between the middle of the cups and the middle of reservoir, respectively, from constant ψ_m that is equivalent to –1.5 kPa (S_1: without stress or control), –4.0 kPa (S_2), and –10.0 kPa (S_3). These represented ψ_m values at the porous cup walls and consequently of the soil water when in equilibrium (soil's matric potential). As the plant roots absorbed water a potential gradient developed, inducing water flow from cup to soil. For this reason the ψ_m values resultant on soils were –7.0, –70.0 and $< –85.0$ kPa from S_1, S_2, and S_3 treatments, respectively (Fig. 1 ψ_m). The S_3 treatment was nominated to be $< –85.0$ kPa because the exact value was not measured due to limitation of the tensiometer used. The water stress imposed by the porous cup system is gradual and the plant is able to gradually adapt to the imposed stress. There are a lot of advantages of the proposed methods such as: (a) accuracy
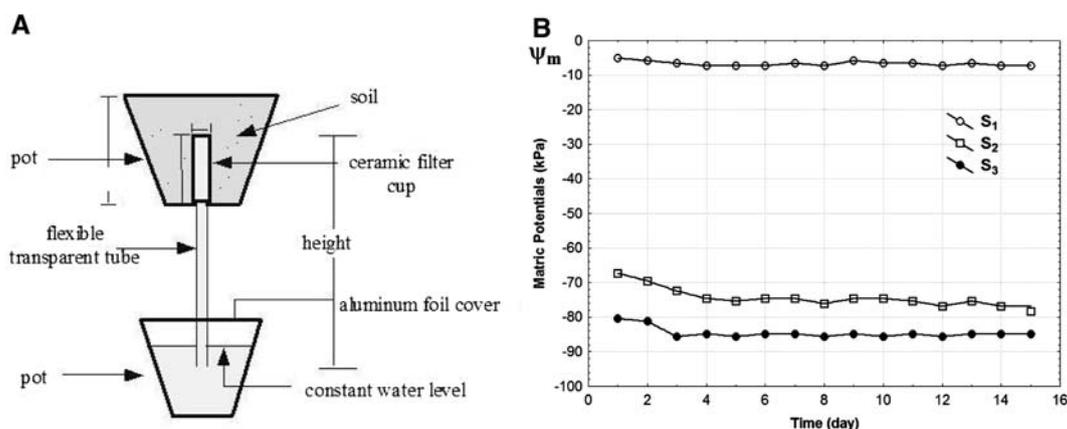
Fig. 1 Layout of irrigation system using a porous cup technique (**A**) and matric potentials (ψ_m) of yellow latosol soil (Araripina Experimental Station, Pernambuco, Brazil) during 15-day-periods. The different levels of drought stress (**B**) ($S1$: without stress, $S2$, and $S3$) were obtained (4 replicates) by setting the vertical height (15, 40 and 100 cm) between the middle of the cups and the middle of reservoir (**A**) associated to plant water demand

for watering (sub-irrigation system); (b) the water stress is "relatively" constant and controlled; (c) the watering is controlled by the plant demand (auto-irrigation); and (d) better and simultaneous control for ψ_m and watering (irrigation system).

Water stress treatments were applied on periods of 15 days at each stage of the symbiotic process of cowpea: (P_1) 0–15 days after seedling emergence, corresponding to initial N_2-fixation and nodules formation; (P_2) 15–30 days, corresponding to N_2-fixation establishment and nodule growth; (P_3) 20–35 days, corresponding to N_2-fixation peak; and (P_4) 30–45 days, corresponding to the bacteriological-cycle diminution and the beginning of nodule senescence. At the end of each stress treatments, sub-samples of plants were harvested for analysis, together with the control S_1 (without stress).

Several aspects of plants associated to water-stress were analysed. Leaf diffusive resistance (Rf) was evaluated (between 09:00 and 10:00 h) on the abaxial side of the more recently expanded leaf from each plant, using the 'Steady State Porometer' (Mod. LI 1600) with a coupled quantic sensor (SRQ 12231) fabricated by LI-COR, Inc. (Lincoln, USA). In order to obtain standardized leaf water potential (ψ_w) measurements, the following precautions were taken according to information in Scholander et al. (1964): (a) the measurements were made to the same leaves at the same time daily; (b) the apical leaves (same age) were enveloped in transparent PVC film with an oblique razor-cut (2 cm) from the petiole; (c) the increase of inlet air pressure (into the test cylinder) was maintained at a constant rate; (d) ψ_m readings were taken daily at 10:00 h throughout the entire drought period; (e) the temperature and relative humidity of the air as

well as soil temperature (at depths of 4.8 cm and 12 cm) were monitored daily with a thermocouple device from 10:00 to 14:00 h. For shoot xylem sap analysis, exudates obtained by pressurization using the Scholander chamber, were collected in calibrated microcapillaries, and stored at –20°C until assayed. The ureide-N concentration was colorimetrically analysed according to Vogels and Van der Drift (1970).

To evaluate enzyme activities, the nodules were kept in liquid N_2 until assayed. Nodule cytosol was prepared in extraction solution according to Farnden and Robertson (1980) and the extraction method according to Hungria et al. (1991). Supernatant was desalted at 4°C on a Sephadex G-25 column equilibrated with a buffer suitable for use in each of the subsequent enzymes assays. The activity of glutamine synthetase GS (EC 6.3.1.2) was measured spectrophotometrically (540 nm) by the biosynthetic hydroxamate assay according to Farnden and Robertson (1980). The activities of NADH-dependent glutamate synthase NADH-GOGAT (EC 1.4.1.14), glutamate dehydrogenase GDH (EC 1.4.1.4) and phosphoenolpyruvate carboxylase PEPC (EC 4.1.1.31) were assayed spectrophotometrically (340 nm) according to Farnden and Robertson (1980), Hungria and Araújo (1994) and Schweizer and Erismann (1985), respectively. Shoot and root dry weights (65°C for 72 h) and the shoot:root ratio were also evaluated.

Statistical design and analysis

The experimental design adopted was a randomized block (4 blocks) with sub-divided plots. Each block contained three plots related to each water-stress treatment (S_1–S_3). The plots consisted of four sub-plots

being at different developmental stages of N_2 fixation (P_1–P_4). ANOVA was applied in conformity with a mathematical model associated to experimental layout (Steel and Torrie 1960), using Statistic soft (Statsoft Inc.,Tulsa) and the effects were evaluated by F-test. The standard error was estimated and the comparisons of treatment means were evaluated ($P < 0.05$) using Tukey's HSD.

Results and discussion

Soil varied in matric potential (ψ_m) from –7.0 kPa to below $<$ –85.0 kPa from S_1 to S_3 treatments, respectively (Fig. 1B). The ψ_m ranges to which plants were subjected with the increase of the head of water can be explained by analysing the unsaturated water flow through the soil as described by Darcy's law: $q = -K$ (ψ_m). $\Delta\psi_{/\ L}$, where: q = water flow through the soil: $K = \psi_m$ at unsaturated hydraulic conductivity, a function of matric potential; and $\Delta\psi_{/\ L}$ = hydraulic gradient. Unsaturated hydraulic conductivity decreases exponentially with soil moisture and consequently with ψ_m. Due to evapotranspiration, an hydraulic gradient is therefore necessary to maintain water flow, and consequently an ever greater difference in ψ_m to compensate for the decrease in hydraulic conductivity. This explains the rising bands of ψ_m as the head of water increased. Since the ψ_m of the porous cup is fixed and equal to the head of water, it is necessary to reduce the soil ψ_m in order to increase the hydraulic gradient.

Kramer (1963) emphasized that measurements of soil water content or soil water potential are not sufficient to determine the effects of water supply on plant processes and yields. Plant water stress can be characterized directly by measuring leaf water potential (ψ_w), but the levels of leaf water potential limiting plant growth are not generally known and must be determined for each species or crop. The ψ_w can be explained in terms of transpiration rate and leaf diffusion resistance, in order to provide principles and means to obtain more effective irrigation (Hsiao 1973).

From Fig. 2 (ψ_w) it can be seen that there was a significant difference ($P < 0.05$) in drought stress levels (but there was not a significant interaction in water stress at different stages of the N_2 fixation development). The ψ_w that the plants were permitted to reach was not extremely low throughout the experiment, through the period of the stress had been relatively long, reaching values of –1.05 MPa. Studies conducted by Ramos et al. (2003) with the common bean (*Phaseolus vulgaris* L.) cv. Carioca presented ψ_w with magnitude similar to that observed in this experiment,

while González et al. (1995) detected ψ_w –1.2 MPa with soybean.

In this work, a correlation was detected between ψ_w and leaf transpiration rate ($r = 0.81**$) by comparing the stressed plants with the control (S_1). An increase in Rf was noted at the most negative ψ_m (S_3) when stress was applied during the P_2 stages. The Rf interacted significantly with the different stages of N_2 fixation development (Fig. 3A). In contrast, the ψ_w did not (Fig. 2).

Stress had a marked effect on soil temperature at 08:00 h, as temperature (28–30°C) increased with depth (4–12 cm) in all the stress treatments. On the other hand, there was a reduction in soil temperature from 32°C to 30°C, with increased depth at 14:00 h. Soil temperature remained lower than air temperature when the ψ_m was close to the capacity of the pot. Because evaporation cooled the soil with reduction of soil hydration, its temperature tended to approach air temperature. This rise in soil temperature can affect the metabolism of roots and *Bradyrhizobium*.

It was also verified that shoot dry matter (Fig. 3B) at the most negative ψ_m (S_3) was more affected in stage P_2 compared to the control indicating that stressed plants in this period suffer a reduction in the leaf area expansion as much as in the dry matter accumulation. Drought stress reductions in the shoot:root ratio (Fig. 2) were similar to those observed in the study of Costa et al. (1997).
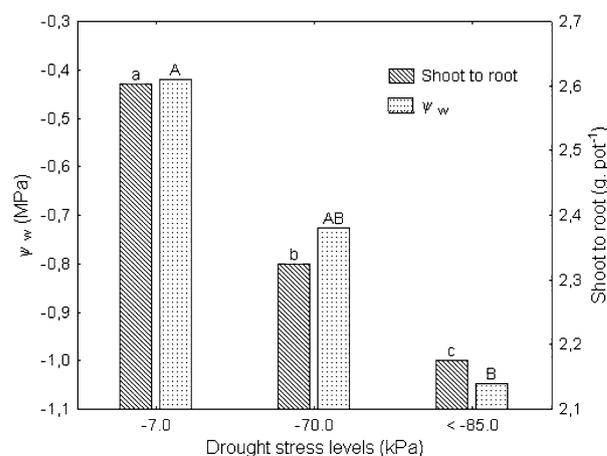

Fig. 2 Leaf water potential (ψ_w) and shoot-to-root ratio in cowpea (*Vigna unguiculata* L. Walp., cv. IPA205) in different drought stress (S_1 = –7.0 kPa (control), S_2 = –70.0 kPa and $S_3 <$ –85.0 kPa). The variation coefficients were 10.9% and 4.7%, respectively, to ψ_w and shoot-to-root ratio. The means (4 replicates) followed by the same letter (lower (shoot-to-root ratio) and capital letters (leaf water potential)) do not differ statistically ($P < 0.05$) according to Tukey's HSD
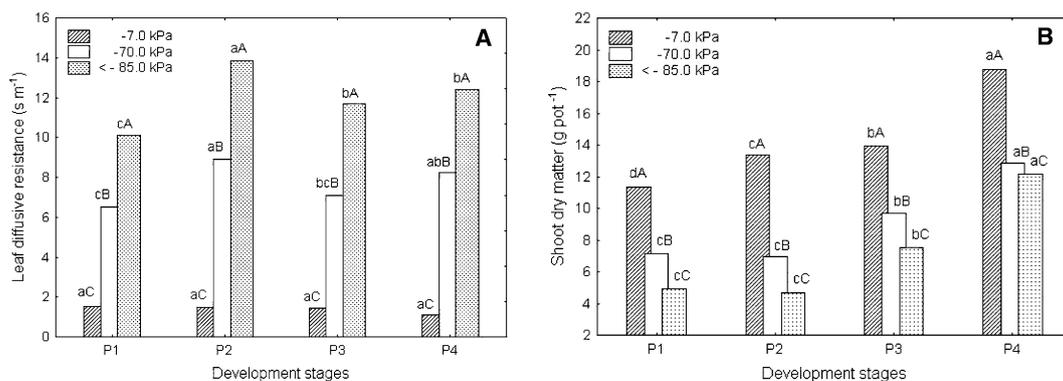
Fig. 3 Leaf diffusive resistance (**A**) and shoot dry matter (**B**) in cowpea (*Vigna unguiculata* L. Walp., cv. IPA205), measured at the end of each development stage (P_1 = 0–15 days, P_2 = 15–30 days, P_3 = 20–35 days, and P_4 = 30–45 days), in different drought stress (S_1 = –7 kPa (control), S_2 = –70 kPa and S_3 < –85 kPa). The variation coefficients were 12.1% and 2.3%, respectively, to A and B variables. The means (4 replicates) followed by the same letter (lower (P_1–P_4) and capital letters (S_1–S_3)) do not differ statistically (P < 0.05) according to Tukey's HSD

Growth and dry matter accumulation of legumes reduced by low drought stress levels has been reported in common bean, cowpea, cunhã *(Clitoria ternatea* L.) and soybean *(Glycine max* [L.] Merrill). Variation among cultivars within legume species in N_2 fixation sensitivity to water deficit (González et al. 1995; Figueiredo et al. 1998b; Diniz et al. 2002; Ramos et al. 2003), indicates that the tolerance trait found in some genotypes may be useful in breeding programs for N_2 fixation drought tolerance in legumes.

Drought was shown to affect xylem water potential, based on the regression equation (y = 4981.92 + 41.51x + 0.25x^2; R^2 = 0.99*). Silveira et al. (2003) found that sap ureide concentration in cowpea cultivar "Vita 7" drastically declined during the water withdrawal period. The drought stress tolerance of N_2 fixation in the soybean cultivar "Jackson" was associated with low concentrations of ureides in xylem exudates and the petiole under well-watered conditions and low ureide accumulation during soil drying (Serraj et al. 2001). Decreased xylem transport in the ureide-transporting species with soil drying might result in a negative feedback on N_2 fixation and, consequently, result in the observed drought sensitivity of several important grain legume crops (Serraj et al. 2001).

The inhibition of N_2 fixation by stress was established, corroborating the observations reported by Diaz del Castillo et al. (1994), but information about the effects of drought stress on enzymes of ammonia assimilation is insufficient and difficult to evaluate. González et al. (1998) did not find any significant difference in the reduction of the GS activity in soybean nodules subjected to –1.2 MPa.

Our results indicate that under drought stress there was a decline in enzyme activity in metabolic paths concerned with N_2 fixation: NADH-GOGAT, GS, PEPC but an increase in GDH. However, it can be noted that the NADH-GOGAT was the most sensitive enzyme under drought stress and its activity in the nodule decreased rapidly with an increase of water stress (Fig. 4).

PEPC in the nodules exhibited a bell-shape tendency with maximal activity around flowering in cowpea plants. These results are similar to those reported by Silveira (1993) and Silveira et al. (1998). There is also a synergism between CO_2 dark fixation and N_2 fixation. PEPC has an important role in energy production and carbon skeleton supply necessary for N_2 reduction and NH_3 assimilation, as well as being a key enzyme for anaplerotic reactions that occur in plants (Silveira 1993). We also observed a positive correlation (r = 0.82*) for cowpea. The positive correlation between PEPC and GS activities and the N_2 fixation suggests a potential use of these enzymes and their specific isoforms in nodules as molecular markers in genetic breeding programs focused on N_2 fixation.

Nodule GDH activity (Fig. 4 GDH) increased with a more negative ψ_m. The higher increase at the P_4 stage may be due to the proximity of the period of nodule senescence. The proportion of NADH-GOGAT to GDH activity varied greatly and presented a low correlation (r = 0.32) indicating that the affinity of the GDH or the NADH-GOGAT can control the synthesis, activation and inhibition of one another.

Our results suggest that drought stress applied at the P_2 stage had the most severe negative affect, indicating that this may be a particularly critical period for the cowpea cultivar IPA 205. Sap ureides concentration in cowpea declined during the drought stress period. GDH activity increased in more negative ψ_m, indicating
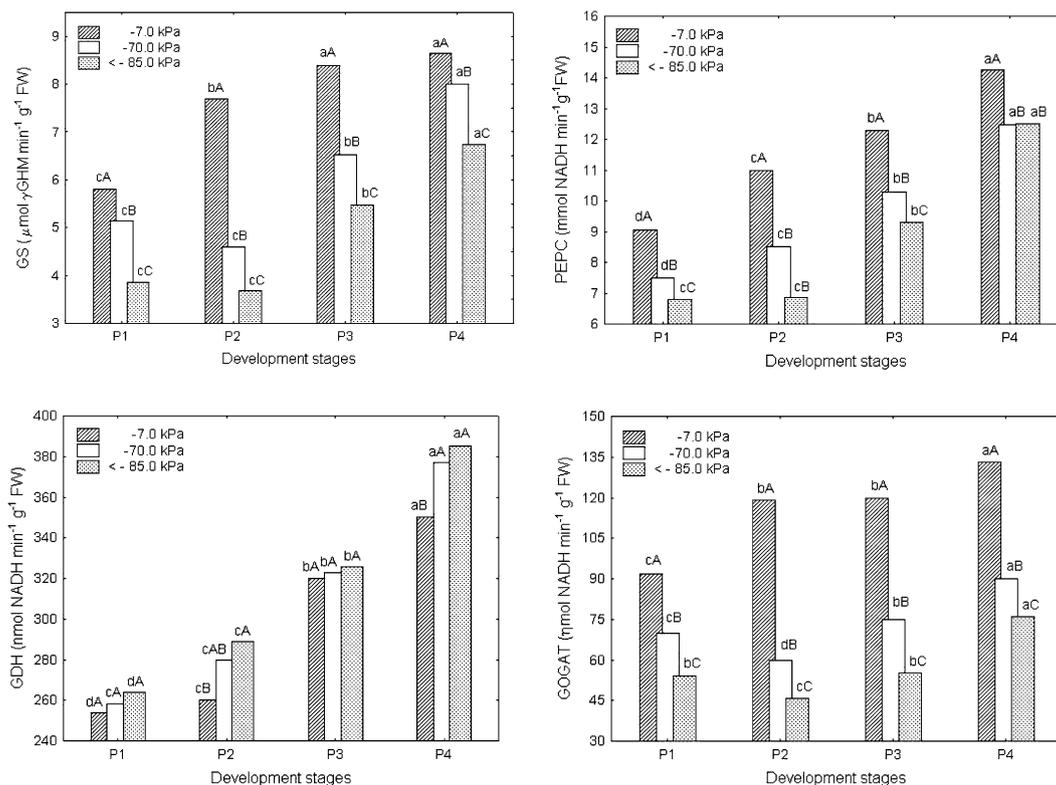
Fig. 4 Glutamine synthetase (GS), phosphoenolpyruvate carboxylase (PEPC), Glutamate dehydrogenase (GDH) and Glutamate synthase (GOGAT) in cowpea's nodule (*Vigna unguiculata* L. Walp., cv. IPA205), measured at the end of each development stage (P_1 = 0–15 days, P_2 = 15–30 days, P_3 = 20–35 days, and P_4 = 30–45 days), in different drought stress (S_1 = –7 kPa (control), S_2 = –70 kPa and $S3$ < –85 kPa). The variation coefficients were 6.7, 5.8, 3.9 and 4.4%, respectively, to GS, PEPC, GDH and GOGAT variables. The means (4 replicates) followed by the same letter (lower (P_1–P_4) and capital letters (S_1–S_3)) do not differ statistically (P < 0.05) according to Tukey's HSD

that the cowpea nodules maintain sufficient enzyme activity under stress. There was a slight reduction in the GS activity and PEPC activity, while NADH-GOGAT activity was the enzyme most sensitive to water stress. From a practical perspective, the negative effects on the metabolism of N_2 assimilation occurred every time that the ψ_w was reduced below –0.73 MPa as had happened in the stressed treatments. However, the stress applied by the porous cup was gradual and the plant recovered its turgor, avoiding deleterious alterations in the cellular metabolism even from the most negative ψ_m.

References

Bataglia OC (1989) Sistemas de irrigação em vasos para experimentos de adubação. Revista Brasileira de Ciências do Solo 13:81–86

Brasil, Ministério da Agricultura (1997) Manual de Métodos de Análises de Solo. 2 ed. EMBRAPA-CNPS, Rio de Janeiro

Costa MMMN, Távora FJAF, Pinho JLN de, Melo FIO (1997) Produção, componentes de produção, crescimento e distribuição das raízes de caupi submetido à deficiência hídrica. Pesquisa Agropecuária Brasileira 32:43–50

Diaz del Castillo L, Hunt S, Layzel DB (1994) The role of oxygen in the regulation of nitrogenase activity in drought stressed soybean nodules. Plant Physiol 106:949–955

Diniz MCNM, Burity HA, Figueiredo MVB (2002) Development and regrowth of cunha (*Clitoria ternatea* L.) under water stress, in association with mycorrhizal fungi-*Bradyrhizobium*. Agrochimica 46:52–58

Farnden KLF, Robertson JG (1980) Methods for studying enzymes involved in metabolism related to nitrogenase. In: Bergersen JF (ed) Methods of evaluating biological nitrogen fixation. John Wiley and Sons Ltd., Chichester, pp 265–316, ISBN 0471277592

Figueiredo MVB, Burity HA, de França FP (1998a) Water deficit stress effects on N_2 fixation in cowpea inoculated with different *Bradyrhizobium* strains. Can J Plant Sci 78:577–582

Figueiredo MVB, Vilar JJ, Burity HA, de França FP (1998b) Alleviation of water stress effects in cowpea by *Bradyrhizobium* spp. inoculation. Plant Soil 207:67–75

González EM, Aparício-Tejo PM, Gordon AJ, Minchin FR, Roynele M, Arrese-Igor C (1998) Water-deficit effects on carbon and nitrogen metabolism of pea nodules. J Exp Bot 49(327):1705–1714

González EM, Gordon AJ, James CL, Arrese-Igor C (1995) The role of sucrose synthase in the response of soybean nodules to drought. J Exp Bot 46:1515–1523

Hoagland DR, Arnon DI (1938) The water-culture method for growing plants without soil. University of California, Berkeley, 39 pp. ASIN B00088TA3W

Hsiao TC (1973) Plant responses to water stress. Ann Rev Plant Physiol Plant Mol Biol 24:519–570

Hungria M, Araújo RS (1994) Metabolismo de carbono e nitrogênio nos nódulos. In: Hungria M, Araújo RS (eds) Manual de Métodos Empregados em Estudos de Microbiologia Agrícola. Embrapa, Brasília, pp 250–283

Hungria M, Barradas CAA, Wallsgrove RM (1991) Nitrogen fixation, assimilation and transport during the initial growth stage of Phaseolus vulgaris L. J Exp Bot 42:839–844

Kramer PJ (1963) Water stress and plant growth. Agron J 55:31–35

Mundree SG, Baker B, Mowla S, Peters S, Marais S, Willigen CV, Govender K, Maredza A, Muyanga S, Farrant JM, Thomson JA (2002) Physiology and molecular insights into drought tolerance. Afr J Biotechnol 1:28–38

Parsons RA, Stanforth A, Raven JA, Sprent JI (1993) Nodule growth and activity may be regulated by a feedback mechanism involving phloem nitrogen. Plant Cell Environ 16:125–136

Ramos MLG, Parsons R, Sprent JI, James EK (2003) Effect of water stress on nitrogen fixation and nodule structure of common bean. Pesquisa Agropecuária Brasileira 38:339–347

Rengel Z (2002) Breeding for better symbiosis. Plant Soil 245:147–162

Scholander PF, Hemmingsen EA, Hammel HT, Bradstreet HD (1964) Hydrostatic pressure + osmotic potential in leaves of mangroves + some other plants. Proc Natl Acad Sci USA 52:119–125

Schweizer P, Erismann KH (1985) Effect of nitrate and ammonium nutrition of non-nodulated Phaseolus vulgaris L. on phosphoenolpyruvate carboxylase and pyruvate kinase activity. Plant Physiol 78:455–458

Serraj R, Sinclair TR, Purcell LC (1999) Symbiotic N_2 fixation response to drought. J Exp Bot 50:143–155

Serraj R, Vadez V, Sinclair TR (2001) Feedback regulation of symbiotic N_2 fixation under drought stress. Agronomie 2:621–626

Silveira JAG (1993) Relações entre metabolismo de C e N: Fixação não-fotossintética de CO2 e assimilação de N inorgânico. In: Fernandes et al (eds) I Simpósio Brasileiro sobre nitrogênio em plantas. Sociedade Brasileira de Fisiologia Vegetal, Itaguaí, pp 217–234

Silveira JAG, Contado JL, Rodrigues JLM, Oliveira JTA (1998) Phosphoenolpyruvate carboxylase and glutamine synthetase activities in relation to nitrogen fixation in cowpea nodules. Revista Brasileira de Fisiologia Vegetal 10:19–23

Silveira JAG, Viegas RA, Figueiredo MVB, Oliveira JTA, Costa RCL (2003) N-compound accumulation and carbohydrate shortage on N_2 fixation in drought-stressed and rewatered cowpea plants. Spanish J Agric Res 3:65–76

Sinclair TR, Purcell LC, Vadez V, Serraj R (2001) Selection of soybean (Glycine max) lines for increased tolerance of N_2 fixation to drying soil. Agronomie 21:653–657

Sprent JI (1981) Nitrogen fixation. In: Paleg LG, Aspinall D (eds) The physiology and biochemistry of drought resistance in plants. Academic Press, New York, pp 131–143, ISBN 0125443803

Steel RGD, Torrie JH (1960) Principles and procedures of statistics. McGraw-Hill, New York, ISBN 007060925X

Streeter JG (1993) Translocation – a key factor limiting the efficiency of nitrogen fixation in legume nodules. Physiol Plant 87:616–623

Venkateswarlu B, Saharan N, Maheswari M (1990) Nodulation and N_2 (C_2H_2) fixation in cowpea and groundnut during water-stress and recovery. Field Crops Res 25:223–232

Vogels GD, Van der Drift C (1970) Differential analyses of glyoxylate derivatives. Anal Biochem 33:143–157