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Drought Stress Response in Enzymatic Activities of Cowpea Nodules

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Summary

The response to drought stress of several nodule enzymes in cowpea (*Vigna unguiculata* (L.) Walp.) cv. IPA 204 at different stages of N_2 fixation development, as well as their changes during the period of stress recovery are described. Stress was applied continuously by the control of water potential through a porous cup. Stress applied during the P_2 stage (15–30 d) interfered most. The recovery in nodule metabolic activity at the P_1 stage (0–15 d) was higher than at other stages. There was a slight reduction in PEPC activity with increased stress. NADH-GOGAT activity was the enzyme most sensitive to drought stress and most closely associated with N_2ase (ARA in closed systems). The coupling between GS and NADH-GOGAT activities was lost as drought stress progressed. GDH activity remained not only insensitive to the stress but its activity even increased, indicating that cowpea nodules maintain sufficient activity of some assimilatory enzymes under drought stress.

Key words: Bradyrhizobium spp., N_2 fixation, nodule metabolism, Vigna unguiculata (L.) Walp., water deficit.

Abbreviations: GS = glutamine synthetase; PEPC = phosphoenolpyruvate carboxylase; GDH = glutamate dehydrogenase; NADH-GOGAT = NADH-dependent glutamate synthase; ψ_m = matric potential; ψ_w = leaf water potential; P_1 = 0–15 d; P_2 = 15–30 d; P_3 = 20–35 d; P_4 = 30–45 d; S_1 = –7.0 kPa; S_2 = –70.0 kPa; S_3 = <–85.0 kPa; ARA = acetylene reduction activity.

Introduction

Under rainfed tropical conditions legume such as cowpea is usually grown in soils subjected to frequent water deficits. Nitrogen fixation, one of the most important physiological processes in this plant, is affected by soil water deficits, and water stress limits N accumulation, dry matter and yields (Venkateswarlu et al., 1989).

The effects of drought stress on a plant's physiology vary depending on the species and the inherent degree of tolerance, as well as the magnitude of the water deficit, and how fast the plant experiences this water deficit. Generally, drought quickly affects cell turgour and the growth of the meristem. If drought persists, other physiological processes will be affected. For example, a change in stomatal closing may lead to a decreased photosynthesis rate, as well as a change in water transport through the xylem, which in turn may decrease transport flux of the absorbed elements in the root and in the whole plant (Hsiao, 1973).

Drought is one of a range of environmental stresses which can cause considerable reductions in N_2 fixation (Pankhurst and Sprent, 1975; Sinclair et al., 1987). However, it is not clear which biochemical processes are actually affecting the nodule (Streeter, 1993; Guerin et al., 1990). The relationship between the water status, photosynthesis and N_2 fixation under drought stress and the changes in nodule morphology

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have been studied for some temperate legumes (Sprent, 1981). However, tropical legumes grown in arid regions, have not received such adequate attention. Even where there was available information, the degree of drought stress in the plants was not clearly defined, and this makes it difficult to compare between cultivars. The physiological and structural basis for the differences in the sensitivity of N_2 fixation in tropical legumes, under drought stress, is not clearly understood (Venkateswarlu et al., 1990). Further, the effects of drought stress on N_2 fixation and its recovery after watering have not received enough study.

The differences in the recovery rate among different crop species and also the cultivars of a species provide useful clues to the N metabolism, growth and the agronomic performance of the field crops under rainfed conditions in the tropics (Venkateswarlu et al., 1990). Several reports on the effects of drought stress on N_2 fixation concentrate on the plant response (Becana et al., 1986). The processes involved in the recovery from stress in the plants and nodules are very important for understanding legume responses to short periods of drought. Some data indicate that drought stress directly separates interactions between the bacteria and the host plant by alteration of nodule structure and enzymatic activity (Sprent, 1981; 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 response in the metabolism of any organism during any environmental stress must reflect changes either in activities of enzymes or in gene expression (Hanson and Hitz, 1982). Although the GS-GOGAT pathway is considered to be the major route for ammonia assimilation under normal growth conditions, the role of 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 (Srivastava and Singh, 1987). The PEPC magnitude on N_2 fixation was presented by Coker and Schubert (1981). The present study was undertaken to evaluate the response to drought stress on enzymatic activities related to nitrogen and carbon metabolism in cowpea nodules at different stages of N_2 fixation development, as well as the changes in these responses during the period of stress recovery.

Materials and Methods

Plants, rhizobia, and growth conditions

The experiment was conducted in a greenhouse at a temperature range of 27 to 35 °C and a relative humidity range of 50 to 80 %. Pots were filled with soil samples (0–20 cm) of a sandy loam Yellow Latosol (Jacomine et al., 1973), from the Araripina Experimental Station, in a semi-arid region of Pernambuco State (latitude: 7° 29′ 00″ S; longitude 40° 36′ 00″ WGR.; altitude 816 m). The soil was air dried, sieved (5.0 mm), corrected to pH 6.3 by the addition of calcium and magnesium oxides in the ratio 3 : 1 (w/w). It was autoclaved (7 kg of soil in each bag) for 30 min at a temperature of 121 °C and a pressure of 101 kPa, once a day for three consecutive days; 14 kg of soil was used in each 15 L pot. 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 (1979) methods and showed the following results: pH ($water^{1:2.5}$) 4.8; Ca^{2+} 7.0 $mmol_c\ kg^{-1}$; Mg^{+} 4.0 $mmol_c\ kg^{-1}$; K^{+} 0.7 $mmol_c\ kg^{-1}$; Na^{+} 0.4 $mmol_c\ kg^{-1}$; Al^{3+} 3.0 $mmol_c\ kg^{-1}$; P 6.1 $mg\ kg^{-1}$; N 0.6 $g\ kg^{-1}$; clay 190 $g\ kg^{-1}$; silt 50 $g\ kg^{-1}$; fine sand 90 $g\ kg^{-1}$; coarse sand 670 $g\ kg^{-1}$; porosity 493 $m^3\ m^{-3}$; particle density 2,650 $kg\ m^{-3}$, and bulk density 1,420 $kg\ m^{-3}$.

The cultivar used was cowpea (*Vigna unguiculata* (L.) Walp.) cv. IPA 204 (L. 1429). The seeds were inoculated with a strain of *Bradyrhizobium* spp. supplied by MIRCEN (Microbiological Resources Center) – Soil Microbiology Section, Porto Alegre-Rio Grande do Sul, Brazil, catalogued under n°. 6145 SEMIA (Agriculture Microbiology Section), origin EMBRAPA/CNPAB (National Agrobiology Research Centre), Rio de Janeiro, Brazil. Five seeds of cowpea were surface-sterilized (Hungria and Araujo, 1994) and sown in each pot, and then inoculated with 5 mL pot^{-1} of liquid culture of *Bradyrhizobium* spp. (10^9 cfu mL^{-1}). After emergence three plants were left per pot. Hoagland and Arnon solution without N was applied weekly at a rate of 2 mL kg^{-1} of soil.

Drought stress application

Drought stress was applied by means of a porous cup arrangement similar to that described by Bataglia (1989). The auto-irrigation system consisted of a porous ceramic filter cup (3.5 cm diameter and 14 cm height) placed in the center of the pot. The porous cup was connected to a constant level water reservoir through a flexible transparent tube (6 mm O.D. and 3 mm I.D.). The porous cup and tubing were filled with distilled water. The different soil water stress values were obtained by setting the vertical distances between the middle of the cups and the reservoir with a constant level of ψ_m at 15, 40 and 100 cm equivalents to $S_1 = -1.5$ kPa, $S_2 = -4.0$ kPa, and $S_3 = -10.0$ kPa representing ψ_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 ψ_m at the treatment S_1 represents a soil ψ_m of –7.0 kPa; at treatment S_2 the soil ψ_m reached –70.0 kPa, and at treatment S_3 the soil ψ_m exceeded –85.0 kPa (the exact value not being measured due to the limited range of the tensiometer) (Fig. 1).

Drought stress was applied during a period of 15 d at different stages of N_2 fixation development: P_1 = 0–15 d, corresponding to the initial period of nodule formation and N_2 fixation; P_2 = 15–30 d, corresponding to the nodule growth period and establishment

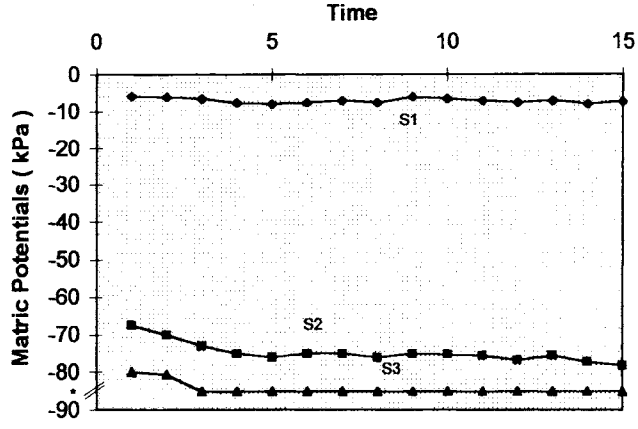

Fig. 1: Soil matric potentials during 15 d periods, in respect to the different levels of drought stress (*matric potentials exceeded –85.0 kPa).

of N_2 fixation; P_3 = 20–35 d, corresponding to the peak of dinitrogen fixation by the nodules; and P_4 = 30–45 d, corresponding to the final stage of the nitrogen fixation and the beginning of nodule senescence. At the end of each stage, the stressed plants were harvested for analysis together with the control S_1 (without stress) and the remaining stressed plants were submitted to watering and harvested when the experiment was finished (45 d after germination) to evaluate the stress recovery in each stage (there was no recovery at P_4 stage, because after this stage the nodules went into senescence, then at the recovery this stage represents the watering experimental period (WEP)). The properties analysed in regard to drought stress were: (1) leaf water potential (Scholander et al., 1964); (2) stomatal diffusive resistance, using the «Steady porometer» LI COR, Mod. LI 1600 with auxiliar quantic sensor LI COR Inc. coupled to the porometer (the readings were taken from 0900 to 1000 h, at the abaxial side of the leaf most recently expanded from each plant); and (3) soil water matric potential, using the tensiometer Soil Moisture, mod. 2725 (the readings were taken daily at 1000 h, throughout the entire drought period). Air relative humidity and temperature was recorded using the thermohygrograph.

Enzyme activities and other analyses

To determine enzyme activities, nodules were kept in liquid N_2 until assayed. Nodule cytosol was prepared using a buffer (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 enzyme assays. GS (EC 6.3.1.2) activity was measured spectrophotometrically (540 nm) by the biosynthetic hydroxamate assay according to Farnden and Robertson (1980). NADH-GOGAT (EC 1.4.1.14) activity was assayed spectrophotometrically (340 nm) according to Farnden and Robertson (1980) and GDH (EC 1.4.1.4) activity was assayed spectrophotometrically (340 nm) according to Hungria and Araujo (1994). PEPC (EC 4.1.1.31) activity was assayed spectrophotometrically (340 nm) according to Schweizer and Erismann (1985). N_2ase activity was determined in nodulated roots by measuring C_2H_4 production after incubation of nodulated roots in a sealed flask under an atmosphere containing C_2H_2 (10 % v.v.) (Hardy et al., 1968) in air for 15 min. (Chamber and Iruthayathas, 1988). Shoot and root dry weights (65 °C for 72 h) and shoot to root ratio were also determined.

Statistical design and analysis

The experimental design adopted randomized blocks with subsubdivided plots, each plot containing a different level of drought stress, one without stress (control S_1) and two with stress (S_2 and S_3) divided in sub-plots containing four different stages of N_2 fixation development (P_1, P_2, P_3, and P_4) and these sub-subdivided plots containing the two evaluation periods (S and R), with four blocks. An analysis of variance was done for each variable studied according to the mathematical model of the adopted experimental layout (Steel and Torrie, 1960). Differences between treatments were analysed through ANOVA with the F test for a $p<0.01$. The significant F test were calculated by Tukey test ($p<0.05$). This experiment was carried out only once.

Results

Drought stress in cowpea, controlled through a porous cup, interfered leaf diffusive resistance and also presented a significant interaction with the different stages of N_2 fixation development (Table 1). Stress applied during the P_2 stage interfered most pronounced, as an increase in the diffusive leaf resistance (13.84 s · cm^{-1}) was noted at the most negative ψ_m (S_3). In contrast, the ψ_w (Table 2) did not present a significant interaction in the stress in relation to the different stages of N_2 fixation development. The ψ_w that the plants were permitted to reach was not extremely low throughout the experiment, though the period of stress had been relatively long, reaching values of –1.0 MPa at the most negative ψ_m (S_3). After watering the ψ_w and leaf diffusive resistance returned to the initial values. In tables 1 and 2 are presented water deficit reductions on shoot dry matter and shoot to root ratio. However, after watering they recovered, even so inferior to the WEP (watering experiment period). The shoot to root ratio in the P_1 and P_3 stages also did not reflect differences in relation to recovery, but in the P_2 stage this relation was more affected in the stressed plants, possibly due to the limited translocation of photosynthates to roots (Babalola, 1980). It can be noted that the most critical period for the synthesis of dry matter occurred in the P_2 stage.

The results indicate a decline in NADH-GOGAT, N_2ase, GS, PEPC activities and an increase in GDH activity (Tables 4 and 5). However, NADH-GOGAT was the most sensitive enzyme under drought stress and its activity in the nodule decreased rapidly with an increase of stress. The results indicate that while the NADH-GOGAT is most closely associated with N_2ase activity ($r = 0.963$) during drought stress, the coupling between GS and NADH-GOGAT activities was lost as drought stress progressed. This observation suggests a very close association between N_2 fixation and the NADH-GOGAT activity in the nodules.

N_2ase activity in the nodules presented a significant interaction of stress in relation to the different development stages, as well as the other enzyme activities. The P_2 stage appeared to be a critical period of water deficit for the cowpea, where the drop in enzymatic activity in relation to the control (S_1) was apparent. A reduction in the PEPC activity oc-

Table 1: Changes in leaf diffusive resistance (Rf) and shoot dry matter in cowpea, measured at the end of each development stage (P_1 = 0–15 d, P_2 = 15–30 d, P_3 = 20–35 d, and P_4 = 30–45 d), in different levels of drought stress (S_1 = –7.0 kPa (control), S_2 = –70.0 kPa and S_3 = <–85.0 kPa).

Stages	Rf (s cm^{-1}) Drought stress levels			Shoot dry matter (g pot^{-1}) Drought stress levels		
	S_1	S_2	S_3	S_1	S_2	S_3
P_1	1.54 a^C	6.54 c^B	10.11 c^A	11.34 d^A	7.15 c^B	4.95 c^C
P_2	1.48 a^C	8.92 a^B	13.84 a^A	13.34 c^A	6.98 c^B	4.70 c^C
P_3	1.42 a^C	7.11 bc^B	11.70 b^A	13.96 b^A	9.72 b^B	7.53 b^C
P_4	1.09 a^C	8.26 ab^B	12.41 b^A	18.77 a^A	12.83 a^B	12.13 a^C
F (stress)		404.44**			1809.79**	
F (stages)		20.93**			4076.90**	
F (interaction)		9.33**			65.36**	
% CV (plot)		21.36			4.70	
% CV (subplot)		12.15			2.30	

** Significant at the 0.01 probability level. In each column (lower letters) and in each line (capital letters), the means (4 replicates) followed by the same letter do not differ statistically ($p<0.05$) from each other, according to Tukey's test.

curred during the most negative ψ_m (S_3), but the activity was more intense after the maximum value of N_2ase was established.

GDH activity (Table 5) in the nodules not only remained insensitive to the stress but actually increased with a more negative ψ_m (S_3) indicating that nodules maintain sufficient enzyme activity under drought stress. The high increase during the P_4 stage may be due to the onset of nodules senescence. The enzymes NADH-GOGAT and GDH presented a low correlation (r = 0.323). In relation to the effect of the stress, we found that the GDH activity was not associated with changes in acetylene reduction activity (r = 0.277). There is evidence indicating that the presence of the GDH activity is incompatible with N_2 fixation by *Rhizobium meliloti* during symbiosis (Osburne and Signer, 1980).

Table 2: Changes in leaf water potential (ψ_w) and shoot-to-root ratio in cowpea, in different levels of drought stress (S_1 = –7.0 kPa (control), S_2 = –70.0 kPa and S_3 = <–85.0 kPa).

Drought stress levels	ψ_w (MPa)	Shoot-to-root (g pot^{-1})
S_1	–0.43 a	2.61 a
S_2	–0.80 b	2.38 ab
S_3	–1.00 c	2.14 b
F (stress)	187.70**	7.04*
F (stages)	3.73**	2.68*
F (interaction)	2.00^{ns}	2.30^{ns}
% CV (plot)	10.89	20.68
% CV (subplot)	9.16	13.81

^{ns} Not significant.
*, ** Significant at the 0.05 and 0.01 probability level. The means (4 replicates) followed by the same letter do not differ statistically ($p<0.05$) from each other, according to Tukey's test.

Table 3: Changes in shoot dry matter and shoot-to-root ratio in cowpea, at the development stage (P_1 = 0–15 d, P_2 = 15–30 d, P_3 = 20–35 d, and P_4 = 30–45 d), measured after recovery from the different levels of drought stress (S_1 = –7.0 kPa (control), S_2 = –70.0 kPa and S_3 = <–85.0 kPa).

Stages	Shoot dry matter (g pot^{-1}) Rec[1]			Shoot-to-root (g pot^{-1}) Rec		
	S_1	S_2	S_3	S_1	S_2	S_3
P_1	19.15 a^A	11.98 b^B	9.16 b^C	3.12 a^A	2.29 a^B	1.69 ab^B
P_2	17.82 b^A	7.93 c^B	6.29 d^C	2.92 a^A	1.83 a^B	1.42 b^B
P_3	18.00 b^A	11.54 b^B	7.57 c^C	2.69 a^A	2.53 a^A	2.23 ab^A
P_4 = WEP[2]	19.24 a	18.12 a	18.23 a	2.76 a	2.60 a	2.52 a
F (Rec)		10317.04**			0.057^{ns}	
F (Rec vs. stages)		417.34**			12.05**	
% CV (sub-subplot)		3.28			18.66	

^{ns} Not significant.
** Significant at the 0.01 probability level. In each column (lower letters) and in each line (capital letters), the means (4 replicates) followed by the same letter do not differ statistically ($p<0.05$) from each other, according to Tukey's test.
(1) Drought stress levels after recovery.
(2) There was no recovery at P_4 stage, as it represents the watering total control (WEP).

Table 4: Changes in nodule enzyme activities, phosphoenolpyruvate carboxylase (PEPC) and nitrogenase (N_2ase) in cowpea, measured at the end of each development stage (P_1 = 0–15 d, P_2 = 15–30 d, P_3 = 20–35 d, and P_4 = 30–45 d), in different levels of drought stress (S_1 = –7.0 kPa (control), S_2 = –70.0 kPa and S_3 = <–85.0 kPa).

Stages	PEPC (µmol NADH ml^{-1} min^{-1}) Drought stress levels			N_2ase (nmol C_2H_4 pl^{-1} h^{-1}) Drought stress levels		
	S_1	S_2	S_3	S_1	S_2	S_3
P_1	9.05 d^A	7.50 d^B	6.80 c^C	7756 c^A	5502 ab^B	4865 ab^B
P_2	11.00 c^A	8.50 c^B	6.85 c^B	10062 b^A	5052 b^B	4385 b^B
P_3	12.30 b^A	10.30 b^B	9.30 b^C	10618 ab^A	6302 ab^B	5490 ab^B
P_4	14.26 a^A	12.47 a^B	12.50 a^B	11643 a^A	6575 a^B	6138 a^B
F (stress)		112.20**			118.66**	
F (stages)		494.52**			13.73**	
F (interaction)		9.47**			2.97*	
% CV (plot)		7.61			19.24	
% CV (subplot)		5.05			17.63	

*, ** Significant at the 0.05 and 0.01 probability level. In each column (lower letters) and in each line (capital letters), the means (4 replicates) followed by the same letter do not differ statistically ($p<0.05$) from each other, according to Tukey's test.

In Tables 6 and 7 can be observed that the P_2 stage after recovery interfered most in the NADH-GOGAT, N_2ase, GDH, GS and PEPC activities, indicating this may be the critical period for the cowpea. However, it is possible that cowpea possesses an ability to support periods of stress during the initial stage of nodule development (P_1 stage) as the recovery of N fixation metabolism was greater than in the other stages, signifying that stress has a greater impact after the primary nodulation stage.

Discussion

The sensitivity of cowpea to water deficit varied with the plant development and with the ψ_w. Studies conduced by Turk et al. (1980) with cowpea and by González et al. (1995) with soybean, presented ψ_w –1.2 MPa with magnitudes almost similar to the data found in this study (Table 2). It is evident that the comparison between minimum values of the ψ_w among different writers is of little significance, since ψ_w is affected by several factors such as climate, soil, the plant itself. In any case, the existing difference found between the ψ_w values of the stressed and control plants is of fundamental importance in indicating to what extent ψ_w fell in the stressed plants.

Here, we imposed a drought stress controlled through a porous cup, which resulted in a decline in the enzymes' activity in metabolic paths concerning the N_2 fixation, NADH-GOGAT, GS, N_2ase, PEPC, and an increase in GDH (Tables 4 and 5). NADH-GOGAT activity was the enzyme most sensitive to drought stress and the most closely associated with N_2ase activity. The levels of NADH-GOGAT activity in nodule cytosol were very low, but study developed by Santos et al. (1996) also found very low levels of NADH-GOGAT

Table 5: Changes in nodule enzyme activities, NADH-dependent glutamate synthase (GOGAT), glutamine synthetase (GS) and glutamate dehydrogenase (GDH) in cowpea, measured at the end of each development stage (P_1 = 0–15 d, P_2 = 15–30 d, P_3 = 20–35 d, and P_4 = 30–45 d), in different levels of drought stress (S_1 = –7.0 kPa (control), S_2 = –70.0 kPa and S_3 = <–85.0 kPa).

Stages	GOGAT (nmol NADH $min^{-1} g^{-1}$ FW) Drought stress levels			GS (μmol γ GDH $min^{-1} g^{-1}$ FW) Drought stress levels			GDH (nmol NADH $min^{-1} g^{-1}$ FW) Drought stress levels		
	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
P_1	92 c^A	70 c^B	54 b^C	5.80 c^A	5.15 c^B	3.85 c^C	254 d^A	258 c^A	264 d^A
P_2	119 b^A	60 d^B	46 c^C	7.70 b^A	4.60 c^B	3.68 c^C	260 c^B	280 c^{AB}	289 c^A
P_3	120 b^A	75 b^B	55 b^C	8.40 a^A	6.52 b^B	5.48 b^C	320 b^A	323 b^A	326 b^A
P_4	133 a^A	90 a^B	76 a^C	8.64 a^A	8.00 a^B	6.73 a^C	350 a^B	377 a^A	385 a^A
F (stress)		2238.72**			463.34**			2.36^{ns}	
F (stages)		322.84**			254.10**			403.15**	
F (interaction)		55.70**			13.84**			8.02**	
% CV (plot)		4.37			5.72			2.25	
% CV (subplot)		4.07			6.71			3.92	

^{ns} Not significant.

** Significant at the 0.01 probability level. In each column (lower letters) and in each line (capital letters), the means (4 replicates) followed by the same letter do not differ statistically ($p<0.05$) from each other, according to Tukey's test.

Table 6: Changes in nodule enzyme activities, NADH-dependent glutamate synthase (GOGAT), nitrogenase (N_2ase) and glutamate dehydrogenase (GDH) in cowpea, at the development stage (P_1 = 0–15 d, P_2 = 15–30 d, P_3 = 20–35 d, and P_4 = 30–45 d), measured after recovery from the different levels of drought stress (S_1 = –7.0 kPa (control), S_2 = –70.0 kPa and S_3 = <–85.0 kPa).

Stages	GOGAT (nmol NADH $min^{-1} g^{-1}$ FW) Rec[1]			N_2ase (nmol C_2H_4 $pl^{-1} h^{-1}$) Rec			GDH (nmol NADH $min^{-1} g^{-1}$ FW) Rec		
	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
P_1	150 a^A	119 b^B	98 b^C	12705 a^A	11250 a^{AB}	10075 ab^B	349 a^A	312 b^B	289 b^C
P_2	133 b^A	90 d^B	81 c^C	12812 a^A	8905 b^B	7912 c^B	337 a^A	255 c^B	248 c^B
P_3	137 b^A	111 c^B	96 b^C	11912 a^A	9950 ab^B	8975 bc^B	350 a^A	315 b^B	306 b^B
P_4 = WEP[2]	151 a	138 a	137 a	12287 a	11300 a	11330 a	349 a	370 a	368 a
F (Rec)		12074.55**			1086.90**			145.92**	
F (Rec vs. stages)		108.05**			2.86**			212.40**	
% CV (sub-subplot)		4.06			15.87			3.33	

** Significant at the 0.01 probability level. In each column (lower letters) and in each line (capital letters), the means (4 replicates) followed by the same letter do not differ statistically ($p<0.05$) from each other, according to Tukey's test.

[1] Drought stress levels after recovery.

[2] There was no recovery at P_4 stage, as it represents the watering total control (WEP).

in soybean nodule (0.12 mmol $min^{-1} kg^{-1}$ FW) in control plants inoculated with strain PJ-17-1 studying the influence of hydrogenase on N_2 fixation. Robertson et al. (1975) suggested that NADH-GOGAT activity, determined in assays, can be less than the activity present *in vivo,* due to the enzyme instability and the presence of inhibitors in the nodule cytosol.

The ψ_w are within the range of values reported for similar experiments and suggest that total N_2ase activity would have declined by 50–70 % (Guerin et al., 1990; Djekoun and Planchon, 1991; Diaz del Castillo et al., 1994). The currently accepted view of the response of nodules to stress is that they are able to increase the resistance to gaseous diffusion, thereby reducing oxygen flux into the nodules in order to match the rate of oxygen utilization, and thus preventing serious damage to the nitrogenase complex (Witty and Minchin, 1990; Hunt and Layzell, 1993), as well as exposure to acetylene, but this problem may not occur with all symbioses (Michin et al., 1983).

There is overwhelming evidence that nodule response to drought (as well as to other stresses) involves a physiologically mediated decrease in permeability to O_2. The current controversies among experts in the field involve the mechanism of permeability decrease and whether this is a primary or secondary response. When establishing primary physiological responses, it is important that the magnitude of the imposed stress be relatively moderate, which will permit the distinguishing between the responses that are produced in this state and those that are produced secondarily. Along these lines, what stands out is the lack of physiological relevance to real situations in nature from the results obtained through the employment of osmotic agents (such as PEG) that cause

Table 7: Changes in nodule enzyme activities, phosphoenolpyruvate carboxylase (PEPC) and glutamine synthase (GS) in cowpea, at the development stage (P_1 = 0–15 d, P_2 = 15–30 d, P_3 = 20–35 d, and P_4 = 30–45 d), measured after recovery from the different levels of drought stress (S_1 = –7.0 kPa (control), S_2 = –70.0 kPa and S_3 = <–85.0 kPa).

Stages	PEPC (μmol NADH $mL^{-1} min^{-1}$) Rec[1]			GS (μmol γ GDH $min^{-1} g^{-1}$ FW) Rec		
	S_1	S_2	S_3	S_1	S_2	S_3
P_1	14.57 ab^A	12.26 b^B	11.34 b^B	8.24 a^A	6.80 b^B	5.87 b^C
P_2	13.50 b^A	11.45 b^B	9.13 c^C	8.38 a^A	5.54 c^B	4.00 c^C
P_3	14.46 ab^A	12.35 b^B	11.00 b^C	9.00 a^A	7.00 b^B	6.20 b^C
P_4 = WEP[2]	14.70 a	14.23 a	15.48 a	8.80 a	8.62 a	8.24 a
F (Rec)		1932.14**			509.60**	
F (Rec vs. stages)		129.76**			81.40**	
% CV (sub-subplot)		6.18			7.05	

** Significant at the 0.01 probability level. In each column (lower letters) and in each line (capital letters), the means (4 replicates) followed by the same letter do not differ statistically ($p < 0.05$) from each other, according to Tukey's test.

[1] Drought stress levels after recovery.

[2] There was no recovery at P_4 stage, as it represents the watering total control (WEP).

sharp drops in the water potential, besides implying physiological mechanisms distinct from those found in normal situations (Aparício-Tejo et al., 1996). The reduction in activity due to water deficit may be also due to some unknown metabolic component (González et al., 1995).

In our work also we found a small decline in the activities of PEPC and GS. The inhibition of N_2 fixation by stress was established, corroborating the observations of Guerin et al. (1990) and Diaz del Castillo et al. (1994), but information about the effects of drought stress on enzymes of ammonia assimilation are insufficient and difficult to evaluate. Sheoran et al. (1981) found that drought 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. (1995) didn't find any significant difference in the reduction of the GS activity in soybean nodules during stress with ψ_w of –1.2 MPa.

There are studies suggesting the possibility that the N_2ase activity follows a similar course to the GS activity (Robertson et al., 1975; Miflin and Lea, 1976). However, GS activity was detected in *Phaseolus vulgaris* in the first days after the emergence of the nodules, prior to acetylene reduction activity (Hungria et al., 1991). Thus the appearence of the GS would not be conditioned by the presence of ammonia derived from N_2ase activity. Other factors, as yet unknown, present in the microsymbiont can be responsible for the GS activity (Nap and Bisseling, 1990). In our study, the correlation obtained for cowpea was r = 0.754, data that presuppose the interdependence of the enzymes in the nodule interior. Information from this experiment also indicates that nodule GS and NADH-GOGAT activities are not strongly coupled. According to Groat and Vance (1981), the GS to NADH-GOGAT coupling would not be required once glutamate was supplied as a substrate to GS by some other mechanism, that is, through NADH-GOGAT.

GDH increased in activity in drought-stressed nodules. It is believed that increased enzyme activity might be due to a measure of detoxification of ammonia released with the degradation of the proteins and amino acids that occurs during drought stress (Srivastava and Singh, 1987). The greater increase during the P_4 stage may be due to the onset of nodule senescence. Groat and Vance (1981) report that GDH can function in ammonia assimilation during nodule senescence of alfalfa (an indeterminate nodule legume).

Our study suggests the lowered soil ψ_m had a direct effect on the N_2 fixation, and the recovery of enzyme activity was below the recovery of the ψ_w. The drought stress applied in the P_2 stage interfered most negatively, indicating a critical period for cowpea. The recovery in nodule metabolic activity in the P_1 stage was higher than that in the other stages. NADH-GOGAT was the most sensitive enzyme under stress. GS and NADH-GOGAT coupling was lost as the drought conditions progressed. There was a slight reduction in the PEPC activity with an increase of stress. GDH activity not only remained insensitive to the stress, but its activity actually increased in more negative ψ_m, indicating that cowpea nodule maintain sufficient enzyme activity under stress and that they can respond to a variety of environmental pertubations.

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