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EFFECTS OF WATER STRESS ON NITROGEN FIXATION OF COMMON BEAN (*PHASEOLUS VULGARIS* L.)

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Abstract: This study was conducted to investigate the effects of water stress and its relief on growth and N_2 fixation of common bean (*Phaseolus vulgaris* L.) and to elucidate the mechanism of regulation of N_2 fixation by water availability. Plants were grown in N-free nutrient solutions and a water stress of -0.5 MPa was applied 3-weeks after germination by polyethylene glycol. Stress was relieved after 5 days. Water stress reduced leaf water potential and plant relative water content, both of which recovered completely after stress relief. Total plant, shoot and nodule dry weights and the relative growth rate (RGR) of the stressed plants were lower than those of the control during stress. RGR showed a complete recovery after stress relief, but nodule dry weight failed to recover completely. Root dry weight and root:shoot ratio were higher in the stressed treatment both during stress and after its relief. Water stress decreased specific nitrogenase activity which recovered partially upon stress relief. There was no correlation between specific nitrogenase activity and total plant soluble sugar concentrations showing that inhibition of specific nitrogenase activity by water stress is not caused by a shortage of assimilate supply to the nodules. Total amino acid concentration increased in shoots and roots, but decreased in nodules in response to stress. This observation suggests that export of reduced nitrogen from nodules was not limited. This is also indicated by the unchanged total nitrogen concentrations in shoots. Based on the evidence of this study, feedback regulation of nitrogenase activity by recycling of amino acids accumulated in the water-stressed shoots back to the nodules is suggested as a possible mechanism of reduction of nitrogen fixation under water stress.

Key words: Nitrogen fixation, *Phaseolus vulgaris*, water stress.

INTRODUCTION

One of the reasons for the popularity of legumes among subsistence farmers in the tropics is their capacity for biological N_2 fixation which reduces the cost of N_2 fertilizer and enhances soil fertility.[1] Water stress, which is one of the major environmental constraints limiting legume yields,[2] has been reported to decrease nitrogen fixation.[3,4] This decrease is partly due to a reduction in nodulation caused by water stress.[5] Drought also causes a decrease in the nitrogenase activity of nodules.[6,7] However, specific information on the effects of

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water stress on nodulation and nitrogenase activity is relatively scarce for common bean.[5] There has been considerable debate on the exact mechanism of the inhibition of nitrogenase activity by water stress.[4] One hypothesis proposed is that nodule activity is dependent upon the supply of assimilates from the shoot.[8] Hence, reduction of photosynthesis by water stress and a consequently reduced carbohydrate supply was thought to be responsible for reduced N_2 fixation. A second hypothesis was advanced by Streeter[9] on the basis of solute transport in and out of nodules. Streeter[9] showed that the assimilates and nitrogenous compounds to the nodules are imported *via* the phloem and products of N_2 fixation are exported *via* the nodule xylem. It was proposed that water stress inhibits the export of reduced nitrogen from the nodules leading to their accumulation and feedback regulation of further nitrogenase activity. Parsons *et al.*[10] put forward a third hypothesis that reduced shoot growth caused by water stress may result in an accumulation of amino acids in the shoot that are recycled *via* the phloem back to the nodules to inhibit further N_2 fixation through feedback regulation.

An important and often less-emphasized aspect of water stress physiology is the recovery of crops from stress. This is especially relevant for crops growing in the field and subjected to alternating periods of drought and rainfall. The specific objectives of the present study were to investigate the effects of water stress and its relief on growth and nitrogen fixation and to elucidate the mechanism of inhibition of nitrogenase activity of common bean which is an important vegetable legume in the tropics including Sri Lanka.[11]

METHODS AND MATERIALS

The experiment was done in the glasshouse at the Institute of Plant Nutrition of the University of Hohenheim, Germany during the period from August to October, 1995. Seeds of common bean (*Phaseolus vulgaris* cv. Brilliant) were germinated in sand and were transferred to pots containing 3.5 l of nutrient solution one week after germination. The sand medium was inoculated with a culture solution of *Rhizobium leguminosarum* (strain DMS, 1982) at sowing. The nutrient solution was free of nitrogen and consisted of the following components: 0.75 mM $MgSO_4$; 0.2 mM KH_2PO_4; 0.9 mM K_2SO_4; 1 mM $CaSO_4$; 0.01 mM KCl; 100 µM Fe-EDTA; 10 µM H_3BO_4; 2 µM $MnSO_4$; 1 µM $ZnSO_4$; 1 µM $CuSO_4$; 0.01 µM Na_2MoO_4; 0.002 µM $CoCl_4$. The nutrient solution was at 1/5 concentration at the time of transfer of seedlings. This was increased to 1/2 strength after 2 days and to full concentration after one week. Thereafter, fresh nutrient solution was applied three times per week until the start of treatments. Three plants were grown per pot.

Experimental treatments: A water stress of -0.05 MPa was applied at 26 d after sowing by adding polyethylene glycol (PEG) 6000 at the rate of 144 g/l of the

nutrient solution. After 5 days of water stress, it was relieved by substituting the PEG-containing nutrient solution by a fresh one and washing the root systems with distilled water. A control treatment was maintained throughout. Measurements were done on two days during the stress period, at 3 and 5 days after application of stress and once more at 3 days after stress relief. Three replicate pots per treatment were used in all measurements on all days.

Measurements of plant water status, nitrogenase activity, plant growth and concentrations of total nitrogen, sugars and amino acids: Leaf water potential was measured with the pressure chamber.[12] Total nitrogenase activity of the three plants in each pot was measured non-destructively (i.e. *in vivo*) by the acetylene reduction assay.[13] In order to make this measurement possible, specially-designed pots which could be sealed air-tight with intact plants were used. At the beginning of the measurement, an exact volume 60 cm^3 of air in the sealed pots were replaced by acetylene. Gas samples of 20 cm^3 for ethylene analysis were drawn 20 min after injection of acetylene. A preliminary testing showed that ethylene production was linear with time until 30 min after the injection of acetylene. The ethylene concentration in the gas samples was measured by gas chromatography using pure ethylene gas as standard.

Plants were subsequently harvested and fresh weights of shoots, roots and nodules were measured. Dry weights were measured after oven drying at 60°C. The relative growth rate (RGR) of plants were computed using eq. 1, to compare the growth rates of different water treatments.

$$RGR = \frac{(\log_e W_2 - \log_e W_1)}{(t_2 - t_1)} \qquad \text{(eq.1)}$$

Where, W_1 and W_2 are the respective total plant dry weights at times t_1 and t_2 after sowing.

Shoot, root and nodule dry matter was milled for chemical analyses. Total shoot nitrogen concentration was analyzed by the standard Kjeldahl method. The total amino acid concentration of shoots, roots and nodules was measured by the ninhydrin method[14] with leucine as the standard. The concentration of soluble sugars (glucose, fructose and sucrose) in shoots, roots and nodules were measured by enzymatic bioanalysis using a test kit (Boeringer Company).

Statistical analysis: Significance of treatment differences were tested by analysis of variance and mean separation by using the least significant difference.

RESULTS

Leaf water potential (ψ) and Relative water content (RWC)

Application of PEG6000 caused significant reductions ($p<0.001$) in both ψ and RWC of the water-stressed plants during the 5 day period of stress (Table 1). However, within 3 days from stress relief both ψ and RWC of the previously-stressed treatments increased up to the level of the control.

Table 1: Variation of plant water stress during the experimental period.

Parameter	Treatment	Time of measurement		
		No. of days after stress imposition		No. of days after stress relief
		3	5	3
Leaf water potential (-MPa)	Control	0.50	0.33	0.31
	Water stress	0.98***	0.78***	0.34ns
Plant relative water content (%)	Control	89.96	89.58	88.31
	Water stress	87.70*	87.14***	88.67ns

Note: Levels of significance of the mean differences between control and water-stressed treatments are given as:

ns - Not significant at P=0.05
* - Significant at P=0.05
** - Significant at P=0.01
*** - Significant at P=0.001

Plant growth

Variation of total dry weights (Table 2) show that water stress significantly reduced ($p<0.05$) overall plant growth even within a time period as short as 3 days after stress imposition. However, plant growth recovered fast after stress relief (Table 2). In fact the relative growth rate (RGR) of the previously-stressed treatment (95.41 mg g^{-1} d^{-1}) was higher than that of the control (72.64 mg g^{-1} d^{-1}) during the 3-day period after stress relief (Table 3). Root dry weight was higher in the stressed treatment throughout the period of measurements. Therefore,

the root:shoot ratio was significantly greater ($p<0.01$) in the water-stressed treatment even after stress relief.

Nodule dry weight decreased significantly ($p<0.05$) under water stress and failed to recover sufficiently even after stress relief. Unlike for overall growth, RGR for nodule growth (Table 3) was lower in the stressed treatment (60.77 mg g^{-1} d^{-1}) during the 3-day period after stress relief as compared to the control (85.31 mg g^{-1} d^{-1}).

Table 2: Response of growth parameters and relative growth rates to water stress and relief.

Parameter	Treatment	Time of measurement		
		No. of days after stress imposition		No. of days after stress relief
		3	5	3
Total dry weight (g/plant)	Control	1.82	2.3	2.86
	Water stress	1.56*	1.72*	2.29ns
Shoot dry weight (g/plant)	Control	1.3	1.69	2.13
	Water stress	0.91**	0.97*	1.54*
Root dry weight (g/plant)	Control	0.33	0.36	0.42
	Water stress	0.49**	0.59*	0.57*
Nodule dry weight (g/plant)	Control	0.19	0.24	0.31
	Water stress	0.16*	0.15*	0.18*
Root:shoot ratio	Control	0.26	0.22	0.2
	Water stress	0.55**	0.64**	0.38***

Levels of significance - see note under Table 1, p. 86.

Table 3: Relative growth rates (RGR) of whole plant and nodules during water stress and its relief.

	Treatment	Period 1	Period 2	Period 3
RGR of whole plant ($mg\ g^{-1}\ d^{-1}$)	Control	179.45	117.04	72.64
	Water stress	174.13	48.82	95.41
RGR of nodules ($mg\ g^{-1}\ d^{-1}$)	Control	180.93	116.81	85.31
	Water stress	175.01	Negative	60.77

Note: Period 1- From stress imposition to 3 days of stress (DOS); Period 2- From 3 to 5 DOS; Period 3- From stress relief to 3 days after stress relief.

Specific nitrogenase activity (SNA)

The specific nitrogenase activity per unit total dry weight (SNA_T) of the stressed treatment was significantly lower ($p<0.001$) than that of the control, both during the stress and relief periods (Table 4). However, the stressed treatment showed some recovery in SNA_T after stress relief. But during the time period measured, recovery was only partial and was not up to the level of the control which was still significantly greater ($p<0.001$). Specific nitrogenase activity per unit nodule dry weight showed a response similar to SNA_T. The recovery of specific nitrogenase activity per unit nodule biomass was proportionately greater than SNA per unit total biomass. The control showed a decrease of both SNA_T and SNA_N during the final period of measurement which coincided with flowering of the plants.

Concentration of soluble sugars

At 3 days after stress, even though nitrogenase activity had decreased significantly, the sum of glucose, fructose and sucrose concentrations was higher ($p<0.05$) in the stressed treatment after 3 days (Table 5). Although the comparative nitrogenase activities of the two treatments at 5 days after stress remained basically similar, the sugar concentrations showed decreased values relative to the control. Despite the 3-fold increase of both SNA_T and SNA_N after stress relief, a proportionate increase in total soluble sugars was not shown.

Table 4: Response of nitrogenase activity to water stress and relief.

Parameter	Treatment	Time of measurement		
		No. of days after stress imposition		No. of days after stress relief
		3	5	3
Total nitrogenase activity (μmol C_2H_4 $plant^{-1}h^{-1}$)	Control	19.39	27.28	29.57
	Water stress	2.49***	2.29**	7.51**
Specific nitrogenase activity per unit total dry weight (μmol C_2H_4 $g^{-1}h^{-1}$)	Control	10.65	12.08	10.38
	Water stress	1.6***	1.31***	3.09***
Specific nitrogenase activity per unit nodule dry weight (μmol $C_2H_4g^{-1}h^{-1}$)	Control	100.39	113.39	94.38
	Water stress	15.96***	14.59***	40.01*

Levels of significance - see note under Table 1, p. 86.

The sugar concentrations in nodules were significantly greater ($p<0.05$) in the stressed treatment than in the control on the 3rd day of stress. However, this trend was reversed by the 5th day of stress and remained so even after stress relief. Therefore, it is clear that the variation of sugar concentrations, both in the whole plant dry matter and in the nodules, do not correspond with the variation pattern of either SNA_T or SNA_N.

Concentration of total amino acids

The total amino acid concentration in the stressed plants showed a significant increase after 5 days of stress but declined significantly on stress relief (Table 6). The higher amino acid concentration at 5 days after stress was due to significantly higher concentrations in the stressed shoots and roots. Despite higher amino acid concentrations in stressed shoots and roots, the stressed nodules had significantly lower amino acid concentrations than the control both during the stress period and after its relief.

Table 5: Response of the sum of glucose, fructose and sucrose concentrations in different plant parts to water stress and relief.

Total soluble sugar concentration	Treatment	Time of measurement		
		No. of days after stress imposition		No. of days after stress relief
		3	5	3
Whole plant (g kg^{-1} dry wt.)	Control	2.03	5.74	5.95
	Water stress	4.5*	4.21*	4.63ns
Shoot (g kg^{-1} DW)	Control	0.78	2.94	3.12
	Water stress	1.86*	1.96*	2.69ns
Root (g kg^{-1} DW)	Control	0.53	0.96	1.62
	Water stress	1.15*	0.63*	1.20ns
Nodule (g kg^{-1} DW)	Control	0.71	1.85	1.21
	Water stress	1.50*	1.63ns	0.74*

Levels of significance - see note under Table 1, p. 86.

Table 6: Effects of water stress and relief on total amino acid concentration (leucine equivalents mmol g^{-1} dry weight) in different plant parts of *Phaseolus vulgaris*.

Total amino acid concentration	Treatment	Time of measurement		
		No. of days after stress imposition		No. of days after stress relief
		3	5	3
Whole plant	Conrol	24.77	19.56	24.64
	Stress	21.35*	24.29*	16.52*
Shoot	Control	7.52	7.15	5.14
	Stress	9.76*	12.72*	3.62ns
Root	Control	1.49	0.22	2.50
	Stress	3.91*	3.41*	4.80*
Nodules	Control	15.76	12.18	17.00
	Stress	7.69*	8.15*	8.10*

Levels of significance - see note under Table 1, p. 86.

Concentration of total nitrogen

The total shoot nitrogen concentration (Table 7) was not significantly different ($p=0.05$) between water stressed and control plants during the stress period. Both treatments showed a decline in total shoot N concentration during the period of stress relief with total N concentration of the control being significantly higher ($p<0.05$).

Table 7: Response of shoot nitrogen concentration (%) to water stress and relief.

Treatment	Time of measurement		
	No. of days after stress imposition		No. of days after stress relief
	3	5	3
Control	3.26	3.23	2.89
Water stress	3.13^{ns}	3.11^{ns}	2.38*

Levels of significance - see note under Table 1, p. 86.

DISCUSSION

Growth response to water stress

The initial decrease of total dry weight at 3 days after stress was not accompanied by a decrease in total soluble sugar concentrations. Therefore, the initial total dry weight decrease in response to water stress was not caused by a shortage of assimilate supply, but probably by a decrease in cellular turgor[15] and/or cell wall hardening.[16, 17] However, the significantly lower sugar concentrations after 5 days of stress show that assimilate shortage may have contributed to lower total dry weights under prolonged stress. The absence of a significant increase in soluble sugars on stress relief further confirms the above conclusion on the greater sensitivity to water stress of growth-related processes as compared to assimilate supply.[5] The greater root:shoot ratio maintained by stressed plants even after stress relief could be a significant factor contributing to the greater tolerance of previously-stressed plants to subsequent stress (i.e. acclimation). However, lower recovery following water stress of nodule biomass as compared to total biomass indicates a greater susceptibility of nodulation to water stress.

Mechanism of inhibition of nitrogenase activity by water stress

The absence of a correlation between reduced nitrogenase activity and a lower concentration of total soluble sugars showed that inhibition of N_2 fixation in response to water stress was not caused by a reduction of assimilates and hence energy supply to the nodules. This agrees with the conclusion of Vance & Heichel[18] who reviewed results from several experiments where the carbohydrate supply to the nodules was manipulated. The amino acids found in the shoot had to originate from the nodules through nitrogen fixation as the plant had no other source of nitrogen. The greater concentrations of amino acids in the shoots of the stressed plants in response to water stress indicated that the export of products of N_2 fixation from nodules was not inhibited by water stress. This was confirmed by the lower amino acid concentrations of nodules of stressed plants because if there had been an inhibition of product export, then amino acids should have accumulated in nodules. Schubert *et al.*[19] observed a similar response in alfalfa. The amino acids accumulating in the shoot in greater concentrations under water stress could be recycled back to the nodules. Parsons *et al.*[10] cite strong experimental evidence on the retranslocation of amino acids to the nodules via the phloem. This higher concentration of amino acids being imported to the nodules could act as a feedback signal to inhibit further fixation of nitrogen because the shoot already contains a high concentration of nitrogenous compounds (i.e. amino acids). The significant reduction of the accumulation of amino acids in shoots of stressed plants following stress relief, and the simultaneous recovery of nitrogenase activity also supports the above hypothesis. The reduction of shoot amino acid concentrations on stress recovery would decrease the amino acid concentrations recycled back to the nodules and would remove the feedback inhibition of nitrogen fixation. Therefore, this feedback regulation is a possible mechanism of reducing nitrogen fixation during periods of water stress. However, the partial recovery of nitrogenase activity on stress relief despite the significant reduction in shoot amino acid concentration, indicates a direct damage to the functioning of nodules by water stress.

Finally, the absence of a significant reduction in shoot N concentration indicated that reduced N_2 fixation was not the primary factor responsible for reduced growth of common bean due to water stress. This agreed with results of Pena-Cabriales & Castellanos[5] and Schubert *et al.*[19]

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