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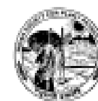

NITROGEN METABOLISM IN THE LEAVES OF *PENNISETUM GLAUCUM* (L) AS AFFECTED BY WATER STRESS

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SUMMARY

Activities of nitrate reductase (NR, both *in vitro* and *in vivo*), nitrite reductase (NiR), glutamine synthetase (GS1 and GS2) and glutamate dehydrogenase (GDH) in the leaves of *Pennisetum glaucum* (Bajra) declined following water stress imposed by withholding irrigation for different duration (24-60 h). Both the catalytic activities of nitrate reductase, *viz*. terminal nitrate reductase and NADH-nitrate reductase (total NR activity) got impeded in water stressed seedlings. Though the enzymes of ammonia assimilation were also affected by water withholding, but the level of reduction was less than that of nitrate assimilation. The isoforms of GS were differently affected by drought stress. GS1 activity declined more (54-63%) as compared to GS2 (21-31%) at 36 and 48 h of water withholding. The enzyme activities were recovered upon supply of water to 36 h-stressed plants. However, the plants subjected to longer period of water withholding (48 and 60 h) could show only the partial recovery of enzymes upon rewatering. The fast response of enzymes to change in water status of the plants could be due to post-transcriptional modification or inactivation of the enzyme(s).

Key words: **Glutamate dehydrogenase, glutamine synthetase, nitrate reductase, nitrite reductase, *Pennisetum glaucum*, water stress**

INTRODUCTION

Water stress is one of the main environmental stresses responsible for reducing crop productivity as it affects growth through various physiological and metabolic processes of plant (Bray 1993). Vital biochemical processes including photosynthesis (Boyer 1976), respiration (Bell *et al*. 1971), protein synthesis (Good and Zaplachinski 1994), nucleic acid synthesis (Deltour and Jacqmard 1974) assimilation of inorganic nitrogen (Munjal *et al*. 1998a) and biological nitrogen fixation (Sprent 1981) have been demonstrated to be adversely affected by water stress.

Nitrogen is a component of wide array of key biomolecules and plays a central role in plant metabolism. Under normal conditions, nitrate is the principal form of nitrogen available to the plant from soil that is reduced to ammonia via the process of nitrate assimilation, which involves sequential participation of two enzymes *viz*. nitrate reductase (NR) and nitrite reductase (NiR). Ammonia so produced is assimilated via GS/GOGAT cycle or via glutamate dehydrogenase (GDH). Limitation of water has been shown to impede capacity of plants to utilize inorganic nitrogen (Greenwood 1976) due to diminished absorption of nitrogen from soil. It may have an adverse effect on the enzymes of nitrate and ammonia assimilation (Reddy *et al*. 1990, Kumar and Dubey 1999). The present investigations were undertaken to ascertain the impact of varying levels of water stress on enzymes of inorganic nitrogen metabolism in leaves of *Pennisetum glaucum.*

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MATERIALS AND METHODS

Plant material and growth conditions: Bajra (*Pennisetum glaucum* L. cv. HHB67) seedlings were raised in plastic pots containing river sand in an illuminated chamber (light intensity 7000 Lux; light/dark cycle 15 h/ 9 h; day/night temperature 35^0C/ 25^0C). Four day old seedlings were provided with modified Hoagland nutrient solution. After 7 days, the seedlings were subjected to water stress of specified duration of 0, 24, 36, 48 and 60 h by withholding irrigation. During this period, control plants were supplied with distilled water at 12 h intervals. At the indicated period, the relative water content (Irgoyen *et al*. 1992) and osmotic potential of leaves (Kumar *et al*. 1984) were recorded.

Extraction of enzymes: Leaves from ten day old seedlings were macerated in chilled pestle and mortar in 0.1M phosphate buffer (pH 7.5) containing 7.5 mM cysteine, 2% (*w/v*) casein, 25 mm FAD and 1mM EDTA for extraction of NiR and NR. For extraction of GS and GDH 0.1M Tris-HCl (pH 8.0) containing 1 mM $MgCl_2$, 1mM cysteine-HCl and 10% glycerol was used. The homogenate was squeezed through four layers of muslin cloth and filtrate was centrifuged at 10,000 x g for 30 min. The supernatant was used as enzyme preparation.

Enzyme assay: *In vitro* NR activity was assayed using slightly modified procedure of Hageman and Flesher (1960) and activity was monitored by determining the amount of nitrite produced according to the procedure of Nicholas and Nason (1957). *In vivo* nitrate reductase activity was assayed by the method of Klepper *et al.* (1971). Leaves cut into small segments were transferred to the test tubes containing infiltration medium composed of 5 ml of 0.1 M phosphate buffer (pH 7.5) and 1.0 ml of 0.2 M KNO_3. The tubes were evacuated and incubated in dark at 30^0C. After 30 min, the suitable aliquots were taken for determination of nitrite using method of Nicholas and Nason (1957). Nitrite reductase activity was estimated by measuring the rate of disappearance of nitrite from the reaction mixture (Sawhney and Naik 1990). One unit of NR/NiR was defined as the µmol of nitrite produced/ utilized in 30 min. Both semisynthetase and transferase activities of glutamine synthetase were assayed by determining γ-glutamyl hydroxamate produced as described by Washitani and Sato (1977). The unit activity was defined as one µmol of γ-glutamyl hydroxamate produced per min. GDH activity was assayed following the formation of glutamic acid from 2-oxoglutarate and oxidation of NADH to NAD^+ was monitored as decrease in absorbance at 340 nm (Murray and Kennedy 1980). One µmol of NAD^+ produced per min denoted one unit of GDH.

Isoforms of GS: The isozymes of GS in crude extract were separated by anion exchange chromatography using DEAE-cellulose. One ml of the leaf homogenate was loaded on to the DEAE-cellulose column pre-equilibrated with 3 bed-volumes of extraction buffer. The elution of the adsorbed proteins was effected in a batch wise manner by using the buffer (0.1 M Tris HCl, pH 7.8) containing firstly 0.15 M KCl and then 0.3 M KCl. The flow rate was maintained at 20 ml h^{-1} and fractions (3 ml each) were collected and GS activity was determined using transferase assay (Washitani and Sato 1977).

RESULTS AND DISCUSSION

Relative water content and osmotic potential: Relative water content (RWC) of the leaves of *Pennisetum glaucum* subjected to water stress by withholding water supply for 0, 24, 36, 48 and 60 h declined progressively with increase in duration of water deprivation. It declined from 88.7% in the non-stressed seedlings to respectively 58.6 and 35.1% in leaves of 36 h and 60 h water stressed seedlings. Decrease in RWC under water deprivation has earlier been reported in other crops (Chandrasekar *et al*. 2000, Phutela *et al*. 2000). Imposition of stress led to development of more negative osmotic potential and magnitude of effect was dependent on duration of the stress. Water stress for 24, 36, 48 and 60 h decreased the osmotic potential of leaf cell sap by 2, 4.5, 5.5 and 6.0 folds respectively (Fig. 1). The osmotic potential tended to become normal rapidly when the plants were relieved of stress. After 6 h of water supply to 36 h stressed plants, RWC increased from 50% to 91% and osmotic potential became less negative, (–3.015 MPa in 36 h stressed plants to –0.72 MPa upon

rewatering) which was almost comparable to the osmotic potential of non-stressed plants (Table 3). The results are in agreement with those reported earlier (Smirnoff *et al*. 1985, Phutela *et al*. 2002).

Nitrate reduction: In conformity with the earlier report (Reddy *et al*. 1990, Kumar and Dubey 1999), *in vitro* and *in vivo* NR activity (NRA) decreased markedly upon subjecting 7 days old *Pennisetum* seedlings to water stress for various periods. The *in vitro* nitrate reductase activity in the leaves of plants subjected to water stress for 24, 36, 48 and 60 h declined by 24, 86, 94 and 98% respectively. The corresponding decrease in the *in vivo* NRA activity in leaves of these seedlings was 18, 57, 70 and 82% respectively (Fig.1, Table 1). Therefore, both the *in vitro* and *in vivo* NR activities diminished quite markedly and most profound effect occurred between 24 and 48 h of stress. The data also tend to suggest that during initial stage of stress, the *in vitro* activity depressed to relatively greater extent than *in vivo* activity. Several other workers have also observed that NR is an extremely sensitive enzyme and that its activity decreases rapidly during water stress (Ourry *et al*. 1992, Barathi *et al*. 2001). Decline in NRA has also been reported under water deficit conditions induced by including polyethylene glycol in the rooting medium (Garg *et al*. 1998, Munjal *et al*. 1998b, Flores *et al*. 2000). In the present investigation, *Pennisetum* seedlings subjected to 24 h water stress resulted in decrease in RWC from 89 to 77% that caused 25% decline in the activity of NRA. For 36 h stressed plants, a 38% recession in RWC was accompanied by 80% drop in the *in vitro* and 57% decline in the *in vivo* NR. These

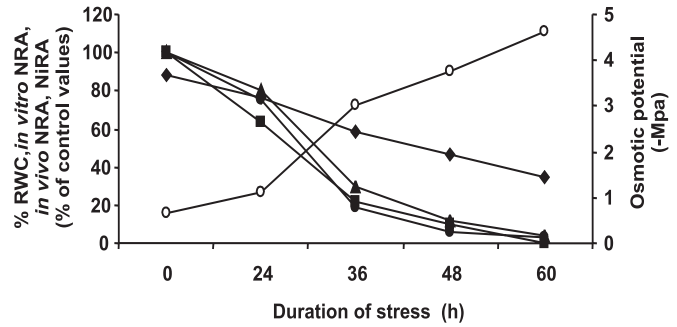

Fig. 1. Effect of water withholding on relative water content (RWC, -u-), osmotic potential (-m-), *in vitro* nitrate reductase activity (NRA -l-), *in vivo* nitrate reductase activity (NRA -s-), and nitrite reductase activity (NiR -n-) in leaf extracts of *Pennisetum glaucum* L.

results are in agreement with those of Maranville and Paulsen (1977).

Nitrite reductase (NiR), the second enzyme of nitrate assimilation pathway that reduces nitrite to ammonia is also deleteriously affected by deprivation of water. Withholding of water supply for 24 and 36 h resulted in respectively 37% and 78% decrease in the activity whereas the seedlings deprived of water for 48 and 60 h barely showed any activity (Fig.1) The adverse effect of moisture stress on this enzyme has also been demonstrated by other workers (Sinha and Nicholas, 1981, Ramanjulu and Sudhakar 1997).

Nitrate reductase is a complex enzyme and is constituted of two distinct catalytic components *viz*. NADH-dehydrogenase and terminal nitrate reductase. The transfer of electron from NADH to nitrate involves

Table 1. Effect of water stress on RWC, osmotic potential, and enzymes of nitrate reduction and ammonia assimilation in the leaves of *Pennisetum glaucum*

Duration of stress (h)	RWC (%)	Osmotic potential (-MPa)	Enzyme activity (Units g^{-1} dry wt)					
			NR		NiR	GS		GDH
			in vitro	*in vivo*		Trans	SS	
0	88.7±3.1	0.67±0.05	41.14±0.38	27.54±1.12	312.5±12.7	88.8±8.0	5.36±0.17	4.31±0.15
24	76.7±2.3	1.13±0.09	31.18±.0.22	22.04±0.99	198.8±14.3	112.3±.2.3	5.08±0.45	4.32±0.29
36	58.6±1.9	3.02±0.08	7.67±0.15	8.29±0.60	67.0±2.8	87.9±2.5	3.75±0.06	3.71±0.12
48	46.4±1.3	3.74±0.10	2.28±0.06	3.32±0.04	30.5±1.5	45.8±1.7	2.73±0.10	2.74±0.21
60	35.1±1.2	4.62±0.17	1.06±0.01	1.07±0.09	ND	8.1±1.3	2.44±0.14	2.03±0.11

participation of both the components (Hewitt *et al*. 1976). In order to identify whether terminal nitrate reductase is affected under water stress, the impact of stress on NADH and $FMNH_2$-dependent (terminal) activities of nitrate reductase was compared. In seedlings subjected to water stress for 36 h, the NADH-NR (total NR) activity was affected by 38% while $FMNH_2$-dependent activity was lowered by about 49%. (Table 2). In the seedlings deprived of water for 48 h, both the activities were impaired by about 97%. The results thus imply that overall activity (NADH-dependent NR activity) and $FMNH_2$-dependent NR activity were affected to more or less the same extent. The results obtained are in accordance with those of Munjal *et al*. (1998b) who also demonstrated that the terminal nitrate reductase activity get impeded in water stressed plants.

Table 2. Total NR (NADH- NR) and terminal NR activities and glutamine synthetase (GS) isoforms in the leaf extract of *Pennisetum glaucum* subjected to water stress.

Enzyme activity (Units g^{-1} dry wt)	Control	Duration of stress (h) 36	48
NADH-nitrate reductase	34.33	21.35 (62.1)	1.26 (3.6)
Terminal nitrate reductase	11.82	5.99 (50.3)	0.35 (2.90)
GS1	48.8	22.6 (46.3)	18.3 (37.4)
GS2	39.9	31.7 (79.2)	27.4 (68.7)
GS1 : GS2	55:45	42:58	40:60

Values in parentheses denote per cent of the control activity

Glutamine synthetase (GS), the key enzyme of ammonia assimilation is the main link between photosynthesis and supply of inorganic compounds (Vance and Griffith 1992). Effect of water deficiency on activity of GS was determined by both semi-synthetase and γ-glutamyl transferase activities. The transferase activity of GS from *Pennisetum* leaves was 20-30 fold higher than semi-synthetase activity (Table 1). Such vast differences in GS activity measured by these two assays have been previously reported by earlier workers (Lea *et al*. 1990b, Yamaya 1995). Withholding irrigation had differential effect on γ-glutamyl transferase and semi-synthetase activities. Water stress for 24 h, enhanced transferase activity by 25% while semi-synthetase activity remained unchanged. Similarly, the semi-synthetase activity in 36 h water deprived plants was 70% whereas transferase activity was comparable to that in the control plants (Fig. 2). Thereafter, both the activities were altered to the same extent. The seedlings subjected to 48 h and 60 h of stress possessed ~50 and 45% of GS activities respectively. Like semi-synthetase activity of GS, GDH activity was also not affected by the stress of 24 h but increase in the period of water deprivation to 36 h and 48 h resulted in decline in activity by 14 and 37% respectively (Fig. 2). The adverse effects of water stress on GS and GDH in leaves have been reported earlier (Diana and Biehler 1997, Sibout and Guerrier 1998, Kumar and Dubey 1999). However, in the present investigations a distinct enhancement in the transferase activity of GS was discerned during the initial stage of the stress. Ramanjulu and Sudhakar (1997) using transferase assay procedure for GS also reported an initial increase in the activity. Not only the activity, the isoforms of GS were differently affected by withholding water for 36 and 48 h. Results presented (Table 2) here show larger decline in GS1 activity (54-63%) as compared to GS2 (20.8-31.4%) under both stress levels, thereby changing the ratio of GS1: GS2. The ratio of GS1: GS2 in the control plants was 55:45 which changed to 40:60 upon withholding irrigation for 48 h. Diminished activities of GS isoforms under water deficit have earlier been reported (Diana and Biehler 1997, Kumar and Dubey 1999).

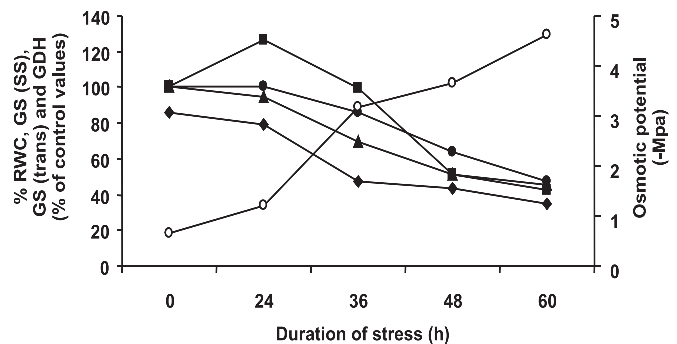

Fig. 2. Effect of water withholding on relative water content (RWC, -u-), osmotic potential (-m-), semi synthetase activity of glutamine synthetase [GS (SS) -s-], transferase activity of glutamine synthetase [GS (Trans), -n-], and glutamate dehydrogenase activity (GDH -l-) in leaf extracts of *Pennisetum glaucum* L.

Effect of supplying water to stressed seedlings on enzyme activities: The seedlings were relieved of the water stress by supplying water for 6 h. Both *in vitro* and *in vivo* NRAs regained quickly after 6 h of irrigation from 18% to about 90% in case of 36 h stressed seedlings compared to that of control plants. However, enhancement in NiRA after 6 h of water supply to 36 h-stressed plants was from 22 to 69% of that in the control. The plants subjected to 48 h of stress did not show any increase in activities of *in vitro* and *in vivo* NR but NiRA increased from 1.5% to 21% after 6 h of supply of water. Both semi-synthetase and transferase activities of GS and GDH showed substantial recovery after 6 h of relief from water stress. After 6 h of irrigation to 48 h-stressed plants, transferase and semi-synthetase activities increased from 50% to 90%. The recovery of GDH activity on supply of water for 6 h was similar to that of GS activity (Table 3).

In conformity with the observations of earlier workers (Garg *et al*. 1998; Munjal *et al*. 1998b, Barathi *et al*. 2001), NRA declined markedly on imposition of water stress. The adverse effect of water deficiency in NADH-NR was accompanied by decrease in activity of terminal moiety of the enzyme. Nitrite reductase has been reported to be more stable than NR during water stress (Sinha and Nicholas 1981, Ramanjulu and Sudhakar 1997). However, in seedlings of *Pennisetum glaucum*, no appreciable difference was perceived in the decline in activity of NR and NiR on imposition of stress. In contrast, GS and GDH were distinctly stable more than NR and NiR and substantial activities of these enzymes were detected in severely stressed seedlings of *Pennisetum glaucum*. Lesser susceptibility of these enzymes to drought than NRA has been reported (Sinha and Nicholas 1981). On the other hand, Reddy *et al*. (1990) reported a slightly more pronounced deleterious effect of PEG-induced water stress on GS than on NRA in seedlings of horsegram.

Upon supply of water to 36 h stressed plants, both nitrate flux and enzymes activities were recovered. Morilla *et al*. (1973) observed that decline in nitrate reductase activity in water stressed maize seedlings was preceded by decrease in polyribosomal level and its recovery on rewatering followed only after rise in polyribosome content. In contrast, fast response of enzyme to change in water status of plant has been assumed to involve its direct inhibition through rapid activation/ inactivation of pre-existing enzyme (Kaiser and Brendle-Behnisch 1991, Munjal *et al*. 1998a, Meyer *et al*. 1999).

Limitation of water has been shown to impede capacity of plants to utilize inorganic nitrogen (Greenwood 1976) that may be due to diminished absorption of nitrogen from soil or its adverse effect on the enzymes of nitrate and ammonia assimilation. The sensitivity of NRA under conditions of moisture limitation has been documented in several crops (Ourry *et al*. 1992, Munjal *et al*. 1998b, Barathi *et al*. 2001). This could be due to cessation of the synthesis of enzyme as a consequence of reduced nitrate flux in to the leaves (Shaner and Boyer 1976) or due to its conversion to inactive form (Aryan *et al*. 1983) via phosphorylation- dephosphorylation mechanism (Kaiser and Brendle-Behnisch 1991) or because of over reduction of the enzyme (Munjal *et al*. 1998a). The results of the present investigations are in

Table 3. RWC, osmotic potential, and enzymes of nitrate reduction and ammonia assimilation in the leaves of *Pennisetum glaucum* after 6 h of relief from water stress

Duration of stress (h)	RWC (%)	Osmotic potential (-MPa)	Enzyme activity (Units g^{-1} dry wt)					
			NR		NiR	GS		GDH
			in vitro	*in vivo*		SS	Transferase	
0	91.8±1.3	0.69±0.06	52.54±0.06	30.00±2.09	228.610.4	5.68±0.18	89.282.66	4.27±0.15
36	91.2±1.4	0.72±0.03	49.42±0.07	26.35±0.43	157.3±2.3	5.54±0.17	92.82±4.34	3.93±0.19
48	50.9±1.5	1.96±0.05	4.35±0.02	4.61±0.12	47.9±1.3	5.09±0.19	85.83±5.28	3.62±0.20
60	39.0±1.0	2.40±0.09	0.92±0.0	1.20±0.03	ND	3.38±0.21	49.93±1.75	2.51±0.17

agreement with those of Munjal *et al*. (1998b) who demonstrated that terminal nitrate reductase gets impeded in the water-stressed plants. This was further confirmed by pre-incubating the enzyme extract of water stressed seedlings with cysteine-HCl required for reduction of sulfhydryl groups that did not restore activity of NR (data not shown). The results indicated that loss of NADH-nitrate reductase activity was probably not due to oxidation of sulfhydryl groups. The impairment of NADH-nitrate reductase could be accounted by the adverse effect of moisture limitation on terminal moiety of the enzyme. The parallel loss of activity of this component of enzyme complex (Table 2) further supports this view. Decline in GS under water stress could be due to decrease in activities of its isoforms as reported by Kumar and Dubey (1999). Sibout and Guerrier (1998) reported that a fall in GS activity under water stress might result from either a decrease in protein level, which may affect the physico-chemical properties of enzymes, or both. The alteration in nitrogen metabolism in leaves of *Pennisetum glaucum* seedlings under water stress and fast response of the enzymes to change in water status of the plant may be due to post-transcriptional modifications or inactivation of the enzymes by a mechanism yet to be resolved.

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