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ACTIVATION OF NITRATE REDUCTASE IN EXTRACTS OF WATER STRESSED WHEAT

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; water stress; nitrate reductase; *in vitro* reactivation; potassium ferricyanide; NADH.

Abstract—Nitrate reductase (EC 1.6.6.1) activity (NRA) in leaves of wheat (*Triticum aestivum* cv. C306) seedlings declined significantly following polyethylene glycol (PEG)-induced water stress. During initial stages of stress, the *in vitro* NRA was affected to a greater extent than the *in vivo* activity. Treatment with potassium ferricyanide of the leaf extracts of plants stressed for 2 and 4 days elevated NRA to the levels almost comparable with that in non-stressed control plants, and in plants stressed for 4 days NADH was also effective. Treatment with EDTA, which promotes conversion of phosphorylated inactive NR to its non-phosphorylated active form, however did not stimulate NRA in these extracts. These results suggest that the decrease in NRA during the initial stages of water stress was due to conversion of the enzyme to an inactive form, which can readily be reactivated *in vitro* by potassium ferricyanide or NADH. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The input of reduced nitrogen to plants via nitrate reduction is determined by activity of nitrate reductase (NR, EC 1.6.6.1), which catalyses the first and the rate limiting reaction of this assimilatory process. Synthesis and activity of this enzyme is influenced by several environmental factors, such as nitrate availability, light, temperature [1–3] and water stress [2, 4–8]. The precise mechanism for rapid decline in its activity under moisture deficit conditions has, however, not been established. The decrease in NRA has been attributed to an impaired protein synthesizing capacity of water stressed plants due to disintegration of polyribosomes [4]. Shaner and Boyer [5] emphasized that the induced synthesis of NR is dependent on nitrate flux to leaves rather than their total nitrate content and ascribed reduced synthesis of the enzyme under moisture stress to diminished flux of nitrate. In contrast, a direct inhibitory effect of water stress on the activity, rather than the synthesis of the enzyme, has also been suggested [9]. A more pronounced increase in NRA on preincubation of leaf extracts of water stressed plants with NADH (Sinha, unpublished, cf. ref. [7]) led to the proposal that NADH acts as a modulator, and the diminished enzyme activity is perhaps due to occurrence of the enzyme in a less active form because of low endogenous concentrations of NADH in water stressed plants [7]. More recently, a sharp decline in extractable NRA in dehydrating leaves has been attributed to covalent modification of the enzyme to its catalytically inactive form [10]. In view of the conflicting postulated mechanisms, the present investigations were undertaken to ascertain whether leaf extracts of water stressed wheat seedlings contain an inactive form of nitrate reductase, whose activity could be restored by treatment with certain reagents.

RESULTS

Effect on relative water content and osmotic potential

Water stress was created by providing 6-day-old wheat seedlings with nutrient solution supplemented with three different levels of PEG. The osmotic potential of these PEG-containing solutions was −2, −4 and −6 bars. As shown in Fig. 1, the relative water content of leaves of stressed plants decreased progressively with increasing intensity as well as the duration of stress. After 6 days of treatment, relative water content of plants growing in PEG-containing media of −2, −4 and −6 bar receded to 74, 65 and 50%, respectively, compared with 98% in control plants. From the results in Fig. 2 it is also evident that the leaf sap of PEG-treated plants developed a more negative osmotic potential which is a characteristic response to stress. Both the relative water content

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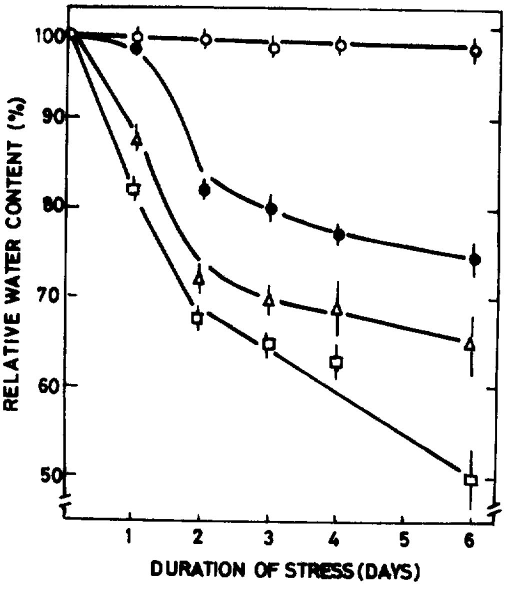

Fig. 1. Effect of water stress on relative water content of leaves. 6-day-old wheat seedlings were supplied with half strength nutrient solution. After 2 days some of these were given PEG-containing nutrient solution of osmotic potential of −2, −4 and −6 bar. The relative water content of leaves was determined on indicated days. Control, ○; −2 bar, ●; −4 bar, △; −6 bar, □.

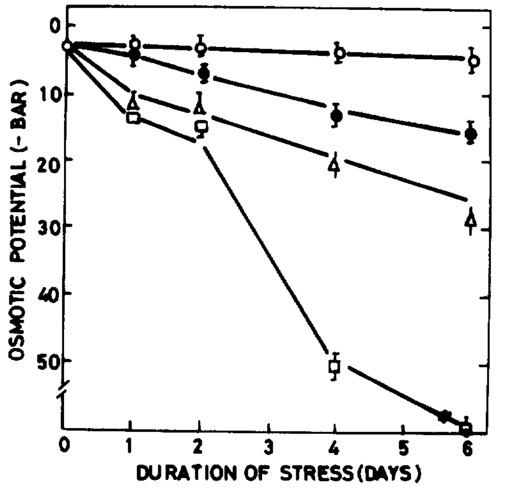

Fig. 2. Effect of water stress on osmotic potential of leaf cell sap of wheat seedlings. The experimental details were as described in Fig. 1. Control, ○; −2 bar, ●; −4 bar, △; −6 bar, □.

and osmotic potential tended to become normal fairly rapidly on transferring the stressed plants to a PEG-free media (Table 1).

Effect on NRA

Imposition of water stress resulted in a pronounced loss of *in vitro* [Fig. 3 (A)] as well as *in vivo* NRA [Fig. 3 (B)]. A stress of −2 bar for 3 days, which lowered relative water content to 81%, compared with 95% in control plants, caused a 60% decrease in *in vitro* activity [Fig. 3 (A)]. The adverse effect on NRA was accentuated with the severity and the duration of stress. After 4 days of stress, enzyme activity in leaf extracts of plants growing in PEG-containing solutions of −2, −4 and −6 bar was only about 25, 20 and 10% of that in control plants, respectively. Comparison of the results in Fig. 3 (A) and (B) reveals that during the initial stages, the deleterious effect of PEG solutions was significantly more pronounced on *in vitro* than on *in vivo* NRA. For instance, after 2 days of stress of −4 bar, *in vivo* activity was depressed by 22% whereas *in vitro* activity showed a reduction of about 55%. This differential effect of stress on these two activities narrowed down with time except at −2 bar where the difference was discernible throughout 6 days of the experiment. From the results in Table 1, it is evident that like relative water content and leaf sap osmotic potential, NRA was also regained to a significant extent during a 6 hr recovery period and the *in vitro* enzyme activity in plants subjected to stress of −2 bar rose from an initial value of 27% to 80% of that in control plants. Also during recovery, the *in vitro* activity was restored to a relatively greater extent than the *in vivo* activity.

Effect of ATP

According to one of the postulated mechanisms, the loss of NRA during moisture stress emanates from covalent modification of the enzyme molecule to its inactive phosphorylated form [10]. Susceptibility of the enzymes of the wheat cultivar used in the present investigations to such a control was first studied. Preincubation of leaf extract with ATP lowered the enzyme activity in concentration (Table 2) and the time-dependent (Fig. 4) manner. Preincubation of enzyme preparation for 20 min with 10 mM ATP caused about 45% reduction in activity. Under identical conditions, 10 mM ADP affected the enzyme activity by 13%, whereas AMP stimulated it by 30% (Table 2). This pre-treatment with ATP caused a parallel loss of NADH-NR and reduced benzyl viologen-terminal NR activities of the enzyme (data not shown). The ATP mediated inactivation of NR was fully reversed by 5 mM EDTA (Fig. 5). A slight stimulation of around 20% by EDTA was recorded in the native enzyme preparation. The decrease in NRA upon incubation with ATP, the complete reversal of this effect by EDTA and the inhibition of terminal NRA are all in conformity with reversible covalent modification of NR via a phosphorylation–dephosphorylation mechanism [11].

Restoration of NRA

For these experiments, the enzyme activity in stressed as well as non-stressed plants was determined after incubating the leaf extracts for 20 min with certain reagents known to stimulate or reactivate inactive

Table 1. Changes in relative water content, osmotic potential and NR activity upon transferring plants stressed for 6 days to PEG-free medium

		Level of stress (−bar)			
		−2		−4	
Parameter	Non-stressed plants	Initial*	Recovery (6 hr)	Initial*	Recovery (6 hr)
Relative water content (%)	96.0±2.0	74.6±1.7	91.2±2.1	65.4±1.4	88.0±1.7
Osmotic potential (bars)	−5.07±0.2	−14.9±0.4	−8.3±0.2	−29.1±1.7	−10±0.4
NRA (% of control					
In vitro	100	27	80	14	69
In vivo	100	44	65	16	58

* Values just prior to recovery treatment.

Experimental details were as described in Fig. 1, except after 6 days of stress, the seedlings were transferred to PEG-free media and various measurements were again made after 6 hr. The *in vivo* and *in vitro* NRA of control non-stressed plants were 2.06 and 4.78 nkat/g dry weight, respectively.

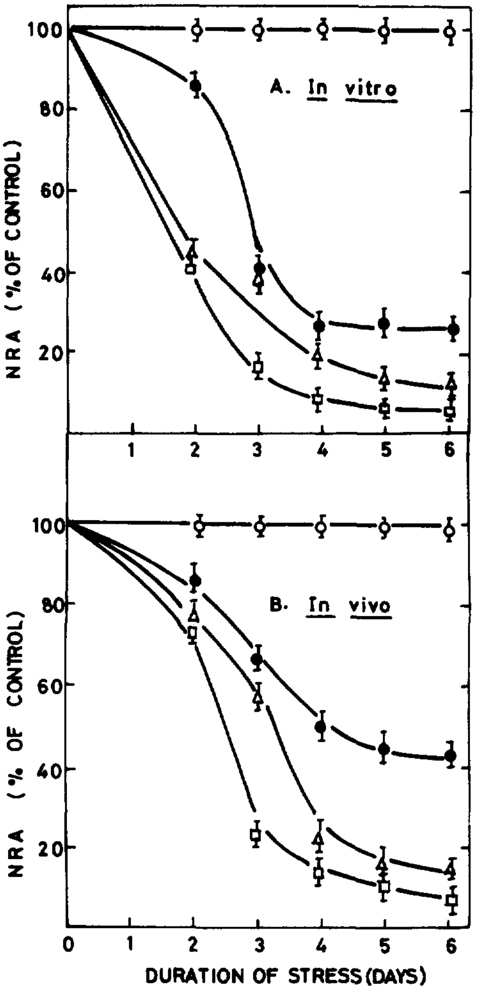

Fig. 3. *In vitro* (A) and *in vivo* (B) NRA in wheat leaves of water stressed plants. The experimental details were as given in Fig. 1. NRA activity was calculated on basis of per g dry wt of tissue and is shown as % of that in control non-stressed seedlings. Control, ○; −2 bar, ●; −4 bar, △; −6 bar, □.

Table 2. Effect of preincubation of leaf extract with varying concentration of ATP, ADP and AMP on NRA

Adenine nucleotide	Concentration (mM)	NRA in kat ml^{-1} of extract	% change in activity
None	0	0.39	0
ATP	1.0	0.35	−9
	5.0	0.32	−18
	7.5	0.25	−33
	10.0	0.21	−46
ADP	5.0	0.39	0
	10.0	0.34	−13
AMP	5.0	0.46	+20
	10.0	0.50	+29

Cell free extract of leaves from non-stressed seedlings was preincubated at 20° for 20 min with the specified concentration of adenine nucleotides, as described in the Experimental, prior to determining *in vitro* NRA.

NR. Treatment of the leaf extracts of non-stressed plants with potassium ferricyanide had a marginal effect on NRA (Table 3); in contrast, that in stressed plants was stimulated by 2.5- to 3.5-fold. Four days of stress had lowered NRA activity to 22% of that in control plants but, on prior incubation of their leaf extracts with 15 μM potassium ferricyanide, the enzyme activity rose to 82% of that in identically treated extracts of non-stressed control plants. A similar type of response was also recorded with 30 μM ferricyanide in plants which had been subjected to stress for 2 days. Evidently treatment of the leaf extracts with potassium ferricyanide was highly effective in elevating the enzyme activity to levels almost comparable to that in non-stressed plants. Preincubation with NADH also caused a marked enhancement of the enzyme activity in leaf extracts of stressed seedlings (Table 3, Expt 2). In the case of non-stressed plants, preincubation with 0.17 and 0.35 μM NADH had either little or slight inhibitory effect on the activity. However, pre-treatment of extracts of seedlings stressed for 4 days with these concentrations of NADH resulted in 2.5- and 3.5-times higher activity, respectively. Following treatment with 0.17

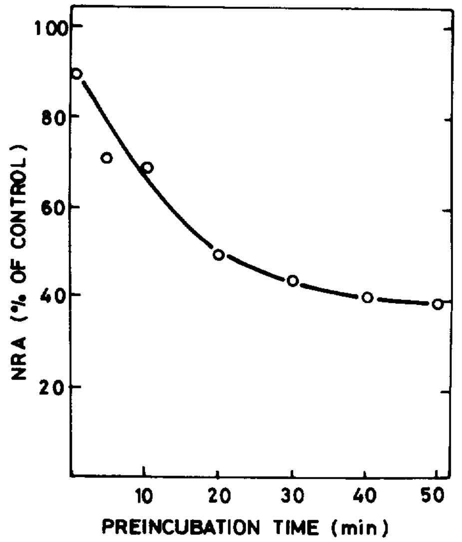

Fig. 4. Time course of ATP-mediated inactivation of NRA. The experimental details were as given in Table 2, except that the leaf extract was preincubated either in the absence (control) or in presence of 10 mM ATP for indicated periods, prior to assaying the enzyme activity. NRA of extract without any preincubation and after 50 min of preincubation in the absence of ATP was 0.42 and 0.40 nkat ml^{-1}, respectively.

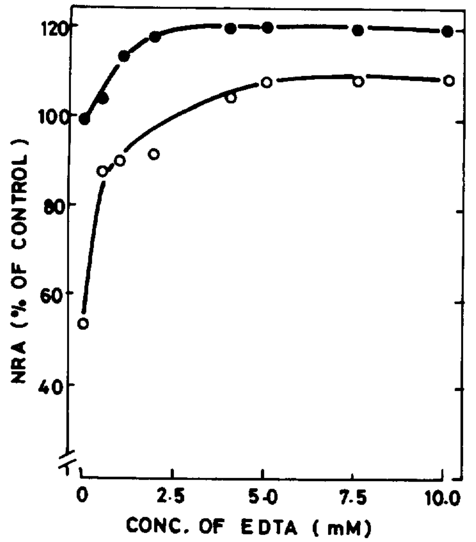

Fig. 5. Reversal of ATP-mediated inactivation of NRA by EDTA. Dialysed leaf extract was preincubated at 20° for 20 min either as such or in the presence of 10 mM ATP. Then EDTA, at the indicated concentration, was added and after further incubation for 20 min, NRA was determined. Enzyme activities of freshly prepared extract, that preincubated for 20 min without ATP (control) and that preincubated with 10 mM ATP were 0.26, 0.21 and 0.12 nkat ml^{-1} of leaf extract, respectively. Results are presented as % activity of control (preincubation carried out in absence of ATP and EDTA).

and 0.35 μM NADH, NRA in leaf extracts of 4 days stressed seedlings rose to 53 and 85%, respectively, of that in similarly preincubated extracts of non-stressed plants. As may be noted, EDTA did not exert any selective stimulatory effect on the enzyme activity in extracts of water stressed seedlings.

DISCUSSION

In agreement with observations of earlier investigators [2, 4–10], NR in leaves of C-306 cultivar of wheat was quite sensitive and its activity declined markedly on imposition of PEG-induced water stress. The magnitude of the adverse effect was dependent both on intensity and duration of stress. In the past, either *in vitro* [4, 5, 6, 8] or *in vivo* [12–14] assay procedures have been employed to ascertain the impact of water stress on NR. In the present studies, significant differences in response of these two activities of the enzyme to water stress discerned [Figs 3 (A) and (B)]. During initial phases of stress, *in vitro* NRA was affected to a considerably greater extent than the *in vivo* activity. This differential effect was perhaps because of inactivation of the enzyme during extraction by elevated concentrations of some inhibitory compounds such as phenols, proteases, etc., in the tissues of stressed plants. Such an inhibition is apt to give an exaggerated effect of water stress on NR. In the *in vivo* assay the NR probably does not come in direct contact with these substances, due to their segregation in different sub-cellular compartments. On the other hand, the slow recovery of *in vivo* activity in plants stressed for 6 days (Table 2) might have been due to diminished capacity of tissues to generate NADH, either because of depletion of respirable substrates or slower repair of NADH generating reactions/systems which may have suffered damage during an extended stress.

Considerable controversy exists about the molecular mechanisms for decline in NRA under water deficit conditions. In the present investigations NRA quite close to that in non-stressed plants were obtained on pre-treatment of leaf extracts of stressed plants with potassium ferricyanide or NADH (Table 3). This observation precludes the possibility that decreased activity was due to impaired synthesis of the enzyme as a consequence of disintegration of polyribosomes [4] or reduced flux of nitrate to leaves [5]. On the other hand the results indicate that the decline, particularly during early stages of stress, was manifested through inactivation of the enzyme.

The loss of NRA in rapidly dehydrating spinach leaves has been attributed to phosphorylation of the enzyme molecule to its inactive form [10]. In preliminary experiments it was demonstrated that NRA, in the wheat cultivar used in the present investigations, was inhibited by ATP (Table 2) and that this inactivation was fully reversed by EDTA (Fig. 5). Such behaviour is consistent with regulation of NR via phosphorylation–dephosphorylation based modification [11]. However, our results show that unlike potassium ferricyanide and NADH, pre-treatment of leaf homogenates with EDTA was completely ineffective in restoring NRA in the extracts of stressed plants.

Table 3. Response of NRA in leaf extracts of stressed and non-stressed plants to various pretreatments

Addition to preincubation mixture	Duration of stress (days) 2 Non-stressed	 Stressed	 4 Non-stressed	 Stressed
	NRA (in kats/g dw)			
Experimental 1				
None	3.30	1.28 (39)*	2.25	0.52 (22)
Potassium ferricyanide				
15 μM	n.d.	n.d.	2.25	1.90 (82)
30 μM	3.54	3.15 (88)	2.20	1.26 (58)
Experiment 2				
None	3.30	0.88 (26)	1.77	0.37 (20)
EDTA (10 mM)	5.31	0.80 (14)	2.76	0.60 (21)
NADH				
0.17 μM	4.25	1.57 (36)	1.66	0.90 (53)
0.35 μM	3.11	1.38 (44)	1.48	1.20 (80)

* Values in parentheses represent activity as % of that of identically treated extracts of non-stressed seedlings.

Six-day-old seedlings were kept in nutrient solution of PEG-containing media of −4 bar for either 2 or 4 days. NRA in leaf homogenates of these seedlings was determined after preincubating the extracts at 20° for 20 min with specified concentration of the indicated reagents as described in the Experimental.

This evidently does not support the view that the diminished activity could have been due to phosphorylation of the enzyme to its inactive form.

NR from higher plants can exist in two redox-mediated interconvertible forms [1, 3, 15]. Pre-incubation of cell free preparations, in the absence of nitrate, with NAD(P)H or any other appropriate reductant, transforms the enzyme to its reduced inactive form which can be reactivated instantaneously by its oxidation with potassium ferricyanide or more gradually with either nitrate or oxygen [15, 16]. Aryan *et al.* [17] had also earlier noted that ferricyanide enhances NRA in extracts of water deprived plants to a relatively greater extent than the non-stressed ones. In their studies, the extent of stimulation in NRA in these two groups of plants was 60 and 30%, respectively. In the present investigations, however, ferricyanide had a much more profound effect and stimulated NRA of stressed plants by 2.5–3.0 fold, while it had little effect in extracts of non-stressed plants. It, therefore, appears that one of the causes for diminished activity of NR in water deficit plants was the redox-mediated transformation of the enzyme to its catalytically inactive reduced form. The concomitant decrease in overall NADH-linked NRA and of $FMNH_2$- and reduced benzyl viologen-dependent activity of the terminal moiety of the enzyme complex in stressed plants (data not shown) is consistent with such an effect. It is relevant that photosynthetic electron transport chain is quite tolerant to water stress [18]. However, operation of the Calvin Cycle may be impeded firstly because of limited availability of carbon dioxide due to closure of stomata [19] and also because of restricted supply of ATP as a consequence of stress-induced damage to the chloroplastic coupling factor [18, 19]. The curtailed utilization of photochemically generated reductant could create more reducing cellular environment in leaves which may promote reduction-mediated inactivation of NR. In fact, an increased ratio of NADPH/NADP and occurrence of ferredoxin in reduced state in leaves of water stressed plants has been documented [18].

In the present investigations, besides potassium ferricyanide, NADH also differentially promoted NRA in stressed plants (Table 3). It is, however, noteworthy that the stimulation by NADH in extracts of plants stressed for 4 days was considerably greater than that in plants NR stressed for only 2 days. It appears that with advancement of stress some NR molecules were converted to a form which could be reactivated by NADH. The results tend to suggest that inactivation of NR might be effected via different mechanisms depending on the stage, the severity and, also perhaps, on the rapidity of stress. This may partly account for the divergent mechanisms advanced by the different workers for the harmful effect of water stress on NR. A more pronounced enhancement in NRA following pretreatment with NADH has been noted earlier by Sinha (unpublished, cf. ref. [7]). It was proposed that activity of NR is modulated by NADH/NAD ratio and decline in its activity might be due to decreased levels of cellular NADH in stressed plants [7]. Alternatively, enhancement by NADH could also be due to protection of the enzyme against a proteinaceous inhibitor which binds reversibly to NR [3, 20, 21].

To summarize, the present investigation indicates that the loss of NRA during early stages of stress occurs predominantly through inactivation of the enzyme. Curtailment of energy dissipating assimilatory processes, including nitrate reduction under unfavourable conditions such as water limitation, could be important for survival of an organism. Restricting nitrate assimilation by inactivation of NR rather than by its removal by degradation could offer an adaptive advantage as it would enable a more rapid resumption of this primary metabolic process following relief of stress. It is perhaps also relevant that the reduced inactive form of NR from *Chlorella* is more stable to attack by proteases such as NR-inactivating corn protease and trypsin [22].

EXPERIMENTAL

Cultural conditions. Wheat (*Triticum aestivum* cv. C 306) seedlings were raised in a growth chamber (light intensity, 7000 lux; light/dark cycle, 15/9 hr; day/night temp. 25°/10°) were grown in plastic trays containing river sand. Six days after sowing, the plants were supplied with half strength modified Hoagland nutrient solution in which $NH_4H_2PO_4$ was replaced by KNO_3 and KH_2PO_4 to give an equivalent amount of PO_4 as in original media and the final concn of NO_3 was 50 mM. Two days later, some of the seedlings were subjected to three different levels of H_2O stress by supplying them with the above nutrient media containing PEG (M_r 6000) to create stress of -2, -4 and -6 bar. Nutrient solns were replenished daily. For experiments in which recovery was examined, the control as well as stressed seedlings were gently removed from sand and kept in beakers under running tap H_2O for 1 hr to thoroughly wash the roots. The seedlings were then given fresh PEG-free nutrients soln, transferred to growth chamber with lights on and after 6 hr their relative H_2O content, sap osmotic potential and NRA were determined as described below.

In vitro *and* in vivo *NRA*. For these leaves were harvested 3–4 hr after the beginning of the light period. For *in vitro* activity extracts were prepared by mascerating 1 g of leaf material in 4 ml of 0.1 M KPi buffer (pH 7.5) containing 7.5 mM cysteine, 2% (w/v) casein, 25 μM FAD and 10% (v/v) glycerol. The homogenate was centrifuged at 10 000 *g* for 20 min. All the above operations were carried out at 4°. The enzyme activity was assayed in the supernatant according to ref. [20]. In some of the experiments, effect of pretreatment of enzyme extract with certain reagents was examined. For this, prior to enzyme assay, 0.2 ml of leaf extract dialysed for 4 hr against 0.05 M extraction buffer, was preincubated at 20° for 20 min in presence of 0.2 ml of KPi buffer (pH 7.5) with 0.1 ml of the specified concn of the indicated reagents. Under the above conditions the enzyme was quite stable during preincubation and retained 90% of its original activity. In preliminary experiments it was first established that the media for extraction of the enzyme was optimal. The enzyme was completely stable up to 8 hr when kept at 4°.

In vivo NRA was determined following the method described in ref. [23] except that the infiltration was carried out in Thunberg tubes and the infiltration media constituted of 4 ml of 0.1 M KPi buffer (pH 7.5), 1 ml of 0.2 M KNO_3 and 0.1 ml of *n*-PrOH. Both *in vitro* and *in vivo* activities were calculated on the basis of dry wt of the tissue.

Relative H_2O *content and cell sap osmotic potential.* These were determined according to refs [24] and [25], respectively.

All the experiments were conducted at least 3 ×. In each experiment relative H_2O content and osmotic potential were measured in four replicates of each treatment and duplicate samples were used for extraction and the enzyme activity in each extract was carried out in duplicate. The data presented is representative of the obtained results.

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