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Short Communication

Fluctuations in Spinach Leaf Nitrate Reductase During Light-Dark Transitions†

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***In vivo* nitrate reductase (NR) activity declined gradually either in absence or presence of Mg^{2+} In dark grown plants of spinach. The increased sensitivity of the extracted NR from the dark grown plants to Mg^{2+} and ATP is indicative of the post-translational modification as one of the mechanisms to control NR activity. The response of extracted NR was gradual and not instantaneous suggesting a complex interplay of NR regulation, as the dark acclimatized plants when exposed to light caused significant nitrate reduction within 15 min of light exposures even in the presence of Mg^{2+} and ATP.**

***Key words*:** spinach, *Spinacia oleracea*, nitrate reductase, light-dark transition.

Nitrate assimilation inside the plant cell involves the activities of two enzymes, nitrate reductase (NR, EC 1.6.6.1) and nitrite reductase (NiR, EC 1.7.7.1). The reduction of nitrate to nitrite by the enzyme nitrate reductase has been considered to be the rate-limiting and controlled step in overall nitrate assimilation process. Nitrate reductase is a very sensitive plant enzyme and it's activity is regulated by several plant and environmental factors (1). Post-translational covalent modification and regulation of NR during light to dark transitions has been reported by earlier researchers which involved phosphorylation of NR by ATP and subsequent complexation with Mg^{2+} ions (2,3). The potential toxicity of possible reaction products of NR such as nitrite, nitric oxide and superoxide anion necessitates the development of a complex and redundant control of NR at the transcriptional and post-transcriptional level (4). We are reporting here the changes in NR activity in spinach leaves during light/dark transition and suggest post-translational modification as one of the mechanisms of enzyme activity.

Seeds of spinach (*Spinacia oleracea cv Mulayam*) were grown in small earthen pots in natural daylight which were periodically irrigated with 15 mM KNO_3 soon after germination so that sufficient nitrate accumulated in their leaves. Leaves from 10-day-old seedlings were used for various *in vivo* and *in vitro* experiments. *In vivo* nitrate reductase activity was assayed as described by Sawhney *et al* (5). Spinach leaves were excised with a sharp razor blade under water with intact petioles and placed vertically in vials with petioles dipped in 5 ml solution of 10 mM $MgCl_2$ and were allowed to absorb the solution for 2 min by vacuum infiltration. Magnesium chloride solution was replaced with distilled water under control. These leaves were then used for *in vivo* NR assays. The extraction and assay of *in vitro* NR activity was performed as described by Salalkar *et al* (6) with slight modifications. All the experiments were conducted in triplicate and the standard error was calculated.

The activity of *in vivo* nitrate reductase declined gradually and the significant decline in activity was observed after 60 min of darkness (Table 1). Further, the decrease in activity to the extent of approximately 42 per cent was noticed after 90 min of darkness. A temporary increase in activity after 15 min of darkness could be attributed to elimination of PS-I dependent nitrite reduction (3). When the plants were again transferred from dark to light, a gradual increase in NR activity was observed. Reins and Heldt (3) have demonstrated that the presence of Mg^{2+} ions is required for rapid inactivation of nitrate reductase after phosphorylation. Therefore, we have examined the effect of Mg^{2+} ions on the activity of *in vivo* and *in vitro* NR. Nitrate reductase activity declined gradually upto 60 min of darkness even in the presence of Mg^{2+} (Table 2), though in the absence of Mg^{2+}, the decline in activity was less pronounced. It was observed that the *in vitro* NR activity

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Table 1. Effect of light/dark and dark/light transition on *in vivo* and *in vitro* NR *activity*

Time in darkness (min)	*In vivo* nitrate reductase activity (μmol NO_2^- g^{-1} fr wt h^{-1})	*In vitro* nitrate reductase activity (μmol NO_2^- g^{-1} fr wt h^{-1})
0	2.15 ± 0.010	5.56 ± 0.121
15	2.99 ± 0.005	4.47 ± 0.031
30	2.55 ± 0.003	3.39 ± 0.061
45	2.19 ± 0.003	2.79 ± 0.031
60	1.95 ± 0.005	2.01 ± 0.093
90	1.24 ± 0.015	
Time in light after 90/60* min of darkness (min)		
15	1.77 ± 0.035	3.25 ± 0.061
30	2.13 ± 0.019	4.30 ± 0.083
45	2.47 ± 0.005	5.56 ± 0.021
60	2.73 ± 0.015	7.24 ± 0.061

*60 min for *in vitro* NR assay

declined gradually upto 60 min of darkness (Table 1). This is due to phosphorylation of NR that takes place when plants were shifted from light to dark. The phosphorylated form of NR is the inactive state of molecule. The decline in activity was to the extent of approximately 64 per cent after 60 min of darkness. Results in Table 2 show that *in vitro* NR activity did not decline markedly even in the presence of Mg^{2+} upto 30 min of darkness. The *in vitro* NR activity declined gradually in presence of ATP upto 60 min of dark period, when the potted plants initially grown in light were shifted to darkness (Table 2). In this case, decline in activity to the extent of approximately 75 per cent was noticed. *In vitro* NR activity declined considerably after 15 min of darkness in presence of Mg^{2+} and ATP. However, in the absence of Mg^{2+} and ATP, there was a gradual decline in NR activity upto 60 min of darkness (Table 2).

Earlier report has clearly indicated that different species of plants can assimilate nitrate in dark at a slower rate than in the light (7). The decline in NR activity in the dark could be due to limitation of NADH, since the supply of redox equivalents either from the chloroplast or mitochondrion will stop upon darkening. Alternative proposals for the regulation of nitrate reductase in light and dark via NADH supply have also been made earlier (8). Hence, critical experiments would involve ^{15}N-nitrate assimilation in plants in light and dark situations. Such experiments previously conducted showed species differences in the rates of ^{15}N-nitrate and nitrite in light and dark conditions (7). The experiments of Yoneyama (9) on *in vivo* NR assays in leaves also reported significant assimilation of ^{15}N-nitrate and ^{15}N-nitrite in complete darkness. Singh *et al* (10) reported in potted spinach plants

Table 2. Effect of magnesium ion on *in vivo* and *in vitro* NR activity and effect of ATP alone and ATP + Mg^{2+} in whole intact leaves during light/dark transition

Time in darkness (min)	*In vivo* nitrate reductase activity		*In vitro* nitrate reductase activity		*In vitro* nitrate reductase activity		*In vitro* nitrate reductase activity	
	(μmol NO_2^- g^{-1} fr wt h^{-1})							
	+Mg (10 mM)	-Mg	+Mg (5 mM)	-Mg	+ ATP (1mM)	- ATP	+(Mg + ATP) 5 mM + 1mM	-(Mg + ATP)
0	1.69 ± 0.031	1.49 ± 0.024	3.14 ± 0.031	3.47 ± 0.024	3.88 ± 0.061	3.51 ± 0.032	4.48 ± 0.062	4.85 ± 0.025
15	1.42± 0.024	1.17 ± 0.010	2.72 ± 0.061	3.18 ± 0.044	3.09 ± 0.078	3.18 ± 0.021	3.92 ± 0.083	4.21 ± 0.044
30	1.17 ± 0.019	1.09 ± 0.020	2.32± 0.031	2.87 ± 0.044	2.20 ± 0.061	2.86 ± 0.061	1.78 ± 0.012	3.01 ± 0.093
45	0.97 ± 0.010	1.00 ± 0.010	1.00 ± 0.019	1.67 ± 0.073	1.51 ± 0.032	2.42 ± 0.061	1.04 ± 0.025	2.47 ± 0.032
60	0.79 ± 0.020	0.98 ± 0.010	0.82 ± 0.061	1.43 ± 0.012	0.99 ± 0.032	1.98 ± 0.032	0.54 ± 0.032	1.20 ± 0.061
Time in light after 60 min of darkness (min)								
15 min	1.13 ± 0.006	1.12 ± 0.010	1.49 ± 0.061	2.12 ± 0.093	1.22 ± 0.083	2.78 ± 0.021	1.61 ± 0.093	2.12 ± 0.044

that leaf NR activity declined by 26 and 55 per cent after 5 and 7 h of darkness, respectively, whereas no enzyme activity was detectable after 36 h of darkness. Remmler and Campbell (11) have shown 30 per cent decline in NR activity in corn leaves within 1 h of dark treatment. However, Riens and Heldt (3) have also shown a rapid decline in *in vitro* NR activity in spinach leaves under dark condition and reported a 50 per cent decline in NR activity within 2 min of light to dark transition, with the activity reducing to 15 per cent within 60 min of incubation. Nitrate is taken up by the plants even in darkness and is reduced to NO_2^- although at a much slower rate than under light. Thus, the decline in nitrate reductase activity in the dark appears to be slow and gradual. The increased sensitivity of NR inhibition to presence of Mg^{2+} and ATP does not rule out the post-translational modification as one of the mechanisms. However, the availability of reductant under dark situation needs to be ascertained.

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