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Toxicity of Selenium on Rate Limiting Step of Nitrogen Assimilation in Excised Greening *Phaseolus vulgaris* Leaf Segments

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***Abstract* – Supply of 0.001mM to 1.0mM sodium selenite in the presence of 10mM KNO_3 inhibited substantially in vivo Nitrate Reductase Activity (NRA), Endogenous nitrate pool and the Chlorophyll content. Further, invitro nitrate reductase and cytochrome C reductase activities were also inhibited by Se, however, Peroxidase activity remained unaltered. Time course study had shown that the inhibitory effect of Se was more pronounced at longer time periods. Further, supply of Selenium (Se) to nitrate induced leaf segments also caused a significant decline in enzyme activity. Induction of NRA by the supply of increasing concentration of nitrate was more pronounced at lower concentration in the presence of Se. Percent inhibition of NRA by Se was almost same in the presence of lincomycin, but was enhanced slightly by cycloheximide and sodium tungstate. Amongst the metabolites tested for their effect on in vivo and in vitro NRA and cytochrome C reductase activity by se, reduced glutathione (GSH) and sucrose were highly effective in protecting the inhibition of all activities. 5.5' Dithio- bis-2 nitro benzoic acid (DTNB) and Molybdenum (Mo) were completely effective against inhibition of cytochrome C reductase activity and cysteine against in vitro NRA. The experiment demonstrate that Se has an inhibitory effect on NRA in bean leaf segments, which is perhaps mediated by affecting nitrate uptake and thiol modulation of the enzyme.**

***Keywords* – Nitrate Reductase Activity, Phaseolus Vulgaris, Selenium.**

I. INTRODUCTION

Selenium (Se) is an essential element and has important benefits for animal and human nutrition but in trace amount, after that limit it causes toxicity in humans and plants (Gupta and Gupta 2017[1], Ellis and salt 2003[2]). Though many studies were carried out on Selenium metabolism in plants, most of the work was concentrated on the nutritional aspects. The effect of Se on processes playing a leading role in plant growth, such as, photosynthesis, respiration and nitrogen metabolism have not been studied in detail. However, decrease in chlorophyll content and inhibition of porphobilinogen synthase activity by Se have been reported in mung bean (Padmaja et al., 1989[3]). Further, strong inhibition of sulfate reduction and decreased levels of sulfhydryl compounds mainly glutathione (GSH) have also been reported (Hugouvieux et al 2009[4], Dekok et al., 1986[5], Dekok, 1989[6] and Bosma et al., 1991[7]). In vivo supply of Selenium in presence of ammonium chloride (NH_4Cl) inhibits total as well as specific NADH-GDH activity in excised bean leaf segments (Sharma Jot 2008[8]) but nitrate reduction, catalyzed by the enzyme nitrate reductase (NR EC 1.6.6.1), is a rate limiting step in overall process of nitrogen assimilation (Srivastava, 1980[9]).Hence we studied the effect of Se on nitrate reductase activity to gain some insight into possible mechanism of inhibition of NRA by Se, the interaction with some inhibitors and metabolites was also analyzed.

II. MATERIAL AND METHOD

2.1. Plant Growth and Treatment

Sterilized seeds of Phaseolus vulgaris cv. Rajmah were raised in small plastic pots containing acid washed sand in continuous light of about $30Wm^{-2}$ radiant flux density supplied by fluorescent tubes. The seedlings were watered daily with half strength minus nitrogen Hoagland nutrient solution (Arditti and Dunn, 1968[10]). For various experiments, segments (about 0.5x0.5 cm) of the excised leaves from 7d old uniformly grown seedlings were floated on the desired solution (pH 6.0) in continuous light of about 40 wm^{-2} intensity supplied by the fluorescent tubes inside the Newtronic growth chamber at 26^0C.

2.2. Assay

2.2.1. Nitrate Reductase Activity was measured either in leaf segment by an in vivo method (Srivastava 1975[11]) or after extraction by an in vitro method of Stevens and Oaks 1973[12]).

2.2.2. Cytochrome C Reductase was extracted in a manner similar to that of NR and total cytochrome C reductase activity was assayed spectrophotometrically by monitoring change in absorbance at 550nm according to Wallace and Johnson's 1978[13].

2.2.3. Peroxidase was extracted and assayed by the method of Maehly 1954[14].

2.2.4. Endogenous Nitrate Pool in the leaf segments was estimated indirectly by the modified method of Aslam 1981[15]. The method involve enzyme reduction of endogenous nitrate to nitrite by dark incubation of the sample in a medium lacking nitrate and secretion of the product into the medium. Nitrite content of the medium was estimated by diazotization.

2.2.5. Total Chlorophyll was estimated by measuring the absorbance of the clear extract in 80% acetone at 645 and 663 nm and using its extension coefficient by the method of Strain and Svec[16] 1966.

III. STATISTICAL ANALYSIS

Each experiment was repeated at least thrice and data presented are the average value and standard deviation of the finding are presented in the tables.

IV. RESULTS

4.1. Effect of Selenite on in Vivo NRA, Endogenous Nitrate Pool and Chlorophyll Content (Table 1A) and in Vitro NRA, Cytochrome C Reductase and Peroxidase Activity (Table 1B) in Excised Bean Leaf Segments

Supply of 0.001-1.00mM Na_2SeO_3 in the presence of 10mM KNO_3 inhibited in vivo NRA, endogenous nitrate pool and chlorophyll content in excised bean leaf segments (Table 1 A). Further, Se inhibited total as well as specific in vitro NRA and cytochrome C reductase activity, but not peroxidase activity (Table 1 B).

4.2. Time Course Effect of Se on NRA in Excised Leaf Segments

When excised leaf segments were incubated with 10mM KNO_3 for different time periods, in vivo NRA increased progressively with time in the absence as well as presence of 0.01mM Se (Figure 1). However, supply of 1mM se caused an abrupt decrease in enzyme activity initially, which persisted throughout the time intervals tested.

4.3. Effect of Supply of Selenium on NRA in Presence of Different Concentration of Nitrate

Supply of 0.01 to 100mM KNO_3increased the in vivo NRA progressively, both in the absence and presence of 0.1mM Se (Table 2). The % inhibition of enzyme activity by Se was less at lower concentration of nitrate, but at higher concentration of nitrate it was almost same as in the absence of nitrate.

4.4. Effect of Se on in Vivo Stability of NRA in the Presence of 10mM KNO_3

When excised leaf segments preincubated with 10mM $KNO_3$for 16hr in light were transferred to fresh ¼th strength Hoagland solution containing 10mM KNO_3, the in vivo NRA increased up to 4 hr at least (Figure 2). However, in the presence of 0.001 and 0.1mM Se, there was a gradual decrease in enzyme activity which was more prominent at 0.1mM KNO_3.

4.5. Effect of Inhibitors on NRA in the Absence and Presence of 0.05mM Na_2SeO_3

When excised leaf segments were incubated with sodium tungstate (10µg/ml), cycloheximide (5µg/ml), cycloheximide (5µg/ml), chloramphenicol (1mg/ml) and lincomycin (5µg/ml), substantial inhibition of NRA was observed in the absence as well presence of 0.05mM Se (Table 3). Percent inhibition of enzyme activity by Se was increased by all the inhibitors tested except lincomycin.

4.6. Effect of Some Compounds on in Vivo and in Vitro NRA and Cytochrome C Reductase Activity in Presence of Na_2SeO_3

When leaf segments were treated with various metabolites in the presence of Se, GSH (1mM) and Sucrose (5mM) caused a tremendous increase in in vivo NRA (Table 4). Further, cysteine (5mM) and DTNB (0.1mM) increased the enzyme activity substantially and Mo (0.1mM) only slightly. In Vitro NRA was increased substantially by all the metabolites. Moreover, substantial increase in cytochrome C reductase activity also resulted by various metabolites except cysteine.

V. DISCUSSION

The results demonstrate a concentration dependent inhibition of in vivo NRA by Se in bean leaf segments (Table 1A). Further, the inhibition of total as well as specific in vitro activities is more severe, suggesting that supply of reductants is not limiting enzyme activity in the presence of Se. Moreover, cytochrome C reductase activity, a partial activity of NR protein, is only slightly affected by Se. The inhibition does not appear to be dependent on the overall metabolic activities of the tissue, as peroxidase is not influenced by the supply of Se and reduction in chlorophyll content by Se supply is less pronounced. Further, the involvement of chloroplastic and mitochondrial protein synthetic activities seems to be only marginal, as lincomycin and chloramphenicol respectively, have either no effect or slight stimulatory effect on % inhibition of enzyme activity by Se (Table 3).

Se may decrease NRA by inhibiting the nitrate uptake and there by suppressing the nitrate induced synthesis of enzyme. Depletion of endogenous nitrate pool during supply of Se and nitrate may involve limited uptake of nitrate. Exogenously supplied selenium elicits a biphasic response on NRA at different concentration of nitrate. Thus % inhibition of enzyme activity is less at lower concentration of nitrate, but remains unaffected at higher concentrations (Table 2). Hence, it is likely that SeO_3 and NO_3 compete with one another for the binding site on the carrier protein responsible for the uptake of nitrate. When NO_3 concentration is low, SeO_3 may enhance nitrate uptake by activating the binding sites but at saturating concentrations of NO_3 positive regulatory effect of SeO_3 is eliminated. Inhibition of activity at earlier time periods (Figure 2) and increase in % inhibition by Se in the presence of cycloheximide (Table 3) suggest suppressed synthesis of NR protein during Se supply.

Selenium inhibition of enzyme may involve mechanisms such as reduced stability of enzyme, thiol modulation, phosphor modulation, impaired electron transport function etc. Thus, the inhibitory effect of Se at longer time periods (Figure 1), decline in activity of preinduced enzyme by Se (Figure 2) and protective effect of sucrose against Se effects (Table 4) indicates that se exerts an effect on stability of enzyme. Supply of sucrose has been reported to enhance the stability of NR (Puranik and Srivastava 1983[16]). As the supply of thio compounds, exert substantial protective effect against inactivation of enzyme by Se (Table 4), enzyme activity modulation involving its thiol group is likely. Further, supply of selenate has been shown to cause a substantial decrease in thiol content in spinach leaf discs (Dekok and Kupier 1986[5]) and spruce needles (Bosma et al 1991[7]).

More ever, NR has been reported to contain reactive –SH groups involved in the binding of NADH (Jones and Mhuimheneachain 1985[18]). Hence, NADH binding requiring participation of thiol groups of enzyme seems to be affected in presence of Se. NR from higher plants is a molybdoprotein (Campbell 1988[19]). In our experiment, exogenously supplied Mo exhibits complete reversal of cytochrome C reductase activity, indicating that electron transport function is affected in presence of Se. However, increase in % inhibition by tungstate, an inhibitor of NR, which inhibits the enzyme activity by competing with Mo for the active site in the molecule, (Notton and Hewitt 1971[20]) suggests that Mo insertion into NR is also influenced by Se. It is worth to discuss here the possible involvement of phosphorylation/d ephosphorylation mechanism of NR modulation also. Although the effect of ATP or any inhibitor of ATP generation was not tested in the present investigation, but deactivation of NR by ATP and activation by AMP has been reported in spinach leaf extracts (Kaiser and spill 1991[21], Kaiser et al 2002[22]). Thus, it is likely that selenite binding to enzyme may inactivate it in a manner analogous to inactivation by phosphorylation.

VI. CONCLUSION

Selenosis or Se toxicity occurs in plants when optimum concentration of Se exceeds. Selenium causes toxicity by two mechanisms, one of which is malformed selenoproteins and another by inducing oxidative stress. Both the mechanisms are known to be harmful for plants in one or other way.

Our experiments demonstrate that Selenium has an inhibitory effect on NRA in bean leaf segments, which is perhaps mediated by affecting nitrate uptake and thiol modulation of the enzyme.

Table 1 A. Effect of selenite on in vivo NRA, endogenous nitrate pool and chlorophyll content in excised bean leaf segments

Na_2SeO_3 Conc.**(mM)**	In Vivo NRA **µ mol NO_2 /h/g.fr.wt**	Endogenous NO_3 pool **µ mol NO_2 /g fr.wt.**	Total chlorophyll **mg/g fr.wt.**
0.00	4.75± 0.09 (100)	12.01± 0.33 (100)	2.11± 0.25 (100)
0.001	3.42 ± 0.03 (72)	10.04± 0.41 (83)	1.96± 0.13 (93)
0.01	2.91± 0.05 (61)	7.76± 0.13 (64)	1.78±0.19 (83)
0.05	2.15± 0.17 (45)	7.48± 0.45 (62)	1.58±0.19 (75)
0.1	2.06±0.66 (43)	4.42 ±0.49 (37)	1.47 ±0.19 (70)
1.00	0.04± 0.01 (0.8)	0.21±0.08 (2)	1.19± 0.21 (56)

Leaf segments from 7d old bean seedlings raised in continues light were floated on 1/4th strength Hoagland solution containing 10mM KNO_3 and desired concentration of Na_2SeO_3 in continuous light inside the Newtronic growth chamber for 24hr at 26^0C. Values relative to control are given in parentheses.

Table 1 B. Effect of sodium selenite on in vitro NRA, Cytochromr C reductase and peroxidase activity in excised bean leaf segments

Na_2SeO_3 Conc. **(mM)**	In vitro NRA **, µ mol NO_2/hr**		Cyto. C. reductase	Peroxidase **ΔA min g fr. wt.**
	g^{-1} fr.wt.	**mg^{-1} protein**	**Δ A /h/g fr. wt.**	
0.00	2.36 ± 0.09 (100)	0.28± 0.03 (100)	83 ± 2.5 (100)	151 ± 2 (100)
0.1	0.85 ± 0.04 (36)	0.04 ± 0.004 (14)	60 ± 1.8 (72)	161 ± 4 (107)

Leaf segments from 7d old bean seedlings raised in continues light were floated on 1/4th strength Hoagland solution containing 10mM KNO_3 and desired concentration of Na_2SeO_3 in continuous light inside the Newtronic growth chamber for 24hr at 26^0 C . Values relative to control are given in parentheses.

Table 2. Effect of supply of selenium on NRA in presence of different concentration of nitrate.

Kno3 Conc., **(mM)**	NRA, **µ mol NO_2 /h/g. fr.wt**		**% Inhibition**
	-Se	+Se (0.1mM)	
0.00	0.84 ± 0.03	0.37 ± 0.02	56
0.01	1.10 ± 0.01	0.76 ± 0.04	31
0.10	1.32 ± 0.02	1.01 ± 0.01	24
1.00	3.75 ± 0.02	1.67 ± 0.03	56
10.00	4.75 ± 0.09	2.06 ± 0.66	57
100.00	8.15 ± 0.29	3.40 ± 0.04	58

Leaf segments from 7d old bean seedlings raised in continues light were floated on 1/4th strength Hoagland solution containing 10mM KNO_3 and desired concentration of Na_2SeO_3 in continuous light inside the Newtronic growth chamber for 24hr at 26^0 C. Values relative to control are given in parentheses.

Table 3. Effect of inhibitors on NRA in the absence and presence of 0.05mM Na_2SeO_3

Treatment	**NRA, µ mol NO_2/h/g fr.wt.**		**% Inhibition**
	-Se	**+Se (0.5mM)**	
control	4.75 ± 0.09	2.15 ± 0.17	55
Lincomycin, 5µg/ml	2.36 ± 0.03	1.12 ± 0.02	53
Chloramphenicol, 1mg/ml	1.34 ± 0.02	0.52 ± 0.02	61
Cycloheximide, 5µg/ml	0.57 ± 0.02	0.21 ± 0.01	67
Na tugstate, 10µg/ml	1.11 ± 0.06	0.25 ± 0.02	78

Leaf segments from 7d old bean seedlings raised in continuous light were floated on 1/4th strength Hoagland solution containing 10mM KNO_3 and desired concentration of Na_2SeO_3 in continuous light inside the Newtronic growth chamber for 24hr at 26^0 C. Values relative to control are given in parentheses.

Table 4. Effect of some compounds on in vivo and in vitro NRA and cytochrome C reductase activity in presence of Na_2SeO_3(0.1mM Se)

Treatment	In vivo NRA **µ mol NO_2 /h/g fr.wt**	In vitro NRA **µ mol NO_2/h/g fr.wt**	Cytochrome C reductase **Δ A /h/g fr.wt.**
Control	4.75± 0.09 (100)	2.36 ±0.09(100)	83 ± 2.5 (100)
None (metabolite) 0.1mM Se	2.06 ± 0.66 (43)	0.85 ± 0, 04 (36)	60 ±1.8 (72)
Cysteine, 5mM	3.22 ± 0.10 (68)	3.03 ± 0.03 (128)	70 ± 2.4 (84)
GSH, 1mM	11.37 ± 0.13 (239)	7.22 ±0.54 (305)	115 ± 3.9 (138)
DTNB, 0.1mM	4.30 ± 0.10 (90)	2.03 ± 0.50 (86)	124 ± 5.6 (149)
Sucrose, 5mM	9.59 ± 0.07 (202)	4.34 ± 0.23 (184)	121 ± 2.4 (145)
Molybdic acid, 0.1mM	2.31 ± 0.13(49)	1.85 ± 0.09 (78)	127 ± 5.1 (153)

Leaf segments from 7d old bean seedlings raised in continuous light were floated on 1/4th strength Hoagland solution containing 10mM KNO_3 and desired concentration of Na_2SeO_3 in continuous light inside the Newtronic growth chamber for 24hr at 26^0 C. Values relative to control are given in parentheses.

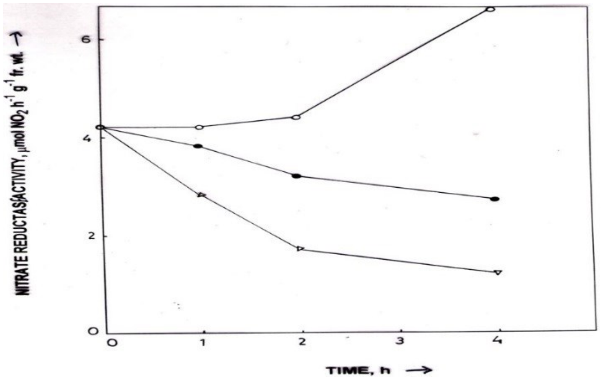

Fig. 1. Time course effect of Se on NRA in excised leaf segments

Leaf segments were floated in the medium containing 10mM KNO_3 either in the absence or the presence of 0.01 and 1.0mM Na_2SeO_3 for the desired time period in continuous light at 26^0 C.

Open circles control; closed circle 0.01mM Se; open triangle 1.0mM Se.

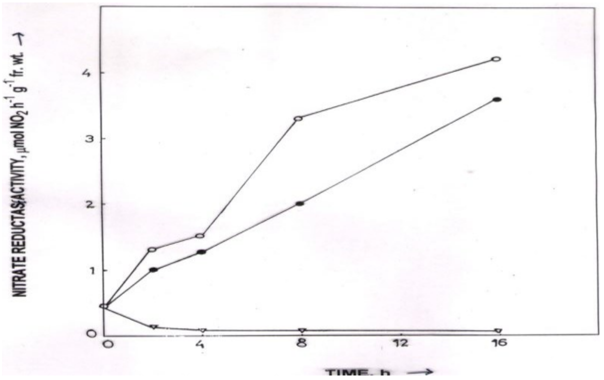

Fig. 2. Effect of Se on in vivo Stability of NRA in the presence of 10mM KNO_3

Leaf segments were treated with 10mM KNO_3 for 16hr in continuous light (0 hr control) and then transferred to the fresh medium with or without Se for the required time period of light.

Open circles 10mM KNO_3; closed circle 10mM KNO_3 +0.001mM Se; open triangle 10mM KNO_3 + 0.1mM Se.

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