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Differential Effect of Aluminium on Enzymes of Nitrogen Assimilation in Excised Bean Leaf Segments

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Abstract

Aluminium is a potent toxicant in acidic soils. The present study was taken up to analyze the effects of Al on enzymes of nitrogen assimilation in excised bean (*Phaseolus vulgaris*) leaf segments so as to gain an insight of the mechanism involved. Supply of 0.001 to 0.1 mM $AlCl_3$ to excised bean leaf segments affected the *in vivo* nitrate reductase activity differently in the presence of various inorganic nitrogenous compounds, being inhibited with 5 mM ammonium nitrate and 10 mM ammonium chloride but enhanced with 10 mM potassium nitrate. Al effect with 50 mM KNO_3 varied with time, showing an increased activity at shorter duration, but decreased at longer duration. Al effect on *in vivo* NRA was dependent upon the nitrate concentration, thus, inhibiting it at 0, 1 and 50 mM KNO_3, while increasing at 2 and 10 mM. Further, saturating and non-saturating effects were observed in the absence and presence of Al. Al supply influenced the *in vitro* NRA also, being increased at 10 mM, but decreased at 50 mM KNO_3. Supply of Al to excised leaf segments substantially inhibited the glutamate dehydrogenase activity in the absence as well as presence of 5 mM NH_4NO_3 but increased the glutamate synthase activity. Inhibition of specific glutamate dehydrogenase activity by Al supply was also observed. However, specific glutamate synthase activity was increased in the presence of NH_4NO_3 only. The experiments demonstrated that effect of supply of aluminium on *in vivo* nitrate reductase activity depended upon nitrogenous source as well as nitrate concentration and it exerted reciprocal regulation of glutamate dehydrogenase and glutamate synthase activities, which depended upon N supply too.

Keywords

Aluminium Effects, Glutamate Dehydrogenase, Glutamate Synthase, Nitrate Reductase, Bean Leaves, *Phaseolus vulgaris*

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1. Introduction

Aluminium (Al), one of the most abundant metals, is not regarded as an essential nutrient for plants, but low concentrations can sometimes increase plant growth or induce other desirable effects [1]. When applied along with ammonia, it has been reported to promote growth in tropical plants adapted to acid soils [2]. Beneficial effects of Al on plant growth have also been reported in *Camellia sinensis* [3], *Miconia albican* Steud [4] and *Pinus radiata* D. Don [5]. Aluminium is one of the most toxic metals for plant growth in acidic soil. Under acidic conditions, it exists as soluble and toxic monomeric Al^{3+} species [6]. Several phytotoxic effects of aluminium have been reported including the inhibition of root growth and nutrient uptake; however, the mechanism is not well understood [7]-[9]. Aluminium causes significant decline in the leaf area, fresh weight and dry weight [10]. It affects mitochondrial dysfunction, which leads to reactive oxygen species production, probably the key critical event in aluminium-induced inhibition of cell growth [11].

Nitrate reductase (NR, EC 1.6.6.1) is a substrate inducible key enzyme of nitrate assimilation. It is regulated by a number of nutritional and environmental factors [12]. Nitrate reductase activity is often correlated with the overall nitrogenous status of the system. Glutamate dehydrogenase (GDH, EC 1.4.1.3) forms a link between carbon and nitrogen metabolism. Further, the enzyme seems to be important in ammonia assimilation under stressful conditions [13]. Glutamate synthase (GOGAT, EC 1.4.1.14) plays a key role in maintaining appropriate levels of glutamate. Results of Al effects on nitrogen assimilation have been found to be inconsistent. Thus, in Sorghum, Al rapidly reduces NO_3^- uptake and enhances NH_4^+ uptake so that total N uptake is almost unaffected [14], while it inhibits nitrate and ammonium uptake in maize [15]. In maize roots, Al induces anaplerotic GDH, while inhibiting glutamine synthetase (GS, EC 6.3.1.2). However, in leaves it does not influence GOGAT and GS activities [15]. Al effects on nitrate reductase activity (NRA) vary from inhibition to stimulation in different systems under different conditions [14] [16] [17]. In the present study, the effect of Al on enzymes of N assimilation in excised bean leaf segments is analyzed with an insight to gain information about the mechanism of Al effect on enzymes of nitrogen assimilation.

2. Materials and Methods

2.1. Plant Material and Treatments

Seeds of *Phaseolus vulgaris* cv. Rajmah purchased from a local dealer were surface sterilized with 0.1% $HgCl_2$ for 1 - 2 minutes followed by thorough washing with distilled water. The seedlings were raised in plastic pots containing acid washed sand for 7 - 8 days in continuous light of intensity 30 Wm^{-2} supplied by fluorescent tubes at 28˚C ± 3˚C. They were watered with 1/2 strength Hoagland's solution (pH 6.0) containing no nitrogen. For various treatments primary leaves from uniformly grown seedlings were cut into about 0.5 × 0.5 cm segments and floated on 1/4 strength Hoagland's solution containing desired compounds, as mentioned in the tables, for required time period in continuous light supplied by fluorescent tubes.

2.2. Enzymatic Analyses

In vivo NRA was assayed by colorimetric estimation of nitrite according to the method of Srivastava [18]. *In vitro* NRA was extracted and assayed by the method of Stevens and Oaks [19]. Cytochrome c reductase activity in extract of NR was assayed spectrophotometrically by monitoring the change in absorbance at 550 nm according to procedure of Wallace and Johanson [20]. Glutamate dehydrogenase preparation was obtained according to the procedures described in Puranik and Srivastava [21] and the activity was assayed by monitoring the decrease in absorbance at 340 nm according to the method of Singh and Srivastava [22]. Glutamate synthase preparation was obtained and assayed for activity based upon the measurement of decrease in absorbance at 340 nm following the method described in Puranik and Srivastava [23]. The unit of enzyme activities of GDH and GOGAT is defined as nmoles of reduced nicitinamide adenine dinucleotide (NADH) oxidized per min. To calculate specific activity, the protein content of the preparations was estimated by Lowry's method [24] after precipitation with trichloro acetic acid.

Results expressed are the average values of at least four independent experiments with ± SE. Difference between means obtained for various treatments was tested by Student's *t* test at level of significance—a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$.

3. Results

3.1. Al Effects on NRA

Supply of 0.001 to 0.1 mM $AlCl_3$ to excised bean leaf segments in the presence of 10 mM KNO_3 gradually increased *in vivo* NRA (Table 1). While in presence of 5 mM NH_4NO_3 and 10 mM NH_4Cl the enzyme activity was gradually decreased by Al supply (Table 1).

Supply of 0.1 mM Al in the presence of 50 mM KNO_3 for short interval up to 4 h maintained a higher level of *in vivo* NRA over control ranging from 15% to 36% (Figure 1).

When leaf segments were treated with Al in presence of varying concentrations of KNO_3, the *in vivo* NRA was inhibited in the absence of nitrate and at 1 and 50 mM KNO_3 (Table 2). However, at 2 and 10 mM KNO_3 the enzyme activity was increased by Al (Table 2). Further, to analyse uptake kinetics, a plot of KNO_3 concentration vs *in vivo* NRA was constructed. It yielded non-saturating effect in the absence of Al, but saturating effect in the presence of 0.1 mM Al (Figure 2).

Treatment of leaf segments with 0.1 mM Al in the presence of 10 mM KNO_3 caused an increase in total as well as specific *in vitro* activity of NR (Table 3). However, in the presence of 50 mM KNO_3, the activity was decreased by Al. Aluminium supply caused a marginal decrease in cytochrome c reductase activity at 50 mM KNO_3 only (Table 3).

3.2. Al Effects on GDH and GOGAT

Supply of 0.1 mM $AlCl_3$ to leaf segments inhibited the NADH-GDH activity significantly (Table 4). However,

Table 1. Effect of supply of Al on inducibility of *in vivo* nitrate reductase activity by different nitrogenous compounds in excised bean leaf segments.

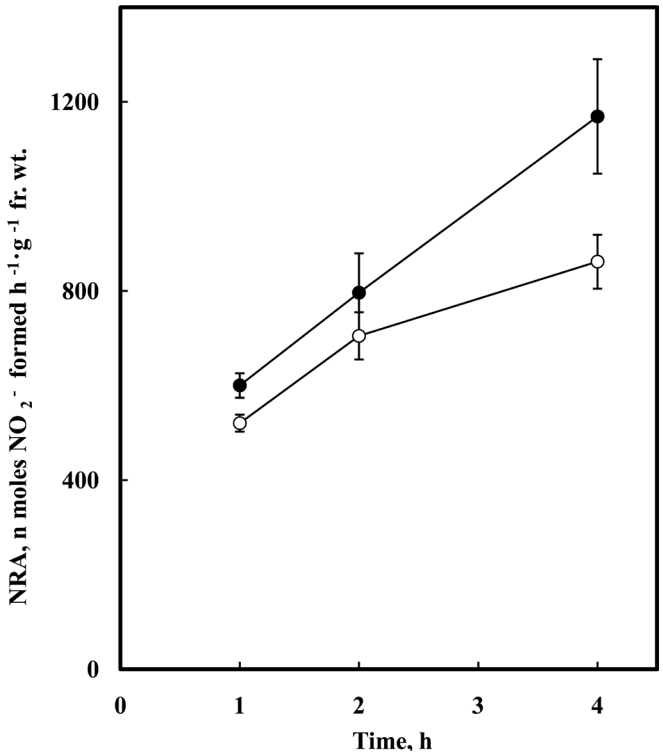
Treatment	NRA, nmoles NO_2 $h^{-1}\cdot g^{-1}$ fr. wt.		
$AlCl_3$ conc., mM	KNO_3, 10 mM	NH_4NO_3, 5 mM	NH_4Cl, 10 mM
0.000	1610 ± 78 (100)	1283 ± 110 (100)	597 ± 79 (100)
0.001	1660 ± 197 (103)	1020 ± 135 (80)	520 ± 94 (87)
0.010	1836 ± 195 (114)	1038 ± 122 (81)	481 ± 44 (81)
0.100	1827 ± 148 (114)	1040 ± 152 (81)	461 ± 60 (77)

Leaf segments were floated on 1/4 strength Hoagland's solution containing the desired nitrogenous compounds in the presence of varying concentrations of $AlCl_3$ for 24 h at continuous light intensity of 30 Wm^{-2} and temperature 26˚C ± 2˚C.
Values relative to control are given in parentheses.

Table 2. Effect of supply of Al on *in vivo* NRA at varying concentrations of KNO_3 in excised bean leaf segments.

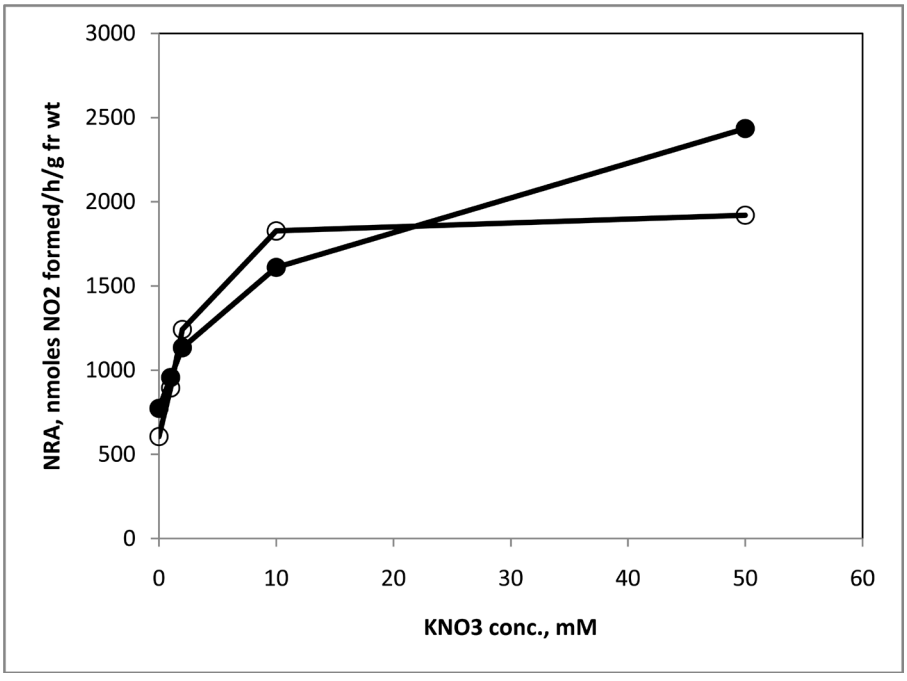
Treatment	*In vivo* NRA, nmoles NO_2 $h^{-1}\cdot g^{-1}$ fr. wt.		
KNO_3 conc., mM	–Al	+Al	% Increase/Decrease
00	774 ±20 (100)	606 ± 31[c] (100)	22% Decrease
01	957 ± 99 (124)	894 ± 75 (147)	7% Decrease
02	1134 ± 82 (146)	1242 ± 112 (205)	9% Increase
10	1610 ± 78 (208)	1827 ± 148 (301)	14% Increase
50	2434 ± 110 (314)	1920 ± 74[c] (317)	21% Decrease

Leaf segments were floated on 1/4 strength Hoagland's solution containing the desired concentrations of KNO_3 in the absence and presence of 0.1 mM $AlCl_3$ for 24 h at continuous light intensity of 30 Wm^{-2} and temperature 26˚C ± 2˚C.
Values relative to control are given in parentheses.
Level of significance—c: $p < 0.001$.



Leaf segments were floated on 1/4 strength Hoagland's solution containing 50 mM KNO_3 in the absence and presence of 0.1 mM $AlCl_3$ for different time intervals at continuous light intensity of 30 Wm^{-2} and temperature 26˚C ± 2˚C.
Symbols used: Open circles (○----○) –A; Closed circles (●----●) +Al, 0.1 mM.

Figure 1. Effect of supply of Al on *in vivo* NRA at 50 mM KNO_3 in excised bean leaf segments at different time intervals.



Leaf segments were floated on 1/4 strength Hoagland's solution containing the desired concentrations of KNO_3 in the absence and presence of 0.1 mM $AlCl_3$ for 24 h at continuous light intensity of 30 Wm^{-2} and temperature 26˚C ± 2˚C.
Symbols used: Closed circles (●----●); –Al; Open circles (○----○); +Al, 0.1 mM.

Figure 2. Effect of supply of Al on *in vivo* NRA at varying concentrations of KNO_3 in excised bean leaf segments.

Table 3. Effect of supply of Al on *in vitro* nitrate reductase and cytochrome c reductase activities in excised bean leaf segments.

Treatment	*In vitro* NRA		Cyt c reductase
	nmoles NO_2 h^{-1}·g^{-1} fr. wt.	nmoles NO_2 h^{-1}·mg^{-1} protein	ΔA_{550} min^{-1}·g^{-1} fr. wt.
KNO_3, 10 mM	664 ± 149 (100)	20 ± 4 (100)	0.882 ± 0.067 (100)
KNO_3, 10 mM +$AlCl_3$, 0.1 mM	874 ± 289 (132)	25 ± 8 (125)	0.844 ± 0.061 (96)
KNO_3, 50 mM	1239 ± 220 (100)	38 ± 6 (100)	0.900 ± 0.035 (100)
KNO_3, 50 mM + $AlCl_3$, 0.1 mM	995 ± 442 (80)	27 ± 9 (71)	0.797 ± 0.061 (89)

Leaf segments were floated on 1/4 strength Hoagland's solution containing 10 and 50 mM KNO_3 in the absence and presence of 0.1 mM $AlCl_3$ for 24 h at continuous light intensity of 30 Wm^{-2} and temperature 26˚C ± 2˚C.
Values relative to control are given in parentheses.

Table 4. Effect of supply of Al on NADH-GDH and NADH-GOGAT activity in excised bean leaf segments.

	NADH-GDH activity		NADH-GOGAT activity	
Treatment	Units ml^{-1} Enzyme	Units mg^{-1} Protein	Units ml^{-1} Enzyme	Units mg^{-1} Protein
Control (-N)	63.1 ± 6.3 (100)	34.2 ± 3.9 (100)	8.4 ± 2.4 (100)	4.4 ± 1.3 (100)
$AlCl_3$, 0.1 mM	31.6 ± 1.9[b] (50)	19.7 ± 2.9[a] (58)	14.6 ± 5.2 (174)	4.2 ± 1.6 (96)

Leaf segments were floated on 1/4 strength Hoagland's solution in either, the absence (–N Control) or presence of 0.1 mM $AlCl_3$ for 18 h at continuous light intensity of 40 Wm^{-2} and temperature 26˚C ± 2˚C inside "Newtronics" growth chamber.
Values relative to control are given in parentheses.
Level of significance—a: $p < 0.05$, b: $p < 0.01$.

Table 5. Effect of supply of Al on NADH-GDH and NADH-GOGAT activity in excised bean leaf segments in presence of 5 mM NH_4NO_3.

Treatment	NADH-GDH activity		NADH-GOGAT activity	
	Units ml^{-1} Enzyme	Units mg^{-1} Protein	Units ml^{-1} Enzyme	Units mg^{-1} Protein
Control (+N)	96.3 ± 11.0 (100)	65.8 ± 7.4 (100)	15.3 ± 3.2 (100)	8.3 ± 1.9 (100)
$AlCl_3$, 0.1 mM	19.7 ± 2.2[c] (20)	26.3 ± 2.9[c] (40)	19.8 ± 6.0 (129)	11.2 ± 3.1 (135)

Leaf segments were floated on 1/4 strength Hoagland's solution in either, the absence (+N Control) or presence of 0.1 mM $AlCl_3$ for 18 h at continuous light intensity of 40 Wm^{-2} and temperature 26˚C ± 2˚C inside "Newtronics" growth chamber.
Values relative to control are given in parentheses.
Level of significance—c: $p < 0.001$.

Al supply increased the NADH-GOGAT activity substantially (**Table 4**). The specific activity of NADH-GDH was also decreased due to inclusion of Al, but that of NADH-GOGAT remained unaltered.

When leaf segments were treated with 0.1 mM $AlCl_3$ containing 5 mM NH_4NO_3, severe inhibition of NADH-GDH activity was observed (**Table 5**). However, NADH-GOGAT activity, in the presence of NH_4NO_3, was increased due to Al supply (**Table 5**). During Al supply, the specific activity of NADH-GDH was decreased while that of NADH-GOGAT increased.

4. Discussion

4.1. Al Effects on NRA

The results demonstrate a differential effect of Al supply on *in vivo* nitrate reductase activity in bean leaf segments depending upon the nitrogenous compound included and nitrate concentration as well. The enzyme activity is increased by Al in the presence of KNO_3, but decreased with NH_4NO_3 as well as NH_4Cl (**Table 1**). Thus, it seems that Al decreases NH_4^+ availability, while increases NO_3^- availability for induction of NRA. However, decreased uptake of NO_3^- and NH_4^+ both by Al in maize roots has been reported [15]. Thus, it is likely that

NR inducibility in the presence of Al depends upon NO_3^- uptake. In cucumber roots and soybean seedlings, aluminium at varying concentration has been reported to affect nitrate uptake-being increased at lower concentration, but decreasing at higher concentration [25] [26] and at very high concentration causing nitrate efflux [25]. The effect of Al on nitrate uptake depends on duration of exposure too. Thus, supply of Al for longer durations has been reported to reduce it [27] [28], while short-term supply induced it [29]. In the present study, the NRA was inhibited by Al in the presence of 1 and 50 mM KNO_3, but it was increased by Al with 2 and 10 mM KNO_3 (**Table 2**). Moreover, Al supplied in the presence of 50 mM nitrate upto 4 h increased the NRA (**Figure 1**) but the activity was decreased at 24 h (**Table 2**).

Plants have multiple nitrate carriers with distinct kinetic properties and regulation. Thus, there are at least three distinct NO_3^- uptake systems, two of which have a high affinity for NO_3^-, while the third has a low affinity. Also, the high-affinity transport system displays Michaelis-Menten kinetics saturating at 0.2 - 0.5 mM nitrate. However, the low-affinity transport system operates at concentrations above 0.5 mM, and usually displays non-saturating uptake kinetics. In the present study, the dependence of Al effect on nitrate concentration suggest that high affinity active transport system of NO_3^- uptake appears to be inhibited by Al, as inhibitory effect of Al on NRA is observed up to 1 mM KNO_3 (**Table 2**) and exhibit saturating effect (**Figure 2**). Al also influences passive diffusion through ion channels negatively, as it inhibited NRA at super-saturating concentration, 50 mM KNO_3 (**Table 2**). On the other hand, low affinity active transport system appears to be activated by Al, as Al increases NRA at 2 - 10 mM KNO_3 (**Table 2**). Further, direct effect of Al on NRA is also likely, as *in vitro* total and specific activities both are altered by Al supply. However, cytochrome c reductase activity of the preparation remains unaltered (**Table 3**) indicating that the terminal nitrate reductase is likely to be affected rather than NADH-dehydrogenase activity.

4.2. Al Effects on GDH and GOGAT

Reciprocal regulation of NADH-GDH and NADH-GOGAT during supply of Al with and without NH_4NO_3 in excised bean leaf segments was demonstrated. Thus, Al stress severely inhibits NADH-GDH activity but activates NADH-GOGAT activity (**Table 1**) and seems to favour GS/GOGAT pathway for ammonia assimilation. Although, two enzymes of ammonia assimilation have been reported to be reciprocally influenced by Cd and glutathione also, but increased NADH-GDH activity by Cd indicated its possible role in ammonia assimilation during metallic stress [30]. In the present study, inhibition of NADH-GDH activity by Al does not appear to result due to overall decrease in metabolic activities, as specific activity of enzyme is also decreased by Al supply (**Table 4**). However, elevated deaminating GDH activity by Al in maize roots was shown to be indicative of metabolic changes associated with plant senescence [15].

In the present investigation, the inhibitory effect of Al on NADH-GDH activity is dependent on the supply of nitrogen in the incubation medium. Thus, stronger inhibition of enzyme activity results in the presence of NH_4NO_3, as N-supply increases the activity in the absence of Al only (**Table 4** and **Table 5**). However, specific activity was increased by N-supply in both, the absence and presence of Al (**Table 4** and **Table 5**). Under Al stress, reduced activity of glutamate dehydrogenase has also been reported in soybean root nodules [31]. Decreased uptake of NO_3^- and NH_4^+ by Al in maize roots has been reported [15]. Hence, decrease in NADH-GDH activity due to Al supply seems to result because of reduced uptake of inorganic nitrogen in particular NH_4^+. Further, Al treatment in wheat has been reported to inhibit Ca^{++} uptake resulting in reduced Ca^{++} influx [32]. So, reduced activity of enzyme due to Ca^{++} depletion is likely, as it is stimulatory for GDH [33]. Inhibition of GDH activity and no change in GS and GOGAT activities by Al treatment has been observed in maize leaves [15]. However, inhibition of GS and NADH-GOGAT activities by Al supply has been reported in soybean root nodules [31]. In the present study, The NADH-GOGAT activity is increased by N-supply in the absence as well as presence of Al, being more prominent for the former (**Table 4** and **Table 5**). Decreased asparagine/glutamine ratio by Al treatment [34] and increased *in vitro* GS activity by Al III complex [35] have been reported. Hence, Al treatment may enhance glutamine level thus increasing NADH-GOGAT activity. Role of GS/GOGAT pathway in ammonia assimilation during Al supply is suggested.

4.3. Conclusion

Effect of aluminium supply on *in vivo* NRA depends upon nitrogenous source as well as nitrate concentration and it exerts reciprocal regulation of NADH-GDH and NADH-GOGAT activities, which depends upon N supply too.

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