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Aluminium stress affects nitrogen fixation and assimilation in soybean (*Glycine max* L.)

Karina B. Balestrasse, Susana M. Gallego and María L. Tomaro*
*Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, (1113) Buenos Aires, Argentina; *Author for correspondence (e-mail: ptomaro@ffyb.uba.ar; phone: +54-11-4964-8237; fax: +54-11-4508-3645)*

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Abstract

Nitrogen fixation and assimilation in nodules and roots were studied in soybean (*Glycine max* L.) exposed to different levels of aluminium (Al) stress (0, 50, 200 and 500 μM). Al at 500 μM induced oxidative stress, which became evident from an increase in lipid peroxidation accompanied by a concomitant decline in antioxidant enzyme activities and leghaemoglobin breakdown. Consequently, there was also a reduction in nitrogenase activity. However, the leghaemoglobin levels and nitrogenase activity were unexpectedly found to be higher in nodules when the plants were treated with 200 μM Al. Of the enzymes involved in nitrogen assimilation, the activity of glutamate dehydrogenase-NADH was reduced in nodules under Al stress, but it was significantly higher in roots at 500 μM Al as compared to that in the control. In nodules, the glutamine synthetase/glutamate synthase-NADH pathway, assayed in terms of activity and expression of both the enzymes, was inhibited at >50 μM Al; but in roots this inhibitory effect was apparent only at 500 μM Al. No significant changes in ammonium and protein contents were recorded in the nodules or roots when the plants were treated with 50 μM Al. However, Al at ≥200 μM significantly increased the ammonium levels and decreased the protein content in the nodules. But these contrasting effects on ammonium and protein contents due to Al stress were observed in the roots only at 500 μM Al. The results suggest that the effect of Al stress on nitrogen assimilation is more conspicuous in nodules than that in the roots of soybean plants.

Abbreviations: APOX – ascorbate peroxidase; BHT – butylated hydroxytoluene; CAT – catalase; EDTA – ethylenediaminetetraacetic acid; GDH – glutamate dehydrogenase-NADH; Gln – glutamine; Glu – glutamate; GOGAT – glutamate synthase-NADH; GS – glutamine synthetase; Lb – leghaemoglobin; NBT – nitroblue tetrazolium; PVP – polyvinylpyrrolidone; SOD – superoxide dismutase; TBA – thiobarbituric acid; TBARS – thiobarbituric acid reactive substances; TCA – trichloroacetic acid

Introduction

Aluminium (Al) is the third most abundant element in the earth's crust after oxygen and silicon. It is found in soils predominantly as insoluble alumino-silicates or oxides. In acid soils, Al is mobilised into soil solution, primarily in the form of Al^{+3}, impairing the growth of most of the plant species. Its harmful effect is initially characterised by a reduction in root growth followed by several other secondary responses (Matsumoto 2000).

The roots of leguminous plants have the ability to develop an endosymbiotic association with nitrogen-fixing bacteria of the genus *Rhizobium*. Rhizobia are taken up into the nodule cells by endocytosis, and there they differentiate into their endosymbiotic form called bacteroid (Whitehead and Day 1997). The legume nodule is the organ of nitrogen assimilation; N_2 is reduced to NH_4^+ in bacteroids by the enzyme nitrogenase (EC 1.18.6.1). It is then exported from bacteroids into the surrounding nodule cells, assimilated into organic compounds and ultimately translocated to other plant parts (Udvardi and Day 1997). However, NH_4^+ is toxic to cells and needs to be readily assimilated. This is achieved by the concerted action of two highly regulated pathways (Loulakakis et al. 1994): i) glutamate dehydrogenase-NADH (GDH; EC 1.4.1.2.) pathway, which synthesises glutamate (Glu) from 2-oxoglutarate and NH_4^+ and ii) glutamate-ammonia ligase [glutamine synthetase (GS); EC 6.3.1.2/glutamate synthase-NADH (GOGAT); EC 1.4.1.14] pathway (Lam et al. 1996). GS catalyses the ATP-dependent amination of Glu producing glutamine (Gln), while GOGAT catalyses the reductive transfer of the amido group of Gln to the α-keto position of 2-oxoglutarate yielding two molecules of Glu (Lancien et al. 2000). GOGAT together with GS maintains the flow of NH_4^+ into Gln and Glu, which are then used in several other aminotransferase reactions for the synthesis of amino acids.

Soybean (*Glycine max* L.), an important crop for both human consumption and industry, is widely cultivated on acid soils (Von Uexküll and Mutert 1995), where Al toxicity is one of the main factors limiting crop productivity (Taylor 1995; Matsumoto 2000). Due to its increased solubility at low pH, plants grown in acid soils have reduced root system and exhibit a variety of nutrient-deficiency symptoms with a consequent decline in yield. Since acid soils comprise a large area of the world's agricultural lands, particularly in the tropics, soil acidity constitutes an important factor limiting food production in developing countries.

Cytotoxicity due to Al has been well documented in plants (Delhaize and Ryan 1995; Horst et al. 1999; Kollmeier et al. 2000; Marienfeld et al. 2000). Several mechanisms have been proposed to explain Al toxicity and its tolerance (Kochian 1995; Taylor 1995; Matsumoto 2000; Mossor-Pietraszewska 2001). However, the physiological basis of both the processes is poorly understood and, therefore, still remains controversial. Al has been shown to adversely affect the nodulation process through inhibition of lateral root extension (Silva et al. 2001) and nodule initiation (Flis et al. 1993) during symbiotic association between *Rhizobium*/*Bradyrhizobium* and legumes. Moreover, it has been demonstrated to induce oxidative stress (Yamamoto et al. 2003; Guo et al. 2004). Nitrogen fixing organisms as well as the whole plant have developed a complex system of antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APOX; EC 1.11.1.11). SOD accelerates the conversion of O_2^- to H_2O_2, while CAT, APOX and a variety of other peroxidases catalyse the breakdown of H_2O_2.

We had previously demonstrated that oxidative stress could affect nitrogen fixation and assimilation (Balestrasse et al. 2001, 2003). In view of these considerations, it is of interest to examine whether Al-induced oxidative stress affects nitrogen fixation and assimilation. In the present study, the effects of Al on oxidative stress and nitrogen fixation/assimilation were determined in the nodules and roots of soybean plants growing in the presence of different levels of Al stress.

Materials and methods

Plant material and growing conditions

Seeds of soybean (*Glycine max* L. cv. AG 445-RG Nidera) were surface-disinfested with 5% v/v sodium hypochlorite for 10 min followed by several washing in distilled water. They were inoculated with 10^8 cells ml^{-1} of *Bradyrhizobium japonicum* (109, INTA Castelar) and planted in vermiculite. The plants were germinated and grown in a controlled climate room at 24 ± 2 °C and 50% relative humidity under a 16-h photoperiod (approximately 175 $\mu mol\ m^{-2}\ s^{-1}$ PPFD). Five days after germination, the plants were removed from the pots, the roots were gently washed and transferred to separate 5 l containers for the establishment of hydroponics. The hydroponic medium consisted of nitrogen-free nutrient solution diluted to quarter-strength standard Hoagland's solution (Hoagland and Arnon 1950). The medium was continuously aerated, replaced

every three days and maintained at a pH of 4.5 ± 0.2 throughout the experiments. After 4 weeks, the plants were treated with 0, 50, 200 and 500 μM of $AlCl_3$. All solutions contained 0.8 mM $CaSO_4$, and the free activity of the Al^{3+} species in solution was predicted with a GEOCHEM-PC (Parker et al. 1995). Root elongation and nodule fresh weight were determined before and after Al treatment. After 48 h of treatment, the roots and nodules were isolated, and used for analytical and enzymatic determinations. A single experimental hydroponic container with 10 plants in each represented one replication.

Determination of lipid peroxidation

Lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS) determined by the thiobarbituric acid (TBA) reaction (Heath and Packer 1968). Fresh control and treated nodules (0.3 g)/roots (0.6 g) were homogenised in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at $3500\times g$ for 20 min. To 1 ml aliquot of the supernatant, 1 ml of 20% (w/v) TCA containing 0.5% (w/v) TBA and 100 μl 4% (w/v) BHT in ethanol were added. The mixture was heated at 95 °C for 30 min, and then quickly cooled on ice. The contents were centrifuged at $10{,}000\times g$ for 15 min, and the absorbance was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Leghaemoglobin estimation

Nodules (0.3 g) were homogenised in 3 ml of extraction medium containing 0.02% (w/v) potassium ferricyanide and 0.1% sodium bicarbonate. Leghaemoglobin was estimated in the red supernatant (nodule cytosol) obtained after centrifugation of homogenates using the fluorometric method as described by La Rue and Child (1979). Bovine haemoglobin was used as the calibration standard.

Nitrogen fixation assay

Nitrogen fixation was measured in terms of acetylene reduction activity (Hardy et al. 1968). Nodules were placed in 100 ml bottles sealed with rubber stoppers containing 10% (v/v) C_2H_2. Gas samples (0.5 ml) were withdrawn 60 min later and analysed for C_2H_4 in a Konik 3000 HRGC chromatograph (Konik Inc., Tokyo) equipped with a hydrogen flame ionisation detector (Hewlett Packard fused silica capillary HP-Plot Al_2O_3 column; oven temperature: 120 °C; carrier gas: N_2 at a rate of 30 ml min^{-1}).

Ammonium determination

Plant material (0.3 g of nodule or 0.6 g of root) were homogenised in 3 ml of 0.3 mM H_2SO_4 and centrifuged at $15000\times g$ for 15 min. Ammonium content was measured in the supernatant by the phenol-hypochlorite method (Weatherburn 1967). A calibration curve with NH_4Cl was used as the standard.

Antioxidant enzyme assays

Extracts for the determination of CAT, SOD and APOX activities were prepared from 0.3 g of nodules or roots homogenised in 3 ml of ice-cold extraction buffer containing 50 mM phosphate buffer (pH 7.4), 1.0 mM EDTA, 1.0 g PVP and 0.5% (v/v) Triton X-100 at 4 °C. The homogenates were centrifuged at $10{,}000\times g$ for 20 min, and the supernatant was used for the assays. CAT activity was determined in the homogenates by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2.0 mM H_2O_2. The pseudo-first order reaction constant ($k' = k$[CAT]) of the decrease in H_2O_2 absorption was determined, and the catalase content in pmol mg^{-1} FW was calculated using $k = 4.7\times10^7$ M^{-1} s^{-1} (Chance et al. 1979). Total SOD activity was estimated by the inhibition of the photochemical reduction of NBT as described by Becana et al. (1986). The reaction mixture consisted of 50–150 μl of enzyme extract and 3.5 ml O_2^- generating solution containing 14.3 mM methionine, 82.5 μM NBT and 2.2 μM riboflavin. Extracts were made up to a final volume of 0.3 ml with 50 mM K-phosphate (pH 7.8) and 0.1 mM Na_2EDTA. The reduction in NBT was followed by reading absorbance at 560 nm. One unit of

SOD was defined as the amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions. APOX activity was measured immediately in fresh extracts, and was assayed as described by Nakano and Asada (1981) using 1.0 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate and 0.1 mM EDTA. The H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε: 2.8 mM^{-1} cm^{-1}). One unit of APOX forms 1 μmol of ascorbate oxidised per min under the assay conditions.

Determinations of nitrogen assimilation

Extracts for the determination of GDH and GOGAT activities were prepared from 0.3 g of nodules or 0.6 g of roots homogenised in 3.0 ml of extraction buffer containing 100 mM MES-NaOH buffer (pH 6.8), 100 M sucrose, 2% (v/v) 2-mercaptoethanol and 15% (v/v) ethylene glycol at 4 °C. Homogenates were centrifuged at 10,000×g for 20 min, and the supernatant was used for the assays. GDH and GOGAT were determined in the homogenates by measuring the decrease in absorption at 340 nm due to NADH oxidation in a reaction medium containing 100 mM potassium phosphate buffer (pH 7.6), 0.1% (v/v) 2-mercaptoethanol, 100 μM NADH, 2.5 mM 2-oxoglutarate and 200 mM $(NH_4)_2SO_4$ or 100 mM glutamine for GDH and GOGAT, respectively (Groat and Vance 1981). One unit of GDH and GOGAT oxidises 1 μmol of NADH per min under the assay conditions. Plant material from GS determination was extracted in 50 mM Tris–HCl buffer (pH 7.5) containing 1.0 mM 2-mercaptoethanol and 2 mM EDTA, and homogenised at 4 °C. Following centrifugation (15,000× g for 30 min), GS activity was measured in an assay buffer consisting of 50 mM Tris–HCl (pH 7.5), 4.0 mM ATP, 80 mM Na-glutamate, 30 mM $MgSO_4$, 10 mM NH_2OH and 30 mM cysteine (Kanamori and Matsumoto 1972). A standard curve was prepared using γ-glutamyl hydroxamate. One unit of GS forms 1 μmol of γ-glutamyl hydroxamate per min under the assay conditions.

Native PAGE and glutamate dehydrogenase activity staining

Gel electrophoresis was performed using a Mini-PROTEAN 3 System (Bio-Rad Laboratories, California). Gels were 0.75 mm thick, and consisted of a separating gel of 7.5% (w/v) acrylamide (prepared at a final buffer concentration of 0.375 M Tris–HCl, pH 8.8) and a stacking gel (1 cm in length) of 4% (w/v) acrylamide (prepared at final buffer concentration of 0.125 M Tris–HCl, pH 6.8) according to the modified method of Laemmli (1970). Gels were run at 200 V for 1.5 h at 4 °C using a running buffer of 0.25 M Tris and 0.96 M glycine (pH 8.5), stained for GDH isozymes (Ju et al. 1997), photographed in a Fotodyne equipment and analysed with Gel-Pro® analyser version 3.1.

Western-blots for GOGAT and GS proteins

Proteins from nodules and roots were subjected to denaturing SDS-PAGE in a Mini-PROTEAN 3 System (Bio-Rad Laboratories, California). SDS-PAGE was performed in 7.5% and 12% gels (4% stacking gels) for GOGAT and GS, respectively and run according to Laemmli (1970). The separated polypeptides were transferred to a nitrocellulose membrane at 25 V/300 mA for 2 h in a Mini-Trans-Blot Electrophoretic System (Bio-Rad Laboratories, California) according to the manufacturer's instructions. The membranes were washed in 25 mM Tris–HCl (pH 8.5), 192 mM glycine and 20% (v/v) methanol. The blots were blocked by incubation for 2 h in 2% (w/v) powdered non-fat dry milk dissolved in TTBS (20 mM Tris–HCl at pH 7.6, 137 mM NaCl and 0.1% (v/v) Tween 20) before reaction with an appropriate antibody. The membrane was incubated with rabbit antibodies against barley leaf GOGAT for GOGAT (dilution 1:1000) or purified GS from *Phaseolus vulgaris* root nodules (Cullimore and Miflin 1984) for GS (dilution 1:1000). The antibodies for GOGAT and GS were diluted in TTBS with 2% (w/v) powdered non-fat dry milk. The blots were washed twice for 10 min each with TTBS. Goat anti-rabbit horseradish peroxidase conjugate was used as a secondary antibody and

incubated for 1 h. The blots were washed twice for 10 min before colour development (ECL inmunodetection system, ECL Western Blotting protocols, Dako). The intensity of bands was analysed with Gel-Pro® analyser version 3.1. The protein concentration was determined according to Bradford (1976) using bovine serum albumin as the calibration standard.

Statistical analyses

The experiment was conducted in a completely randomised design (CRD) with five replications. The one-way ANOVAs were calculated using the standard procedure, and the means were separated by Tukey's test. The term significant has been used to indicate differences for which $p \leq 0.05$.

Results

Effect of Al stress on root and nodule traits

When the plants were exposed to Al stress, the roots became short, curled and highly branched with blighted tips. The effect of Al stress on root length and nodule fresh weight is shown in Table 1. Al up to 200 μM did not have any adverse effect on root and nodule traits. There was only a significant decline in both root length and nodule fresh weight when the plants were treated with 500 μM Al.

Oxidative stress generation

TBARS formation in plants exposed to adverse environmental conditions is a reliable indicator of free radical generation in the tissues. In the present study, the TBARS content remained unaltered in nodules and roots when the plants were exposed up to 200 μM Al (Figure 1). However, Al at 500 μM increased its content by about 30% in both the tissues. Oxidative stress may be defined as an increment of oxidant species and/or a depletion of antioxidant defences. Therefore, the activities of three antioxidant enzymes were also measured in the present study. The results showed that there was a decline in SOD, CAT and APOX activities in soybean nodules by about 50 and 75% at 200 and 500 μM Al, respectively as compared to the controls (Table 2). However, in roots, a decline in SOD (57%), CAT (56%) and APOX (64%) activities occurred only at the highest concentration of Al (500 μM).

Table 1. Effect of aluminium on root elongation and nodule fresh weight of *Glycine max*.

Aluminium (μM)	Root length (cm)[a]	Nodule fresh weight (g plant^{-1})[a]
0	20.5 ± 0.2 a	0.21 ± 0.01 a
50	20.2 ± 0.1 a	0.20 ± 0.02 a
200	22.1 ± 0.2 a	0.23 ± 0.01 a
500	12.4 ± 0.1 b	0.15 ± 0.01 b

[a] Means with common letters within a column are not significantly different at $p \leq 0.05$, according to Tukey's test.

Ammonium and protein contents

In both the nodules and roots, Al at 50 μM did not have any effect on ammonium content as compared to the controls (Figure 1). In the presence of 200 μM Al, there was no significant difference in root ammonium content, but it was significantly higher (25%) in the nodules. Ammonium content in both the nodules and roots increased by 45% as compared to the controls when the plants were treated with 500 μM Al. However, an opposite trend was observed in protein contents of both the nodules and roots at 200–500 μM Al (Figure 1). The protein content in nodules declined by about 25 and 40% at 200 and 500 μM Al, respectively; however, Al could reduce the protein content in the roots only at 500 μM. No significant change in protein content occurred in nodules or roots, when the plants were exposed to 50 μM Al.

Leghaemoglobin content and nitrogen fixation

Leghaemoglobin content and nitrogenase activity were measured as indicators of nodule effectiveness. Both the parameters exhibited similar trend at all the concentrations of Al. No significant changes were observed either in leghaemoglobin content or in nitrogenase activity at 50 μM Al as compared to the controls (Figure 2). However, with 200 and 500 μM Al, a 20% increment and 30% decrement, respectively occurred in leghaemoglobin concentrations. Similarly, nitrogenase activity also

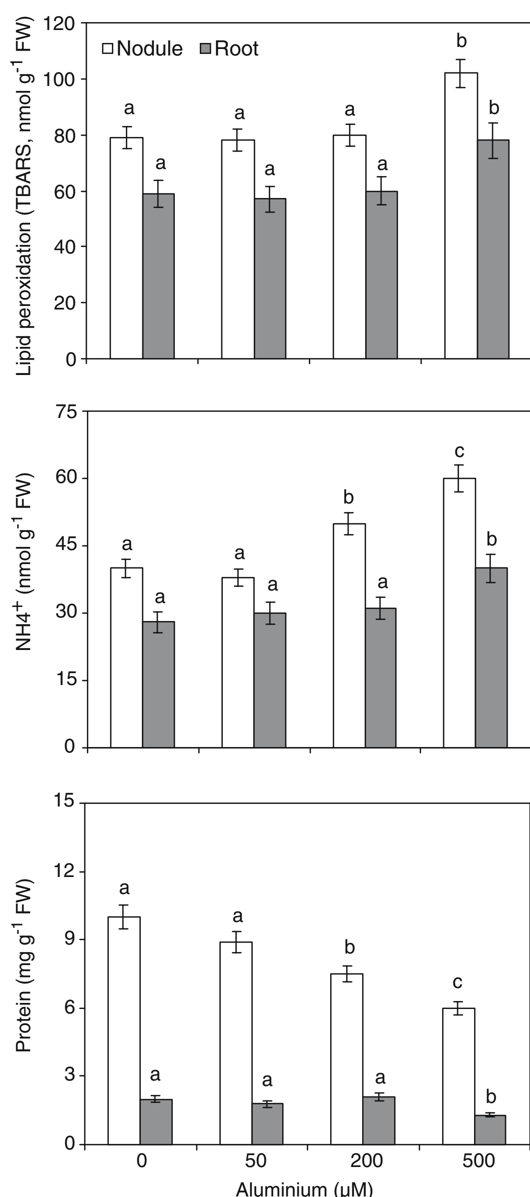

Figure 1. Effect of aluminium stress on lipid peroxidation (*top*), ammonium content (*middle*) and protein content (*bottom*) in soybean nodules and roots. Means with common letters are not significantly different at $p \leq 0.05$, according to Tukey's test.

recorded about 35% increment and 34% decrement at 200 and 500 μM Al, respectively.

Nitrogen assimilation

The control activities of different enzymes involved in ammonium assimilation were higher in nodules than that in the roots (Table 3). In nodules, about 24, 36 and 47% decline in GDH activity was recorded at 50, 200 and 500 μM Al, respectively as compared to the control. Al at 50 μM increased the GOGAT activity by about 35%, but there was a decline in its activity with 200 μM (25%) and 500 μM (35%) Al. A major increment (120%) in GDH activity and a corresponding decline in GOGAT activity (45%) were observed in roots when the plants were exposed to 500 μM Al. However, at 50 and 200 μM Al, both the enzymes showed the activities close to those of the controls (Table 3). Similar trend was also recorded for GS activity at 500 μM Al with 36 and 67% decline in nodules and roots, respectively. In both the tissues, no significant difference in GS activity occurred at 50 and 200 μM Al as compared to the control.

The activity staining on native gels for GDH showed the presence of its single isoform in soybean nodules and roots (Figure 3). In nodules, there was a decline in GDH activity with increasing Al stress, while in roots an increase in its activity occurred only at higher Al concentrations (200–500 μM). These results are in agreement with those presented in Table 3. Western-blots for GOGAT and GS showed similar results to those obtained when their activities were measured, indicating that enzyme activity and protein abundance were closely correlated (Table 3; Figures 4 and 5).

Discussion

Oxidative stress induces the degradation of a variety of biologically important molecules such as lipids, amino acids, proteins and carbohydrates resulting in the release of malondialdehyde (Alaiz et al. 1999). Therefore, the increase in TBARS content is a precise indicator of general oxidative damage. The present study showed that Al at higher concentrations induced oxidative stress in nodule and root tissues of soybean plants as evident from a significant but moderate increase in TBARS content. Superoxide dismutase, catalase and ascorbate peroxidase are essential components of antioxidative defence system, and, therefore, their decrease is also symptomatic of oxidative damage. In soybean nodules and roots, there was a significant decline in the activities of these antioxidant enzymes at higher Al concentrations, demonstrating that Al-induced oxidative stress was mediated by an increase in TBARS content and

Table 2. Effect of aluminium on antioxidant enzyme activities[a] in nodules and roots of *Glycine max*.

Aluminium (μM)	Nodule: Superoxide dismutase (U g^{-1} FW)	Nodule: Catalase (pmol mg^{-1} FW)	Nodule: Ascorbate preoxidase (U g^{-1} FW)	Root: Superoxide dismutase (U g^{-1} FW)	Root: Catalase (pmol mg^{-1} FW)	Root: Ascorbate preoxidase (U g^{-1} FW)
0	250.1 ± 21.0 a	24.1 ± 2.1 a	49.2 ± 4.3 a	115.3 ± 11.2 a	14.1 ± 1.2 a	5.9 ± 0.3 a
50	241.2 ± 20.1 a	23.2 ± 2.3 a	50.1 ± 4.6 a	110.1 ± 11.0 a	13.9 ± 1.1 a	5.2 ± 0.4 a
200	120.9 ± 20.3 b	12.3 ± 2.2 b	24.6 ± 4.2 b	120.2 ± 12.1 a	14.4 ± 1.3 a	5.6 ± 0.5 a
500	62.5 ± 6.2 c	6.5 ± 0.5 c	12.3 ± 1.2 c	50.2 ± 5.4 b	6.2 ± 0.7 b	2.1 ± 0.1 b

[a] Means with common letters within a column are not significantly different at $p \leq 0.05$, according to Tukey's test.

depletion of enzymatic antioxidant system. The present results are in agreement with those reported earlier (Yamamoto et al. 2003; Guo et al. 2004), and may perhaps be responsible for the inhibition of root elongation observed under 500 μM Al. The reduction of leghaemoglobin content in nodules at a very high Al concentration as observed in the present study may also be due to Al-induced oxidative stress. Richards et al. (1998) have also reported similar results in *Arabidopsis thaliana*.

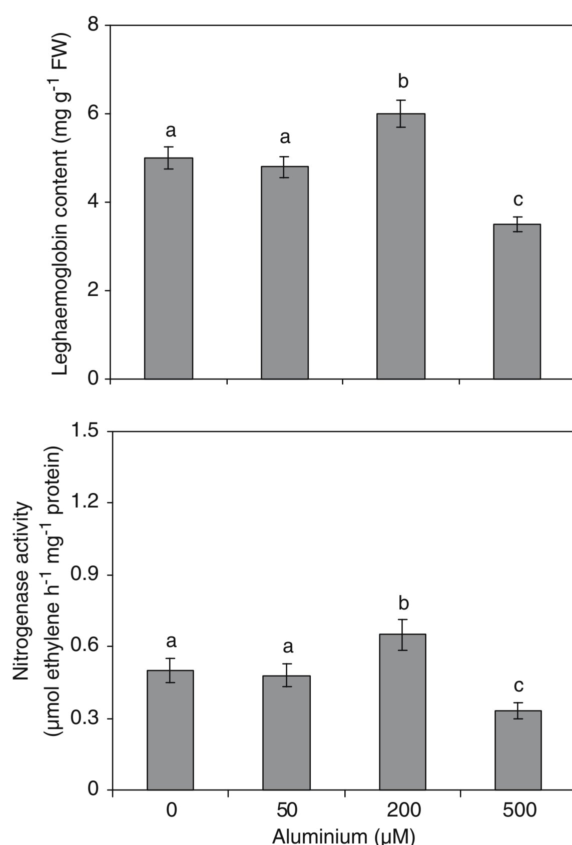

Figure 2. Effect of aluminium stress on leghaemoglobin content (*top*) and nitrogenase activity (*bottom*). Means with common letters are not significantly different at $p \leq 0.05$, according to Tukey's test.

It is well known that the ascorbate-glutathione pathway is critical for optimum functioning of the nodules. Superoxide dismutase and catalase protect nitrogen fixation, and are present in both the symbiotic partners (Matamoros et al. 2003). Many reports have confirmed a positive correlation between leghaemoglobin content and nitrogenase activity (Comba et al. 1998; Balestrasse et al. 2001). In the present study, we have also observed a correlated effect of Al stress on both leghaemoglobin content and nitrogenase activity. The inhibition of acetylene reduction under severe Al stress could result from the effect of metal ions on bacteroid O_2 uptake, because bacteroid respiration provides the energy and reducing power that nitrogenase employs for efficient nitrogen fixation. This event is similar to that occurs under cadmium stress (Balestrasse et al. 2001). Igual et al. (1997) have also reported similar effects of Al on nodulation and nitrogen fixation in *Casuarina cunninghamiana*. Unexpectedly, an increase in leghaemoglobin levels and nitrogen fixation was observed under 200 μM Al^{3+}, possibly because Al at this concentration could not affect lipid peroxidation, but could reduce at least two antioxidant enzyme activities such as SOD and CAT. Despite a decline in nitrogenase activity in nodules treated with 500 μM Al, ammonium content was found to be increased. Ammonium is released by plant tissues during senescence (Loulokakis et al. 1994). Al induces oxidative stress in soybean nodules and roots, and it is well known that oxidative stress is responsible for the induction of senescence (Sandalio et al. 2001). Therefore, increased

Table 3. Effect of aluminium on enzyme activities[a] involved in ammonium assimilation in nodules and roots of *Glycine max*.

	Nodule			Root		
Aluminium (μM)	Glutamate dehydrogenase (U g^{-1} FW)	Glutamate synthase (U g^{-1} FW)	Glutamine synthetase (U g^{-1} FW)	Glutamate dehydrogenase (U g^{-1} FW)	Glutamate synthase (U g^{-1} FW)	Glutamine synthetase (U g^{-1} FW)
0	0.45 ± 0.01 a	0.20 ± 0.04 a	3.27 ± 0.18 a	0.14 ± 0.01 a	0.09 ± 0.01 a	0.45 ± 0.04 a
50	0.34 ± 0.03 b	0.27 ± 0.02 b	3.25 ± 0.25 a	0.13 ± 0.02 a	0.08 ± 0.01 a	0.41 ± 0.03 a
200	0.29 ± 0.02 b	0.15 ± 0.01 c	3.22 ± 0.17 a	0.15 ± 0.01 a	0.08 ± 0.01 a	0.43 ± 0.02 a
500	0.24 ± 0.01 c	0.13 ± 0.02 c	2.11 ± 0.20 b	0.31 ± 0.01 b	0.05 ± 01 b	0.15 ± 0.01 b

[a] Means with common letters within a column are not significantly different at $p \leq 0.05$, according to Tukey's test.

ammonium levels in nodules at 200–500 μM Al and in roots at 500 μM Al, as observed in this study, may be due to the generation of oxidative stress. In contrast, Al caused a reduction in total protein content in nodules at 200–500 μM and in roots at 500 μM, and this detrimental effect on total protein content could be the consequence of an increase in protein degradation or a decline in protein synthesis.

Al ions are known to have a toxic effect on both plant and animal cells. In mammalian systems, Al can specifically accumulate in neuronal cells leading to a variety of cognitive disorders. A possible explanation for its toxicity is that Al modifies glutamate metabolism (Struys-Ponsar et al. 2000). The present study showed that in soybean the enzymes responsible for ammonium assimilation were more affected in nodules than that in the roots. The enzyme GDH catalyses a reversible enzymatic reaction involving the assimilation of ammonium into glutamate and the deamination of glutamate into 2-oxoglutarate and ammonium (Lancien et al. 2000). We found that GDH activity was reduced in nodules at all the levels of Al stress. This unexpected decline in GDH may be because of its inactivation by Al, which induces a conformational change (induced by Al binding) similar to that observed in human GDH (Yang et al. 2003) or a correlated inhibition resulting from a concomitant inhibition of nodule GS (Glevarec et al. 2004). However, there was a significant increment in GDH activity in roots at the highest level of Al stress, although only one isoform could be detected. GDH activity is induced when the rates of amino acid catabolism are high.

In the present study, Al at 500 μM had an adverse effect on GS/GOGAT activity and expression in nodules and roots of soybean plants. Purcino et al. (2003) have reported that Al affects the enzymes involved in nitrogen assimilation in maize genotypes with different degrees of Al-tolerance. In plants, ammonium is primarily

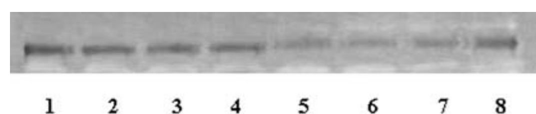

Figure 3. Effect of aluminium stress on glutamate dehydrogenase-NADH (GDH) activity. Proteins (40 μg protein per well) were separated by native-PAGE and stained for GDH activity. Lanes 1, 2, 3 and 4: GDH activity in nodules at 0, 50, 200 and 500 μM Al, respectively. Lanes 5, 6, 7 and 8: GDH activity in roots at 0, 50, 200 and 500 μM Al, respectively.

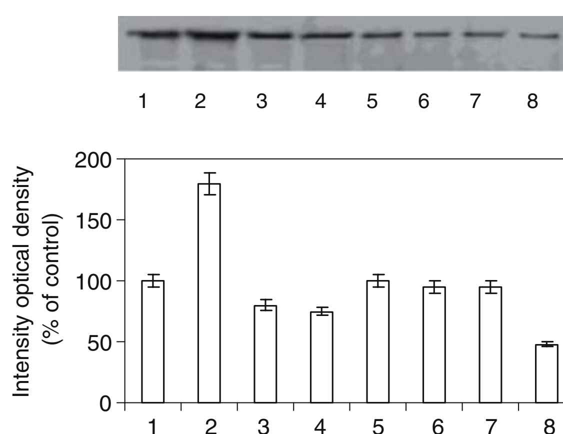

Figure 4. Effect of aluminium stress on glutamate synthase-NADH (GOGAT) expression in soybean nodules and roots. Proteins (40 μg protein per well) were separated by native-PAGE and GOGAT expression was detected by Western blotting. *Top*, lanes 1, 2, 3 and 4: GOGAT expression in nodules at 0, 50, 200 and 500 μM Al, respectively; lanes 5, 6, 7 and 8: GOGAT expression in roots at 0, 50, 200 and 500 μM Al, respectively. *Bottom*, quantification of protein levels by Gel-Pro® analyser.

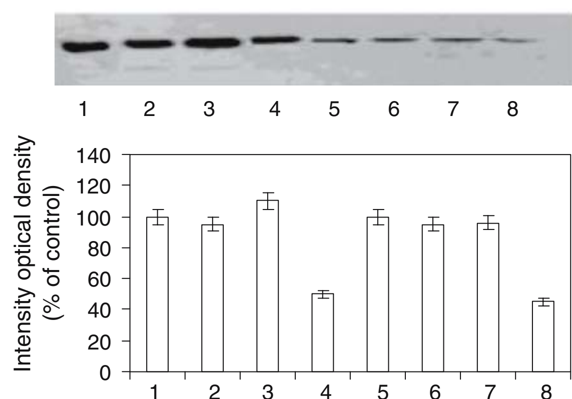

Figure 5. Effect of aluminium stress on glutamine synthetase (GS) expression in soybean nodules and roots. Proteins (40 μg protein per well) were separated by native-PAGE and GS expression was detected by Western blotting. *Top*, lanes 1, 2, 3 and 4: GS expression in nodules at 0, 50, 200 and 500 μM Al, respectively; lanes 5, 6, 7 and 8: GS expression in roots at 0, 50, 200 and 500 μM Al, respectively. *Bottom*, quantification of protein levels by Gel-Pro® analyser.

assimilated by GS/GOGAT pathway. Since Al generates oxidative stress in nodules and roots, oxidative modification of GS has been implicated as the first step in its turnover. Ortega et al. (1999) have demonstrated oxidative inactivation of GS in soybean roots, and oxidised GS has been shown to be inactive and more susceptible to degradation that non-oxidised form. Chien et al. (2002) reported that oxidative damage in cadmium-treated rice leaves was associated with alterations in ammonium content and GS activity. The inhibition of the pathways responsible for ammonium assimilation accompanied by an increase in nitrogenase activity may be assumed to be responsible for the reported increase in NH_4^+ content in soybean nodules, as recorded in the present study. This enhancement of nodule NH_4^+ content was, however, associated with a significant decline in protein content, indicating that Al at higher concentrations affects the protein balance resulting in a corresponding decline in GOGAT/GS levels.

A proposed mechanism for Al tolerance is the production and excretion of organic acid by the root. In soybean roots, Al-tolerance has been shown to result in the release of citrate into the external solution with its high accumulation in the root tip over time (Silva et al. 2001; Abdullahi et al. 2004). Abscisic acid, protein kinases and up-regulation of plasma membrane H^+-ATPase activity are associated with the secretion of citrate from soybean roots (Shen et al. 2004, 2005). Furthermore, it has been demonstrated that Al-tolerance is associated with high antioxidant levels (Rama Devi et al. 2003; Darkó et al. 2004). Here, we propose that Al toxicity could be associated with the generation of oxidative stress through the production of increased reactive oxygen species and/or depletion of antioxidant enzymatic defence system. Al-induced oxidative stress produced an alteration in nitrogen metabolism and caused root growth inhibition. The present study shows that Al stress affects nitrogen assimilation to a greater extent in soybean nodules than that in the roots. Similarly, Al at higher concentrations reduces the rate of nitrogen fixation in soybean.

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