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DOES ALUMINIUM PHYTOTOXICITY INDUCE OXIDATIVE STRESS IN GREENGRAM (*VIGNA RADIATA*)?

S.K. Panda*, L. B. Singha and M.H. Khan

Plant Biochemistry Laboratory, School of Life Sciences, Assam (Central) University, Silchar – 788 011, Assam, India.

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Summary. To verify whether aluminium phytotoxicity causes oxidative stress in developing greengram seedlings, the present investigation was carried out. An uniform decrease in root and shoot elongations was marked as the primary signs of aluminium injury. A significant increase in lipid peroxidation measured in terms of TBARS content was noticed which was also correlated with an increase in membrane injury index. The increase in peroxide content was accompanied by a decrease in catalase (CAT, EC 1.11.1.6) activity. However, superoxide dismutase (SOD, EC 1.15.1.9), peroxidase (POX, EC 1.11.1.6) and glutathione reductase (GR, EC 1.6.4.2) activities increased with increasing aluminium concentrations. Both glutathione and ascorbate contents showed a decrease at a higher metal concentration. These results suggested an induction of oxidative stress in developing greengram seedlings under aluminium phytotoxicity.

Key words: aluminium, greengram, oxidative stress, phytotoxicity

Abbreviations: CAT – catalase, EC – electric conductivity, EDTA – ethylenediaminetetraacetic acid, GPx – guaiacol peroxidase, GR – glutathione reductase, MDA – malondialdehyde, ROS – reactive oxygen species, TBARS – thiobarbituric acid reactive substances, TCA – trichloracetic acid, SOD – superoxide dismutase.

Introduction

Aluminium is the most abundant metal and the third most common element in the earth's crust. Aluminium toxicity is the primary factor limiting crop productivity in

* Corresponding author, e-mail: drskp_au@yahoo.com

acidic soils, which comprise large areas of the world's land, particularly in the tropics and subtropics (Foy et al., 1978, Foy et al., 1984). Thus it is an important factor limitting food production in many developing countries. As soil becomes more acidic, phytotoxic forms of Al are released into soil to levels that affect root system, plant growth and seed yield. Direct evidence has been demonstrated that the root apex is the primary site of Al-induced root growth inhibition. Aluminium can interact with a number of extracellular and intracellular substances like interaction within the root cell walls, disruption of plasma membrane and plasma membrane transport system, interaction with symplastic constituents such as calmodulin etc. (Kochian, 1995). In the external environment, plants are under various abiotic and biotic stress. An important response to stress by aerobic cells is the production of reactive oxygen species (ROS), like superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), alkoxy radical ($^{\cdot}RO$), singlet oxygen ($^1O_2$), and toxic hydrogen peroxide (H_2O_2) molecules (Salin, 1988, Luna et al., 1994, Asada, 1999, Breusegem et al., 2001). These ROS produced in the cell are detoxified by both non enzymic and enzymic antioxidant system. ROS if not detoxified cause serious damage to proteins, lipids and nucleic acids (Elstner, 1984), Alscher et al., 1997). Metals including Al are known to induce lipid peroxidation and oxidative damages in various plant systems and act as catalysts in ROS production (Aust, 1989, Cakmak and Horst, 1991, Luna et al., 1994, Gallego et al., 1996, Weckx and Clijsters, 1997, Subrahmanyam, 1998, Dietz et al., 1999, Piexoto et al., 1999, Panda and Patra, 2000, Shah et al., 2001).

As greengram is a stress sensitive legume, the present investigation was undertaken to analyse whether aluminium (Al) produces oxidative stress during early stages of seedling development.

Materials and Methods

Uniform seeds of greengram (*Vigna radiata* L. cv. Wilckzeck var. K_{851}) were surface sterilised with 0.1% mercuric chloride and germinated at 25 ± 2 °C in petri dishes in the darkness containing Whatman No. 1 filter paper moistened with Hoagland nutrient solution (Hoagland and Arnon, 1950). After 48 h of germination, seeds were transferred to plastic glasses containing Hoagland nutrient solution and kept in a growth chamber under continous white light with a photon flux density of photosynthetic active radiation (PAR) of 52 $\mu mol.m^{-2}.s^{-1}$. Aluminium (in the form of aluminium chloride, $AlCl_3$) was given to the Hoagland's nutrient solution at increasing concentrations (0, 0.001, 0.01, 0.1, 1.0 mM). Root length and shoot length were measured using a standard centimeter scale at an interval of 48 h and the rates of elongation were calculated.

Extraction and estimation of metabolites were done in the primary leaves of 9-day-old seedlings growing in Hoagland's nutrient solution at different metal concentrations. Primary leaves were homogenised in 5% (w/v) trichloroacetic acid (TCA) and

centrifuged at 17 000 rpm at 4 °C for 10 min. The supernatant was used for the estimation of total peroxide content following ferri-thiocyanate method by Sagisaka (1976). Reaction mixture contained 1.6 ml leaf extract, 0.4 ml of 50% TCA, 0.4 ml ferrous ammonium sulphate and 0.2 ml potassium thiocyanate. The absorbancy of the ferrithiocyanate complex was measured at 480 nm and compared to the hydrogen peroxide (H_2O_2) standard.

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid reactive substance (TBARS) as described by Heath and Packer (1968). After homogenising the primary leaves with 5% (w/v) trichloroacetic acid (TCA), the homogenate was directly used for MDA estimation. 1 ml of 5% TCA and 4ml of TBA reagent (0.5% in 20% TCA) was mixed and used as a blank. For correction blank, 1 ml of homogenate and 4 ml of 20% TCA and for sample 1ml of homogenate and 4 ml TBA reagent were mixed. After heating for 30 min at 95 °C in a water bath the mixture was cooled and centrifuged for 10 min at 4000×g. The absorbancy was measured at 532 nm and corrected for non-specific absorbancy at 600 nm and for the absorbancy at 532 nm of the correction blank. The concentration of MDA was calculated by using an extinction coefficient at 155 $mM^{-1}.cm^{-1}$.

Ascorbate was estimated by the method of Oser (1979) and glutathione was assayed by the modified Griffith (1930) method. Primary leaves were homogenised in 5% (w/v) sulphosalicylic acid and the homogenate was centrifuged at 10000×g for 10 min. The supernatant was neutralised with 0.5 ml of potassium phosphate buffer (pH 7.5). Total glutathione was measured by adding 1 ml of neutralised supernatant to a standard solution mixture consisting of 0.5 ml of sodium phosphate buffer (pH 7.5) containing EDTA, 0.2 ml of 6 mM 5,5´-dithiobis(2-nitrobenzoic acid), 0.1 ml of 2 mM NADPH and 0.1 ml of $1 - U^{-1}$ yeast GR type III (Sigma Chemical, USA). The change in absorbance at 412 nm was followed at 25 ± 2 °C until the absorbance reached 0.5 U.

Sampled primary leaves were dipped inside 15cm^3 of deionized water and incubated for 24h at 25 ± 2 °C. The electrical conductivity (EC) of the bathing medium was measured at room temperature by a conductivity meter. The leaf tissue and leachate were autoclaved at 1 $kg.cm^{-2}$ pressure and EC was measured. The injury index was calculated using the formula of Sullivan (1972).

For the extraction and assay of enzymes, primary leaves were homogenised with 0.1 M potassium phosphate buffer (pH 6.8) in a pre-cooled mortar and pestle. The extract was centrifuged at 4 °C for 5 min. at 17000×g in a cooling centrifuge. The supernatant was used for the assay of catalase (CAT) peroxidase (POX), superoxide dismutase (SOD) and glutathione reductase (GR). The catalase and peroxidase activities were assayed according to the method of Chance and Maehly (1955).

The assay mixture for CAT comprised of 3.0 ml of phosphate buffer (pH 6.8), 1.0 ml (30 mM) H_2O_2 and 1.0 ml enzyme extract. The reaction was terminated by adding 10 ml 2% H_2SO_4 (v/v) followed by 1 ml 0.01 N $KMnO_4$ to determine the quantity of the residual H_2O_2. The CAT activity was expressed as µmol of H_2O_2 destroyed

min^{-1} g dry $weight^{-1}$. A control was run simultaneously in which enzyme activity was stopped at zero time.

The assay mixture for the estimation of peroxidase (POX) comprised of 2.1 ml (0.1 M) phosphate buffer (pH 6.8), 0.3 ml 1.6% guaiacol, 0.3 ml 0.04 M H_2O_2 and 0.3 ml enzyme extract. The rate of change in absorbance at 470 nm was determined. A zero time control was run with water substituting the enzyme extract.

The assay of superoxide dismutase (SOD) was done by the method of Giannopolitis and Ries (1977). The 3.0 ml reaction mixture consisted of 2.5 ml Tris buffer (pH 8.9), 0.1ml bovine serum albumin (3.3×10^{-3}% w/v), 0.1 ml NBT (6 mM), 0.1 ml riboflavin (600 µM in 5 mM potassium hydroxide) and 0.2 ml of the enzyme extract. The reaction mixtures were illuminated in glass tubes selected for uniform thickness and colour, identical unilluminated assay mixtures as blanks. Test tubes were exposed to light by immersing in a beaker 2/3 filled with clean water, maintained at 27 °C. The increase in absorbance due to formazan formation was read at 560 nm. Under these conditions the increase in absorbance in the absence of enzyme was taken as 100% and 50% initiated and taken equivalent to one unit of SOD activity. Glutathione reductase (GR) activity was determined according to Smith et al. (1988) by monitoring the increase in absorbance at 412 nm when DTNB is reduced by GSH to produce 2-nitro-5-thiobenzoic acid (TNB). Enzyme units denote $\mu mol.min^{-1}.g$ d.w.$^{-1}$ for CAT, POX, SOD whereas for GR it is ΔA min^{-1}g. d.w.$^{-1}$.

Results and Discussion

Fig. 1a depicts the changes in the shoot and root length of greengram at different Al concentrations. A gradual decrease in root and shoot elongation rate were observed with the increase in aluminium concentration. The reduction in root and shoot elongation with an increasing concentration of aluminium has also been observed for many other crops, as the first sign of Al toxicity appears in the root system which becomes stubby as a result of inhibition of elongation of root main axis (Foy et al., 1974, Zaifnejad et al., 1997, Patra and Panda, 1998; Subramanyam, 1998). Changes in total peroxide, TBARS content and membrane injury index (%) were shown in Fig. 1b,c,d. An uniform increase in total peroxide content, TBARS content and membrane injury index was recorded with the increase in Al concentrations. As reported earlier, increase in Al concentration enhanced the lipid peroxidation in greengram leaves measured in terms of an increase in TBARS contents, which may be due to an excessive generation of hydroxyl radicals [$^{\bullet}$OH] (Cakmak and Horst., 1991. Gallego et al., 1996, Mazoudi et al., 1997, Subrahmanyam, 1998; Peixoto et al., 1999, Shah et al., 2001, Sakihama and Yamasaki, 2002). Similar to lipid peroxidation a loss in membrane integrity was found with increasing Al concentration as judged by the increase in membrane injury index (De and Mukherjee, 1996, Yamamoto et al., 2001).

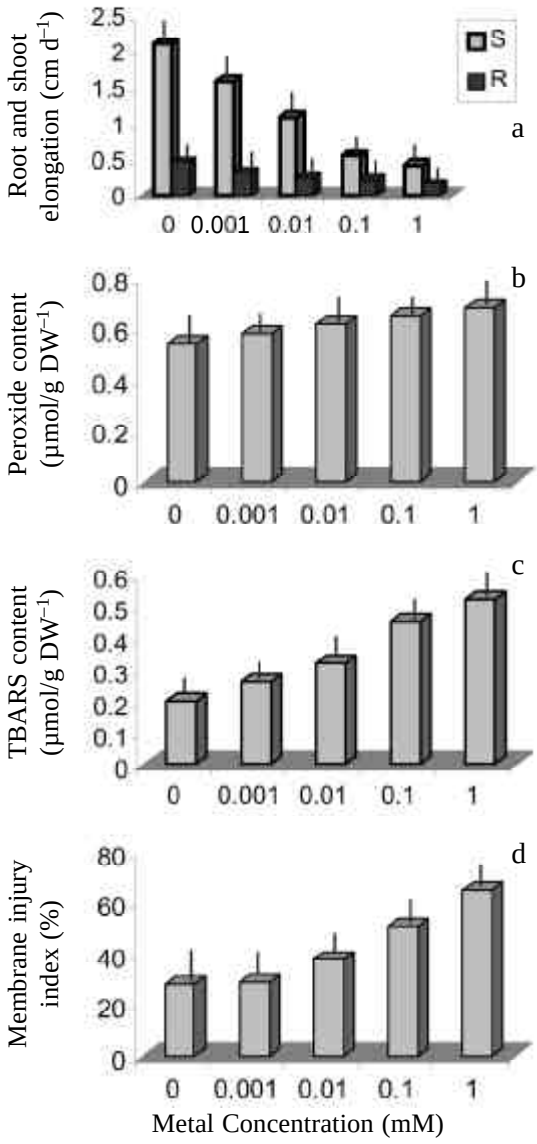

Fig.1. Effect of aluminium treatment on the rate of elongation (a) of root (R) and shoot (S), (b) peroxide content, (c) TBARS content and (d) membrane injury index of developing greengram seedlings. Data presented are means +SE. For details see "Materials and Methods".

An uniform increase in SOD, POX and GR activities parallled with a gradual decrease in CAT activity was detected with the increase in Al concentrations

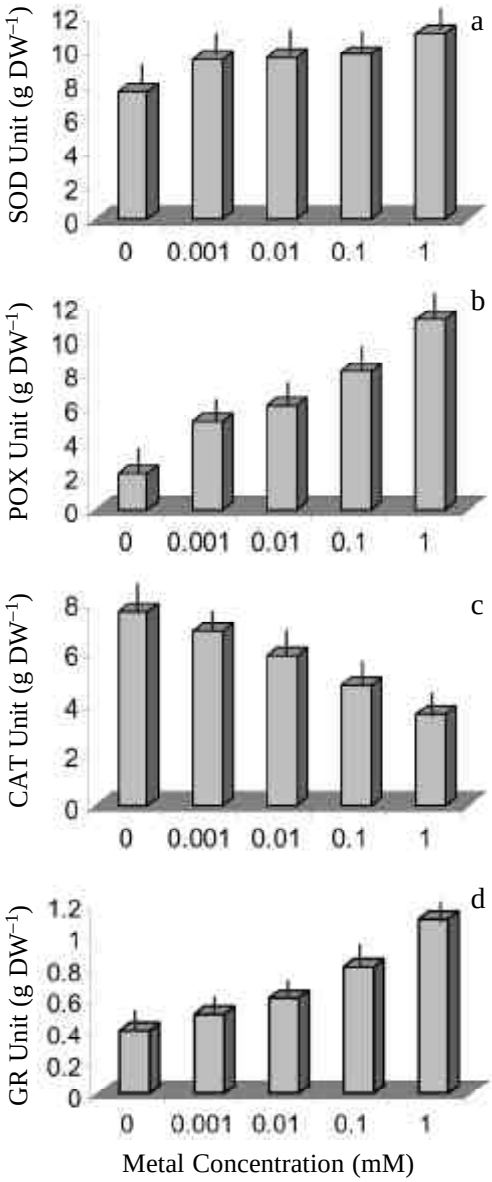

Fig. 2. Changes in SOD (a), POX (b), CAT (c) and GR (d) activities in developing greengram seedlings under aluminium treatment. Others are the same as in Fig. 1.

(Fig. 2a,b,c,d). The changes in non-enzymic antioxidants like ascorbate and glutathione showed a decrease with increasing aluminium concentrations in greengram (Fig. 3a,b).

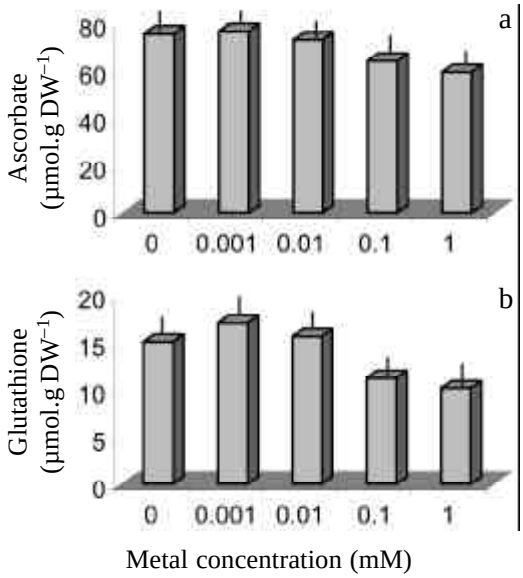

Fig. 3. Changes in ascorbate (a) and glutathione (b) content of developing greengram seedlings under aluminium treatment. Others are the same as in Fig. 1.

Plants have developed a complex antioxidant system against the reactive oxygen species generated in plant tissue during normal and stressful condition (Alscher et al., 1997). A moderate increase in SOD activity with a greater increase in POX and GR activity was recorded with increasing Al concentrations . An increase in SOD activity will result in a higher hydrogen peroxide level as substantiated in our result with higher peroxide content in increasing Al concentration. Hydrogen peroxide which is cytotoxic and acts both as an oxidant and reductant is detoxified by CAT activity which dropped at higher Al concentration. The decline in CAT may be due to the fact that the enzyme being photosensitive, needs constant synthesis as reported for Al^{3+} (Feierabend et al., 1992) and for other metals (Maksymiec and Baszynski, 1996, Shaw and Rout, 1998, Prasad et al., 1999). Though a decrease in CAT activity poses an oxidative threat an increase in POX and GR activities may play a role in H_2O_2 detoxification (Peixoto et al., 1999).

A small decrease in ascorbate content was visible at 0.1 and 1 mM aluminium concentrations whereas lower aluminium concentrations didn't affect it. Though a brief increase in glutathione content was seen at 0.001 mM concentration at 0.1 and 1 mM concentrations it decreased. The decreasing trend in the cellular non-enzimatic antioxidants like glutathione and ascorbate at higher aluminium concentration may suggest their inability to detoxify the reactive oxygen species directly (Rennenberg, 1982, Gallego et al., 1996).

From the present investigation, it is evident that Al phytotoxicity induces oxidative stress in growing greengram seedlings and that SOD, POX and GR may serve as important defensive antioxidants to combat Al induced oxidative damage.

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