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Aluminum Induced Biochemical Alterations in *Amaranthus tricolor*

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Abstract

Background and Objective : The current study reveals the effect of Aluminum (Al) on *Amaranthus tricolor L* and subsequent biochemical changes on the plant. In this study, the effect of Al stress on growth and other related antioxidants and enzymes were investigated. ***Methodology*** : This was achieved by investigating plant growth parameters (root/shoot length, dry weight) in presence and absence of various concentrations of Al. The antioxidant activity was determined by estimating proline, ascorbate. The effects of scavenging enzymes were determined by Catalase, Glutathion and SOD activity. The accumulation of Al in root and shoot was determined by FAAS (Flame Atomic Absorption Spectroscopy). ***Results:*** Al induced growth retardation observed more in roots (52.2%) compared to shoots (35.3%). Total concentration of proline increased (18 folds) compared to the control. The antioxidants and enzymes exhibited initial increase and subsequent decrease in response to Al stress. Accumulation of Al (by FAAS) was predominantly in root (70.44ppm ± 0.420).***Conclusion***: Result thus suggested Al induced growth inhibition in *Amaranthus tricolor* and the primary affected region is the root. Al accumulation in the roots could play an important role in the acclimation of the genotype to Al stress, and could be used as physiological markers for Al-tolerance. Metal induced scavenging mechanism observed both at enzymatic and antioxidant molecular level.

Keywords: *Amaranthus tricolor*, Aluminum, Antioxidant, ROS, Scavenging enzyme

1. Introduction

Pollution of the biosphere with toxic metals has accelerated dramatically since the beginning of the industrial revolution[1]. Not only the humankind, but also plants and other organisms are affected by metal pollution. Trace metals such as Aluminum, Zinc, Arsenic, Cadmium, Manganese, Nickel, and Selenium etc., have been considered to be the major environmental pollutants and their phyto - toxicity is well established[2]. Aluminum (Al) is the most abundant metal in the world and the third most common element in the earth crust. It is the primary limiting factor in crop productivity particularly in acid soils in the tropic and sub tropics[3]. Evidences have demonstrated that the root apex is the primary site of Al-induced root inhibition[4]. Al can interact with root cell walls, disrupts plasma membrane transport system and interacts with calmudulin and therefore plant signaling system[5]. Al stress induced genes are identified and characterized from wheat, tobacco and *Arabidopsis*[6,7,8].

A common aspect of all environmental stresses is the enhanced production of reactive oxygen species (ROS) within several subcellular compartments of the plant cell[9]. ROS, if not detoxified cause serious damage to proteins, lipids and nucleic acids[10]. To minimize the harmful effects of ROS, plants have evolved an effective defense system. This includes both enzymatic antioxidants and non-enzymatic antioxidants. Among the antioxidant defense system, ascorbate (AsA), glutathione (GSH) and related enzymes (such as ascorbate peroxidase, glutathione reductase and superoxide dismutase) play a pivotal role in scavenging of ROS from plant cells[11].

Amaranthus tricolor L belongs to amaranthaceae family, commonly known as "Dantu sappu" (in Southern part of India) is a widely cultivated plant in the world, particularly in tropical Asia[12]. It is one of the major leafy vegetable available in hot summer. It is an upright branched annual herb, which grows about 0.45m – 1m[12]. The seeds are small and brown. Mature leaves contain red-violet pigments, Betacyanin, Amaranthin and Isoamaranthin[13]. The plant leaves contains 38.3% dry matter as protein, 0.25% fats and 6.6% carbohydrates, along with other minerals and vitamins[14]. The role of *Amaranthus* as an under exploited plant with promising economic value has been recognized by the National Academy of Sciences[12].

A wide range of environmental stresses, such as extreme temperatures, drought, salinity, UV radiation and metals are potentially harmful to plant growth[15]. Hence, the present study aimed to investigate the toxicity of Al on growth, antioxidant profile of *Amaranthus tricolor*.

2. Materials and Methods

2.1 Seed germination and seedling growth

Seeds of *Amaranthus tricolor L* were purchased from the local market, Bangalore city, Karnataka, India and verified by Dr. Shiddamallayya N, National Ayurveda Dietetics Research Institute, Bangalore (Voucher no. Drug Authentication/SMPU/NADRI/BNG/2013-14/418). The healthy and uniform sized seeds were separated. The seeds were washed and surface

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sterilized with 1.0 % mercuric chloride. The seedlings were watered with Hoagland nutrient solution[16] for 14 days, and then stress treatments were started.

2.2 Stress treatments

Hoagland nutrient solution (1X) was supplemented with $AlCl_3$ solution at different concentrations of 20 µM, 60 µM, and 100 µM (pH-5.5). The control seedlings were maintained by watering normal Hoagland nutrient solution. Stress treatments were continued for, 3, 6, 9, and 12 days.

2.3 Assay of the Plant growth parameters

2.3.1 Root, shoot length and dry weight

Root and shoot lengths were measured in the intact seedlings with the help of a scale and thread. The measurements were determined for 5 seedlings from each treatment and controls, then average values were calculated. Dry weight of detached shoots and roots of both control and Al stressed plants were determined separately. The samples were oven-dried at 80°C for 15 min, then vacuum-dried at 40 °C to constant weight and the dry weights (DW) were measured.

2.3.2 Estimation of Total Protein

Two fifty (250) mg of root and shoot extract was homogenized separately in phosphate buffer (1.5ml) under ice cold conditions. The homogenate was centrifuged and the supernatant was estimated for total protein content spectrophotometrically. The concentration of the protein in supernatant was calculated using the formula as per the method of Layne, E. 1957[17] and UV spectrophotometrically. Here the O.D of the protein solution was read at 280 nm and 260 nm for possible nucleic acid contamination using the phosphate buffer (20mM) as the blank. Then the following formula was used to estimate the protein concentration of the enzyme sample.

2.3.3 Estimation of Proline

Proline was estimated by Bates method[18]spectrophotometrically. The amount of proline in the test sample was calculated from the standard curve constructed by appropriate concentration of proline. Express the proline content on fresh-weight-basis as follows:

$$\text{mmoles per g tissue} = \frac{\text{mg proline/mL} \times \text{mL toluene}}{\text{molecular weight of proline}} \times \frac{5}{\text{g sample}}$$

2.3.4 Ascorbate assay

Ascorbate was determined at 525nm spectrophotometrically according to the modified procedure of Law et al[19]. Fresh root and shoot samples (500 mg) of both control and Al treated were separately homogenized into 3.0 ml of 5% metaphosphoric acid and centrifuged at 22,000×*g* for 15 min at 25 °C, using cooling centrifuge (REMI, India; Model C-24 BL). Supernatant was saved and used for the estimation of ascorbate.

The supernatant was initially treated with dithiothreitol (for reducing dehydroascorbate to ASC). The supernatant (0.2 ml) was added to 0.5 ml of 150 mM phosphate buffer (pH 7.4) containing 5mM EDTA and 0.1 ml of 0.5% (w/v) *N*-ethylmaleimide. After adding 0.4 ml of 10% (w/v) trichloroacetic acid, 0.4 ml of 44% (v/v) orthophosphoric acid, 0.4 ml of 4% (w/v) 2,2'-bipyridyl in 70% (v/v) ethanol and 0.2 ml of 3% (w/v) ferric chloride, the mixture was incubated at 40 °C for 40 min. The absorption of the color developed was measured at 525 nm using a UV-Visible spectrophotometer (Systronics -117). Total ascorbate was calculated using a standard curve for pure ascorbate.

2.4 Antioxidant Assay

2.4.1 Glutathion assay

Extraction and estimation of total glutathione in both control and stressed tissues was carried out according to Griffith[20] at 412 nm spectrophotometrically using 5,5'-dithiobis-2-nitro benzoic acid (DTNB) reagent. The concentration of the glutathione was calculated by using a standard calibration curve constructed by appropriate concentrations of glutathione (reduced; GSH) standard.

2.4.2 Catalase assay

Catalase was estimated in the tissue extract by the method of Aebi[21] using a UV visible spectrophotometer. The amount of H_2O_2 reduced was determined by using molar extinction coefficient of H_2O_2. The activity was expressed in terms of µmol of H_2O_2 reduced $min^{-1}g^{-1}$ tissue fresh weight at 25 ± 2°C.

2.4.3 Superoxide dismutase (SOD) assay

Superoxide dismutase activity was assayed by using the photochemical NBT method[22]. The homogenate was filtered through four layers of muslin cloth and centrifuged at 10,000 rpm for 20 min at 4°C, using cooling centrifuge and the supernatants were used for protein concentration determination and enzyme assays. Fresh roots and shoots (500 mg) of both control and aluminum stressed were separately homogenized into 4 ml extraction buffer consisting of 100 mM potassium phosphate buffer (pH 7.0) containing 1% PVP. The assay mixture in 3 ml contained 50 mM phosphate buffer, pH 7.8, 9.9 mM L-methionine, 57 mM NBT, 0.025% (w/v) Triton X-100, and 0.0044% (w/v) riboflavin. The contents were mixed rapidly and kept below 30 cm of light (at the light intensity of 300 mmol $^{-1}$ m^{-2} s^{-1}) for 10 minutes along with enzyme control lacking extract. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm using a UV-Visible spectrophotometer. An inhibition curve was made against different volumes of extract. One unit of SOD is defined as being present in the volume of extract that causes inhibition of the photo-reduction of NBT by 50%, that was calculated with the help of following formula.

$$\text{SOD (units)} = \frac{1.000000000000000000000000000}{\text{Vol. of extract (ml) required to cause 50\% of NBT inhibition} \times 10^{-3}}$$

2.5 Estimation of Al accumulation in plant root and shoot

Plant samples were gently ground using electrical grinder. Three gm of plant sample was digested with 20ml of HNO_3:$HClO_4$ (2:1). The mixture was heated in a beaker until dissolved and then cooled. The digested plant samples were then re-dissolved in 10% $HClO_4$ and filtered through Whatman no. 40 filter papers, and the volume was adjusted to 50ml with 10% $HClO_4$ in polyethylene volumetric flask. Reagent blanks for plant filters were also prepared by carrying out the whole extraction procedure, but without samples[23]. The Al in plant samples were estimated using flame atomic absorption spectroscopy method (GBC AVANTA Model 324).

2.6 Statistical analysis

Statistical analysis of data was conducted using one-way Analysis of Variance (ANOVA) using SPSS 17.0 software. Values in the figures indicate the mean values±SD based on independent three determinations (n = 3). Least Significant

Difference (LSD) test was used to assess the differences between control and different treatments; $p<0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Plant growth

The growth parameters were used as useful bio-indicators of Al toxicity in *Amaranthus* seedlings. These parameters are expressed as root and shoot length and dry weight. A gradual reduction in growth parameters were observed with increased Al^{3+} concentration (Fig. Ia-c).

Al^{3+} exhibited injurious effects followed by the death of *Amaranthus* seedlings when added at the highest (100 µM) level. Inhibition of growth and reduction of biomass production are general responses of some plants to metal toxicity and are often a reliable indication of plant's sensitivity to their stresses[24]. Our result also suggested that higher concentration of Al^{3+} (60-100) µM, exerts stress effect on *Amaranthus* and inhibits all the growth parameters.

The total protein contents both in root and shoot were increased especially under (20-40) µM concentration of Al^{3+} upon 3-6 days treatment (Fig Id, e). However with higher concentration of Al^{3+} and longer period of incubation the total protein content decreased. The decrease in protein content may be due to the enhanced of protein degradation as a result of increased protease activity under Al^{3+} stress condition[25].

3.1.1 Total Proline Content

The accumulation of proline has been considered as a result of metal stress in plant[26]. Schat H et al, (1997) suggested that the increased level of proline enhanced the plant's tolerance level through mechanisms like osmoregulation, stabilization of protein etc[27]. In the present study the total proline content (Fig. II a, b) has increased as the concentration of Al^{3+} was increased both in root and shoot samples. This is an indication of metal stress and plant's tolerance level. The elevated level of proline

Figure Ia: Effect of Al on root length

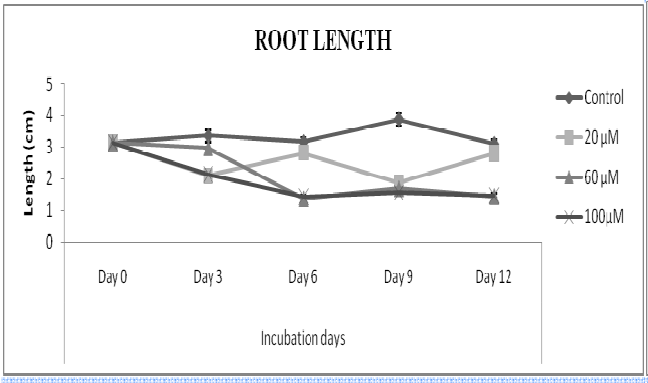


Figure Id: Effect of Al on total protein in root

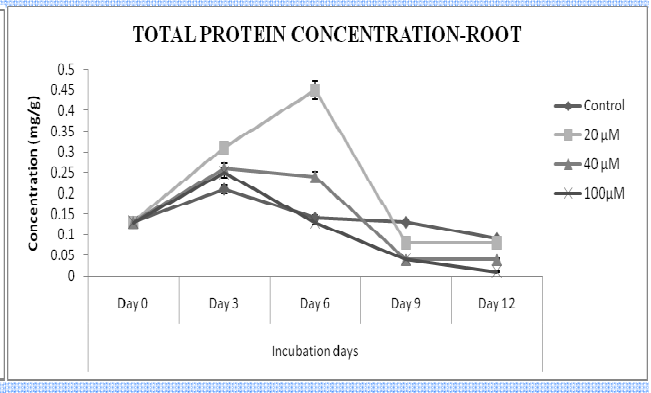


Figure Ib: Effect of Al on shoot length

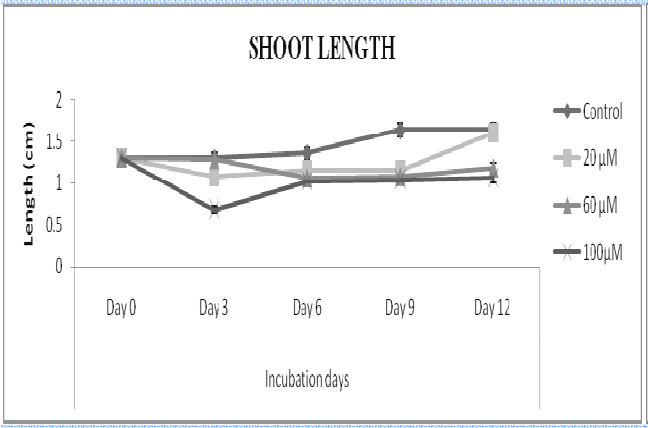


Figure Ie: Effect of Al on total protein in shoot

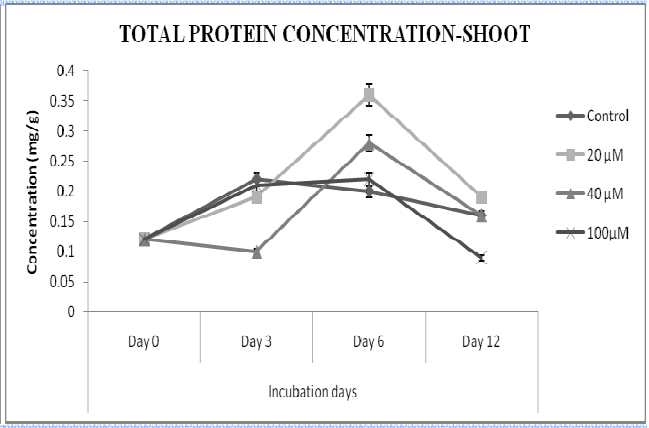


Figure Ic: Effect of Al on dry weight

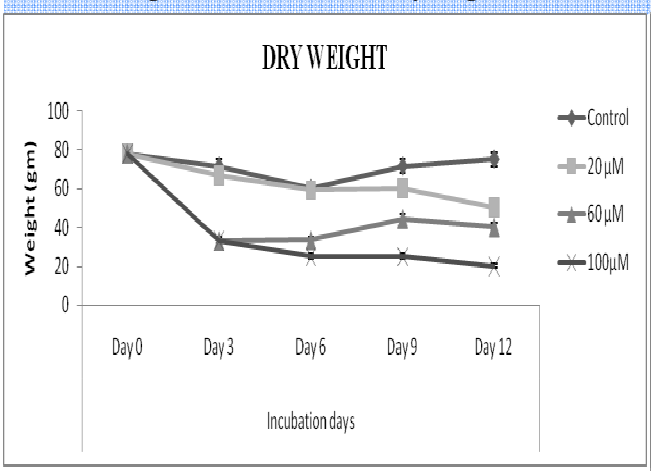


Figure IIa: Effect of Al on Proline concentration in root

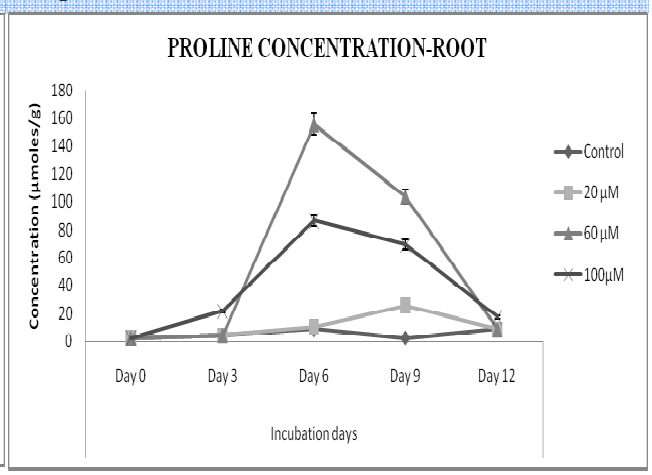
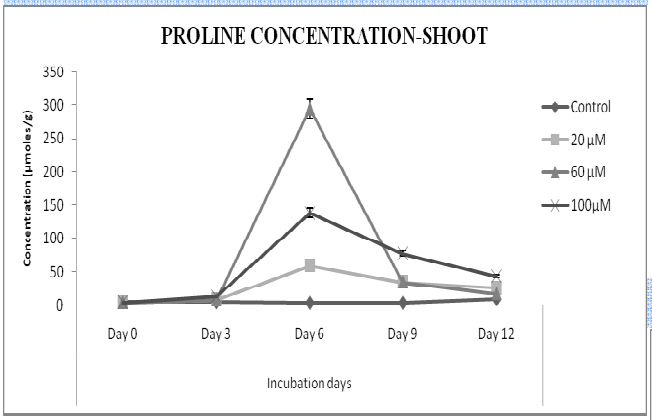


Figure IIb: Effect of Al on Proline concentration in shoot



could be either due to *de novo* synthesis or decreased degradation or both as suggested by Kasai Y et al (1998)[28].

3.1.2 Ascorbate Estimation

Ascorbate is an essential compound in plant tissues, reacts rapidly with superoxide and singlet oxygen (chemically), and hydrogen peroxide (enzymatically). Ascorbate content in the roots and shoots of *Amaranthus tricolor* exhibited a significant increase at metal stress (40-100 µM) on 6th day as compared to

Figure IIIa: Effect of Al on Ascorbate concentration in root

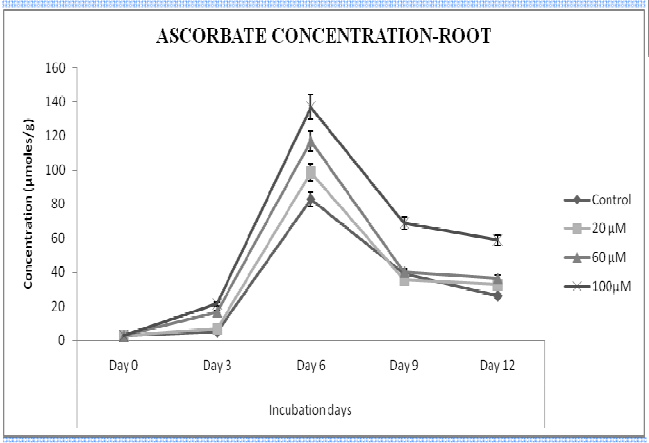
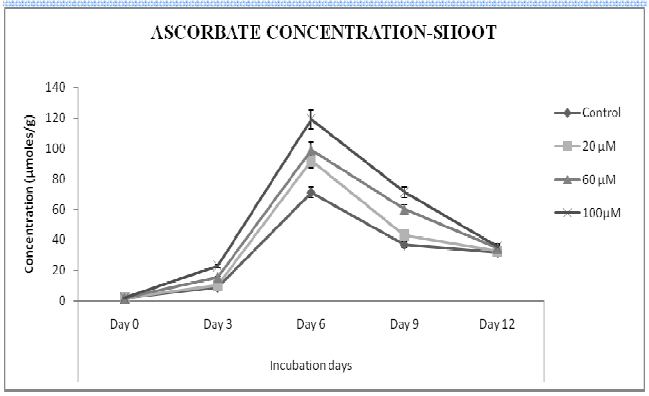


Figure IIIb: Effect of Al on Ascorbate concentration in shoot



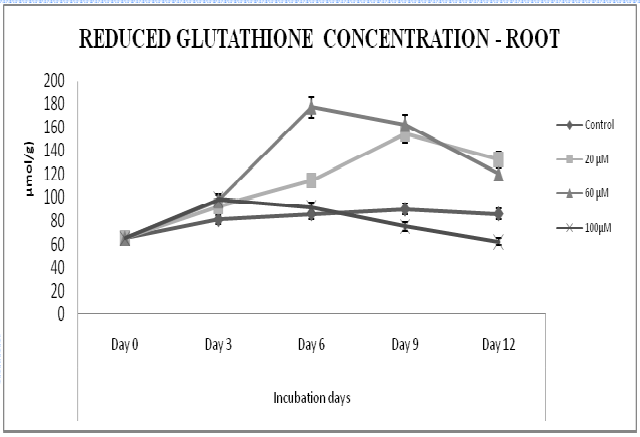
their respective controls (Fig. IIIa, b).

3.2 Antioxidant assay

3.2.1 Glutathion Assay

GSH plays several roles in cell metabolism such as redox state regulation, oxidative stress control, and defense against heavy metals[29]. Induction of GSH level is an important protective mechanism to minimize oxidative damage in plants exposed to metals. A marked increase in GSH content of the *amaranthus* root samples was observed under Al stress (60µM) on 6th day when compared to the controls (Fig. IV). This result is in agreement with other plants exposed to metals[30].

Figure IV: Effect of Al on Reduced Glutathione concentration in



3.2.2 Catalase and SOD Estimation

The SOD converts superoxide radical into hydrogen peroxide and molecular oxygen, whereas the catalase converts hydrogen peroxide into water. In this way, two toxic species, superoxide radical and hydrogen peroxide are converted to the harmless product water. Induction and activation of superoxide dismutase (SOD) and catalase are some of the major metal detoxification mechanisms in plants. Gwozdz et al. (1997) found that at lower heavy metal concentrations, activity of antioxidant enzymes increased, whereas at higher concentrations, the SOD activity did not increase further and catalase activity decreased[31]. Our result showed an initial increase, and subsequent decrease in SOD level (Table I), which actually supports Gwozdz et al's (1997), finding. Peixoto P H P et al (1999) suggested that catalase enzyme activity decreased after Al treatment both in roots and shoots of the plant[32]. Similar results have been obtained by Richards et al. (1998) who has also observed a reduction in specific mRNA for these catalases in Al^{3+} treated plants[8].The present study shows a decrease in catalase activity (Table I) upon (100 µM) Al^{3+} treatment in *Amaranthus* which supports the previous findings.

3.3 Al Accumulation

The toxic effects of Al are primarily root related. The present study confirmed the primary site of Al accumulation in *Amaranthus tricolor* is in the root (Table II). A higher amount of Al accumulation was observed upon 9-12 days incubation with (20-60) µM Al^{3+}. Ciamporova M (2002) suggested that longer Al treatment is required to reduce cell division or to interfere with nucleic acids in the root apex[33]. Further investigation is required to elucidate the genomic interference of Al at root apex in *Amaranthus tricolor.*

Table I: Levels of antioxidant enzymes in the root of *Amaranthus tricolor* in control and Al exposed

Factors AOA	Control			Experimental 100 µM		
	Days 3	Days 6	Days 12	Days 3	Days 6	Days 12
SOD µgm/ min/mg protein	0.005	0.0031	0.0069	0.02	0.022	0.0063
Catalase µmol/min/gm	7213.9	7201	3078.8	2773.4	2989.1	3182.8

Table II. Accumulation of Al in root

Conc. Of Al in (ppm)	Incubation Days		
	Day 3	Day 9	Day 12
Control	0.09 ± 0.01	0.255 ± 0.25	0.18 ± 0.09
20 µM	0.12 ± 0.03	5.192 ± 0.10	66.385 ± 10.94
60 µM	0.097 ± 0.050	25.579 ± 0.61	70.44 ± 0.42

Conclusion

The results obtained showed that *A.tricolor has* Al-tolerant mechanism. Al accumulation in the roots as a result of metal stress appeared to play an important role in the acclimation of the genotype to Al stress, suggesting that they could be used as physiological markers during the screening for Al-tolerance. Total protein, proline, ascorbate and scavenging enzymes responded distinctly to $AlCl_3$ stress, suggesting divergent of response mechanisms in the species. Genetic evaluation of genotypes based on Al tolerance indices could be exploited in the breeding of Al tolerant genotypes.

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