FINAL REPORT OF WORK DONE ON THE MINOR RESEARCH PROJECT

ON

To study the changes in antioxidant gene expression and induction of oxidative stress in aluminium toxicity in Sorghum

Submitted

To

UNIVERSITY GRANTS COMMISSION, NEW DELHI-110002

Vijay Kumar

Principal Investigator

Department of Biochemistry

M.D. University, Rohtak-124001, Haryana

Annexure III

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002.

Final Report of the work done on the Minor Research Project.

1. Project report No. : **Final**

2. UGC Reference No. **:** **F. No 40-478/2011 (SR) dated 13.07.2011**

3. Period of report: from **:** **13/07/2011 to 12/07/2013**

4. Title of research project **:** **To study the changes in antioxidant gene expression and induction of oxidative stress in aluminium toxicity in Sorghum**

5. (a) Name of the Principal Investigator **: Dr. Vijay Kumar**

(b) Deptt. and University/College where work has progressed :

Deptt. of Biochemistry, M.D. University, Rohtak-124001

6. Effective date of starting of the project : **13/07/2011**

7. Grant approved and expenditure incurred during the period of the report:

a. Total amount approved Rs. : 1**,25,000/- (Rs. 1,16,500/- received)**

b. Total expenditure Rs. : **1,11,134/-**

Report of the work done: (Please attach a separate sheet) **Annexure I**

(i) Brief objective of the project : **Annexure 1**

(ii). Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication : **Annexure II & IV**

(iii) Has the progress been according to original plan of work and towards achieving the objective. if not, state reasons: **Yes**

(iv) Please indicate the difficulties, if any, experienced in implementing the Project: Nil

(v) If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet : **NA**

(vi). If the project has been completed, please enclose a summary of the findings of the study. Two bound copies of the final report of work done may also be sent to the Commission : **Annexure III**

(vii). Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as (a) Manpower trained (b) Ph. D. awarded (c) Publication of results (d) other impact, if any NA

SIGNATURE OF THE PRINCIPAL INVESTIGATOR

REGISTRAR/PRINCIPAL

Annexure I

Objectives of the Project:

1. **To study the aluminium induced oxidative stress in Sorghum seedlings**
2. **To study the changes in gene expression of antioxidant enzymes in aluminium treated Sorghum seedlings**

Introduction

Aluminium (Al) is one of the most abundant elements of the planet and constitutes about 8% of the earth crust. The almost ubiquitous presence of this element has so heavily contaminated the environment that exposure to it, is virtually inescapable. Al compounds are released into the atmosphere during process of smelting and may enter the soil either directly in precipitation or indirectly through contaminated litter. Although Al occurs as harmless oxides and alumino-silicates but in the acidic soil, Al may be solubilized into toxic forms, such as $[Al(H_2O)_6]^{3+}$. Al toxicity is considered as one of the major factors leading to decreased crop production on the acid soils which comprise almost half of the arable land (Panda and Matsumoto 2007; Panda et al. 2009). Al occurs as harmless oxides and alumino-silicates but in the acidic soils, it is solubilized into soil solution from aluminosilicates, inhibiting root growth and function (Ma et al. 2001; Kochian 2005).

Reactive oxygen species (ROS) are continuously generated as byproducts in oxygen metabolism in plants. The balance between generation of ROS and their degradation is required to maintain normal metabolic functions under the stress conditions. Under normal conditions, ROS level in plant tissues is controlled by antioxidant enzymes present in several organelles (del Rio et al. 2006). The incomplete reduction of ROS may result in a state of oxidative stress leading to the oxidation of biomolecules (lipids, proteins and DNA) or even cell death. Although Al itself is not a transition metal and cannot catalyze redox reactions, the involvement of oxidative stress in Al toxicity has been suggested (Boscolo et al. 2003; Pereira et al. 2010). The Al-induced ROS generation may be one of the decisive factors for Al-induced inhibition of

root elongation (Yamamoto et al. 2001; Panda et al. 2009). Cakmak and Horst (1991) first showed the enhancement of lipid peroxidation and small increases in activities of SOD and peroxidases by Al induced ROS production in the root tips of soybean (Glycine *max*). Studies have shown that Al induced oxidative stress leads to the alteration in the expression patterns of genes, some of which are important in the oxidative stress response (Richards et al. 1998; Thirkettle -Watts 2003; Maron et al. 2008). Recent study carried out by Panda and Matsumoto (2010) has also shown the induction of oxidative stress and changes in gene expression of antioxidant enzymes in Pea plant under Al-toxicity. Earlier, similar results were observed by Sharma and Dubey (2007) in Al treated rice seedling. While increase in antioxidant enzyme activities as a stress response to Al exposure are well documented in some crop species but, there is little or no information present on the molecular response of antioxidant enzymes of sorghum plant under Al induced oxidative stress. The purpose of the present work was therefore to contribute to a better understanding on the possible ability of Al to generate the oxidative stress and on the response of antioxidant enzymes in sorghum roots and leaves.

Materials and Methods

Growth and collection of plant material

Seeds of grain sorghum (cv AN 2000) were surface sterilized in 0.1% $HgCl_2$ for 5 min and washed in sterile distilled water. The sterilized seeds were germinated on moistened filter paper lined in plastic petridishes. The seedlings were treated with various regimes of aluminium chloride i.e. 0, 10, 25, 50, 100, 250, 500 and 1000 µM at pH 4.5 for preliminary screening of the tolerance range of plant in seed germinator at 25°C. The pre-soaked and sterilized seeds were then regerminated in petriplates in nutrient solution containing 8 mM KNO_3, 2mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$ and micronutrients: 30 µM H_3BO_4, 5 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $(NH_4)_6Mo_7O_{24}$ and 1µM $ZnSO_4$. The solution with conc. of 100 µM or more produced visible morphological symptoms of toxicity in sorghum and hence 100 µM and 250 µM solutions of $AlCl_3$ were

used to irrigate two sets of plants besides a third set of plants without aluminium as control.

Measurement of growth

During growth, the root and shoot samples were taken for fresh weight determination and compared with those of controls. The root and shoot length assessment was done at both levels of Al treatments at 3 days interval upto 12 days using ten random samples in triplicate.

Preparation of crude extract

Fresh plant tissue was homogenized (1:5 w/v) in ice-cold 0.1 M potassium phosphate buffer of pH 7.0 containing 2% PVP in prechilled mortar and pestle. The homogenate was centrifuged at 4°C for 15 min at 12,000 x g and supernatant was used for enzyme assays. The protein content of the supernatant was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Determination of $O^{\cdot}_2{}^{-}$ and H_2O_2 content

Extra-cellular generation of $O^{\cdot}_2{}^{-}$ was measured according to Kiba et al. (1997) with minor modifications. 10 excised root and leaf tips of equal length were incubated in 3 ml of the reaction mixture containing 50 mM Tris-HCl buffer (pH 6.5), 0.2 mM nitroblue tetrazolium, 0.2 mM NADH and 250 mM sucrose for 24 h at room temperature in dark. The absorbance of the blue monoformazan thus formed was measured at 530 nm and its concentration was calculated using an extinction coefficient of 12.8 mM^{-1} cm^{-1}.

The H_2O_2 content of both control and Al treated sorghum roots and leaves were determined according to Sagisaka (1976). One gram of tissue was homogenized in 5% trichloroacetic acid (TCA) and the homogenate was centrifuged at 16,000 x g at 4°C for 10 min. The reaction mixture contained 1.6 ml supernatant, 0.4 ml TCA (50 %), 0.4 ml ferrous ammonium sulfate and 0.2 ml potassium thiocyanate. The absorbance was recorded at 480 nm.

Antioxidant enzymes assays

Catalase activity was determined by consumption of H_2O_2 in absorbance at 240 nm by the method of Vitoria et al. (2001). The assay mixture consisted of 0.1 ml extract and 25 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2. The decreases

in absorption were recorded at 240 nm and quantified from the extinction coefficient of 0.036 mM^{-1} cm^{-1} and activity expressed as µmol H_2O_2 oxidized/ min/ mg protein

Total SOD activity was assayed by monitoring inhibition of photochemical reduction of nitroblue tetrazolium (NBT) as described by Beauchamp and Fridovich (1971). The 3 ml reaction mixture consisted of 2.9 ml 50 mM potassium phosphate buffer (pH 7.8) containing 10 mM methionine, 168 µM NBT, 0.025 % Triton X-100, 1.17 µM riboflavin, and 0.1 ml enzyme. The assay was carried out by placing the test-tubes below a 20W fluorescent lamp for 30 min. The amount of formazan formed was measured at 560 nm compared with amount of formazan formed in the absence of enzyme. One unit of SOD was defined as the enzyme causing 50 % NBT reduction and activity was expressed as U/mg protein.

Guaiacol peroxidase was estimated with guaiacol as substrate according to the method of Vitoria et al. (2001). The assay mixture contained 0.1 ml extract in 25 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and 9 mM guaiacol. The formation of tetraguaiacol was monitored by noting increase in absorbance at 470 nm and quantified using the extinction coefficient 26.6 mM^{-1} cm^{-1} and activity expressed as µmol guaiacol oxidized/ min/ mg protein.

Ascorbate peroxidase activity was estimated by the method of Nakano and Asada (1981) with modification by monitoring the rate of ascorbate oxidation (extinction coefficient = 2.8 mM^{-1} cm^{-1}). The assay mixture was 0.1 ml extract added to 50 mM potassium phosphate buffer (pH 7.0) containing 0.1mM H_2O_2, 0.5 mM ascorbate and 0.1 mM EDTA. The change in absorbance was monitored at 290 nm and activity expressed as µmol ascorbate oxidized/min/mg protein.

Lipid peroxidation and proline content

The level of lipid peroxidation in sorghum roots and leaves was determined as the amount of 2-thiobarbituric acid-reactive substances (TBARS) mainly malondialdehyde (MDA) content formed as described by Dhindsa et al. (1981). 1 g of tissue was homogenized in 5 ml 0.1% TCA and centrifuged at 10,000 rpm for 15 min. To 2 ml supernatant, 2 ml of 20 % TCA containing 0.67 % TBA was added. The mixture was heated at 90°C for 30 min for formation of pink-colored 1:2 adduct between MDA and TBA and then quickly cooled on ice. After centrifugation at 10,000 rpm for 10 min, the

absorbance of the supernatant was read at 532 nm and the value for the non specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as n mol/g fresh weight (FW).

Proline concentration in sorghum roots and leaves was determined following the method of Bates et al. (1973). 0.5 g sample was homogenized with 5 ml of sulfosalicylic acid (3 %) using mortar and pestle and filtered through Whatman No. 1 filter paper. The volume of filtrate was made up to 10 ml with sulfosalicylic acid and 2.0 ml of filtrate was incubated with 2.0 ml glacial acetic acid and 2.0 ml ninhydrin reagent and boiled in a water bath at 100°C for 30 min. After cooling the reaction mixture, 6.0 ml of toulene was added and after cyclomixing it, absorbance was read at 570 nm.

Protein oxidation

The oxidation of proteins was assesses in terms of reaction of carbonyl resulting from modification of proteins and 2,4-dinitrophenyl hydrazine (DNPH) (Levine et al. 1994). In brief, two equal aliquoits each containing 1 mg protein of roots and leaves were precipitated with equal volume of 20% (w/v) trichoroacetic acid and supernatant was discarded. The pellet was resupended with 2 N HCL was incubated for 1 h at room temperature after DNPH reaction. The samples were then precipitated with 20% TCA and supernatant was discarded. After washing three times with ethanol;ethyl acetate (1:1), the pellet was dissolved in 20 mM sodium phosphate buffer (pH 6.8) containing 6 M guanidinimum hydrochloride. Carbonyl concentration was calculated from the difference in absorbance recorded at 380 nm for DNPH-treated and HCl-treated (blank) samples (ε= 22 $mM^{-1}cm^{-1}$) and expressed in nmol of carbonyl content/mg protein.

Antioxidant gene expression

Frozen root and leaf tissue (approximately 100 mg) was ground thoroughly in liquid nitrogen using a mortar and pestle. RNA was extracted using the Plant total RNA Kit (Sigma-aldrich, St Louis USA) according to manufacturer's instructions. RNA was reverse transcribed in a total volume of 20 µl (RevertAid cDNA synthesis kit, Fermentas) according to manufacturer's instructions. For amplification, cDNA products (1µl) were subjected to reverse transcriptase PCR analysis on a gradient thermal cycler instrument (PEQLAB, Germany). PCR cycle comprised of initial denaturation at 94°C for 2 min. The amplification was then carried out for 30 cycles consisting 30 sec each for 94°C

(denaturation) and 72°C (annealing), 1 min (extension). Final extension was done at 72°C for 10 min. β-Actin was used as internal control. The following genes were amplified: CAT (sense: 5'-GTGAATGCACCAAAATGTGC-3') and (antisense: 5'-ACCAGCCTGCTTGAAGTTGT-3'), cAPX (sense: 5'- TGCTGGTCTTGTGAATGCTC-3') and (antisense: 5'- ATTGTTCAGGGGCAGTAACG-3'), GPX (sense: 5'-ATGTGGGTTGACAACAGCAA-3') and (antisense: 5'- GGGGGCTGTATTAGGTCCAT -3'), SOD (sense: 5'- TGCTGGTCTTGTGAATGCTC-3') and (antisense: 5'-CTTGCTCGAAAGGGTAGTGC -3'), Actin (sense: 5'- TTGGGTCAGAAAGGTTCAGG -3') and (antisense: 5'- TGCTCATTCGATCAGCAATC -3'). The PCR products were applied to 1% (w/v) agarose gel electrophoresis and stained with ethidium bromide.

Statistical analysis

All the assays and estimations were done in triplicates. The mean and standard deviations were calculated and the significance of difference between control and treatment mean values was determined by Student's *t*-test. Differences at $p \leq 0.05$ were considered significant.

Results

Growth parameters

The treatment of sorghum with 100 and 250 µM Al affected the plant growth that is shown in terms of inhibition of root and shoot length of growing sorghum. A decrease of fresh mass of root and shoot under Al treatment besides root growth inhibition was also observed (Table 1). Treatment of sorghum with 100 and 250 µM Al for 12 days also resulted in changes in morphology of plant. As early as 7th day following Al treatment, morphological symptoms of Al toxicity in terms of leaf necrosis were observed at both the treatments of Al.

Table 1: Effect of aluminium treatment on root length, shoot length and fresh mass of sorghum plant.

Time	Treatment	Root length (cm)	Shoot length (cm)	Fresh mass of Root (mg)	Fresh mass of Shoot (mg)
3rd day	Control	2.5	3.0	8.0	16
	100µM	2.4^{NS}	3.0^{NS}	8.5^{NS}	15^{NS}
	250 µM	2.4^{NS}	2.8^{NS}	8.0^{NS}	15^{NS}
6th day	Control	4.8	5.5	15.5	28
	100µM	4.2^{NS}	6.0^{NS}	13.8^{NS}	24^{NS}
	250 µM	3.7*	5.3*	12.5*	20^{NS}
9th day	Control	6.0	8.8	24	42
	100µM	5.0*	8.0^{NS}	20*	35^{NS}
	250 µM	4.5*	7.2*	19*	32*
12th day	Control	8.0	12.5	35	60
	100µM	5.6**	10.0*	28**	50*
	250 µM	5.4**	9.4**	24**	45**

The values are mean of ten random samples in triplicate. *$p<0.05$ significantly different from control. **$p<0.01$ significantly different from control. NS-not significant

Effect of Al on $O^{\cdot}{}_2^-$ and H_2O_2 generation

Al treatment (100 and 250µM) enhanced the generation of $O^{\cdot}{}_2^-$ in sorghum roots and leaves. There was 50% ($p\leq0.05$) and 65% ($p\leq0.01$) increase in $O^{\cdot}{}_2^-$ content in

sorghum roots treated with 100 and 250 µM of Al respectively compared to controls. Similarly, the increase in $O^{\cdot}{}_2^-$ content was about 40% ($p \leq 0.01$) and 62% ($p \leq 0.01$) in leaves (Fig 1). The content of H_2O_2 also increased significantly both in sorghum roots and leaves following Al treatments. The H_2O_2 content was observed to be increased by 52% ($p \leq 0.01$) and 70% ($p \leq 0.01$) in Al treated roots while this increase was about 28% ($p \leq 0.05$) and 45% ($p \leq 0.01$) in sorghum leaves treated with 100 and 250 µM of Al respectively compared to controls (Fig 2).

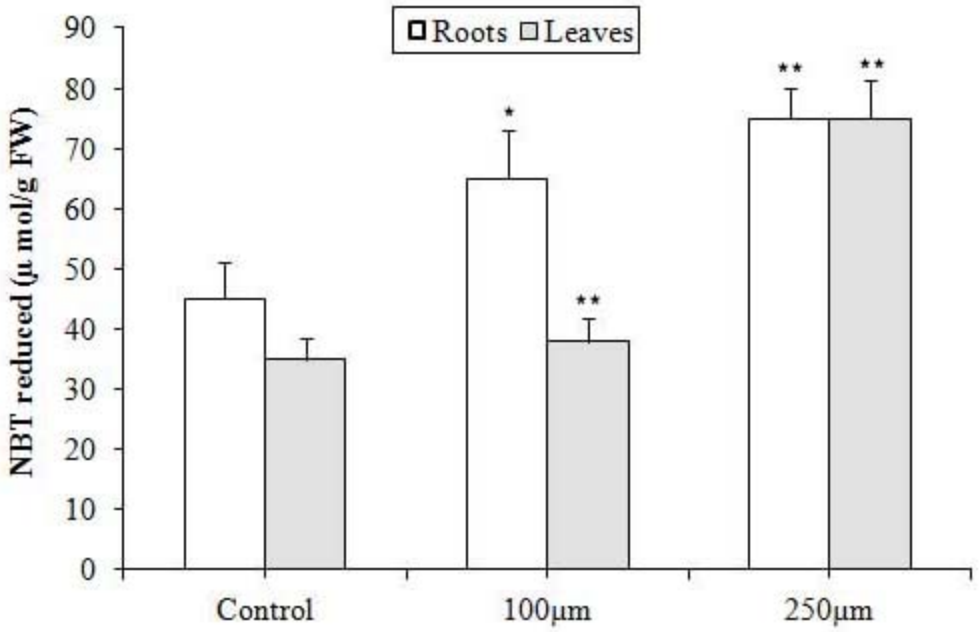

Fig 1: Superoxide content in sorghum roots and leaves treated with 100 and 250µM Al for 12 days. Values are mean ± S.D. (N=3). **p<0.01 significantly different from control; *p<0.05 significantly different from control.

Antioxidant enzyme assays

There was decrease in CAT activity in sorghum roots and leaves following 100 µM and 250 µM Al exposure. The activity of CAT was decreased by 55% ($p \leq 0.01$) and 68% ($p \leq 0.01$) in roots. In leaves, there was about 40% ($p \leq 0.05$) and 65% ($p \leq 0.01$) decrease in CAT activity after Al treatment (Fig 3). APX activity also decreased in Al treated roots. We observed about 35% ($p \leq 0.05$) and 56% ($p \leq 0.001$) decrease in APX

activity in roots treated with 100 µM and 250 µM Al. In contrast to roots, the constitutive activity of APX was observed to be increased by 42% ($p \leq 0.05$) and 70% ($p \leq 0.01$) in leaves (Fig 4). A profound dose dependent increase in GPX activity was observed in Al treated roots and leaves. There was about 68% ($p \leq 0.01$) and 80% ($p \leq 0.001$) increase GPX activity in roots while in leaves this increase was about 75% ($p \leq 0.01$) and 90% ($p \leq 0.01$) compared to the controls (Fig 5). A trend, similar to GPX was observed for SOD activity in Al treated sorghum roots and leaves. SOD activity was increased about 95% ($p < 0.01$) above the control level in roots treated with 100 µM Al for 12 days, while further increase in Al concentration caused reduction in SOD activity to normal level. In leaves, we observed about 110% ($p < 0.001$) and 140% ($p < 0.01$) increase in SOD activity above the control level under 100 µM and 250 µM Al treatments respectively (Fig 6).

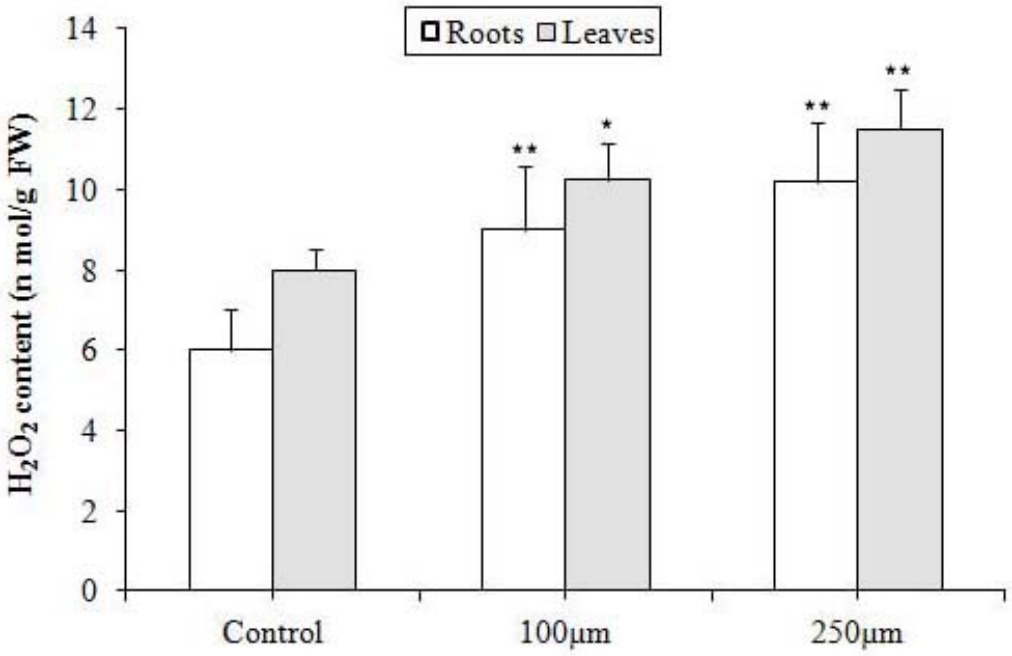

Fig 2: H_2O_2 content in sorghum roots and leaves treated with 100 and 250µM Al for 12 days. Values are mean ± S.D. (N=3). **$p < 0.01$ significantly different from control; *$p < 0.05$ significantly different from control.

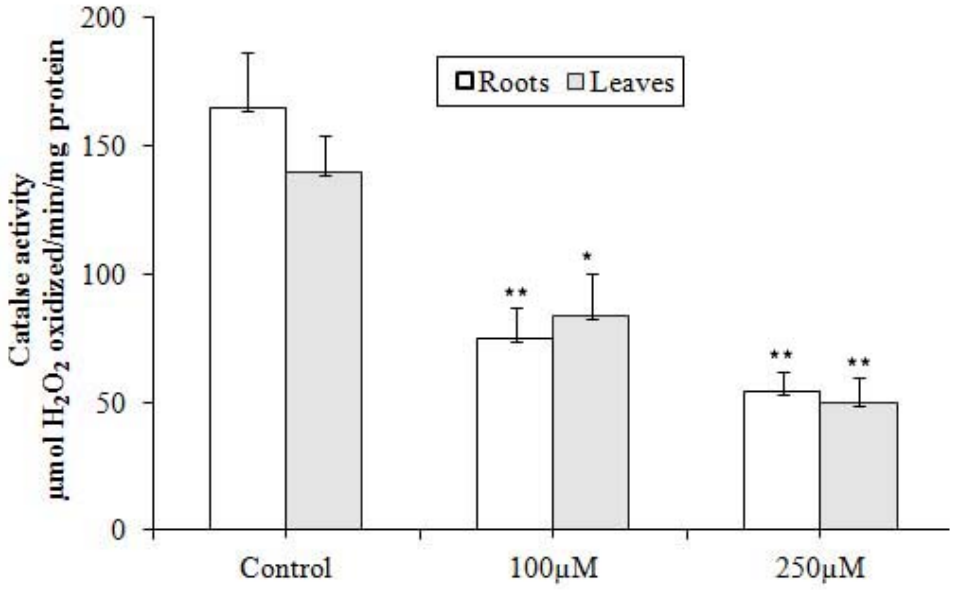

Fig 3: Catalase activity in sorghum roots and leaves treated with 100 and 250µM Al for 12 days. Values are mean ± S.D. (N=3). **p<0.01 significantly different from control; *p<0.05 significantly different from control.

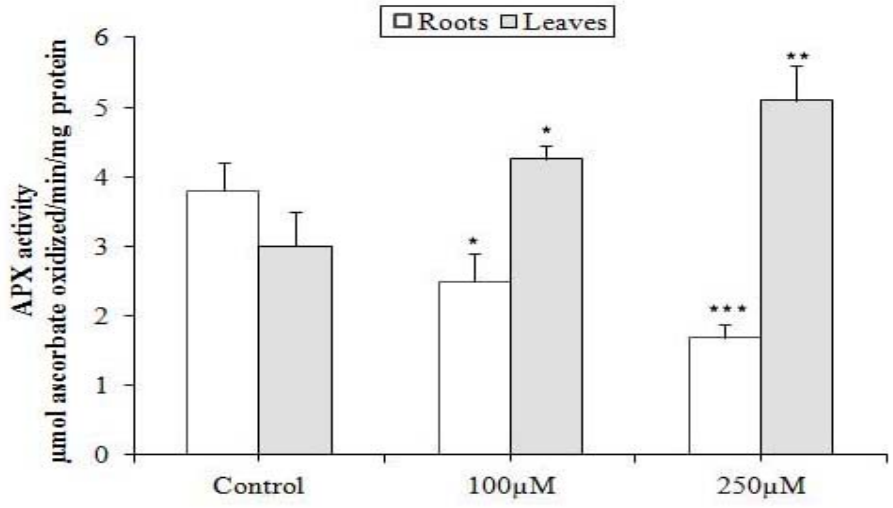

Fig 4: Ascorbate peroxidase activity in sorghum roots and leaves treated with 100 and 250µM Al for 12 days. Values are mean ± S.D. (N=3). ***p<0.001 significantly different from control; **p<0.01 significantly different from control; *p<0.05 significantly different from control

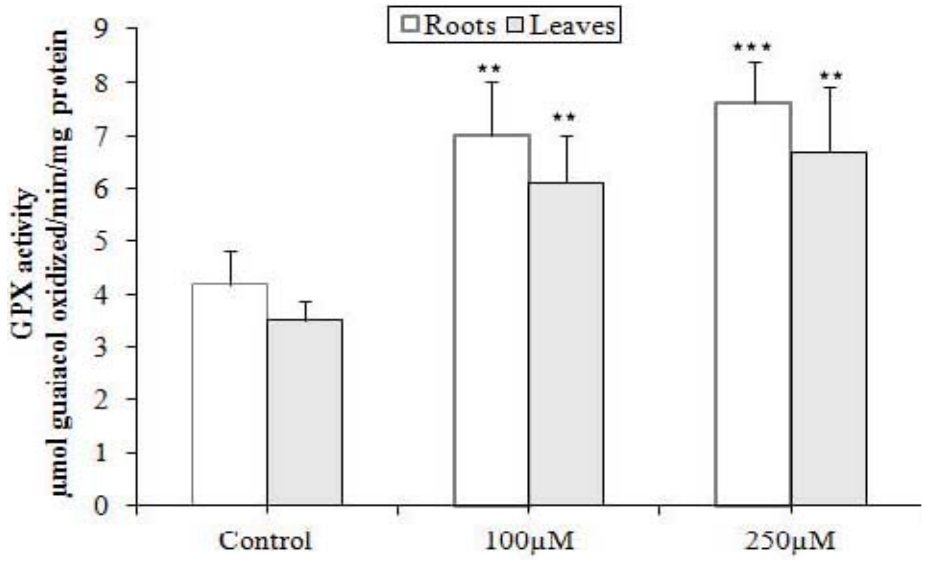

Fig 5: Guaiacol peroxidase activity in sorghum roots and leaves treated with 100 and 250µM Al for 12 days. Values are mean ± S.D. (N=3). ***p<0.001 significantly different from control; **p<0.01 significantly different from control

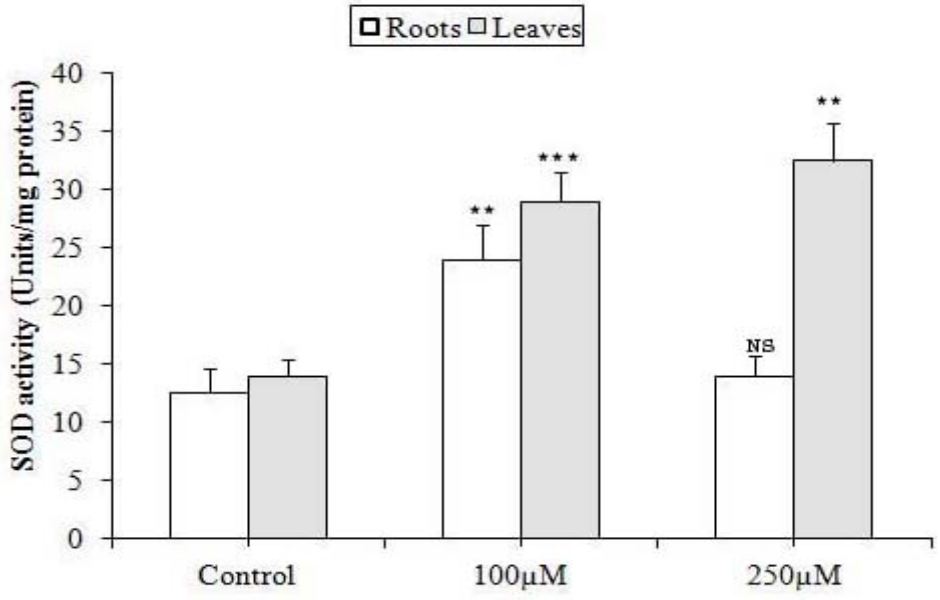

Fig 6: Superoxide dismutase activity in sorghum roots and leaves treated with 100 and 250µM Al for 12 days. Values are mean ± S.D. (N=3). ***p<0.001 significantly different from control; **p<0.01 significantly different from control; NS-not significant.

Antioxidant Gene expression

It may be possible that the decrease or increase in antioxidant enzymes might result from altered gene expression. To determine the possibility whether Al induced oxidative stress in sorghum roots and leaves were regulating the expression of antioxidant genes, we performed semi-quantitative RT-PCR analysis. Our study showed that there was decrease in CAT gene expression both in roots and leaves which is in concordance to the biochemical study for this enzyme. The expression of SOD and GPX were up regulated in Al treated roots and leaves. In case of APX, the expression was decreased in roots and stimulated in leaves (Fig7a & b).

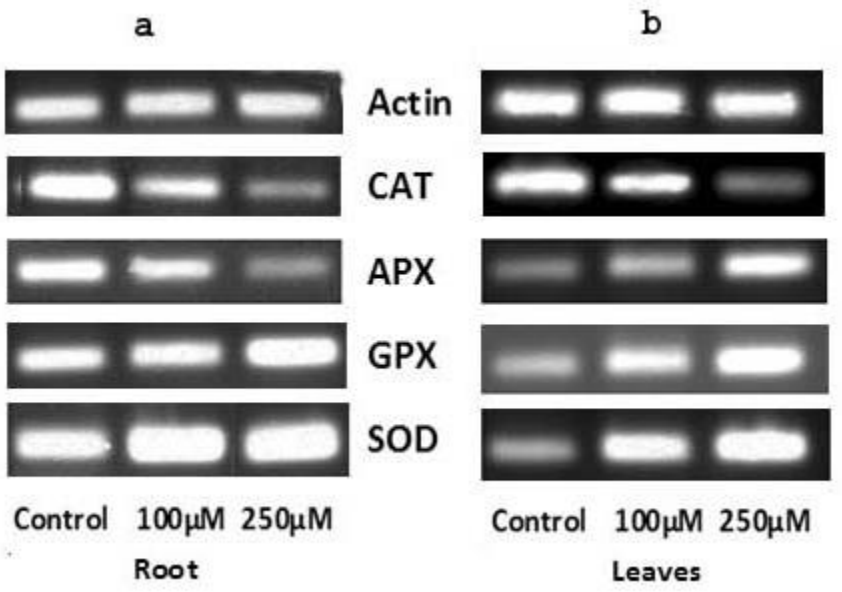

Fig 7: Reverse Transcriptase (RT)-PCR showing changes of antioxidant genes expression in the root (a) and leaves (b) of Sorghum under 100 and 250µM Al treatment.

Effect of Al treatment on lipid peroxidation and proline content

Lipid peroxidation, measured as MDA levels, was observed to be elevated only in Al exposed roots and this increase in MDA level was more with increase in Al concentration. The increase in MDA level in sorghum roots was found to be about 35% ($p \leq 0.05$) and 52% ($p \leq 0.05$). In contrast to roots there was no significant change in lipid peroxide content in leaves compared to controls (Fig 8). The proline content was found to be accumulated both in roots and leaves. A significant increase of about 50% ($p \leq 0.01$) in

proline was observed in 100 µM Al treated roots while increase was about 65% ($p \leq 0.05$) in 250 µM Al treated roots (Fig 9). Similarly, a significant increase of 40% ($p \leq 0.01$) and 70% ($p \leq 0.01$) in proline was observed in leaves following 100 and 250 µM Al treatments (Fig 9).

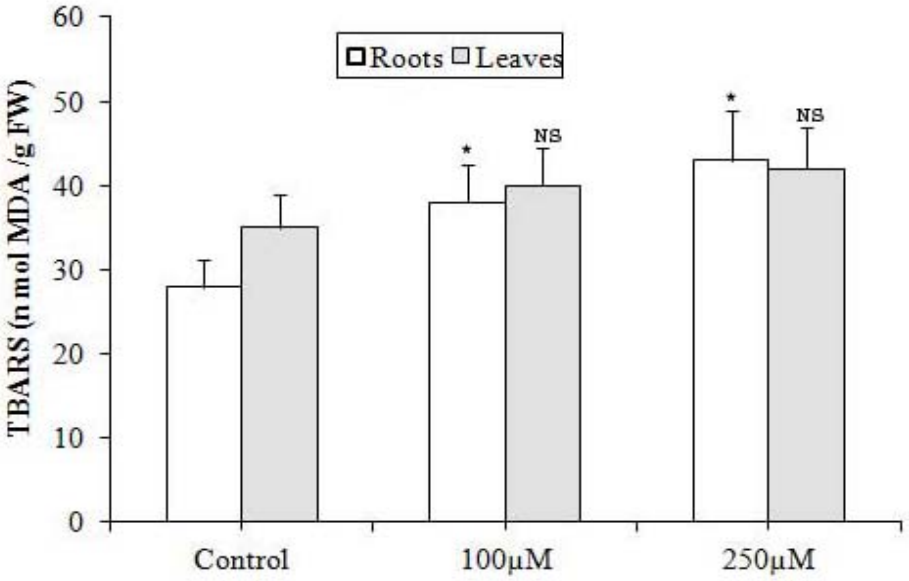

Fig 8: Lipid peroxidation measured as MDA level in sorghum roots and leaves treated with 100 and 250µM Al for 12 days. *p<0.05 significantly different from control; NS – not significant

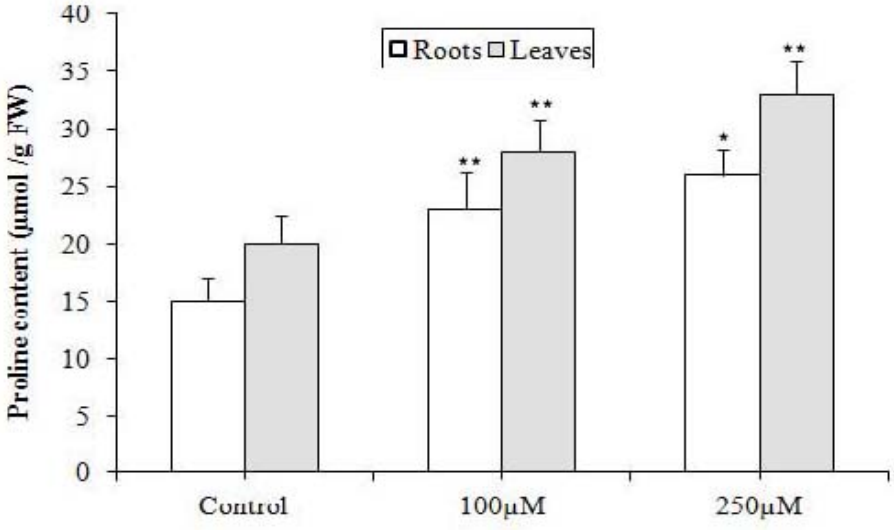

Fig 9: Proline content in sorghum roots and leaves treated with 100 and 250µM Al for 12 days. Values are mean ± S.D. (N=3). **p<0.01 significantly different from control; *p<0.05 significantly different from control.

Effect of Al treatment on protein oxidation

Al treatment to sorghum caused increase in protein carbonyl content in roots and leaves. In roots, the carbonyl content increased significantly by 48% ($p \leq 0.01$) with 100 µM Al treatment while carbonyl content was increased by 72% ($p \leq 0.001$) with 250 µM Al treatment. In leaves, there was increase in carbonyl content of about 36% ($p \leq 0.01$) and 52% ($p \leq 0.05$) compared to controls (Fig10).

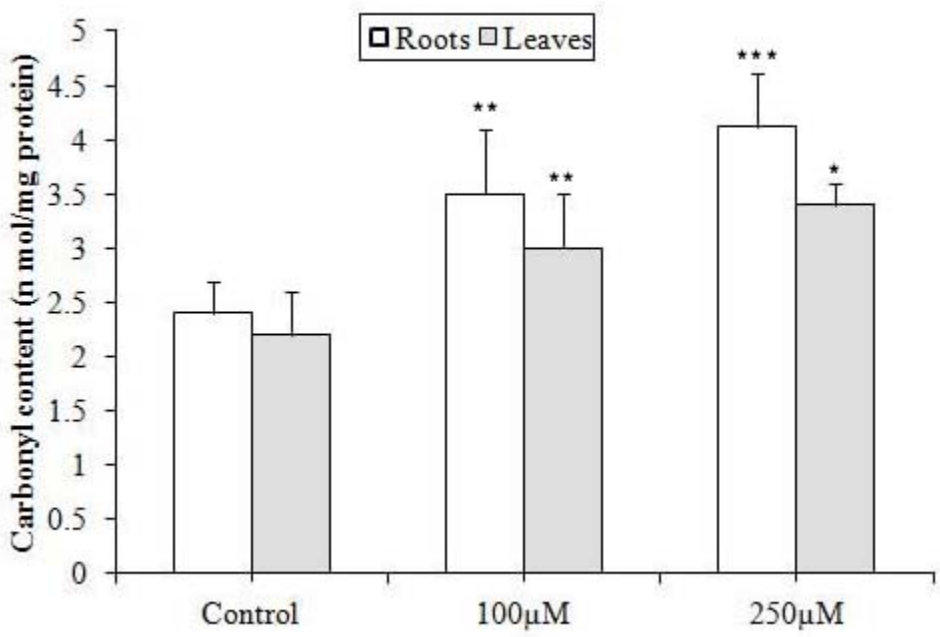

Fig 10: Protein carbonylation content in sorghum roots and leaves treated with 100 and 250µM Al for 12 days. Values are mean ± S.D. (N=3).). ***p<0.001 significantly different from control; **p<0.01 significantly different from control; *p<0.05 significantly different from control.

Discussion

Al toxicity is one of the major factors that inhibit plant growth and development in many acid soils (Kochian 1995). In our study, we also observed that Al toxicity affected the plant growth that is shown in terms of inhibition of root and shoot length of growing sorghum. The decrease in root or shoot length has been reported earlier in many plants (Ryan et al. 1992; Matsumoto 2000; Alvim et al. 2012) under Al toxicity. Various studies of Al toxicity in the roots suggest that production of ROS may significantly contribute to Al-induced inhibition of root elongation (Pereira et al. 2010; Yamamoto et al. 2001; Panda et al. 2009). We also observed increase in ROS in our study as evident by increased H_2O_2 and $O^{\cdot}{}_2^-$ levels in sorghum roots and leaves, suggesting generation of oxidative stress in sorghum.

The production of ROS in plants is counteracted by antioxidant enzymes such as SOD, CAT, APX, GPX or other antioxidant enzymes. SOD is considered the first line of defense against $O^{\cdot}{}_2^-$ by rapidly converting $O^{\cdot}{}_2^-$ to O_2 and H_2O_2 (Alscher et al. 2002). Our results indicate increased activity of SOD in Al treated roots and leaves. This increase in SOD activity might be attributed to the elevated production of superoxides, resulting in increased activity of enzyme or up regulated expression of the gene as was observed in our study. We also observed that the activity of SOD decreased to control level at higher Al treatment that may be due to increase in ROS i.e. excessof ROS might have declined SOD activity. Similar to our results, Pereira et al. (2010) showed that SOD activity was stimulated upto 500 µM Al treatments in cucumber and then declined with increase in Al conc. Al has been shown to enhance SOD activity in root tips of soybean (Cakmak and Horst 1991), roots of *Arabidopsis* (Richards et al. 1998) and roots of barley (Simonovicova et al. 2004). Lee et al. (2001) suggested that enhanced activity of SOD may function in signaling of oxidative stress, which leads to the induction of antioxidant enzymes associated with H_2O_2 scavenging system. In higher plants, a number of enzymes regulate intracellular H_2O_2 levels. CAT and APX are considered the most dominant enzymes in the removal of excess H_2O_2 from plants (Nakano and Asada 1981; Mittler et al. 2004; del Rio et al. 2006). We observed decrease in CAT activity in our study indicating inefficient removal of H_2O_2 under Al stress. The expression of CAT gene was also decreased suggesting that decline in CAT activity might be due to decrease of CAT

at molecular level. Sharma and Dubey (2007) have also shown the decline in CAT activity in rice seedlings with 80 µM of Al treatment. Recently, the study carried out by Panda and Matsumoto (2010) has also depicted decrease in CAT activity in Pea shoots following Al treatment. However, induction of CAT activity was shown by Pereira et al. (2010) in Al induced oxidative stress in cucumber. This difference in activity pattern may be attributed to different treatment conditions. APX has higher affinity for H_2O_2 than any other H_2O_2 scavenging enzymes (Sharma and Dubey 2007). In the present study, both activity and transcripts of APX decreased in roots that may indicate accumulation of ROS in cells. In contrast, Al treatment increased APX activity in leaves which was also supported by increased APX expression at transcriptional level. The activation of APX activity may suggest that it is involved in removal of excessive ROS in leaves. The reports available on effect of Al on APX activity have also been shown to be contradictory. Al has been reported to enhance the activity of APX in Cucurbita pepo (Dipierro et al. 2005) and rice (Sharma and Dubey 2007). The activity of chloroplastic-APX was shown to be inhibited by Al in rice (Sharma and Dubey 2007). Panda and Matsumoto (2010) did not observe any change in APX activity in Pea following Al exposure. The decline in APX activity in the roots and CAT activity in roots and leaves indicates insufficient removal of H_2O_2 and this in turn may induce SOD as observed in our study. GPX have been used as potential biomarkers for assessing metal-induced injury. The increase in GPX activity in present study indicates that GPX helps in tolerance and scavenging of H_2O_2 to some extent in sorghum roots and leaves. The increased activity might also be attributed to a damage response to Al. Jan et al. (2001) have shown that Al induced POD activity in Al-sensitive rice cultivar whereas in Al-tolerant cultivar they were unaffected by Al treatment. Recently, GPX activity was also shown to be increased in both roots and shoots of Al stressed Pea seedlings (Panda and Matsumoto 2010).

Production of ROS has been shown to cause lipid peroxidation, enzyme inactivation and oxidation of proteins (Dat et al. 2000). Lipid peroxidation is an effective indicator of cellular oxidative damage (Verma and Dubey 2003). The observed increase in lipid peroxide content with increasing Al treatments suggests that Al induces oxidative stress in growing sorghum roots which coincide with decrease in CAT and APX activities

and gene expression. Many recent studies have also shown the increase in lipid peroxidation and elevated ROS levels in many plant species exposed to toxic levels of Al (Yamamoto et al. 2001; Pereira et al. 2010; Navascues et al. 2012). In leaves, there was no significant change in lipid peroxide content suggesting that biochemical and genetic activation of APX, GPX and SOD conferred protection from Al induced ROS generation. The increased carbonyl content is a result of protein modification both in roots and leaves as evident by increased ROS under Al stress. Proline occurs widely in plants and normally accumulates in large quantities in response to environmental stresses (Rhodes et al. 1999; Hsu et al. 2003; Kavi Kishore et al. 2005). Proline accumulation in plants may be attributed to a protection from ROS under oxidative stress conditions. Although, proline was found to be accumulated in roots but it was inefficient to provide protective mechanism from ROS in roots as suggested by increased MDA levels in roots.

In conclusion, our results suggest that Al toxicity induces oxidative stress in sorghum roots and leaves resulting in increased generation of ROS which in turn; might alter antioxidant enzyme system. The inefficient removal of these ROS may further induce oxidative damage to membranes as evident by increased lipid peroxidation in roots but the stimulation of SOD, APX and GPX both at activity and gene level combated protection against oxidative damage in leaves.

References

1. Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J Exp Biol 531: 1331–1341. doi: 10.1093/jexbot/53.372.1331
2. Alvim MN, Ramos FT, Oliveira DC, Isaias RMS, França MGC (2012) Aluminium localization and toxicity symptoms related to root growth inhibition in rice (Oryza sativa L.) seedlings. J Biosci 37: 1079–1088. Doi: 10.1007/s12038-012-9275-6
3. Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39: 205-207. doi: 10.1007/BF00018060
4. Beauchamp CH, Fridovixh I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276-287. doi: 10.1016/0003-2697(71)90370-8
5. Boscolo PRS, Menossi M, Jorge RA (2003) Aluminum-induced oxidative stress in maize. Phytochemistry 62:181-189. doi.org/10.1016/S0031-9422(02)00491-0

6. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

7. Cakmak I, Horst WJ (1991) Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). Physiologia Plantarum 83:463-468. doi: 10.1111/j.1399-3054.1991.tb00121.x

8. Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Van Breusegem F (2000) Dual action of the active oxygen species during plant stress responses. Cell Mo. Life Sci 57:779-795. Doi: 10.1007/s000180050041

9. del Rio LA, Sandalio LM, Corpas FJ, Palma JM, Barroso JB (2006) Reactive Oxygen Species and Reactive Nitrogen Species in Peroxisomes. Production, Scavenging and Role in Cell Signaling**.** Plant Physiol 141: 330–335. doi: 10.1104/pp.106.078204

10. Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 32:93-101. doi: 10.1093/jxb/32.1.93

11. Dipierro N, Mondelli D, Paciolla C, Brunetti G, Dipierro S, (2005) Changes in the ascorbate system in the response of pumpkin (*Cucurbita pepo* L.) roots to aluminium stress. J of Plant Physio 162:529-536. doi: 10.1016/j.jplph.2004.06.008

12. Ezaki B, Gardner RC, Ezaki Y, Matsumoto H, (2000) Expression of aluminium-induced genes in transgenic Arabidopsis plants can ameliorate Al stress and/or oxidative stress. Plant Physiol 122:657-665. doi: 10.1104/pp.122.3.657

13. Hsu SY, Hsu YT, Kao CH (2003) The effect of polyethylene glycol on proline accumulation in rice leaves. Biol Plant 46:73-78. doi: 10.1023/A:1022362117395

14. Jan F, Yamashita K, Matsumoto H, Maeda M (2001) Protein and peroxidase changes in various root-cell fractions of two upland rice cultivars differing in Al tolerance. Environ Exp Bot 46: 141-146. doi: 10.1016/S0098-8472(01)00091-0

15. Kavi Kishor PB, Sangam S, Amruth RN, Sri Laxmi P, Naidu KR, Rao KRSS et al. (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Current Science 88:424-438.

16. Kiba A, Miyake C, Toyoda K, Ichinose Y, Yamada T, Shiraishi T (1997) Superoxide generation in extracts from isolated plant cell wall is regulated by fungal signal molecules. Phytopathology 87: 846- 852. doi: 10.1094/PHYTO.1997.87.8.846

17. Kochian LV (1995) Cellular mechanisms of aluminum toxicity and resistance in Plants. Annu Rev Plant Physiol Plant Mol Biol 46:237-260. doi: 10.1146/annurev.pp.46.060195.001321

18. Lee DH, Kim YS, Lee CB (2001) The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.). J Plant Physiol 158:737-745. doi: 10.1078/0176-1617-00174
19. Levine RL, Stadtman ER, Shacter E (1994) Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 233:346-357.
20. Ma JF, Ryan PR, Delhaize E (2001) Aluminum tolerance in plants and the complexing role of organic acids. Trends Plant Sci 6: 273–278. doi: 10.1016/S1360-1385(01)01961-6
21. Maron LG, Kirst M, Mao C, Milner MJ, Menossi M, Kochian LV (2008) Transcriptional profiling of aluminum toxicity and tolerance responses in maize roots. New Phytologist 179: 116–128. doi: 10.1111/j.1469-8137.2008.02440.x
22. Matsumoto H (2000) Cell biology of aluminum toxicity and tolerance in higher plants. Int Rev Cytol 200:1-46. doi: 10.1016/S0074-7696(00)00001-2
23. Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9: 490–498. doi: 10.1016/j.tplants.2004.08.009
24. Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867-880.
25. Navascues J, Perez-Rontome C, Sanchez DH, Staudinger C, Wienkoop S, Rellan-Alvarez R, Becana M (2012) Oxidative stress is a consequence, not a cause, of aluminum toxicity in the forage legume Lotus corniculatus. 193: 625-636. doi: 10.1111/j.1469-8137.2011.03978.x.
26. Panda S, Matsumoto H (2007) Molecular physiology of aluminum toxicity and tolerance in plants. Bot Rev 73: 326–347. doi: 10.1663/0006-8101(2007)73[326:MPOATA]2.0.CO;2
27. Panda SK, Baluska F, Matsumoto H (2009) Aluminium stress signaling in plants. Plant Signal Behav 4:592-597.
28. Panda SK, Matsumoto H (2010) Changes in antioxidant gene expression and induction of oxidative stress in pea (*Pisum sativum* L.) under Al stress. Biometals 23:753-762. doi: 10.1007/s10534-010-9342-0
29. Pereira LB, de A Mazzanti CM, Gonçalves JF, Cargnelutti D, Tabaldi LA, Becker AG et al. (2010). Aluminum-induced oxidative stress in cucumber. Plant Physiol Biochem 48: 683-689. doi: 10.1016/j.plaphy.2010.04.008
30. Rhodes D, Verslues PE, Sharp RE (1999) Role of amino acids in abiotic stress resistance. In: Singh BK (ed.) Plant Amino Acids Mercel Dekker Inc, New York, 319-356.
31. Richards KD, Schott JE, Sharma YK, Davis KR, Gardner RC (1998) Aluminum induces oxidative stress genes in Arabidopsis thaliana. Plant Physiol 116:409-418. doi: org/10.1104/pp.116.1.409

32. Ryan PR, Shaff JE, Kochian LV (1992) Aluminium toxicity in roots: correlation among ionic currents, ion fluxes, and root elongation in aluminium sensitive and aluminiumtolerant cultivars. Plant Physiol 99:1193-1200. doi: 10.1104/pp.99.3.1193

33. Sagisaka S (1976) The occurrence of peroxide in a perennial plant Populas gelrica. Plant Physiol 57:308-309.

34. Sharma P, Dubey RS (2007) Involvement of oxidative stress and role of antioxidative defense system in growing rice seedlings exposed to toxic concentrations of aluminum. Plant Cell Rep 26:2027-38. Doi: 10.1007/s00299-007-0416-6

35. Simonovicova M, Tamas L, Huttova J, Mistric I (2004) Effect of aluminium on oxidative stress related enzymes activities in barley roots. Biologia Plantarum 48:261-266. Doi: 10.1023/B:BIOP.0000033454.95515.8a

36. Thirkettle-Watts D, McCabe TC, Clifton R, Moore C, Finnegan PM, Day DA, Whelan J (2003) Analysis of the alternative oxidase promoters from soybean. Plant Physiol 133: 1158–1169. doi: 10.1104/pp.103.028183

37. Verma S, Dubey RS (2003) Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci 164:645-655. doi: 10.1016/S0168-9452(03)00022-0

38. Vitoria AP, Lea PJ, Azevedo RA (2001) Antioxidant enzymes responses to cadmium in radish tissues. Phytochemistry 57:701-10. doi: org/10.1016/S0031-9422(01)00130-3

39. Yamamoto Y, Kobayashi Y, Matsumoto H (2001) Lipid peroxidation is an early symptom triggered by aluminum, but not the primary cause of elongation inhibition in pea roots. Plant Physiology 125:199-208. doi: 10.1104/pp.125.1.199

Annexure III

Summary of the Findings

The earth is gradually polluting with the releasing of several heavy metals from industries. These metals are accumulated and showed their toxic effect in plant system. Aluminium (Al) is distributed in water, soil and air but most of the Al is stored as aluminosilicate soil minerals in the earth and toxic to plants only in acidic solid. Al toxicity promotes oxidative damage in plants. Being amongst the most abundant elements of the planet, Al exposure can cause oxidative stress and lead to various signs of toxicity in plants. The toxicity of Al results in decreased crop productivity. In this study we have studied the effect of Al treatment (100 µM and 250 µM) on induction of oxidative stress and changes in antioxidant gene expression in sorghum bicolor (cv AN 2000). Al treatment increased superoxide and H_2O_2 content and protein oxidation in both roots and leaves of sorghum. The lipid peroxide measured as MDA levels increased only in roots. The catalase (CAT) activity decreased both in roots and leaves while ascorbic peroxidase (APX) activity decreased in roots and increased in leaves. The activities of guaicol peroxidase (GPX) and superoxide dismutase (SOD) were found to be increased in dose dependent manner both in roots and leaves. The proline content accumulated with increase in aluminium conc. Further, to get insights into the molecular response, the antioxidant gene expression profile was also evaluated at both the treatments. The expression of SOD and GPX genes was up-regulated in roots and leaves while CAT showed decrease in expression. APX was observed to be down regulated in roots and up regulated in leaves. Our results indicate that Al toxicity generated oxidative damage in

roots as indicated by increased level of lipid peroxidation but stimulated antioxidant enzymes conferred protection from oxidative damage in leaves.

Annexure IV

CONTRIBUTION TO THE SOCIETY

Sorghum is the most important cereal crop grown not only in India, Africa and Australia but also in America. Sorghum bicolor is an important crop in semiarid regions. . It is well suited to regions of moderate rainfall. There is little information on response of antioxidant enzymes under aluminium toxicity induced oxidative stress in this plant. It has been expected that some of the stress-induced genes can relate to the resistant mechanism for the stress. It might therefore become imperative to understand the mechanism of the gene-induction by aluminium stress that may be important in unrevealing the mechanism of the resistance to Al. By exploring the mechanism of changes in Al-induced oxidant stress and antioxidant systems might help to verify the hypothesis that some antioxidants besides their function in detoxification, may also be sensitive targets of Al toxicity in plants. Moreover, the basic information about the response mechanism to Al stress could be applied to other stresses that have similar response mechanisms to Al stress.

PUBLICATIONS OUT OF THE PROJECT

1. Chandra Prakash and **Vijay Kumar.** Transcriptional and enzymatic regulation of antioxidant enzymes in aluminium induced oxidative stress in Sorghum roots and leaves. *International journal of current research. 2014:6(2); 4858-65.*