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Influence of triacontanol on glutathione metabolism in radish (*Raphanus sativus* L.) seeds under Cd-induced oxidative stress

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Abstract
The present study was undertaken to study the effect triacontanol (TRIA) on the growth, antioxidant enzyme activities and glutathione metabolism in radish (*Raphanus sativus* L.) seedlings subjected to cadmium (Cd) stress. Cd at 1mM conc decrease thiol content and total glutathione levels, a clear indication of oxidative stress. TRIA supplemented to Cd (1mM) treated seeds effectively restored total glutathione levels and maintained cellular redox status in equilibrium. Cd (1mM) induced lipid peroxidation and increased H_2O_2 level but inhibited glutathione reductase (GR, E.C. 1.6.4.2) enzyme activity. Further, supplementation of TRIA restored and enhanced the functional activity of ascorbate peroxidase (APX, E.C. 1.11.1.11), γ-Glutamylcysteine synthetase (γ-GCS, E.C. 6.3.2.2) and glutathione-*S*-transferase (GST, E.C. 2.5.1.18) activity to a greater extent then Cd-alone treatment. These results clearly indicate the protective role of TRIA in modulating the redox status of the plant system through the antioxidant pathway for combating Cd-induced oxidative stress.

Keywords: cadmium, glutathione metabolism, oxidative stress, radish, redox status, triacontanol

Introduction

Among many abiotic stresses influencing plant growth and development, heavy metal toxicity is very important, especially if crop species are grown on sites in the vicinity of heavy industry (Bi *et al*., 2006) [1]. Heavy metals hamper the growth of plants by disturbing many biochemical, physiological and metabolic processes. Cadmium (Cd) is a non-essential heavy metal pollutant naturally present in the environment. Cd is considered the most toxic heavy metal, causing a significant damaging effect on general plant metabolism (Benavides *et al*., 2005), including oxidative stress. Mining and smelting industries are the main sources of Cd. The relationships between Cd toxicity and oxidative stress have been studied in many systems and heavy metal contamination has often been implicated as the root cause of oxidative injury to the plants. The production of reactive oxygen species (ROS), key step in oxidative stress initiates a variety of auto oxidative chain reactions on membrane unsaturated fatty acids, producing lipid hydroperoxides and thereby cascade of reactions ultimately leading to destruction of organelles and macromolecules (Shaw *et al*., 2004) [15]. Oxidative stress also causes alteration in cellular redox homeostasis by disturbing the GSH/GSSG ratio. Under excessive stressful conditions, cells try to remove ROS by a complex network of non-enzymic (ascorbate, glutathione, carotenoids) and enzymic (SOD, GPX, APX, GR, CAT) antioxidant systems (Veena *et al*., 1999). Out of these antioxidant systems, glutathione has been proposed to be a signal molecule in defense against most of the biotic and abiotic stresses (Noctor *et al*., 2002). Recently glutathione has been shown to regulate the expression of genes whose products are involved in redox regulation or enhancement of stress tolerance. All cells exhibit two forms of glutathione, oxidized (GSSG) and reduced (GSH), interchanging with each other to maintain redox potential under different conditions. GSH is used directly by glutathione peroxidase (GPX), glutathione-S-transferase (GST) and indirectly by ascorbate peroxidase (APX) as a result of which oxidized (GSSG) level rises which is toxic to the plant cell. Therefore, glutathione homeostasis that involves high level of reduced to oxidized glutathione should be maintained inside the cell for their survival. Glutathione reductase (GR) reduces GSSG to GSH and that helps in regaining the cell of their homeostatic GSH/GSSG ratio. In addition to this, glutathione performs a number of functions like maintenance of active form of proteins, xenobiotic conjugation, heavy metal detoxification and others. In view of its main role in various metabolic functions, manipulation of glutathione levels can be used to improve the tolerance of plants against abiotic stress.

Plant growth regulators (PGRs) play an important role in agriculture and proper applications of PGRs not only regulate plant growth but also enhance resistance to various environmental stresses. Triacontanol (TRIA), a saturated primary alcohol, is a natural component of plant epicuticular waxes (Crosby and Vlitos, 1959) [5] and has plant growth enhancing properties (Ries *et al*., 1977) [23]. TRIA is used to increase crop yields on millions of hectares, particularly in Asia (Chen *et al*., 2002) [4]. Apart from their role in plant growth and development, TRIA are involved in protecting plants from a broad spectrum of stresses such as salinity, high temperature and heavy metal stress (Krishnan and Kumari, 2008) [21]. Although much efforts have been made to recommend TRIA as highly promising and eco-friendly plant growth regulator for a wide spread utilization in agriculture, horticulture and to protect against environmental stresses. However, the underlying mechanism for TRIA-mediated plant growth and development, stress tolerance are still poorly understood. Considering the protective role of TRIA against stresses the purpose of present work was to examine the role of TRIA in

protecting radish seedlings by regulating both glutathione metabolism and antioxidative system under Cd stress.

Material and Methods

Plant material

Seeds of radish were purchased from National Seeds Corporation, Hyderabad, India. Cadmium in the form of cadmium chloride ($CdCl_2.H_20$) was used. Different concentrations used for cadmium- 0.5mM, 1mM and 1.5mM to find out where the substantial reduction, but not complete suppression was observed in radish seedlings. Cd at 1.0mM concentration caused substantial reduction in the radish seedlings, and this concentration were selected as stress concentration for the experiment. TRIA was procured from Godrej Vipul, Mumbai, India. Similarly, from a wide range of concentrations 10 µg and 20 µg of TRIA were selected where significant growth stimulation was observed. Radish seeds were surface sterilized with 0.5% (v/v) sodium hypochlorite solution and thoroughly washed with sterile distilled water. Seeds were soaked for 24 h in either (i) distilled water (control), (ii) 10/20 µg TRIA (iii) 1mM cadmium solution (stress control), (iii) 1mM Cd supplemented with 10/20 µg TRIA. For each treatment 20 seeds were placed per sterile petri plates of 15cm diameter, layered with Whatman No.1 filter papers. The plates were kept in a dark room at 25±1ºC. On 6th day estimation of antioxidant enzymes were assayed. At the end of the experiment (7th day) seedling length, fresh mass and dry mass of the seedlings were recorded.

Lipid peroxidation

Lipid peroxidation in radish seedlings was determined by estimating the malondialdehyde (MDA) content following the method of Hearth and Packer (1968). 1 g of seedlings was homogenized in 5ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000g for 15min. For every 1ml of aliquot of the supernatant, 40 ml of 0.5% thiobarbituric acid in 20% TCA was added. The mixture was heated at 95ºC for 30 min and then quickly cooled in an ice bath. After centrifugation for 10 min at 10,000 g, the absorbance of the supernatant was measured at 532nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated by using extinction coefficient of $155mM^{-1}$ cm^{-1}.

Hydrogen peroxide (H_2O_2) level

H_2O_2 was calorimetrically measured by Jana and Choudhuri (1981) [12] method. 50 mg of the seed material was homogenized with 3 ml of phosphate buffer (50mM pH 6.8) and centrifuged for 25 min at 6,000 g. 3 ml of the extract solution was mixed with 1ml of 0.1% titanium chloride in 20 % v/v H_2SO_4 and the mixture was then centrifuged at 6,000g for 10 min. The intensity of yellow color of the supernatant at 410nm was measured. H_2O_2 was calculated using the extinction co-efficient 0.28 $\mu mol^{-1}cm^{-1}$.

Lipoxygenase (LOX, E.C. 1.13.11.12) activity

The assay of lipoxygenase activity performed according to Ederli *et al*. (1997) [6]. 300 mg of seedling material was homogenized in 50mM sodium phosphate buffer (pH 7.0), 1mM EDTA, 0.1mM PMSF, 2 % (w/v) polyvinyl pyrrolidone (PVP), 1% (v/v) glycerol and 0.1 % (v/v) tween 20. The extract was centrifuged at 15,000 x g for 20 min and the supernatant was immediately used for the assay of lipoxygenase activity. Lipoxygenase was measured spectroscopically at room temperature by addition of 1 mM linoleic acid in 0.1 M sodium acetate buffer (pH 5.6) to the extract and measuring the increase of absorbance at 240 nm. The extinction coefficient of $(25\ mM\ L^{-1})^{-1}\ cm^{-1}$ was used to convert absorbance values to µmoles of conjugated diene. One unit of activity was defined as the amount of enzyme catalyzing the synthesis of 1µmol of hydroperoxide (HPOD) min^{-1}

Measurement of total, protein bound and non-protein thiols

The treated seedlings were homogenized in 0.02 M EDTA under cold conditions. The thiol contents of the homogenates were measured using Ellman's reagent [5, 5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB)] (Sedlak and Lindsay 1968) [24].

Total thiols (T-SH)

Aliquots of 0.5 ml of the homogenates were mixed with 1.5 ml of 0.2 M Tris buffer (pH 8.2) and 0.1 ml of 0.01 M DTNB. The colour was allowed to develop for 15 min. The absorbance of the clear supernatant was read at 412 nm (ε=13,100)

Non-protein thiols (NP-SH) and protein bound thiols (PB-SH)

Aliquots of 5 ml of the homogenates were mixed with 4 ml of distilled water and 1 ml of 50% TCA. The contents were mixed and after 15 min the tubes were centrifuged at 10.000 x g for 15 min. Two ml of the supernatant was mixed with 4 ml of 0.04 M Tris buffer pH 8.9), 0.1 ml of DTNB and absorbance was read within 5 min at 412 nm against a reagent blank. The protein bound thiols (PB-SH) were calculated by subtracting the non-protein thiols from protein thiols (Sedlak and Lindsay, 1968) [24].

Cellular glutathione content

Total glutalthione [GSH (reduced) + GSSG (oxidized)] content was estimated according to the method of Hissin and Hilf (1976) [10]. One gram of seedlings were homogenized with 10 ml Tris EDTA (pH=8.2) and centrifuged at 25,000 g for 30 minutes at 4^0 C. From the homogenate about 0.3 ml (300 µl) was pipetted into 1 ml of tube to which 60 µl of 25% phosphoric acid is added and kept in ice for 5 minutes, centrifuged at 25,000g for 30 minutes at 4^0 C. Supernatant was collected for the estimation of GSH and GSSG. For reduced glutathione (GSH) estimation, to 50 µl of supernatant, 0.45 ml (450 µl) of cold phosphate EDTA buffer (pH=8) was added and mixed thoroughly. 25 and 50 µl aliquots of this was taken into 5 ml test tubes and made up to 100 µl with cold glass distilled water. 1.8 ml of phosphate EDTA buffer was added to the tubes and mixed. 100 µl of OPT (ortho-phthalaldehyde) solution was then added and after thorough mixing, incubated at room temperature (25^0C) for 15 minutes fluorescence was measured in JASCO, FP-750 Spectrofluorometer at wavelength of 350 nm and 420 nm. For Oxidized glutathione (GSSG) estimation, 50 µl aliquot was incubated at room temperature (25^0C) with 20 µl of NEM (N-ethyl malcimide) reagent for 30 minutes. To the above mixture 0.43 ml (430 µl) of 0.1 N NaOH was added and mixed thoroughly. From this 50 and 100 µl aliquots were taken into 5 ml test tubes and made up to 100 µl with

glass distilled water. 100 µl of OPT was added and to this mixture 1.8 ml of 0.1 N NAOH was added and incubated at room temperature for 15 minutes. After incubation, fluorescence was measured in JASCO, FP-750 spectrofluorometer at 350 nm and 420 nm.

Ascorbate glutathione cycle

Ascorbate peroxidase (APX, E.C. 1.11.1.11)

The reaction mixture contained 50mM phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 250 mM H_2O_2 and 50µg of protein. The activity of APOX was measured spectophotometrically by measuring the rate of ascorbate oxidation at 290 nm for 1min. The amount of ascorbate was calculated from the extinction coefficient of 2.6 mM^{-1} cm^{-1} by the method of Nakano and Asada (1981) [19].

Glutathione reductase (GR, E.C. 1.6.4.2)

The assay was performed according to Jiang and Zhang (2001) [13]. The reaction mixture contained 500µL of sodium phosphate buffer pH 7.0, 100µL each of 10mM GSSG, 1mM NADPH and 180 µL of distilled water. The reaction was started by addition of protein and NADPH oxidation was recorded as the decrease in absorbance at 340nm for1 min. The activity was calculated using the extinction coefficient for NADPH ε= 6.22 $mM^{-1}cm^{-1}$.

Glutathione metabolism enzymes

γ-Glutamylcysteine synthetase. (γ-GCS, E.C. 6.3.2.2)

The assay performed following the methodology of Nagalakshmi and Prasad (2001) [18]. The reaction mixture for assaying γ-GCS contained 50µL each of 0.2 M sodium glutamate, 0.2 M L-amino butyrate, 40 mM Na_2-EDTA, 0.4% BSA, 100µL each of 0.2M $MgCl_2$, 50mM Na_2-ATP and 500µL of 0.2 M Tris-HCl (Ph 8.2). It was pre incubated for 2 min at room temperature and the reaction was started by addition of 50µg protein.

Glutathione S-transferase (GST, E.C. 2.5.1.18)

The assay was performed according to Habig and Jacoby (1981) [8]. The reaction mixture for assaying GST consisted of 50 µl of 0.2M potassium phosphate buffer pH (7.0), 100 µl of 0.1M 1-chloro, 2,4-dinitrobenzene and 390 µl of distilled water. The reaction was started by the addition of protein and the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction coefficient of the conjugate ε= 9.6 $mM^{-1}cm^{-1}$.

The data were analyzed by one-way ANOVA, followed by Post Hoc Test (Multiple Comparisons). The differences were considered significant if P was at least ≤ 0.05. The mean values have been compared and lower case alphabets are used in the figures to highlight the significant differences between the treatments.

Results

In the present study, Cd toxicity resulted in a substantial reduction in seedling growth and the inhibitory effect on seedling growth was ameliorated by the application of TRIA (Fig. 1A-C). TRIA at both conc. (10µg/20µg) caused a considerable increase in seedling growth under Cd stress and restored the growth to the level of unstressed control seedlings.

The content of MDA was greatly reduced in radish seeds treated with TRIA under Cd stress (Fig.2A). MDA content increased gradually under Cd stress by 196% when compared to control plants. However no such increase was found in TRIA treated seedlings in which the content was decreased gradually.

H_2O_2 content increased significantly under Cd stress (Fig.2B). Both the conc. of TRIA decreased the H_2O_2 content under stress conditions and alleviated Cd stress in radish seedlings.

The results of the activity of LOX also confirm the role of TRIA in membrane stabilization. Cd showed a very high increase (118%) in activity of lipolytic enzyme. This was decreased in Cd-treated seeds supplemented with TRIA up to 73% from the increased activity, indicating the inhibitory effect of TRIA on this fatty acid oxidizing enzyme and restoring the membrane stability (Fig. 2C). Seedlings with TRIA treatments only showed a very little decrease in LOX activity.

The levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) directly point towards the induction of oxidative stress by Cd. GSH levels showed a 48% decrease in Cd-alone treated seedlings and 56 % increase in the level of GSSG, over control they by altering the GSH/GSSG ratio more towards the oxidized form. TRIA supplementation to Cd-treated seedlings was effective not only in restoring the GSH but also indicated an increase in GSH level over Cd stressed seedlings (Fig.3B)

The activity of APX increased by 46% in Cd treated seedlings compared to control treatment (Fig.3C). Supplementation of TRIA to Cd-treated seeds further enhanced the APX activity.

The toxicity imposed by Cd on radish seedlings did not affect γ-GCS, an important enzyme involved in GSH biosynthesis. There was only a slight decrease in Cd-treatments which was restored by TRIA (Fig.4A)

The GR activity important for maintaining the reduced pool of glutathione was drastically reduced (25 %) in seeds treated with Cd (Fig.3D). Supplementation of Cd-treated seedlings with TRIA restored this impairment in GR activity over control.

GST an important cellular detoxifier of metabolites involved in oxidative stress, further it indicated the role of TRIA in alleviating Cd-induced oxidative stress (Fig.4B). GST showed a slight 15 % increase in seedlings with TRIA (µg) over control.

The total thiol content (T-SH) indicating the level of sulfydryl groups showed 35% reduction in Cd (1.0mM) treatment, while protein bound thiol (PB-SH) and non-protein thiol (NP-SH) showed 72% and 61% decrease respectively in Cd treated seeds in comparison with control. Cd- treated seedlings with TRIA supplementation showed 80% restoration of the T-SH levels and 95% in PB-SH and 74% in NP-SH levels (Fig. 3A)

Discussion

There was a considerable decrease in length, fresh and dry mass of radish seedlings when subjected to Cd stress as compared to unstressed radish seedlings. Similar decrease in seedlings growth of *Arabidopsis thaliana* due to heavy metal stress was reported by Li *et al*. (2005). However, the loss of plant growth under Cd stress was overcome by the supplementation on the stressed seedlings with TRIA, resulting in an increased biomass in terms of shoot growth (length, fresh and dry mass). Enhanced plant growth in stressed seedlings might be due to the primary effect of TRIA in promoting cell elongation and cell division, thus

alleviating metal stress. Similarly, TRIA was also found to ameliorate the impact of chilling stress on growth in sweet basil (Borowski and Blamowski, 2009) [3].

There is increasing evidence that metal stress induces the elevated levels of ROS, which causes oxidative damage to the plants (Shaw *et al*., 2004) [15]. Plant hormones are integrated in the regulation of stress response and plant development. In the present study the level of H_2O_2 increased significantly in the radish seedling under Cd stress; which showed the obvious decline in TRIA treated seedlings. TRIA slowed down the increase of ROS levels and alleviated the Cd-induced oxidative damage.

ROS accumulation may cause oxidative damage to plant cell membrane, forming toxic products such as MDA, a secondary end product of polyunsaturated fatty acid oxidation. Thus, MDA is an indicator of the degree of plant oxidative stress (Hodges *et al*., 1999) [11]. Treatment with TRIA resulted in a decrease in MDA content, which clearly suggested that TRIA may reduce metal injury of plant cell membrane due to lipid peroxidation therefore protecting the structural integrity of the membranes and resulting in the enhancement of metal tolerance. Ramanarayan *et al*. (2000) [22] also demonstrated that TRIA inhibits lipid peroxidation in tomato plants and increase in stress tolerance.

Increase in LOX activity by Cd clearly suggests higher lipolytic activity on the membrane and oxidation of membrane bound fatty acids leading to lipid peroxidation (Lacan *et al*., 1998) [14]. It is evident from the results that TRIA supplementation reduced both lipid peroxidation and LOX activity and ameliorated the effect of Cd. Mishra and Choudhuri (1999) [16] also reported that salicylic acid ameliorated the damaging effects of metal-induced membrane deterioration mediated by LOX in rice.

Cd toxicity affected the ascorbate-glutathione cycle (AGC) to a great extent. The first enzyme of AGC-ascorbate peroxidase (APX), which utilizes ascorbic acid in reduced form as a reductant is one of the most potent H_2O_2 scavenger in the plant cell (Shigeoka *et al*., 2002) [26]. Cd-treatments did not enhance APX to a great extent probably high levels of H_2O_2 formed in Cd-treatment became inhibitory to APX. Over expression of APX enhanced the tolerance to salt stress and water deficit in tobacco chloroplasts (Badawi *et al*., 2004) [2]. In TRIA supplemented Cd-treated seeds, a high induction of APX, much higher than Cd-alone treatment, were observed indicating the efficient control of H_2O_2 levels thereby preventing H_2O_2 mediated cell damage. The activity of GR is extremely sensitive to inhibition by heavy metals and by compounds that react with the –SH groups due to the presence of thiol groups at the active site of the enzymes (Nagalakshmi and Prasad, 2001) [18]. GR was inhibited by Cd, shifting the redox equilibrium of GSH to a more oxidative side (GSSG). TRIA enhanced the activity of this enzyme ensuring efficient cycling and utilization of the pyridine nucleotide reducing power. TRIA at 20µg increased GR by over Cd-treated seedlings.Results of thiols estimation and total glutathione estimation in Cd-1mM treatment indicated a drastic reduction of protective thiol groups with decrease in reduced glutathione and simultaneous increase in oxidized glutathione (GSSG) a true indication of oxidative stress. The toxicity of Cd is checked by various endogenous antioxidants where by the thiol pool of the plants play an important role (Potters *et al*., 2002) [20]. GSH, an important component of the thiol pool, functions as a stress indicator, promptly responding to oxidative stress. The formation of GSSG in Cd treated seeds could be due to the reaction of GSH with oxy radicals generated by the Cd, as in any other metal stress resulting in elevated GSSG. TRIA clearly restored the thiol pool and also restored GSH, with simultaneous decrease in the oxidized form (GSSG) as seen from the results of Cd-treated seedlings with supplementation of TRIA. The present study further indicated that radish seedlings tried to cope Cd-induced oxidative stress by increasing their antioxidant capabilities. Since, the AsA-GSH, the major cellular antioxidants in plants was significantly reduced under Cd stress. Both AsA and GSH are major cellular antioxidants and redox buffers; they also play important role in growth, development and stress responses (REF). The cellular content of AsA can be determined by DHAR, MDHAR and APX, which are the enzymes for glutathione biosynthetic pathway. APX is recognized as the most important peroxidase in H_2O_2 detoxification (Michalak 2006). Under Cd stress, increased activity of APX was observed, probably due to high levels of H_2O_2 detoxified by APX. TRIA treatment to Cd stressed seedlings further enhanced APX activity indicating the efficient scavenging of H_2O_2, preventing H_2O_2mediated cell damage. Like AsA, GSH is another important compound of plant antioxidant system. The cellular content of GSH can be determined by γ-GCS and GR, which are the enzymes for glutathione biosynthetic and recycling pathway respectively. In the present study under Cd stress GSH pool shifted to a more oxidized form as evidenced from the increase in GSSG/GSH ratio.

Upon TRIA treatment the cellular GSH pool shifted towards reduced form with concomitant decrease in GSSG/GSH ratio. This must be through active GSH biosynthesis and/or recycling capacity of γ-GCS and /or GR activity respectively. REFs GST is an important enzyme as it protects the cell by detoxifying harmful compounds. It catalyses the conjugation of GSH to a variety of electrophilic cytotoxic compounds, free radicals induced toxic compounds like membrane lipid peroxidase, products of oxidative DNA damage and degradative components of proteins that are finally targeted to the vacuole (Foyer *et al*., 2001) [7]. In the present study increase in GST activity by TRIA indicate enhanced activity of the enzyme to catalyze the conjugation of GSH to a variety of toxic compounds and protect the cell from harmful compounds,

Conclusion

The data given in the present study gives a clear impression that the presence of Cd causes stress and adversely affects both plant growth and metabolism. Further stress also activates the system to improve resistance capacity in plants under stress. Supplementation of TRIA, has an added effect both on growth, metabolism and enhanced the resistance capacity in the plants to over come the toxic effect of Cd. The study clearly demonstrated that TRIA is involved in the elevation of Cd stress by preventing the formation of ROS by inducing antioxidant enzymes. Further TRIA, also influenced glutathione pool that plays a crucial role in removing ROS and in maintaining cellular redox status. Thus, TRIA truly acts an antioxidant, modulating Cd toxicity through its antioxidant properties and can be used as potential growth regulator to improve plant growth under metal stress.

Acknowledgement

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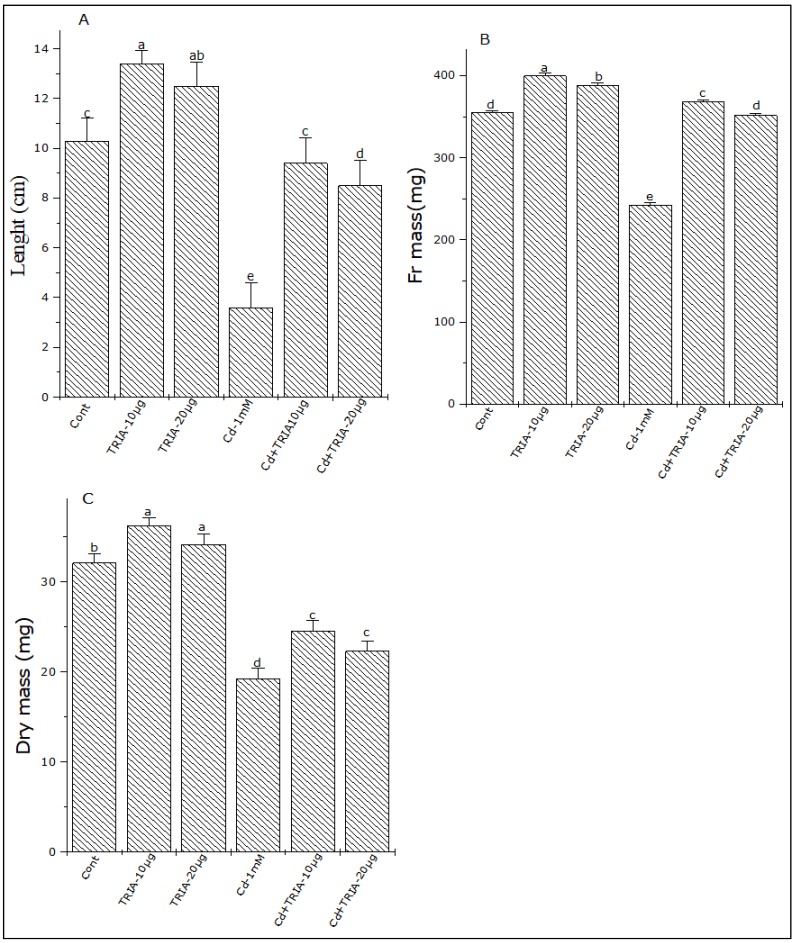

Fig 1: Effect of TRIA on the seedling growth (A-C) of radish seedlings under Cd stress. The values are means of three individual experiments. *N*=3 for ± SE.The differences were considered significant if *P* was at least ≤ 0.05. the mean values have been compared and lower case alphabets are used in the figures to highlight the signifcant differences between the treatments.

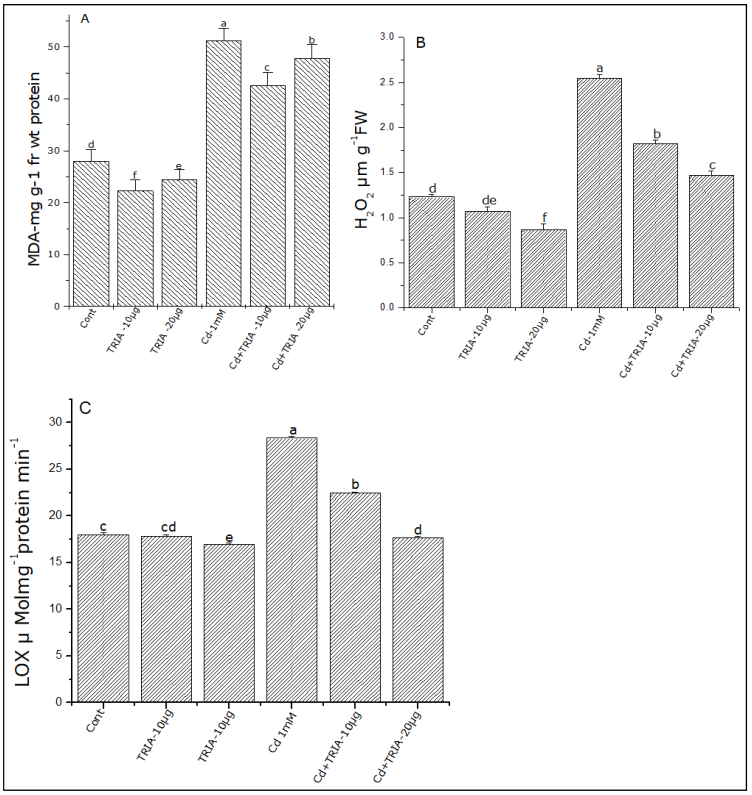

Fig 2: Effect of TRIA on (A)-lipid peroxidation; (B)-levels of hydrogen peroxide and (C)-lipoxygenase activity of radish seedlings under Cd stress. The values are means of three individual experiments. *N*=3 for ± SE.The differences were considered significant if *P* was at least ≤ 0.05. the mean values have been compared and lower case alphabets are used in the figures to highlight the signifcant differences between the treatments.

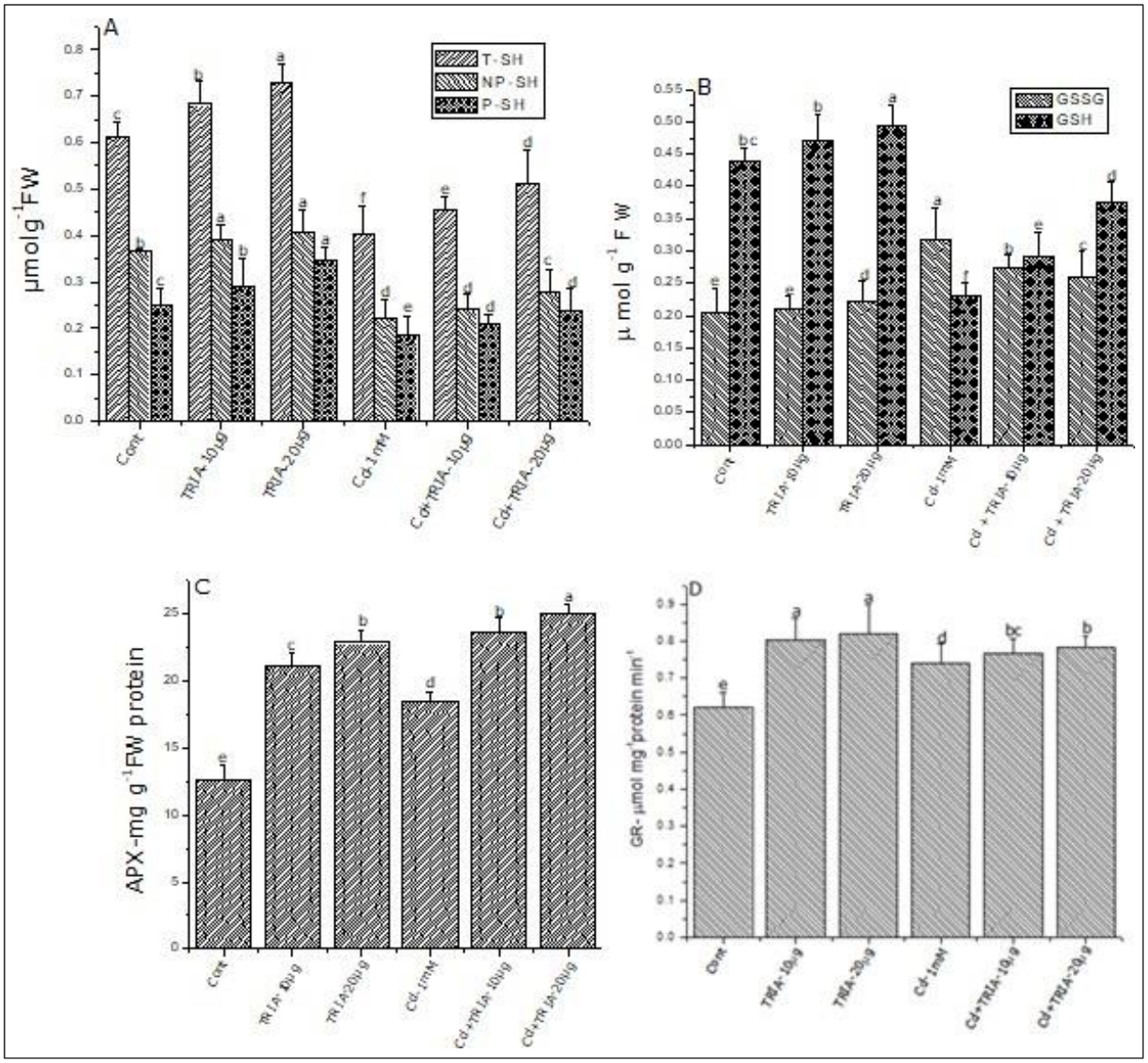

Fig 3: Effect of TRIA on (A)-levels of total, protein bound and non-protein thiols; (B)-total glutathiones, (C)-ascorbate peroxide and (D) – glutathione reductase of radish seedlings under Cd stress. The values are means of three individual experiments. *N*=3 for ± SE.The differences were considered significant if *P* was at least ≤ 0.05. The mean values have been compared and lower case alphabets are used in the figures to highlight the signifcant differences between the treatments.

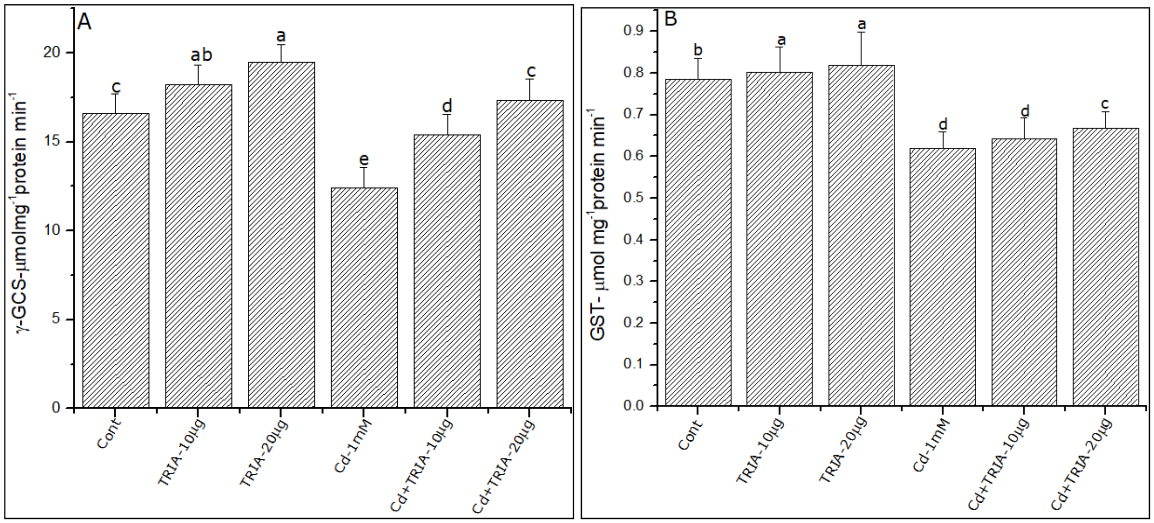

Fig 4: Effect of TRIA on (A) –γ-glutamylcysteine snthetase and (B)-glutathione-S-transferase of radish seedlings under Cd stress. The values are means of three individual experiments. *N*=3 for ± SE. The differences were considered significant if *P* was at least ≤ 0.05. The mean values have been compared and lower case alphabets are used in the figures to highlight the signifcant differences between the treatments.

References

1. Bi X, Feng X, Yang Y, Qui G, Li G, Li F *et al*. Environmental contamination of heavy metals from zinc smelting areas in Hezhang County, western Guizhou, China. Environment International,2006:32:883-890.
2. Badawi GH, Kawano N, Yamauchi Y, Shimada E, Sasaki R, Kubo A *et al*. Over–expression of ascorbate peroxide in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiol. Plant,2004:121:231-238.
3. Borowski E, Blamowski ZK. The effect of triacontanol 'TRIA' and Asahi SL on the development and metabolic activity of sweet basil (*Ocimum basilicum* L.) plants treated with chilling. Folia Horticulturae Ann,2009:21:39-48.
4. Chen EL, Chen YA, Chen LM, Lin ZM. Effect of copper on peroxidase activity and lignin content in *Raphanus sativus*. Plant Physiol. Biochem,2002:40:439-444.
5. Crosby DG, Vlitos AJ. Growth substances from Maryland Mammoth tobacco: Long chain alcohols. Contrib. Boyce Thompson Inst,1959:20:283.
6. Ederli L, Pasqualini S, Batini P, Antonielli M. Photoinhibition and oxidative stress: effects on xanthophylls cycle, scavenger enzymes and abscissic acid content in tobacco plants. J. Plant Physiol,1997:151:422-428.
7. Foyer CH, Theodoulou F, Delrot S. The functions of inter and intra cellular glutathione transport system in plants. Trends Plant Sci,2001:6:486-492.
8. Habig WH, Jakoby WB. Assay for differentiation of glutathione-s-transferase. In: Jakoby, W.B. (Ed.), Methods in Enzymology, Academic Press, New York,1981:77:398-405.
9. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts 1. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem. Biophys,1968:125:189-198.
10. Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem,1976:74:214-226.
11. Hodges MD, DeLong JM, Forney CF, Prarge PK. Improving the thio-barbituric acid reactive substances assay for lipid peroxidation in plant tissues containing anthocyanine and other interfering compounds. Planta,1999:207:604-611.
12. Jana S, Choudhuri MA. Glycolate metabolism of three submerged aquatic angiosperm during aging. Aquatic Botany,1981:12:345–354.
13. Jiang M, Zhang J. Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. Plant Cell Physiol,2001:42:1265-1273.
14. Lacan D, Baccou JC. High levels of antioxidant enzymes correlate with delayed senescence in non-netted muskmelon fruits. Planta,1998:204:377-382.
15. Li W, Khan MA, Yamaguchi S, Kamiya Y. Effects of heavy metals on seed germination and early growth of *Arabidopsis thaliana*. Plant Growth Regul,2005:46:45-50.
16. Mishra A, Choudhuri MA. Effect of salicylic acid on heavy metal-induced membrane deterioration mediated by lipoxygenase in rice. Biol Plant,1999:42:409-415.
17. Mishra A, Choudhari MA. Differential effect of Pb^{2+} and Hg^{2+} on inhibition of germination of seeds of two rice cultivars. Indian J.Plant Physiol,1997:2:41-44.
18. Nagalakshmi N, Prasad MNV. Reponses of glutathione cycle enzymes and glutathionecycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus.* Plant Sci,2001:160:291-299.
19. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol,1981:22:867-880.
20. Potters G, De Gara L, Asard H, Horemans N. Ascorbate and glutathione: guardians of the cell cycle, partners in crime. Plant Physiol. Biochem,2002:40:537-548.
21. Krishnan R, Kumari BDR. Effect of N-Triacontanol on the growth of salt stressed soybean plants. J Biosci,2008:19:53-62.
22. Ramanarayan K, Bhat A, Shripathi V, Swamy GS, Rao KS. Triacontanol inhibits both enzymatic and non enzymatic lipid peroxidation. Phytochemistry,2000:55:9-66.
23. Ries SK, Wert VF, Sweelev C, Leavitt RA. Triacontanol: a new natural occurring plant growth regulator. Science,1977:195:1339-1341.
24. Sedlak J, Lindsay RH. Estimation of total, protein-bound and non protein sulfhydryl groups in tissue by Ellman's reagent. Anal. Biochem,1968:25:192-208.
25. Shaw BP, Sahu SL, Mishra RK. Heavy metal induced oxidative damage in terrestrial plants. In: Prasad, M.N.V. (Ed.), Heavy metal stress in plants from biomolecules to Ecosystem. Springer-Verlag, Narosa, New Delhi, Second ed, 2004, 84-126.
26. Reugsegger Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y. Regulation and function of ascorbate peroxidase iso enzymes. J. Exp. Bot,2002:53:1305-1319.
27. Zhang J, Kirkham MB. Enzymatic responses of the ascorbate-glutathione cycle to drought in sorghum and sunflower plants. Plant Sci,1996:113:139-147.
28. Adua EA, Adzitey F, Abu A. Nutrient composition of whole grain and processed "niri"(Citrullus vulgaris) seeds. Int. J. Food Sci. Nutr. 2019;4:76-9.