INSIGHT INTO ALUMINIUM-INDUCED ANTIOXIDANT ENZYME RESPONSES IN BLACKGRAM [*Vigna mungo* (L.) HEPPER]

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Abstract

Blackgram [*Vigna mungo* (L.) Hepper] is an important pulse legume due to its high nutraceutical value and also has the nitrogen fixing bacterial association. But its yield is highly compromised by abiotic stressors, especially in acidic environment caused by aluminium (Al^{3+}) toxicity. This work is an attempt to understand the effects of Al^{3+} regarding the antioxidant enzymes under acidic (pH-4.7) condition. From this investigation, it was observed that, under pH-4.7+Al^{3+} stress, lipid peroxidation and lipoxygenase (LOX) activity increased which indicates Al^{3+} causes oxidative stress and induces cellular damage under acidic medium. In blackgram root and shoot, enhanced level of certain antioxidant enzymes was also observed, which might be very much indicative of their involvement in detoxification of oxidative stress induced by Al^{3+}. Among the antioxidative enzymes, peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD) showed significant involvement, with their enhanced activity for Al^{3+} stress in both root and shoot of blackgram. Isoenzyme profile analysis also indicated enhanced POX activity under stress condition in both root and shoot, which was found to be increased with the concentration of Al^{3+}. From this study, we report that under low pH condition Al^{3+} can trigger oxidative damage in blackgram, simultaneously triggering antioxidative enzyme coupled defence system.

Keywords: Aluminium toxicity, antioxidant, isoenzyme, oxidative damage, ROS scavenger.

1. Introduction

Aluminium (a light metal) is the most abundant (8%) in the earth crust after oxygen and silicon. Agricultural field gets exposed to it through human and activities of nature. Al^{3+} can be highly phytotoxic to plants under acidic soil conditions (Kochian 1995; Ma 2000; Matsumoto 2000; Ma et al. 2001). Aluminium trivalent cation (Al^{3+}) is the most toxic state among all the available forms (Parker et al. 1988). Al^{3+} directly induces root growth inhibition when soil pH is below 5.0, in most of the crop plants (Ryan et al. 1993). Al^{3+} enters into the plant cell and disturbs cell cycle by interacting with the cytoskeletal system which are regulatory elements for cell division. Al^{3+} is more interactive with the major functionaries of a cell such as the cell wall (Horst 2010), plasma membrane (Dellers et al. 1986; Jones and Kochian 1997), symplasm (Clarkson 1995) and callose synthesis pathway (Wissemeir et al. 1992).

It has been reported that in response to Al^{3+} stress plant induces certain avoidance mechanism such as exudation of various organic acids (ex.-Citrate, Malate and Oxalate) after formation of complexes with Al^{3+}. The regeneration of primary metabolites was also found to be involved in providing tolerance to Al^{3+} stress such as ascorbate, glutathione etc. by diluting ROS accumulation and also working as a precursor for some stress regulatory mechanisms (Panda et al. 2010). Ascorbate directly scavenges ROS molecules and are also involved in regeneration of alpha-tocopherol; a lipophilic antioxidant (Noctor et al. 1998; Smirnoff et al. 1996). Glutathione, helps to reduce the ROS activity by keeping a check on H_2O_2 content in plants under different environmentally challenging conditions (Kuzniak et al. 2001; Kocsy et al. 2001). In previous studies, it had been reported that oxidative stress was induced by Al^{3+} toxicity in plants (Ezaki et al. 2000; Milla et al. 2002). It had also been reported that in barley (Pan et al. 2001), and wheat (Deslile et al. 2001) cell death in root tip was induced by Al^{3+} triggered oxidative stress. In response to Al^{3+} stress the excess ROS production can be reduced by antioxidant enzyme activity to a certain extent, *viz.*, superoxide dismutase (SOD-EC 1.15.1.1), peroxidase (POX-EC 1.11.1.7) and catalase (CAT-EC 1.11.1.6) (Williams 1999; Diao et al. 2011). Among peroxidases, nonspecific, guaiacol peroxidases (GPX) are well distributed in all higher plants and can act as effective ROS scavenger and peroxy radicals induced under stress conditions in the cells (Vangronsveld and Clijsters 1994). SOD scavenges $O_2^{\cdot-}$ radicals by converting it into H_2O_2 and O_2. SOD had been reported to exist in three different isoforms, *viz.*, Cu-Zn SOD which is CN^- sensitive and mostly present in chloroplast, Fe-SOD which is also present in the chloroplast but CN^- insensitive and, last is Mn-SOD which is CN^- insensitive but found in mitochondria (Ushimaru et al. 1999). CAT can also scavenge H_2O_2 by oxidising it into H_2O and O_2, similar to that of SOD (Willekens et al. 1997). Whereas, POX oxidizes phenolic compounds to catalyse the H_2O_2 scavenging process. Lipooxygenase (LOX-EC. 1.13.11.13, linoleate: oxygen oxidoreductase) enzyme ubiquitous in plants, has got important roles in many physiological processes and stress responses (Ye et al. 2000; Suzuki and Matsukura 1997).

Ascorbate-glutathione cycle (AGC) plays a major role in scavenging ROS under stress condition. It was proposed that AGC includes certain interlinked redox molecules such as AA/DHA, GSH/GSSG, and NADPH/NADP with regulatory enzymes, ascorbate peroxidase (APX-EC1.11.1.11), monodehydroascorbate reductase (MDHAR-EC1.6.5.4), dehydroascorbate reductase (DHAR-EC1.8.5.1) and glutathione reductase (GR-EC1.6.4.2) (May et al. 1998).

Blackgram is the most widely used food crop among the pulses. In many parts of the world, blackgram is being cultivated for food and fodder. In India, South and North-East regions are the most preferred destinations for cultivation of blackgram. Blackgram is cultivated as a rotation crop because of its role as a green manure. Blackgram plants have nitrogen fixing bacterial association, so it can increase the

soil fertility by increasing the soil nitrogen content. Acidic soil and Al^{3+} toxicity is becoming a major constraint to blackgram cultivation, as the native places of this crop is getting contaminated with Al^{3+} and other elements that contribute soil acidity (Chowra et al. 2016). So far report says aluminium phytotoxicity is limiting the productivity of many other crops day by day (Panda et al. 2009; Singh et al. 2011). In our previous work the physiological changes and metabolic shifts under Al^{3+} stress in blackgram was analysed (Chowra et al. 2016). A few researchers have reported about the effect of Al^{3+} toxicity in pulse crop and also about ROS content, antioxidant enzyme activity and ascorbate glutathione cycle has not yet been well described for pulse crop in response to Al^{3+} stress (Panda et al. 2003; Panda and Matsumoto 2010; Chowra et al. 2016). Moreover, the isoenzyme profile analysis for different isoforms of antioxidative isoenzymes in response to Al^{3+} stress is also not well explored by researchers. Therefore, in this study responses of antioxidant enzymes, primary metabolites, ascorbate-glutathione cycle and some other important parameters under Al^{3+} stress was investigated in blackgram. The isoenzyme profile further validated the antioxidative enzymes content (quantification data) data. The study was performed to understand the predominant antioxidative enzymes and its regulatory responses under Al^{3+} stress in blackgram. In this study it was analysed that low pH alone can't induce more phytotoxicity as the low pH with Al^{3+} can do. It is because we kept low pH (pH-4.7) as control and low pH with different concentrations of Al^{3+} as stress condition.

2. Materials and method

2.1. Plant growth and stress conditions

Seeds of blackgram were surface sterilized with 0.1% mercuric chloride and placed for germination in petri plates containing water soaked cotton bed at 30^0C for two days. When the seeds germinated, the seedlings were transferred to Hoagland nutrient solution pH-6.2 and grown under plant growth chamber at 25-30º C with a 16 hours' light and 8 hours' dark photoperiod condition and light illumination at 52µmol/m/s. After growing in nutrient solution for 4 days, 100 µM of $CaCl_2$ (pH-4.7) pre-treatment was given for 24 hrs, followed by Al^{3+} treatment (0, 10, 50 and 100 µM in 100 µM $CaCl_2$, pH-4.7). After 24, 48 and 72 hrs of Al^{3+} treatment, samples were harvested for analysis, snap frozen in liquid nitrogen and stored in -80^0C deep freezer for further use.

2.2. Lipid content analysis in response to Al^{3+} stress

The lipid peroxidation of Al^{3+} stressed blackgram was quantified as malondialdehyde (MDA) content, described by Heath and Packer (1968). The absorbency was measured at 532nm and subtracted for any nonspecific absorbency at 600nm. The MDA content was calculated by taking extinction coefficient (€) = $155mM^{-1}cm^{-1}$ and expressed as µmol/gm fresh weight.

2.3. Non-enzymatic antioxidant analysis in response to Al^{3+} stress

Total glutathione and ascorbate contents were determined and expressed (µmol/gFW) as per the method of Griffith (1980) and Oser (1979). Absorbency was measured at 660nm. On the other hand, glutathione assay mixture contained plant extract, 0.1M phosphate buffer (pH6.8), 5-5/ DTNB (10µM) and the absorbency was recorded at 412nm.

2.4. Enzymatic antioxidant analysis in response to Al^{3+} stress:

POX activity was measured and expressed (µmol/gFW) by the method of Kar and Mishra (1976). The activity of GPX was assayed according to Egley et al. (1983). Increase in absorbance due to for- mation of tetraguaiacohinone was measured at 420 nm. Enzyme-specific activity is expressed µmol/gFW. LOX activity was determined and expressed (µmol/gFW) by the method of Axelrod et al. (1981). Catalase (CAT) activity was measured and expressed (µmol/gFW) by the method of Chance and Maehly (1955). SOD activity was measured and expressed (µmol/gFW) by the method of Gianopolitis and Reis (1977).

2.5. Measurement of Ascorbate –Glutathione cycle components in response to Al^{3+} stress:

GR activity was analysed and expressed (µmol/gFW) by the method of Smith et al. (1988). APX activity was determined and expressed (µmol/gFW) by the method of Nakano and Asada (1981). MDHAR activity was analysed and expressed (µmol/gFW) by the method of Miyake and Asada (1992). DHAR activity was measured and expressed (µmol/gFW) by following the method of Ma and Cheng (2004).

2.6. Isoenzyme pattern detection under aluminium stress:

The enzyme extraction procedure was followed after Larkindale and Huang (2004). By using the enzyme extract, CAT, SOD, APX and POX were determined. The changes of certain proteins having isoenzyme activity was analysed by native PAGE under non-reduced, non-denatured conditions at 4^0C according to the method described by Laemmli (1970). To keep native protein intact certain specific conditions were maintained. Native PAGE was carried out for SOD by the method of Rucinska et al. (1999), APX by the method of Gara et al. (1993), GPX by the method of Cakmak and Horst (1991), CAT by the method of Lee (2003).

2.7. Total protein content analysis:

The extracted enzyme mixture was used for total protein content analysis according to Bradford (1976) with bovine serum albumin (BSA) as the standard.

2.8. Statistical analysis:

Experiments were performed triplicate to get a significant variation between the control and the stress samples. All statistical analysis of the data was performed by using Microsoft Excel and data were expressed as mean ±standard error of mean (±SE). Significant differences between the stress and control samples were determined by Student's t-test at $p \leq 0.05$.

3. Results

3.1. Non-enzymatic antioxidant analysis in response to Al^{3+} stress

Among the non-enzymatic antioxidants, ascorbate and glutathione are the most important. In this study, ascorbate content was found to be increased with the increase in concentration of Al^{3+} after 48 hrs of treatment in the shoot (Fig 1A) as compared to low pH. But, no such elevated ascorbate content was observed after 72 hrs of treatment in shoot. Similarly, less elevated ascorbate content was observed as compared to control after 24 and 72 hrs. In contrast, significant enhancement of ascorbate content was observed in root after 48 hrs of 50 and 100µM Al^{3+} treatment (Fig 1B). In shoot changes in glutathione activity was observed in 10, 50 and 100 µM Al^{3+} concentration, significantly (Fig. 1C). After 48hrs of treatment the glutathione content was significantly found to be elevated under 10, 50 and 100 µM Al^{3+} concentration. In root, the total glutathione content was found to be elevated under Al^{3+} + pH-4.7 condition as compared to pH-4.7 alone (Fig. 1D). Under 10, 50 and 100 µM Al^{3+} concentration the glutathione content was more elevated as compared to low pH treatment (4.7) after 48 hrs, in root. Whereas, after 72 hrs of Al^{3+} treatment, roots did not show a marked difference in glutathione content as compared to low pH.

3.2. Effect of Al^{3+} toxicity on MDA content

Under Al^{3+} stress, increase in MDA content was observed in blackgram. In shoots, very less enhancement of MDA content was observed under Al^{3+} stress as compared to low pH (control) with the duration of stress and concentration of Al^{3+} (Fig. 1E). In root, after 24, 48 and 72 hrs of treatment marked elevation of MDA content was observed (10, 50 and 100 µM of Al^{3+}) as compared to pH 4.7 (0 µM of Al^{3+}) (Fig.1F).

3.3. Lipoxygenase activity analysis under aluminium stress

Changes in LOX activity was observed in shoot (Fig. 1G) which was found to be increased with the increase in Al^{3+} concentration after 24, 48 and 72 hrs of treatment. Similarly, in roots elevated LOX activity was observed (Fig. 1H). With the increase in Al^{3+} concentration, the activity of LOX was also increased in roots after 72hrs of treatment. LOX activity was found to be more significantly elevated under 100 µM Al^{3+} stress in root.

3.4. Effect of aluminium on total protein content

In response to the different concentration of Al^{3+}, the total protein content was found to be altered. In shoot sample decrease in total protein content was observed as the concentration of Al^{3+} increased (Fig. 1I). In root, total protein content was found to be increased under Al^{3+} stress which was more after 72hrs as compared to 24 and 48hrs. Under Al^{3+} stress condition, higher in total protein content was observed in root as compared to shoot (Fig. 1J).

3.5. Enzymatic antioxidant analysis in response to Al^{3+} stress:

In this study, certain enzymatic antioxidant activity was evaluated. In shoot, increased CAT activity was observed (Fig. 2A) with the increase in concentration of Al^{3+} dose, which was more prominent in 100 µM Al^{3+} dose. On the other hand, root samples also showed similar CAT activity (Fig. 2B). Most importantly, gradual increase of CAT activity was observed with the increase in duration of stress only under 50 µM Al^{3+} treated root.

In addition, SOD showed significant activity in response to Al^{3+} stress in blackgram. In shoot, significantly elevated SOD level was observed as compared to low pH (0 µM) (Fig. 2C). The content of SOD increased with increase in concentration of Al^{3+}. No gradual increment of SOD activity was observed as the duration of stress increased. In case of root, increase in SOD activity was observed as the concentration of Al^{3+} increased (Fig. 2D). Further, higher SOD activity was observed at 100 µM Al^{3+} after 24 hrs of stress. After 48 hrs of treatment a gradual increase of SOD content was observed with the increase in the concentration of Al^{3+}. Whereas, after 72 hrs of stress the SOD content declined as the concentration of Al^{3+} increased.

GPX activity was measured in terms of Al^{3+} stress in blackgram root and shoot tissue. In shoot the GPX activity was found to be elevated only after 48 and 72hrs (Fig. 2E). In addition, 50 and 100µM Al^{3+} dosed samples showed high GPX content as compared to control i.e. under pH4.7. Whereas, in root the GPX content was remarkably less than that of shoot (Fig. 2F). Under Al^{3+} treated samples also showed less increased GPX content but it was found to be increased with the duration of stress condition increased.

POX activity was also analysed in both shoot and root. Both shoots and roots showed, marked gradual increment of POX activity with the increasing Al^{3+} concentration (Fig. 2 G-H). Under 100 µM Al^{3+} concentration higher POX activity was observed in both shoot and root. In shoot gradual increase of POX activity was observed with the duration of stress condition increased.

3.6. Measurement of Ascorbate – Glutathione cycle components in response to Al^{3+} stress

APX activity was evaluated in response to Al^{3+} stress in blackgram. Under Al^{3+} stressed condition, enhanced APX content was observed as compared to control (low pH). In both shoot and root, APX content was enhanced on exposure to Al^{3+}stress after 24 hrs of treatment. Gradual increase in APX content was observed with increment in duration and the concentration of Al^{3+} in shoot (Fig. 3A-B) whereas in root it was only for 24 and 48hrs sample.

In blackgram plants, after aluminium treatment marked enhancement of GR activity was observed. The GR activity was found to be increased with time and concentration of Al^{3+}, in both shoot and root (Fig. 3C-D). It was also observed that GR content was more elevated in shoot after 48 and 72hrs of stress as compared to 24 hrs.

DHAR activity was also analysed in response to Al^{3+} stress. In both shoot and root, elevated DHAR activity was observed in treated samples as compared to control (low pH) (Fig. 3E-F). In shoot, DHAR activity was more under 50 µM Al^{3+} then the 10 and 100 µM Al^{3+}. After 24 hrs of treatment gradual increase in DHAR activity with the increasing in Al^{3+} concentration was recorded. In case of root also a similar gain of DHAR activity was observed with the gradual increment in time and concentration of Al^{3+}, except for the plants under 100 µM Al^{3+} after 48hrs of stress.

MDHAR activity in response to Al^{3+} stress was evaluated spectrophotometrically and was found to be altered in Al^{3+} treated plants. MDHAR content was found to be profoundly elevated after 24 and 48hrs of stress in shoot (Fig. 3G). Whereas, somewhat different pattern of MDHAR activity was observed in root (Fig. 3H). 10 and 100 µM Al^{3+} treated plants showed decreased MDHAR activity in root with the increased duration of stress, whereas, roots under 50 µM Al^{3+} showed increased MDHAR activity with the increased duration of stress.

3.7. Isoenzyme pattern analysis in response to Al^{3+} stress

In this study, we subsequently investigated the patterns of certain isoenzymes, *viz*., APX, CAT, POX and SOD. In response to different concentrations of Al^{3+}, the expression of isoenzymes also varied which was very much similar to our quantification data. The number of bands and its' intensity varied in response to different concentration and duration of Al^{3+} stress. In shoot, very less significant pattern of isoenzymes were observed, except for POX isoenzyme which showed higher band intensity in treated plant when compared to the (0 µM +pH-4.7) control (Fig.4). In root, POX isoenzyme showed dose dependent expression pattern. After 48hrs of stress the intensity of POX isoenzyme was more as compared to 24 and 72 hrs (Fig.5).

4. Discussion

Al^{3+} toxicity and Al^{3+} activated defence system has been explored by many researchers in different crop plant but still very less information is available regarding pulse crops. Among the pulses, blackgram, being enriched with nutrients and vitamins, is one of the important pulse crop in Asia. Geographical distribution of acidic soil has extensively increased in recent years which includes blackgram cultivation areas also. Due to higher accumulation of aluminium and other acidic cations in agricultural fields it creates a major constraint to blackgram production. In this study, we understand distinguishable adverse effects of low pH alone and low pH with Al^{3+} in blackgram. In this work we used blackgram seedlings (5^{th} day old) to impart aluminium stress under acidic condition to have a clear understanding of stress response at very initial development stage, in addition Al^{3+} always present in soil in its different form and impose stress to plants from the very beginning of seed germination. We previously reported the phytotoxic effects of aluminium in blackgram plant (Chowra et al. 2016). Another important point of this study is to understand the responses under acidic medium low pH (pH-4.7) between without Al^{3+} (control) and with Al^{3+} (stress). In this way we will be able to understand the responses of blackgram in presence of Al^{3+} and in absence of Al^{3+} under acidic medium.

Ascorbate (AA) is involved in physiological and stress related defence mechanism processes through direct interaction with ROS molecules (Noctor et al. 1998; Smirnoff 1996; Gupta et al. 1999). In shoot, slightly elevated AA content was observed after 48hrs, which indicated plant defence system activated the ROS scavenging system with the production of AA (Fig.1A). In this study very less elevated AA content was observed in treated root as compared to control (low pH) root, this might indicate that Al^{3+} toxicity reduced the production of AA in root system (Fig.1B). Panda and Matsumoto (2010) also found no change in AA content after 48hrs of Al^{3+} stress in pea. Ascorbate can interact with many enzyme and reduces cellular damage caused by ROS molecules through synergistic activity with other antioxidants (Millar et al. 2003). Schutzendubel and polle (2001) observed that ROS accumulation is favoured by lack of gluthathione content and results in disintegration of developmental processes. Root growth inhibition induced by Al^{3+} toxicity actually works with different physiological and intercellular processes. Reduction or no change in glutathione level might be one of them. In this study, increased glutathione content was observed with increase in duration of stress, but it was more pronounced after 48 hrs of stress for both shoot and root (Fig. 1C-D). Glutathione was found to be involved in detoxification of oxidative stress induced by heavy metals in plants (Xiang 1998). An increased glutathione level was observed in pea root system in response to Al^{3+} stress while in shoot it was vice versa (Panda and Matsumoto 2010).

In our study, enhanced lipid peroxidation in terms of MDA content was observed in root whereas in shoot very less elevated MDA content was observed (Fig.1E-F) in Al^{3+} treated plant. This might be because of root being the primary target of Al^{3+} toxicity. Lipid peroxidation is a primary symptom of any kind of stress and can be induced by Al^{3+} toxicity which is being already reported in pea and soybean (Yamamoto et al. 2001; Cakmak and Horst 1991). Basu et al. (2001) showed the correlation between root growth inhibition and increase in lipid peroxidation in *Brassica* spp. Wu et al. (2012) observed that MDA level elevated for a certain duration of stress and later declined. In this study, similar consequences were also observed in root which indicates that aluminium can induce lipid peroxidation at the early stage of stress and might be by altering Ca^{2+} from the membrane lipids (Delhaize et al. 1995). After long treatment with Al^{3+} certain defence mechanism might be getting activated by ROS complex, thus promoting stress recovery mechanisms.

From this study, it was observed that LOX activity was directly proportional with time and concentration of Al^{3+} treatment which indicated that long time exposure to Al^{3+} increases LOX activity causing lipid peroxidation (Fig. 1G-H). MDA content increases due to lipid peroxidation of disintegrating membranes and making it dysfunctional where increased LOX activity is considered to be the main precursor of lipid peroxidation (Einsparh et al. 1988). Macri et al. (1994) suggested similar view on LOX activity and MDA content. Increased level

of LOX activity can catalyse the dioxigenation of polyunsaturated fatty acids and are toxic to cellular system. From this study, increased MDA and LOX activity was observed which might be induced by Al^{3+} toxicity in blackgram (Fig. 1e-f).

Alteration in total protein content was also observed under Al^{3+} stress in blackgram (Fig. 1I-J). In root, it was found to be increasing with time and concentration which is indicative of Al^{3+} mediated induction of protein synthesis in root synchronizing with the exponential increase in antioxidant enzyme activity. More protein content indicates more cellular metabolic activity under Al^{3+} stress which might be a part of defence system in blackgram. On the other hand, in shoot, total protein content declined but after 72 hrs it was found to be increased, which indicates activation of defence system in shoot is quite slow than root. In contrast, Sharma and Dubey (2007) found increased protein activity after 15 days of Al^{3+} stress in rice seedlings but decreased protein content was observed after long-term exposure to Al^{3+}.

In this study certain antioxidative enzyme assay in response to Al^{3+} stress was performed for blackgram. The assay includes POX, GPX and CAT antioxidant enzymes. These are so far being well known as oxidative stress scavengers. The toxic free radicals, in response to abiotic stress, such as $O_2^{\cdot-}$ and H_2O_2 can be scavenged by CAT and POX in plants (Willekens et al. 1997).

Increased CAT content (Fig. 2A-B) in response to Al^{3+} stress was observed in blackgram which might be due to increment of H_2O_2 content (Chowra et al. 2016). Excessive ROS produced under stress condition can be scavenged by CAT activity (Davis and Swanson 2001). Darko et al. (2004) observed that Al tolerant wheat plant showed elevated CAT activity under stress condition to detoxify the excessive ROS molecules induced by Al^{3+}, but Panda et al. (2003) showed that Al^{3+} induces reduction in CAT activity in green gram except root samples treated with 50 µM Al^{3+} which showed enhanced CAT activity. Similarly, Sharma and Dubey (2007) found declined CAT activity in rice under Al^{3+} stress. Regarding this kind of ununiformed CAT activity, Ushimaru et al. (1999) suggested that due to inhibition of enzyme synthesis or modification in the assembly of enzyme subunits, certain plants shows declined CAT activity. In contrast, we observed increased CAT activity under aluminium stress in blackgram.

From this investigation, it was observed that under Al^{3+} stress, SOD activity increased (Fig. 2C-D) in blackgram. SOD consist of different metalloenzymes which are Cu-Zn SOD, MnSOD and FeSOD isoforms. SOD is involved in catalysing the reaction of dismutation of ROS molecules ($O_2^{\cdot-}$ to H_2O_2 and O_2; Alscher at al. 2002). Increase in SOD activity in blackgram might be attempting to dismute the excess ROS molecules. Achary et al. (2008) observed increased SOD activity in response to Al^{3+} stress in *Allium cepa* L. Previous reports also support our finding regarding increased SOD activity in response to Al^{3+} stress (Cakmak and Horst, 1991; Boscolo et al. 2003; Tamas et al. 2007). The activities of SOD isoforms which was found to be increased under stressful conditions, indicate its involvement in defence mechanism against oxidative stress in cytosol, mitochondria and chloroplast (Ushimaru et al. 1999). In contrast, Meriga et al. (2003) observed decreasing activities of SOD under prolonged exposure to Al^{3+} and it was suggested that the amount of increased free radicals beyond the cell's capacity may reduce enzymatic activities and inhibits cellular metabolic functions thereafter leading to DNA damage.

GPX is well known to be involved in lignifications of cell wall, degradation of IAA, biosynthesis of ethylene, wound healing, and defense against pathogens (Klotz et al. 1998). May because of this reason more GPX content was observed in shoot under stress condition (Fig. 2 E-F). Increased GPX content might be helping in defence system to scavenge Al^{3+} stress in blackgram.

POX is another well-known antioxidant enzyme involved in scavenging the ROS molecules and protects from oxidative injury (Smeets et al. 2008). By oxidizing phenolic compounds POX can catalyse H_2O_2 degradation. We observed significantly increased POX activity in blackgram plants under Al^{3+} stress (Fig. 2. G-H). Our quantification data of POX and isoenzyme profiling of POX clearly proven that POX has lots to do under Al^{3+} stress in blackgram. Similar result was also reported in maize, pea and rice in response to Al^{3+} stress (Boscolo et al. 2003; Panda and Matsumoto 2010; Sharma and Dubey 2007).

Here we report that APX activity increased under Al^{3+} stress in blackgram (Fig. 3A-B). It was suggested that APX has a major role in scavenging H_2O_2 molecule under stressed condition. APX is one of the important antioxidant that decomposes H_2O_2 more efficiently than CAT (Wang et al. 1999). APX reduces H_2O_2 to H_2O molecule by using ascorbic acid as an electron donor and this step is considered as the first step of ascorbate-glutathione cycle (Noctor et al. 2005). Sharma and Dubey (2007) found elevated APX activity in 160 µM Al^{3+} treated rice seedlings similarly, Dipierro et al. (2005) also observed enhanced APX activity induced by Al^{3+} stress in *Cucurbita pepo*. Enhanced APX activity under Al^{3+} stress in blackgram is indicative of the presence of an active ascorbate-glutathione cycle.

Also investigated the GR activity, another enzyme of ascorbate-glutathione cycle, which showed increased activity under Al^{3+} stress in blackgram (Fig. 3C-D). Similar result was also reported by Sharma and Dubey (2007) in rice. GR catalyses CoA-SSG to form GSH and maintains GSH/GSSG ratio which is an important part of AGC to detoxify oxidative stress (Foyer and Noctor 2005). In this study, we observed time dependent enhancement of GR activity under aluminium stress but, in root, the GR activity was more at the early stage (24 hrs) of Al^{3+} stress. This finding is indicative that the primary site of Al^{3+} induced oxidative damage is root resulting in initiation of the antioxidant responses to alleviate the Al^{3+} induced phytotoxicity. Previously, it has been already reported that root is the primary target of Al^{3+} stress. Prolonged treatment with Al^{3+} can also induce oxidative damages in shoot.

DHAR contributes to ascorbate production by reducing itself by using glutathione as reductant. Further ascorbate is utilized by APX to detoxify H_2O_2 to H_2O. In this work we have observed slightly elevated DHAR activity in treated root (Fig. 3F) as compared to untreated root. But there was no marked increase with time and concentration in shoot (Fig. 3E). We also observed that DHAR activity was induced by Al^{3+} at the early stage (24-48 hrs) of stress in blackgram. Drazkierwicz et al. (2002) reported elevated DHAR activity at early stage of copper stress in Arabidopsis.

In this work MDHAR activity was evaluated in response to Al^{3+} stress in blackgram. The main purpose of MDHAR in AGC is to regulate the level of MDHA radical involved in non-enzymatic disproportionation and generate DHAR (Gara et al., 1994). In our findings we have observed increased MDHAR activity in treated samples when the concentration of Al^{3+} increased (Fig. 3G-H). Higher MDHAR activity can regulate the MDHA content by reducing it into AA by using NADPH (Noctor et al., 1998).

In this work we have analysed some isoenzyme patterns in response to Al^{3+} stress in blackgram. We observed that root samples showed CAT, POX, SOD and APX isoenzyme band (Fig. 5) in response to different concentration of Al^{3+}. Similar result has been already reported in rice under Al^{3+} stress (Sharma and Dubey 2007). Most importantly, the POX isoenzyme activity was the most significant one in both

shoot and root. With the increase in concentration of Al^{3+} the band intensity of POX isoenzyme was found to be increased in blackgram plant. This result also synchronizes with the quantification data of POX antioxidant enzyme. As compared to shoot, another significant observation was the prominent and high intensity band of POX isoenzyme in root, the major and primary site of Al^{3+} toxicity. High POX antioxidant content and high expression of POX isoenzyme in root indicates its significant role in response to Al^{3+} stress in blackgram. In this work two distinct bands for SOD isoenzyme were observed, indicating that two SOD isoforms were expressed under Al^{3+} stress in blackgram (Fig. 4 & 5). In control (low pH) plant also, two SOD isoforms were detected whereas three SOD isoform should have observed in general as per previous report (Ushimaru et al., 1999), but variation in band intensity was observed in blackgram. In shoot, two distinct SOD isoform's band was observed. In shoot, variation in band intensity of SOD isoform was observed after 24 hrs of Al^{3+} stress (Fig. 4). The quantitative data of SOD and its isoform's band intensity indicates similar SOD activity under Al^{3+} stress in blackgram. Remarkably variation in band intensity was not observed for 48 and 72 hrs shoot as compared to control shoot. After 24 hrs of Al^{3+} treatment, increased band intensity was observed in 50 µM Al^{3+} treated root which very much corresponds to quantification data. In root, after 48 and 72 hrs of treatment band intensity of SOD activity was increased with increment of Al^{3+} concentration and this was very much similar to quantification data.

Figures and tables:

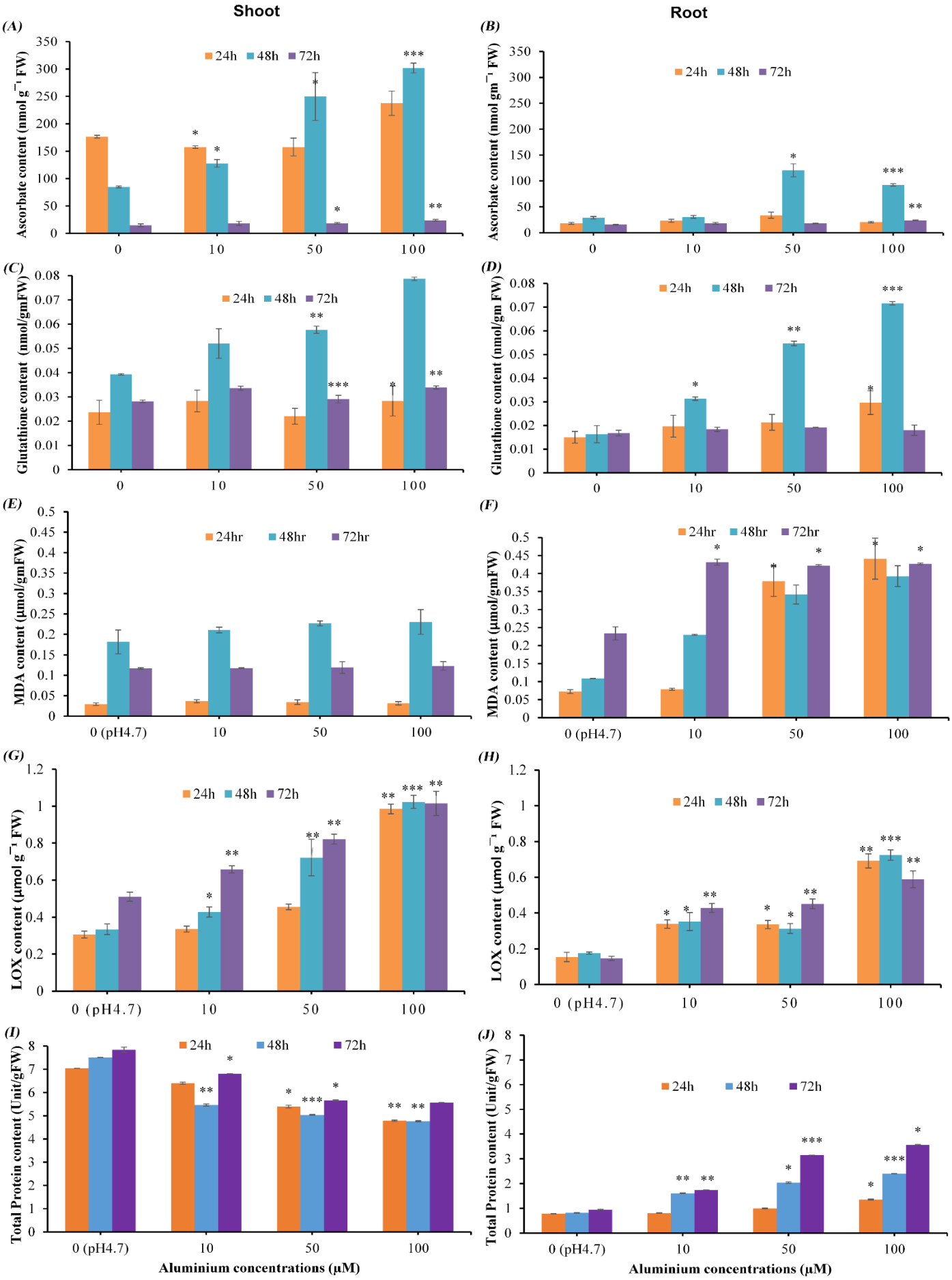

Figure 1: Changes in the contents of Ascorbate (A-B), Glutathione (C-D), lipid peroxidation (E-F) (MDA), Lipoxygenase (G-H) (LOX) and Total Protein in shoot and root of blackgram under aluminium stress. Varying concentration of Al^{3+} (0, 10, 50 and 100 µM with pH-4.7) and duration (24, 48 and 72 hrs) of stress were used for the analysis. Data presented are mean ±SE. '*' indicating significant differences from control at P<0.05 by student's t-test (*p<0.05, **p<0.01 and ***p<0.001).

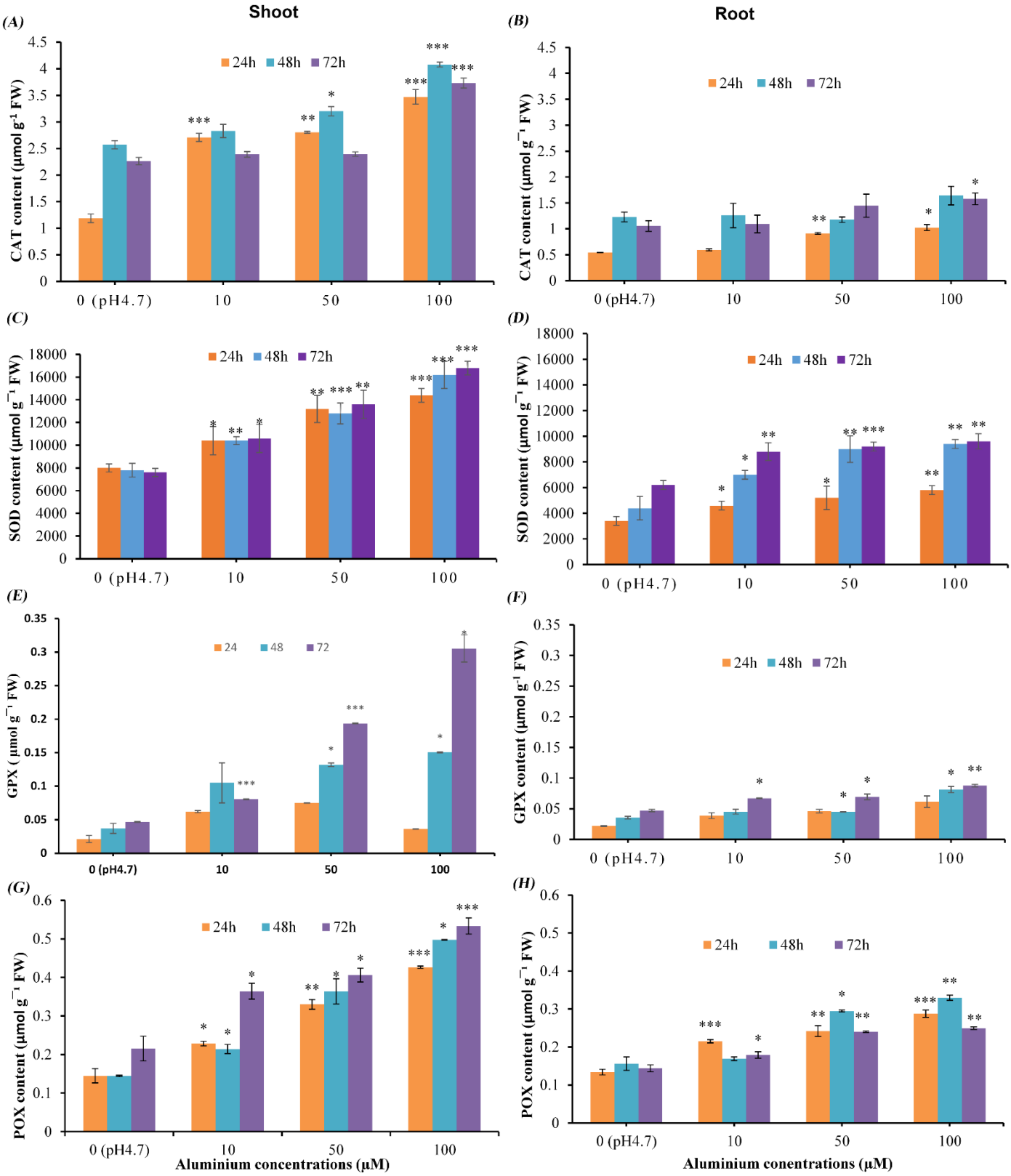

Figure 2: Changes in the activity of catalase (A-B) (CAT), superoxide dismutase (C-D) (SOD), guaicol peroxidase (E-F) (GPX) and peroxidase (G-H) (POX) in shoot and root of blackgram under aluminium stress. Different concentration of Al^{3+} (0, 10, 50 and 100 µM with pH 4.7) and duration (24, 48 and 72 hrs) of stress were used for the analysis of the same. Data presented are mean ±SE. '*' indicating significant differences from control at $P<0.05$ by student's t-test (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

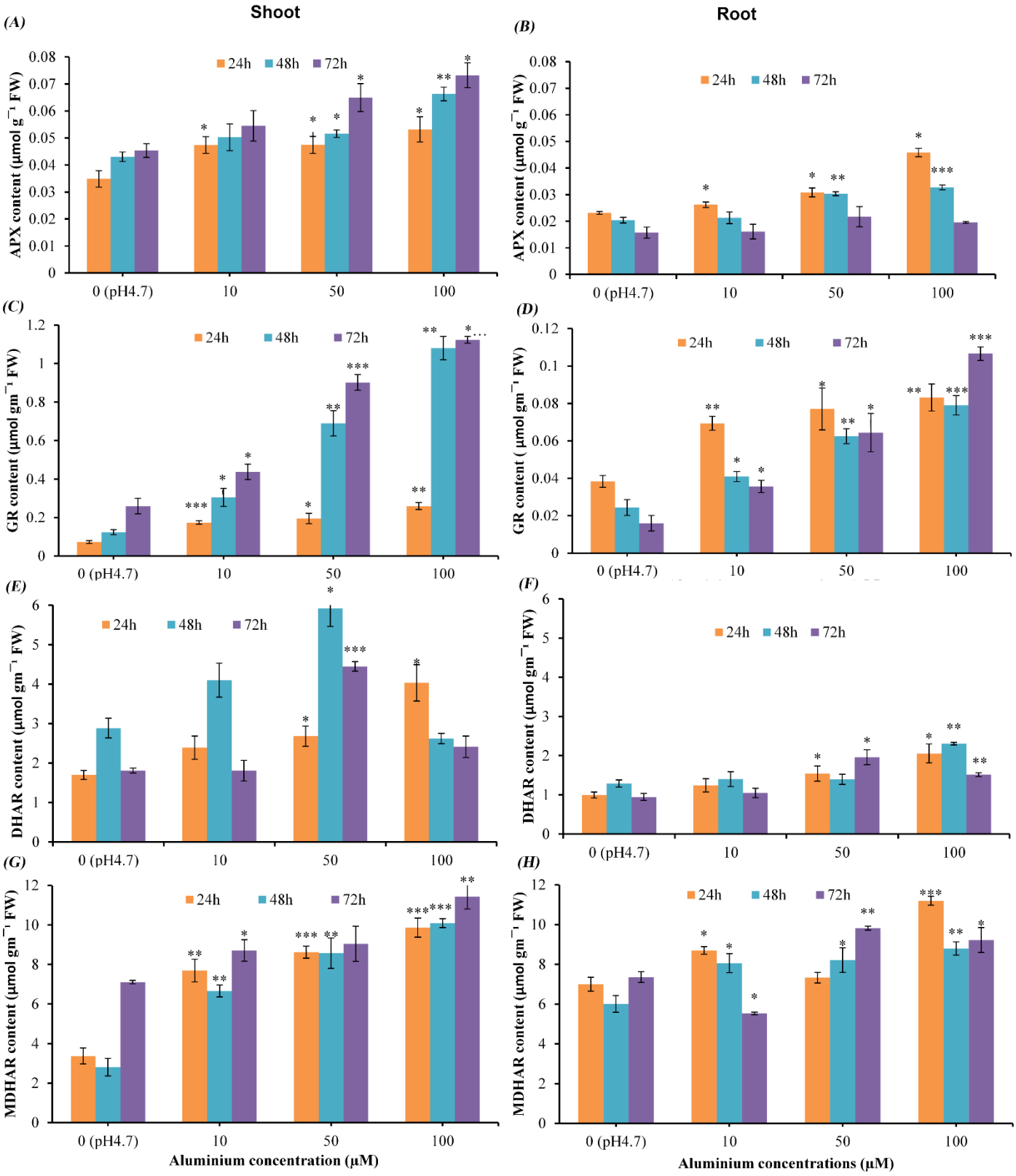

Figure 3: Changes in the activity ascorbate peroxidase (A-B) (APX), glutathione reductase (C-D) (GR), dehydroascorbatereductase (E-F) (DHAR) and monodehydroascorbate reductase (G-H) (MDHAR) in shoot and root of blackgram under aluminium stress. Different concentration of Al^{3+} (0, 10, 50 and 100 µM with pH-4.7) and duration (24, 48 and 72 hrs) of stress used for the analysis of the same. Data presented are mean ±SE. '*' indicating significant differences from control at $P<0.05$ by student's t-test (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

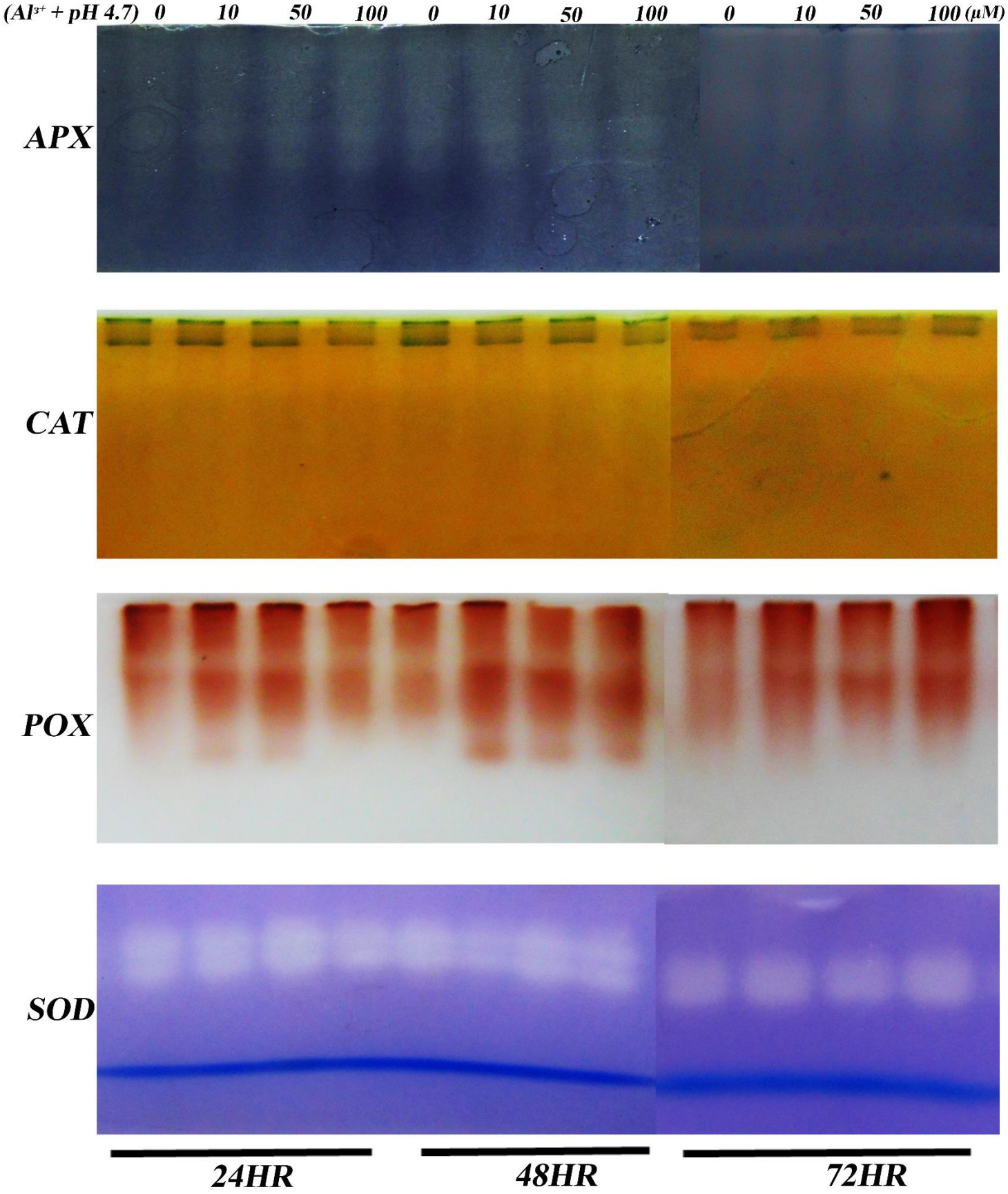

Figure 4: Isoenzyme profiling of ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) in shoot of blackgram under aluminium stress. Different concentration of Al^{3+} (0, 10, 50 and 100 µM with pH-4.7) and duration (24, 48 and 72 hrs) of stress used for the analysis of the same.

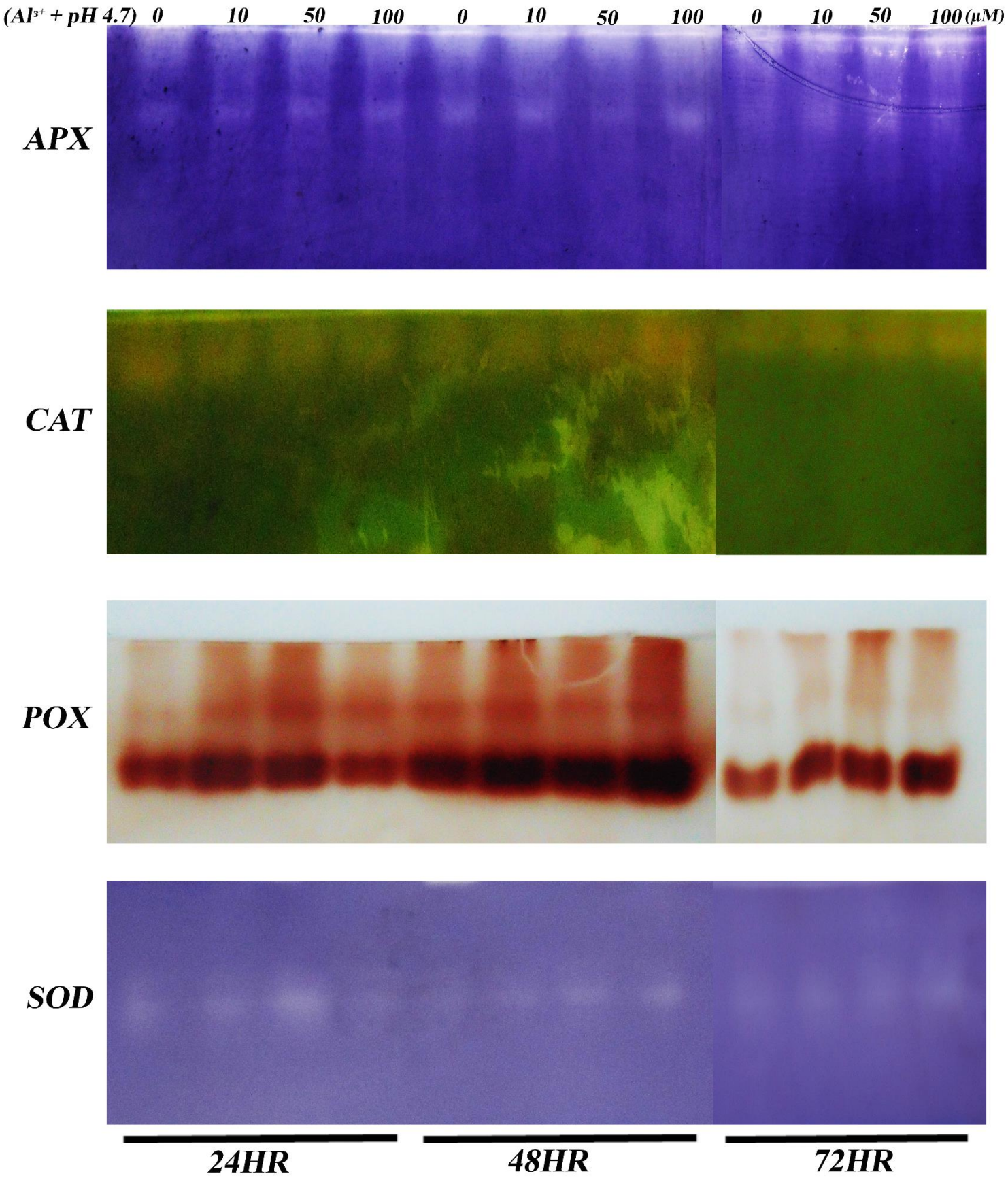

Figure 5: Isoenzyme profiling of ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) in root of blackgram under aluminium stress. Different concentration of Al^{3+} (0, 10, 50 and 100 µM with pH-4.7) and duration (24, 48 and 72 hrs) of stress used for the analysis of the same.

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