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ORIGINAL ARTICLE

Aluminium-induced excessive ROS causes cellular damage and metabolic shifts in black gram *Vigna mungo* (L.) Hepper

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Abstract Aluminium-induced oxidative damage caused by excessive ROS production was evaluated in black gram pulse crop. Black gram plants were treated with different aluminium (Al^{3+}) concentrations (10, 50 and 100 μM with pH 4.7) and further the effects of Al^{3+} were characterised by means of root growth inhibition, histochemical assay, ROS content analysis, protein carbonylation quantification and ^{1}H-NMR analysis. The results showed that aluminium induces excessive ROS production which leads to cellular damage, root injury, stunt root growth and other metabolic shifts. In black gram, Al^{3+} induces cellular damage at the earliest stage of stress which was characterised from histochemical analysis. From this study, it was observed that prolonged stress can activate certain aluminium detoxification defence mechanism. Probably excessive ROS triggers such defence mechanism in black gram. Al^{3+} can induce excessive ROS initially in the root region then transported to other parts of the plant. As much as the Al^{3+} concentration increases, the rate of cellular injury and ROS production also increases. But after 72 h of stress, plants showed a lowered ROS level and cellular damage which indicates the upregulation of defensive mechanisms. Metabolic shift analysis also showed that the black gram plant under stress has less metabolic content after 24 h of treatment, but gradually, it was increased after 72 h of treatment. It was assumed that ROS played the most important role as a signalling molecule for aluminium stress in black gram.

Abbreviations

ROS	Reactive oxygen species
Al^{3+}	Aluminium
PC	Protein carbonylation
H_2O_2	Hydrogen peroxide
FW	Fresh weight
DW	Dry weight

Introduction

Aluminium toxicity and acidic soil are becoming the major constraints in the field of agricultural productivity worldwide (Panda et al. 2009). Acid soil causes 25–80 % yield losses in various crop plants (Singh et al. 2011). Von Uexkull and Mutret (1995) found that the acid soil level globally expands up to 3950 million ha of total ice-free land in the world. Forests and woodlands covered 66.3 % acid soil globally; on the other hand, 17.7 % of acid soils is covered by savanna, prairie and steppe vegetation. 5.4 % of acid soil is used to cultivate crop plants. In soil, clay particles help to maintain soil acidity, because they can hold alkaline cations to maintain the soil pH. Due to high rainfall, alkaline cations like Ca^{2+}, Mg^{2+} and K^+ leach from soil, and instead it deposits certain acidic cations to soil such as Al^{3+} and H^+. Field application of lime with gypsum can reduce the acidity of soil and increases crop

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productivity (Carvalho and van Raij 1997). However, it is an expensive approach for local farmers.

Aluminium is non-toxic to plants until and unless soil becomes acidic (pH < 5). In normal soil, aluminium is present as silicates, phosphate sulphides and oxides. But under an acidic soil condition, it becomes toxic to plants and occurs as a toxic Al^{3+} form. High rainfall, Earth crust mining and other human activities are the major sources of acidic soil contamination. Any kind of stress induces oxidative stress in plants, but generation of excessive reactive oxygen species (ROS) indicates stress conditions in plants. Different abiotic and biotic stress conditions can induce ROS ($O_2^{\cdot-}$, H_2O_2, ^-OH and $^1O_2$) production in plants, which is a major toxic substance for plant system (Elstner et al. 1988). In normal conditions, ROS production also occurs in plants via different metabolic pathways (Hippeli et al. 1999) but its production increases in response to various stress conditions in plants (Shao et al. 2008) and that leads to severe oxidative damages in plants. Boscolo et al. 2003 (in maize), Achary et al. 2008 (in *Allium*) and Panda and Matsumoto 2010 (in pea) found that Al^{3+} can induce dose- and time-dependent ROS accumulation in cells. Several research groups found significant ROS production associated with oxidative damage and antioxidative activity in response to Al^{3+} stress in plants (Yamamoto 2001; Boscolo et al. 2003; Ikegawa et al. 2000; Meriga et al. 2004; Wang et al. 2004; Panda et al. 2009).

Black gram is an important pulse crop which is used as food in many parts of the world, mostly in the southern part of India. Moreover, black gram plants can maintain the nitrogen requirement in the soil environment because they have a root nodule bacterial association to fix atmospheric nitrogen. But unfortunately, acidic soil is a major constraint for crop productivity in its native place. So far, from previous reports, it has been confirmed that Al^{3+} induces root growth inhibition and modifies root morphology (Panda et al. 2003; Sharma et al. 2015; Wang et al. 2015). From this point of view, we tried to understand the morphological behaviour on black gram in response to Al^{3+} stress. This impact is entirely related to the soil environment. Very less information is known about the stress responses and loss of productivity of this crop under an acid soil situation where Al^{3+} toxicity is the major constraint. Understanding the Al^{3+} stress responses and Al^{3+} tolerance can be a solution and informative work of this major pulse crop.

Materials and method

Root growth, fresh weight and dry weight measurement

The black gram seeds were procured from the Indian Institute of Pulse Research (IIPR), Kanpur, India. Black gram seeds were surface sterilised with 1 % NaClO and kept for germination; the germinated seedlings were transferred to Hoagland nutrient solution (pH 6.2). After 3 days of growth, seedlings were treated with 100 μM of $CaCl_2$ (pH 4.7) for 24 h. Subsequently, seedlings were treated with different concentrations of Al^{3+} (10, 50 and 100 μM) in 100 μM of $CaCl_2$ (pH 5) and root growth and biochemical parameters were tested after 24, 48 and 72 h of treatment. Fresh weight and dry weight were measured to analyse the effect of Al^{3+} stress in regulating the biomass of black gram seedlings.

Histochemical assay

Black gram plants were grown in Hoagland nutrient solution and treated with different concentrations of Al^{3+} as mentioned earlier. After 0, 24, 48 and 72 h of treatment, plant tissues were used for the following histochemical assays. Evan's blue test, to analyse plasma membrane integrity, was done as per Ikegawa et al. (1998). Haematoxylin assay for aluminium uptake was done as per Ownby (1993). Schiff's reagent test for lipid peroxidation test was followed from Pompella et al. (1987). After staining, samples were observed under a compound light microscope. Photographs were taken with a Fujifilm camera under ×40 microscopic resolution.

Superoxide, H_2O_2 and protein carbonylation analysis under Al^{3+} stress

Superoxide production was measured following Elstner and Heupel (1976). Plant tissue (200 mg) was homogenised with 3 ml of 65 mM (pH 7.8) and centrifuged at 5000 rpm for 10 min. The supernatant was added with 0.9 ml of 65 mM phosphate buffer and 0.1 ml of 10 mM hydroxylamine hydrochloride and incubated at room temperature for 20 min. Then, 1 ml of sulphonylamide and 1 ml of 7 mM ∞-napthyl ethylene dihydrochloride were added. After incubation at room temperature, 1 ml of diethyl ether was added. Centrifugation was at 1500 rpm for 5 min. Then, absorbency was observed at 530 nm. H_2O_2 was determined by the method of Sagisaka (1976).

Plant tissue (200 mg) was homogenised in 5 % trichloroacetic acid (TCA) followed by centrifugation at 15,000 rpm for 20 min at 4 °C. Thereafter, the reaction mixture was prepared by adding 50 % TCA, 2.5 M potassium thiocyanate (KSCN) and 10 mM ferrous ammonium sulphate ((NH_4)$_2$Fe(SO_4)$_2$·$6H_2O$). The absorbency was observed at 480 nm by using a spectrophotometer.

Protein carbonylation was done with spectrophotometric DNPH assay of Levine et al. (1994) with some modifications. Total protein was extracted by homogenising 500 mg of root in phosphate buffer (25 mM, pH 7.0) followed by DNPH reaction. The carbonyl content was calculated by measuring absorbency

at 374 nm. The extinction coefficient of hydrazones, i.e. 221 m/mol/cm, was used for the calculation of carbonylated proteins.

Metabolic profile analysis under Al^{3+} stress by an ^{1}H-NMR approach

Black gram plants were grown under the above-mentioned hydroponic culture condition for further ^{1}H-NMR analysis following Kruger et al. (2008) with some modifications. NMR signals were analysed after Kim et al. (2010) to detect the primary metabolites under normal and stress conditions. Two hundred milligrams of fresh treated and untreated root and shoot tissues was homogenised with liquid nitrogen (LN2). The ground sample was added with 1 ml of 3 M $HClO_4$ and vortexed to mix well. Samples were incubated on ice for 15 min. Again, 1 ml of 1 M $HClO_4$ was added and the solution was shaken vigorously to get a homogenised suspension. Thereafter, samples were incubated on ice for 30 min and then centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant was collected in a new tube. The pellet was added with 2 ml of 1 M $HClO_4$ to re-suspend the pellet and centrifuged again. Both supernatants were combined together and pH was adjusted to 6.0 to neutralise the supernatant with 2 M KOH. Then, samples were kept on ice for 30 min to precipitate the $KClO_4$. After incubation, the pH was again adjusted to 6.0. The upper solution part was taken in a centrifuge tube and centrifuged at 15,000 rpm for 15 min at 4 °C. Again, the supernatant was collected and lyophilised. The freeze-dried samples were dissolved in 1 ml of 25 mM Na-phosphate buffer containing EDTA. After that, samples were centrifuged at 10,000 rpm for 15 min at 4 °C to remove $KClO_4$ precipitation. The supernatant was again lyophilised and dissolved in 0.3 ml of 10 % D_2O in Na-phosphate buffer (pH 6.8) containing trimethyl silyl propionic acid sodium salt 2,2,3,3-D_4 (TMSP), as a concentration standard and chemical shift reference ($\delta = 0$ ppm). Thereafter, the samples were centrifuged at 10,000 rpm for 30 min at 4 °C to get a final supernatant and to remove probable remaining $KClO_4$. Required amounts of samples were transferred into a NMR tube (Shigemi), and spectra were measured and recorded in ^{1}H-NMR (Bruker Biospin AVANCE III 600). Measurement parameters were as follows: probe temperature—299 K, scan number—256, receiver gain—203.

Statistical analysis

All experiments were performed thrice in replicate to get a significant variation between the control and stress samples. All statistical analyses of the data were performed by using Microsoft Excel and data were expressed as means $\pm$ SE. Significant differences between the stress and control samples were determined by Student's t test at $p \leq 0.05$.

Results

Root growth, fresh weight and dry weight measurement

The root growth inhibition test was performed in hydroponic culture with different concentrations of Al^{3+} (10, 50 and 100 μM, at pH 5). In this study, 10 μM of Al^{3+} also induces root growth inhibition (Fig. 1a). The rate of root growth inhibition increases as the time interval of stress and concentration of Al^{3+} increased. The low pH root growth inhibition analysis (Fig. 1b) showed that only low pH can reduce the root elongation rate, but with aluminium, it was more toxic to black gram. Similarly, under aluminium stress, decreased fresh (FW) and dry weight (DW) of root was observed in black gram. As the concentration of aluminium increased, the reduction of mass also increased, whereas significant increased DW was observed after 72 h of treatment among all aluminium concentrations in this study.

Histochemical assay

Histochemical assay can visualise the plasma membrane integrity lost. Evan's blue test is well known to check the integrity of the plasma membrane. It can only enter through a disintegrated plasma membrane caused by different stress media. Control roots did not show Evan's blue uptake whereas the Al^{3+}-treated roots showed gradual Evan's blue uptake, and in response to an increase in Al^{3+} concentration, an increased intensity of the blue colour was also observed (Fig. 2a). Haematoxylin test is specific to aluminium ion uptake by plant root systems, which is being represented by haematoxylin brown-coloured regions in the root tip zone. In black gram, the haematoxylin assay (Fig. 2b) reveals Al^{3+} ion uptake through the root system. Higher concentrations of Al^{3+} (50 μM)-treated plants showed more haematoxylin (~Al^{3+}) uptake in the root tip region. Schiff's reagent test was done specially to detect lipid peroxidation in the cell membrane. Due to Al^{3+} toxicity, the lipid molecules were peroxidised and some free aldehydes were released. Schiff's reagent binds with those free aldehydes and forms a magenta-coloured product, i.e. fuchsin. In the black gram plant, under Al^{3+} stress, in the root tip zone, a distinct magenta colour was spotted, while in the normal root tip, no such colour was observed. The production of magenta colour was greater in the 48-h-treated root (Fig. 2c).

Superoxide, H_2O_2 and protein carbonylation analysis under Al^{3+} stress

Superoxide ($O_2^{\cdot-}$) content was measured in response to different Al^{3+} concentrations as well as with different time intervals in both root (Fig. 3a) and shoot (Fig. 3b). Al^{3+} stress-induced ROS activity was observed as the concentration and duration
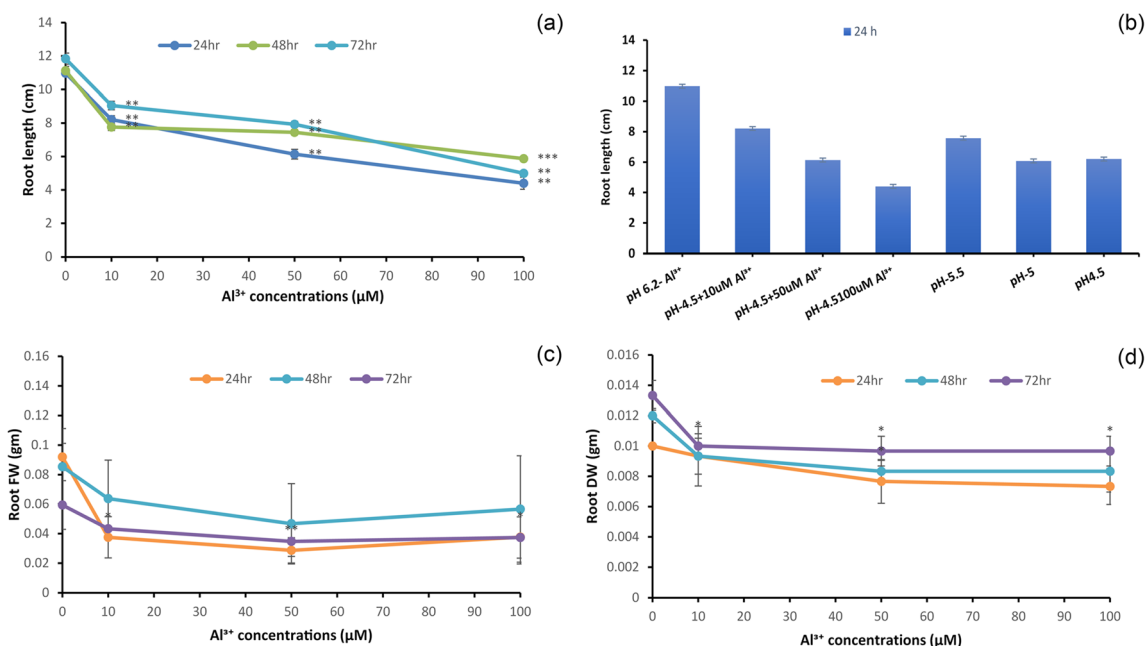
Fig. 1 **a** Root growth analysis of black gram plant in response to Al^{3+} (10, 50 and 100 μM with pH 4.7) after 24, 48 and 72 h. **b** Comparative root growth inhibition analysis between different pH values (pH 6, 5.5, 5, 4.5) and different Al^{3+} concentrations (10, 50 and 100 μM with low pH 4.7) for 24 h only. **c** Fresh weight and **d** dry weight of root were measured in response to Al^{3+} (10, 50 and 100 μM) after 24, 48 and 72 h. Data presented are means ± SE. *Asterisks* indicate significant difference from control at $p < 0.05$ by Student's t test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$)

of stress gradually increased. But a decreased superoxide content was observed after 48 h of treatment as compared to control in shoot only. It was also observed that at 10 and 50 μM, the superoxide content was greater as compared to that at 100 μM of Al^{3+} in both root and shoot. Most interestingly, in both root and shoot, a highly increasing order of superoxide content was observed after 24 h of treatment, but it was more significant in root rather than shoot.

Hydrogen peroxide (H_2O_2) content was also measured in both root (Fig. 3c) and shoot (Fig. 3d) under different Al^{3+} concentrations. The H_2O_2 content was increased gradually in all treated samples with the increasing duration of stress and concentration of Al^{3+}. But under 100 μM of Al^{3+} stress, less H_2O_2 content was observed than the other two concentrations (10 and 50 μM) in both root and shoot. Under 50 μM of Al^{3+} stress, a high level of H_2O_2 content was quantified in both root and shoot. Time-dependent analysis showed an increasing order of H_2O_2 level even in the control, but the amount was lesser than that in the stressed one in both root and shoot. A significant Al^{3+} stress-induced H_2O_2 production was observed in this study. In root, H_2O_2 production was greater under 10 and 50 μM Al^{3+}, whereas in shoot, a high level of H_2O_2 production was observed under 50 μM Al^{3+} only.

Carbonylated protein (PC) content was also measured in both root (Fig. 3e) and shoot (Fig. 3f) under different Al^{3+} stress conditions. The stressed plants showed a greater content of carbonyl proteins. The level of carbonyl-bound proteins increased with increasing Al^{3+} concentration, after 24 h of

treatment. After 48 and 72 h of treatment, the carbonylated protein content was greater as the concentration increased. But as compared to control plants at 48 and 72 h, the carbonylated protein content was less in the 24 h control plant. Under 100 μM Al^{3+} concentration, the plant showed more carbonyl-bound proteins in root. In shoot, after 48 h of treatment at higher concentration, carbonyl-bound protein content was decreased, whereas in root, after 48 h of treatment, the carbonyl-bound protein content was greater and gradually increased as the concentration increased.

Primary metabolite content analysis under Al^{3+} stress by the ^{1}H-NMR approach

^{1}H-NMR spectra of the stressed and control (root (Fig. 4) and shoot (Fig. 4)) samples were obtained based on their X: ppm value with standard spectra as per Kim et al. 2010. The spectra obtained from control samples showed a characteristic difference with the stress samples. Time-dependent analysis of metabolite change was also done and a significant difference was observed. Under stress condition, very low metabolite synthesis was observed after 24 h as compared to that in control samples. Based on the peak intensity of different samples, the level of expressed metabolites can be measured. Characteristic NMR signals (δ) are the determinants of a particular metabolite in NMR spectra. In this NMR analysis, we tried to detect certain amino acids, organic acids, sucrose and non-phenolic compounds. In this study, we were able to measure
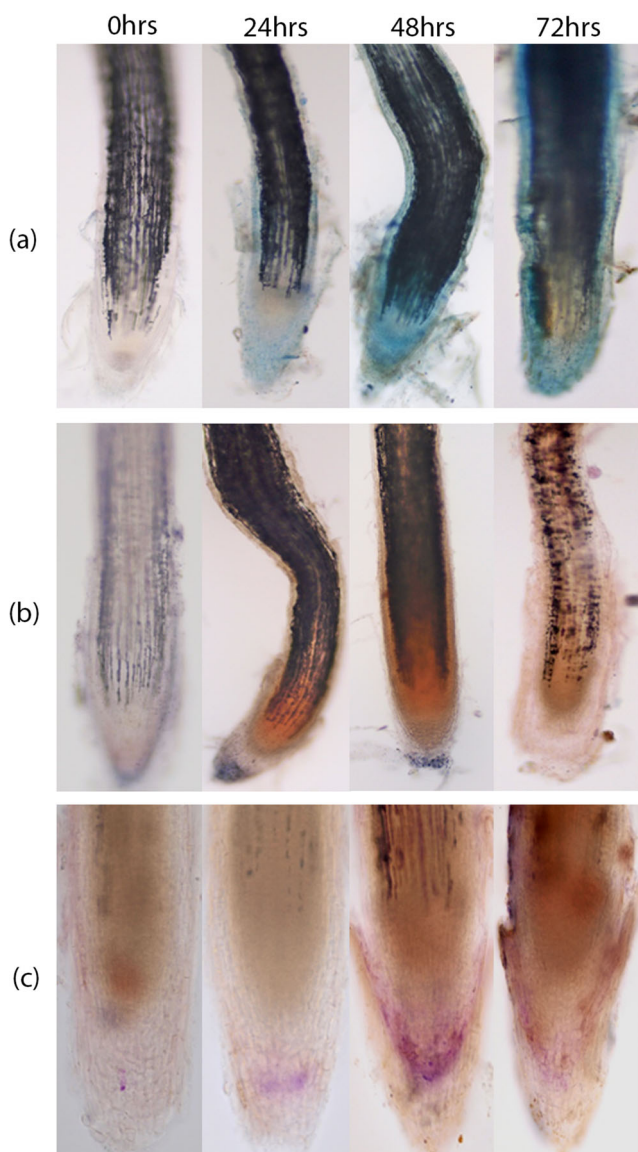
Fig. 2 Histochemical assay: **a** Evan's blue assay, **b** haematoxylin staining assay and **c** Schiff's reagent test of aluminium (+50 μM)-treated and untreated black gram plant roots observed under a compound light microscope after 0, 24, 48 and 72 h

the characteristic ^{1}H-NMR signals for tryptophan ($\delta = 7.20$), proline ($\delta = 4.08$), tyrosine ($\delta = 6.86$), alanine ($\delta = 1.48$), valine ($\delta = 1.00$), threonine ($\delta = 1.32$), formic acid ($\delta = 8.46$), fumaric acid ($\delta = 6.56$), malonate ($\delta = 3.36$), citric acid ($\delta = 2.74$), succinic acid ($\delta = 2.56$), glutamine ($\delta = 2.15$), malic acid ($\delta = 2.68$), γ-amino butyric acid ($\delta = 1.90$), choline ($\delta = 3.24$), methanol ($\delta = 3.36$), inositol ($\delta = 4.0$) and sucrose ($\delta = 5.4$). Most of them are interrelated and connected to the citric acid cycle which is a major concern for aluminium stress. Most of the metabolites get lowered in both root (Fig. 4) and shoot (Fig. 5) after 24 h of aluminium treatment as compared to control samples. But after 72 h of treatment, all peaks were detected to be increased in the stressed samples as compared to the control samples.

Discussion

Aluminium responses in pulse crop are not yet well understood by researchers. Little work has been done by some researchers in pulse crops in response to proton and aluminium toxicity. Because of aluminium contamination in agricultural fields, the productivity of pulse crops is decreasing. Our soil analysis data showed (data not shown) that in the north-east region (Assam), aluminium content in soil increased to 10–11 %, whereas in normal soil, it should be 7–8 %. This elevated percentage of aluminium added more acidity to this region. Field observation of this area proves that the productivity of crops is decreasing in order every year. In this study, we tried to understand the aluminium responses in black gram. We tried to understand the primary responses of the black gram plant under aluminium stress conditions. More than 50 % root growth inhibition was observed under the 100 μM Al^{3+} stress condition in black gram (Fig. 1a). Aluminium-induced root growth inhibition was increased by a low pH condition. Our proton toxicity analysis showed that root growth inhibition under low pH (5–4.5) is less toxic to black gram than aluminium with low pH (50 μM Al^{3+} + pH 4.5) (Fig. 1b). Similar results on root growth inhibition induced by Al^{3+} were also reported in previous studies in other plant species such as sorghum (Ohki 1987), soybean (Hai et al. 1989), wheat (Ryan et al. 1992), squash (Van et al. 1994), green gram (Panda et al. 2003), maize (Boscolo et al. 2003) and pea (Panda and Matsumoto 2010). Besides root growth inhibition analysis, we also observed a decrease in fresh and dry weight of root samples under aluminium stress. Under 50 μM Al^{3+} concentration, plants were found with decreased fresh and dry weight (Fig. 1c, d). Time interval analysis showed that after 24 h of treatment, the fresh and dry weight of plants decreased more than that of plants after 48 and 72 h of treatment. Meriga et al. (2003) suggested that the root is the major site of cytokinin-like hormones, and under aluminium stress, the production of such hormone can be reduced, which results in reduced root growth and dry mass production.

Histochemical analysis of aluminium-treated roots revealed the primary target of aluminium toxicity. From this study, it was observed that the root tip and elongation zone are the major sites of aluminium interaction (Taylor 1995). Foy (1988) found that aluminium-like toxic species can cause reduced root cell division by increasing the rigidity of the double helix of DNA. Aluminium causes plasma membrane integrity loss which was confirmed by Evan's blue test. In this assay, a gradual uptake of the blue colour indicates that long treatment with aluminium causes plasma membrane disintegration (Fig. 2a). This result was also supported by the haematoxylin assay (Cançado et al. 1999), where it was observed that with the increased duration of aluminium treatment, the uptake of aluminium was also increased in terms
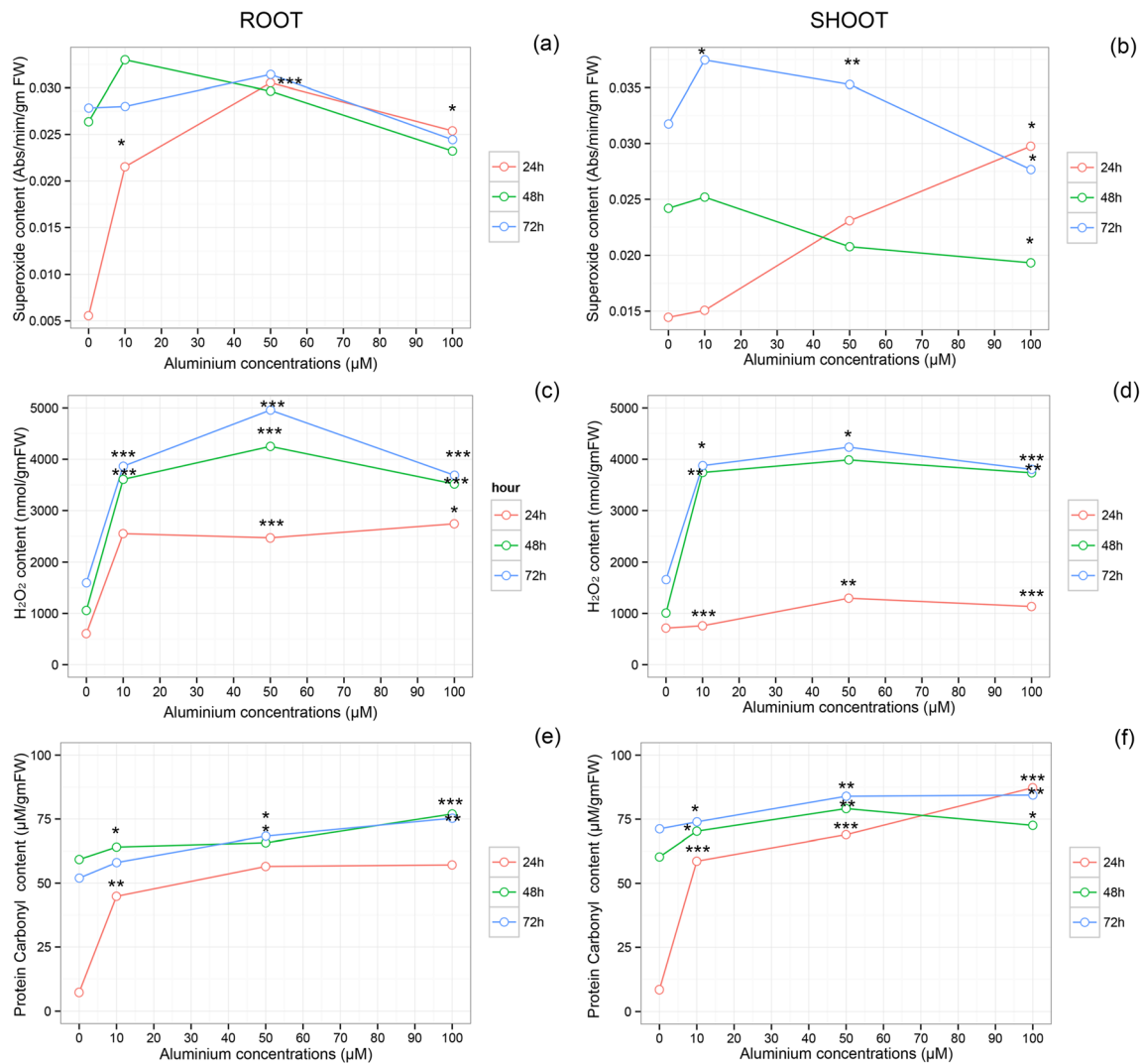
Fig. 3 Changes in the activity of superoxide content in root (**a**) and shoot (**b**), H_2O_2 content in root (**c**) shoot (**d**) and protein carbonyl content in root (**e**) shoot (**f**) of black gram seedlings after 24, 48 and 72 h of aluminium treatment [control (0), 10, 50 and 100 μM]. Data presented are means

$\pm$ SE. *Asterisks* indicate significant difference from control at $p < 0.05$ by Student's t test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). The graphical representation of these data was created by using ggplot2 package of R programme (Wickham 2009; R Core Team 2013)

of haematoxylin colour (Fig. 2b). Haematoxylin can bind to aluminium to form a complex which gives a reddish brown colour. The brown colour that appeared in the root tips of aluminium-treated plants after 24, 48 and 72 h indicates the uptake of aluminium in the root tip region at the preliminary stage of stress. But more interestingly, it was observed that after 48 h of stress condition, the root tips showed a more intense colour which was less after 72 h of stress. Wu et al. (2012) found that ROS production and lipid peroxidation were higher during the early stage of aluminium treatment and they gradually declined as the duration of stress increased. After entering into the root tip of the plant, aluminium starts modifying the cellular content and integration, which was characterised by Schiff's reagent test. This test showed that under aluminium stress, roots undergo lipid peroxidation. After 24 h of treatment, root tips showed pink-coloured lesions.

These lesions are the complexes of Schiff's reagent, and free aldehydes are released from the lipid peroxidation process, which was caused by aluminium. In the 0-h-treated root, no such lesions were observed. Moreover, the pink-coloured lesions were more prominent in the 48-h-treated root samples (Fig. 2c). From this point, we can suggest that more aluminium uptake can cause more lipid peroxidation in cells, due to oxidative damage of polyunsaturated fatty acids by excessive ROS production induced by aluminium in cells promoting lipid peroxidation (Andrews et al. 1965).

ROS can be generated by cellular metabolism in plants (Kim et al. 2008; Song et al. 2010; Elstner 1982). But excess ROS production can be induced by certain stresses, which is toxic to plants. Most plant defence mechanisms can scavenge the excess ROS activity via various metabolic processes. In this study, it was observed that the ROS level was induced by
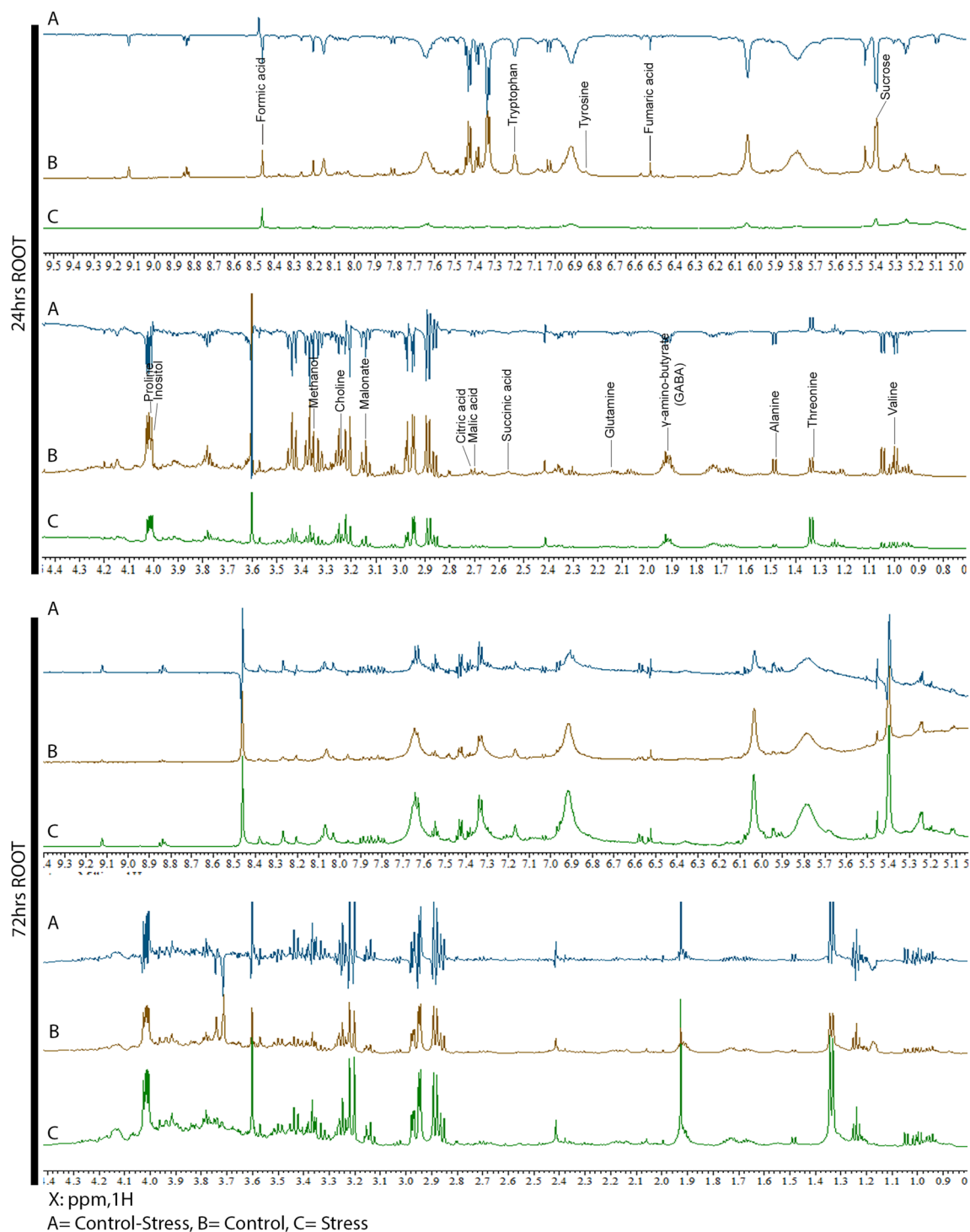
Fig. 4 ^{1}H-NMR spectra of black gram root extract after 24 and 72 h under $+Al^{3+}$ and $-Al^{3+}$ treatment. The δ values representing the specific metabolites. The ^{1}H-NMR spectra were obtained by using a ^{1}H-NMR (Bruker Biospin AVANCE III 600) spectrometer

aluminium. Under 10 μM Al^{3+} concentration, plants showed a high level of superoxide content, which indicates that a lower concentration of aluminium can also enhance the production of ROS and can cause oxidative damages. As compared to shoot (Fig. 3b), the superoxide level was higher in root (Fig. 3a), because the root is the primary site of aluminium

toxicity. In this study, we observed a significant increment of H_2O_2 after 24 h of aluminium treatment as compared to control ($-Al$) in both root and shoot. For 24 h of treatment, H_2O_2 content was less increased than that for 48 and 72 h of treatment in both root (Fig. 3c) and shoot (Fig. 3d). A gradual increased level of H_2O_2 was observed with the duration of
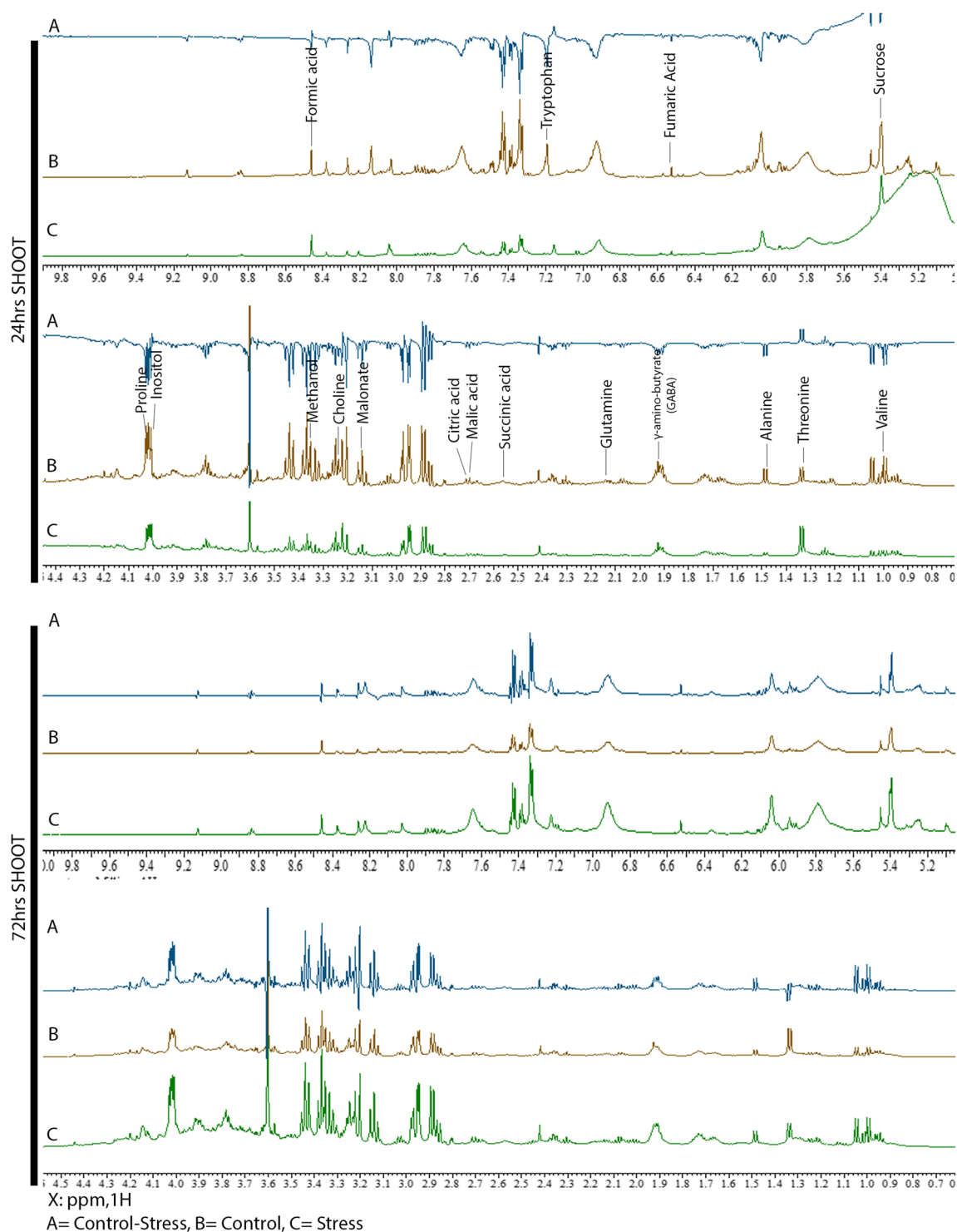
Fig. 5 ^{1}H-NMR spectra of black gram shoot extract after 24 and 72 h under +Al^{3+} and −Al^{3+} treatment. The δ values representing the specific metabolites. The ^{1}H-NMR spectra were obtained by using a ^{1}H-NMR (Bruker Biospin AVANCE III 600) spectrometer

stress increased. From this data, we assumed that aluminium can induce more ROS activity. Wu et al. (2012) suggested that H$_2$O$_2$ might work as a signalling molecule to activate the tolerance mechanism. The high amount of H$_2$O$_2$ can cause stomata closure and decrease the photosynthetic rate; as a result, crop productivity becomes less (Aftab et al. 2010).

Another effect of excessive ROS production was observed by means of carbonyl content in both root and shoot. Interestingly, after aluminium stress, an increased level of carbonyl protein content was observed. When excessive ROS production is not scavenged by antioxidant regulatory mechanisms, protein carbonyl content is suggested to be an indicator for

oxidative damage (Juszczuk et al. 2008 and Achary et al. 2008). Accordingly, in this study, we observed that after 24 h of treatment, the protein carbonyl content was greater in the control than that in both root (Fig. 3e) and shoot (Fig. 3f), but it was found to be less increased after 48 and 72 h of treatment. This might be because of the induction of antioxidative defence mechanism that may give resistance to aluminium stress in black gram. This was also supported by our ^{1}H-NMR analysis of the metabolic profile of black gram. We observed that after 24 h of aluminium treatment, black gram plants showed a decreased level of certain metabolic products, but after 72 h of treatment, it was increased as compared to untreated samples. In our ^{1}H-NMR data, we have observed certain metabolites which play a critical role in metabolic processes in plants, such as tryptophan which acts as a precursor in auxin, protein biosynthesis. Fumarate is an intermediate in the citric acid cycle. Fumarate can be converted into malate with the help of the enzyme fumarase and gives malate as a product, which is a major detoxifying organic acid involved in aluminium stress in plants. Changes in sucrose content were also observed in our data, which is a major source for energy production. On the other hand, proline (a major non-enzymatic antioxidant in plants) content was also increased with the time of stress increased. But, as compared to control, the content was less/decreased at the initial stage of stress; it indicates that aluminium stress induces proline production after long exposure. It can act as an osmo-protectant and keeps proteins safe against a stressed environment (Pollard and Wyn Jones 1979). Proline also helps to scavenge hydroxyl radicals produced from stressful conditions (Smirnoff and Cumbes; 1989). Proline formation is implicated as a mechanism of alleviating cytoplasmic acidosis and may maintain NADPH/NADP$^+$ ratios at values compatible with metabolism (Hare and Cress 1997). Another important metabolite that has been detected by our study is GABA, which can be converted into succinic acid semialdehyde and take part in the citric acid cycle by oxidising itself into succinic acid. This condition was the same in both root and shoot. Metabolic processes get interrupted after treating with aluminium stress which caused damage to black gram, but as long as we kept the stress on the plants, defence mechanisms also activate possibly to revive its metabolic homeostasis.

Conclusion

From this investigation, we conclude that aluminium-induced ROS production caused major oxidative damage like lipid peroxidation and protein carbonylation and ROS activity and also altered the metabolic status at the early stage of stress. From the ROS and NMR quantification data, it was observed that prolonged aluminium treatment may be managed by the activation of metabolic processes that may have a chance of either tolerating or falling prey to Al stress. Our investigation also supports the previous findings in terms of root growth inhibition and primary root cell injury. Histochemical assay indicates the post-detoxification mechanism involved in black gram, which means long treatment with aluminium in black gram may be managed by several detoxification mechanisms that again will depend on the genotype and severity of Al stress.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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