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ORIGINAL PAPER

Ameliorative capacity of salicylic acid and nutrients (Ca, P, and Mg) against aluminum toxicity in sensitive pea (*Pisum sativum* L.)

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Abstract

Aluminum (Al) toxicity is a limiting factor for crop growth, especially legumes in acid-affected soil of the world. The present study was done with the objective to study the comparative ameliorative property of exogenously added Ca, Mg, P, and salicylic acid on Al stress in pea (*Pisum sativum*) grown in nutrient media. Treatment of seedling with 24 ppm Al resulted in an inhibition of root/shoot growth with less dry matter production along with an enhanced level of superoxide, H_2O_2, and lipid peroxidation. Al toxicity in a pea seedling caused significant variation in the activity of an antioxidative enzyme. The application of 1 mM $CaCl_2$, 0.5 mM H_3PO_4, $MgSO_4$ (0.5 mM and 0.25 mM), and SA (100 µM) reduced the negative effect of Al stress in the pea seedling marked by the restoration of seedling growth and suppression of Al accumulation, superoxide, and H_2O_2 content. $MgSO_4$ and SA treatment elevated the Al-induced decline in photosynthetic pigment. Exogenously, an addition of Mg, P, and SA elevated the Al-induced decrease in the activity of SOD, catalase, vitamin C, and carotenoids. Application of 0.5 mM H_3PO_4 resulted in 47% recovery of root growth and 60% of shoot growth along with the highest suppression of Al uptake and ROS accumulation. Among all the treatments, the application of the 0.5 mM P under Al stress appeared a potential tool in restoring the growth and physiological activities in sensitive pea genotype (AP-3) indicating the best ameliorating agent under Al toxic condition.

Keywords : Aluminum toxicity · Phosphorus · Magnesium · Calcium · Enzymatic and non-enzymatic antioxidant

Introduction

Aluminum (Al) is one of the most abundant metals found in the Earth's crust, contributing nearly 7% of its mass. Generally, it occurs in non-phytotoxic forms like aluminosilicates and precipitates, but its toxicity occurs mainly in acidic soil. Acidic soils constitute 40% of total cultivated land across the world and pose a major threat to agricultural crop production (Kochian 1995). Soil acidification occurs naturally when basic cations are leached out from soils, and the process is accelerated by heavy rainfall. Various plant species are sensitive to micromolar concentration of Al which hampers the physiological processes and growth. The Al-sensitive plant species express oxidative stress and very low antioxidative property under toxic Al concentration which creates abiotic stress and drastically restrict the crop growth and development (Naz et al., 2022). Al-induced acidity causes oxidative injury in leaf and root cells, which leads to permanent damage, or death of the entire plant (Ahmad et al., 2019). In Al-induced abiotic stress acid soils, change in the ion distributions and water availability retraction within plant

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cells causes the plant to grow slowly, produce less, and be of lower quality (Khalid et al., 2020).

Pea (*Pisum sativum* L.) is an important legume crop grown all over the world, and it provides important dietary protein to millions of people. Many abiotic stresses affect the production of peas, of which Al toxicity in acidic soil is a major constraint for pea growth and physiology (Kichigina et al. 2017). The primary effect of phytotoxic Al is the inhibition of root growth in peas and injury to root tips making it hard and subtle (Motoda et al. 2010). Due to Al stress, reactive oxygen species (ROS) viz. superoxide radical, hydrogen peroxide (H_2O_2), and hydroxyl radical is enhanced in plants (Borgo et al. 2020) which causes damage to protein and causes lipid peroxidation. Plants have a mechanism to defend Al toxicity through various methods viz. secretion of organic acids from roots which capture free Al toxic to plants making it unavailable, increasing the soil pH around the roots, internal detoxification by Al chelation inside plant tissues, compartmentalization of Al in the vacuole (Liu et al. 2009), and enzymatic and non-enzymatic antioxidants which scavenge the ROS (Pandey et al. 2014).

It is reported that the growth and productivity of peas may enhance either by screening for Al tolerance (Singh and Choudhary 2010; Kichigina et al. 2017) or by incorporation of remedial measures such as liming and use of boron (Yu et al. 2009) and mucilage (Geng et al. 2012). The macronutrients viz. P, Mg, and Ca have been reported by many researchers as potential ameliorating agents for Al toxicity (Iqbal 2014; Guo et al. 2006; Bose et al. 2011). Calcium is known to ameliorate Al stress in plants by increasing the soil pH and reducing the uptake of Al (Guo et al. 2006). In peas, Ca treatment has decreased the negative effect of Al on K uptake by roots (Matsumoto and Yamaya 1986). Magnesium (Mg) can prevent Al migration through the cytosolic plasma membrane in root tips. Mg plays an important role in the amelioration of Al toxicity by competing for membrane transporters and metal binding sites on enzymes (Bose et al. 2011). Kinraide et al. (2004) have shown that a higher concentration of Mg in the growth medium helps in the amelioration of Al toxicity in plants. Phosphorus is an important macronutrient for root growth in plants and alleviates Al toxicity by forming a complex with Al thus inhibiting the uptake by plants (Iqbal 2014). Salicylic acid (SA), a phyto-protectant and hormone, plays an important role in various kinds of environmental stress. It inhibits the Al uptake in the plant by enhancing the citrate efflux of the roots (Yang et al. 2003). Application of SA in plants has been shown to protect the cell from oxidative stress by activating the enzymatic defense response (Pandey et al. 2014; Nawaz et al. 2021).

In recent years, a lot of work has been put into discovering an efficient method for creating abiotic Al-induced stress tolerance in plants, which will ultimately assist plants in thriving and surviving in situations that are caused by Al-induced acidity. The most promising technique for enhancing plant tolerance under acidic circumstances is the exogenous application of plant phytohormones, particularly the use of salicylic acid and different nutrients. The action of different nutrients and hormones has been studied individually to observe the ameliorative effect on Al toxicity, but comparative studies among them to depict better ameliorative capacity were limited in pea crops.

In order to examine whether Ca, Mg, P, and salicylic acid could be used to alleviate Al toxicity and prevent oxidative damage to pea seedlings the present study was done with the objective to study the comparative effects of exogenously added Ca, Mg, P, and salicylic acid in the growth medium on Al accumulation, ROS production, and the response of antioxidative defense in pea seedling grown under Al stress.

Materials and methods

Plant material and experimental site

The experiment was carried out at the College of Horticulture and Forestry CAU, Pasighat, Arunachal Pradesh, India, during 2019–2020. The plant materials for the present study comprised one susceptible genotype (Azad Pea-3). The susceptibility/tolerance of the genotype was accessed by hematoxylin staining and growth (data not reported here).

Table 1 Treatment details

Treatment number	Treatment details*
T1 (control)	Only the Hoagland nutrient solution
T2 (Al)	24 ppm Al
T3 (Ca+Al)	1 mM $CaCl_2$+24 ppm Al
T4 (0.25 mM P+Al)	0.25 mM KH_2PO_4+24 ppm Al
T5 (0.5 mM P+Al)	0.5 mM KH_2PO_4+24 ppm Al
T6 (0.25 mM Mg+Al)	0.25 mM $MgSO_4$+24 ppm Al
T7 (0.5 mM Mg+Al)	0.5 mM $MgSO_4$+24 ppm Al
T8 (50 µM SA+Al)	50 µM Salicylic acid+24 ppm Al
T9 (100 µM SA+Al)	100 µM salicylic acid+24 ppm Al

*Seedling supplied with only Hoagland nutrient solution served as control and nutrient solution containing the above chemicals served as treatment

Experimental setup and plant growth

The present study was planned under a completely randomized design (CRD) with 9 treatments and three replications (Table 1). For each treatment, 15 seeds were taken, and after sterilization with sodium hypochlorite, 3% (v/v) was soaked in water for 12 h. The seeds were sown in plastic pots of 1.5 l capacity containing inert sand and allowed to germinate and establish for 6 days. On the 6th day after sowing, seedlings were supplied with only the Hoagland nutrient solution (Hoagland and Arnon 1950) served as control or the Hoagland nutrient solution containing 24 ppm Al,1 mM $CaCl_2$+24 ppm Al, 0.25 mM KH_2PO_4+24 ppm Al, 0.5 mM KH_2PO_4+24 ppm Al, 0.25 mM $MgSO_4$+ppm Al, 0.5 $MgSO_4$+24 ppm Al, 50 µM SA+24 ppm Al, and 100 µM SA+24 ppm Al which served as treatment (Table 1). The pH of the Hoagland nutrient solution was maintained at 4.5 with 1 N HCl. During the growth period, the plants were kept under artificial light (3000 lux) for 14 h photo-period at a temperature of 24/18 °C (day/night) and 70% relative humidity. Nutrient solution was replenished every 5 days. And after 14 days of treatment, plants were uprooted carefully and stored at −80 °C for further analysis. For dry matter content, the sample was kept in a hot air oven and measured in an analytical balance. The dried sample was also used for Al uptake estimation.

Statistical analysis

The analysis of variance was done according to the standard method given by Gomez K. and Gomez A. (1984). The mean comparison within the treatment was done by Tukey's HSD test at a 5% probability level using the SPSS software (version 21). In the graph, different letters indicate significant differences at $p>0.05$, and the error bar = mean ± SD ($n = 3$).

Protein and lipid peroxidation estimation

The protein in the pea sample was estimated by Lowry's method (Lowry et al. 1951). The lipid peroxidation was estimated in terms of the thiobarbituric acid reactive substance (TBARS) method as suggested by Heath and Packer (1968). The TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}, and it was expressed as nmol TBARS g^{-1} fresh weight (FW).

Fresh samples (100 mg) were homogenized in 5 ml of 10% trichloro-acetic acid (TCA) containing 0.25% 2-thiobarbituric acid (TBA). The mixture was heated for 25 min at 95 °C and immediately cooled in an ice bath. Then, the aliquot was centrifuged for 10 min at 10000 rpm, and the supernatant was collected for reading absorbance at 532 nm using 0.25% TBA in 10% TCA as blank. For the values of non-specific absorption recorded at 600 nm, values recorded at 532 nm were subtracted. Lipid peroxide concentration was calculated using the following formula:

$$\text{nmol TBARS/g fresh weight} = \frac{(\text{Abs@532nm} - \text{Abs@600nm}) \times \text{Vol. of reaction mixture} \times 1000}{\text{extinction coefficient} \times \text{weight of the sample (g)}} \quad (1)$$

Estimation of reactive oxygen species (ROS)

The superoxide anion content in the plant tissue was assayed by the epinephrine method (Mishra and Fridovich 1972) based on its capability to inhibit the autoxidation of adrenaline to adrenochrome. Plant sample (100 mg) was cut into pieces (2–4 mm) and was kept overnight in the reaction mixture containing 100 µmol/l disodium-EDTA, 20 µmol/l NADH, and 20 mmol/l NaH_2PO_4 NaH_2PO_4 buffer pH (7.8). Fresh epinephrine was prepared in 0.1 M HCl and was added to the samples for initiating the reaction. The samples were shaken at 150 rpm and 28 °C for 5 min. The absorbance of the supernatant was read at 480 nm for 10 min. The formation of O_2 was expressed as ΔA [480 nm] min^{-1} g^{-1} tissue fresh weight.

Hydrogen peroxide content was estimated according to Jana and Choudhuri (1981). Hydrogen peroxide was measured both in the shoot and roots using the UV-Vis spectrophotometer (Hitachi U-1900) after a reaction with titanium sulfate. The level of H_2O_2 production was calculated using an extinction coefficient of 0.28 μM^{-1} cm^{-1} and expressed as nmol per g fresh weight of the tissues.

The content of hydroxyl free radical was estimated by the deoxyribose assay given by Halliwell and Gutteridge (2007). The absorbance was measured at 532 nm.

Determination of enzymatic and non-enzymatic antioxidants

The catalase activity in the root and shoot was measured by the procedure outlined by Beers and Sizer (1952). For the assay, 200 µl of the extracted enzyme was taken and mixed with 100 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of 50 mM H_2O_2. The change in absorbance was recorded at 240 nm at an interval of 5 min at 25 °C. The enzyme activity was expressed as unit mg^{-1} protein min^{-1} and calculated using an extinction coefficient of 0.036 mM^{-1} cm^{-1}.

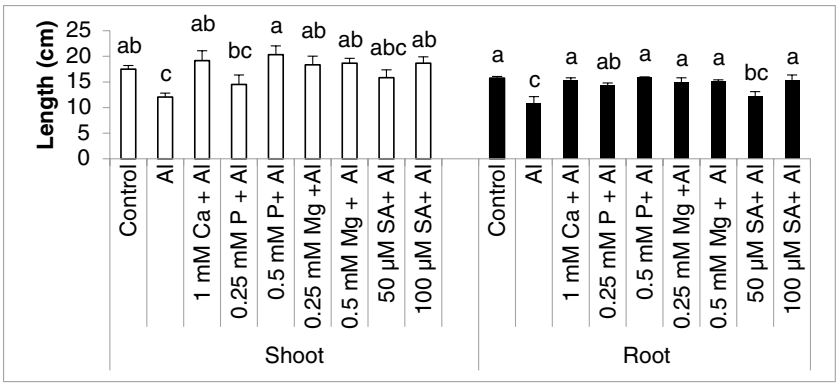

Fig. 1 Effect of treatment on length of shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

The activity of superoxide dismutase (SOD) was estimated based on the photochemical reduction of riboflavin as superoxide and the inhibition of nitroblue tetrazolium (Beauchamp and Fridovich 1971). Samples (50 mg) were grounded in a condition with 100 mM potassium phosphate buffer (5 ml, pH 7.8) containing 0.1 mM EDTA, 2% (w/v) PVP, and 0.5% (v/v) Triton X-100. Centrifugation of homogenate was done for 10 min at 10000 rpm and 4 °C. The supernatant was dialyzed in cellophane membrane tubings for 6 h against the extraction buffer in cold with 3–4 changes of the buffer. SOD activity was determined in supernatants. One unit of enzyme activity is expressed as the amount of enzyme giving a 50% inhibition of the NBT reduction. The absorbance was measured at 560 nm, and the values were expressed in units per mg protein.

APX catalyzes the reduction of H_2O_2 using the substrate ascorbate, and it was estimated using the method of Nakano and Asada (1981). The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM AsA, and 1 mM H_2O_2. Diminishing absorbance was recorded at 290 nm at 30-s intervals for 5 min. The enzyme activity was expressed as µmol ascorbate oxidized min^{-1} mg^{-1} protein and calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

The estimation of guaiacol peroxidase (GPX) was determined by the method given by Egley et al. (1983). The homogenate was centrifuged at 22000×*g* for 10 min at 4 °C, and the supernatant after dialysis was used for enzyme assay. An increase in absorbance due to the formation of tetraguaiacohinone was measured at 420 nm (extinction coefficient of 26.6 mM^{-1} cm^{-1}) at 30-s intervals up to 3 min using a spectrophotometer (Hitachi U-1900).

Vitamin C content was estimated by the method of Jagota and Dani (1982), and photosynthetic pigment was done by following Lichtenthaler's method (Lichtenthaler 1987).

Al uptake estimation

Plant samples were dried at 70 °C, powdered, and sieved through a 0.5-mm sieve. One gram of plant sample was digested in a 10-ml tri-acid mixture (9:4:1 mixture of nitric acid, perchloric acid, and sulfuric acid), and the volume was made up to 100 ml using distilled water.

The aluminum in the plant sample was determined colorimetrically using an aluminon-acetate buffer (Barnhisel and Bertsch, 1982).

Fig. 2 Growth inhibition due to Al toxicity and recovery using different ameliorating agents

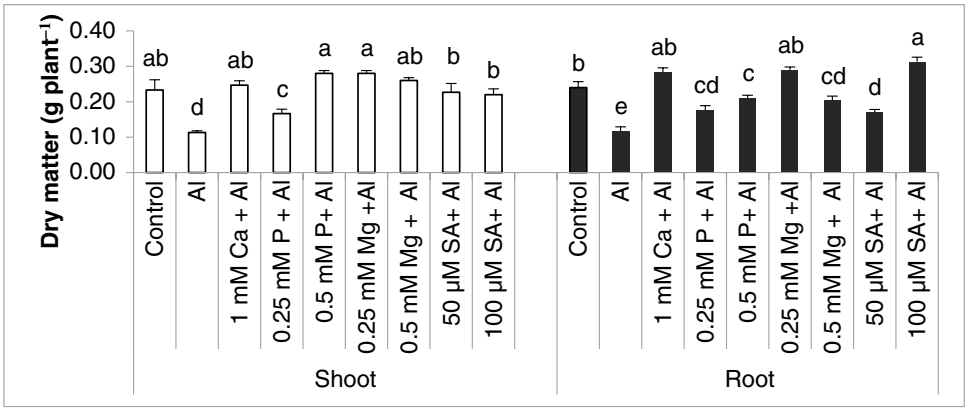

Fig. 3 Effect of treatment on dry matter content of shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

Results

Effect of Al on growth and ameliorative capacity of different chemical agents

The present study revealed that Al in sand culture had a significant decline in pea seedling growth. Al treatment reduced the root length of the pea seedling by 31% ($p<0.05$) (Fig. 1). Root length recovery in the presence of 24 ppm Al was nearly 47% ($p<0.05$)with the application of 0.5 mM KH_2PO_4 which was statistically similar with 0.25 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.25 mM $MgSO_4$, and 100 µM salicylic acid (Fig. 2). Al treatment reduced shoot length of the pea seedling by 31% ($p<0.05$) (Fig. 1). Shoot length recovery in the presence of 24 ppm Al was nearly 60% ($p<0.05$) with the application of 0.5 mM KH_2PO_4 which was at par with 1 mM $CaCl_2$.

Al treatment reduced the shoot and root dry weight of the pea seedling by 51% ($p<0.05$) (Fig. 3). In the presence of Al, application of nutrients and SA had significant restoration of dry weight in the pea seedling. The restoration of shoot dry weight was nearly 1.5-fold ($p<0.05$) with the application of 0.5 mM KH_2PO_4 and 0.25 mM $MgSO_4$ in the presence of 24 ppm Al.

Photosynthetic pigment and total soluble protein

Pea seedlings grown for 14 days in the presence of Al resulted in nearly 26% ($p<0.05$) and 29% ($p<0.05$) decrease in chlorophyll a and chlorophyll b, respectively compared to the control (Fig. 4). In the presence of 24 ppm Al, nearly 105% ($p<0.05$) chlorophyll a was observed with the application of 50 µM SA (2.24 mg g^{-1}) which was at par with the application of 0.5 mM $MgSO_4$ (2.10 mg g^{-1}), and chlorophyll b was observed highest in 50 µM SA (1.03 mg g^{-1}) Al followed by 0.5 mM $MgSO_4$ (0.86 mg g^{-1}). Presence of 24 ppm Al in the growing media had no significant effect on the protein content of the shoot but a nearly 49% ($p<0.05$) decrease in soluble protein content in the root, compared to the control (Fig. 5). Application of nutrient and SA along with 24 ppm Al resulted in a significant increase in soluble protein content in the shoot, and the highest was observed with the application of 0.5 mM $MgSO_4$ (29.6 ± 1.3 mg g^{-1}) which was at par with 0.25 mM $MgSO_4$ (28.6 ± 1.4 mg g^{-1}). In the root of pea grown in the presence of 24 ppm Al, application of 1 mM Ca, 0.25 mM and 0.5 $MgSO_4$, 0.5 mM KH_2PO_4, and 50 µM and 100 µM SA was able to restore soluble protein to

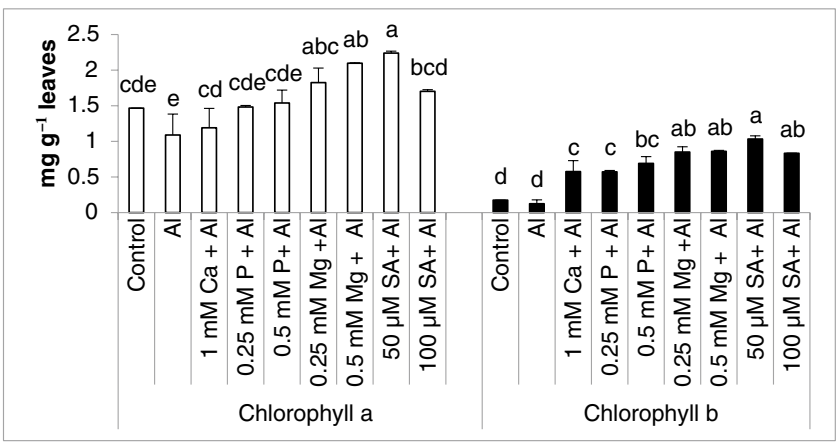

Fig. 4 Effect of treatment on chlorophyll a and chlorophyll b in leaves. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

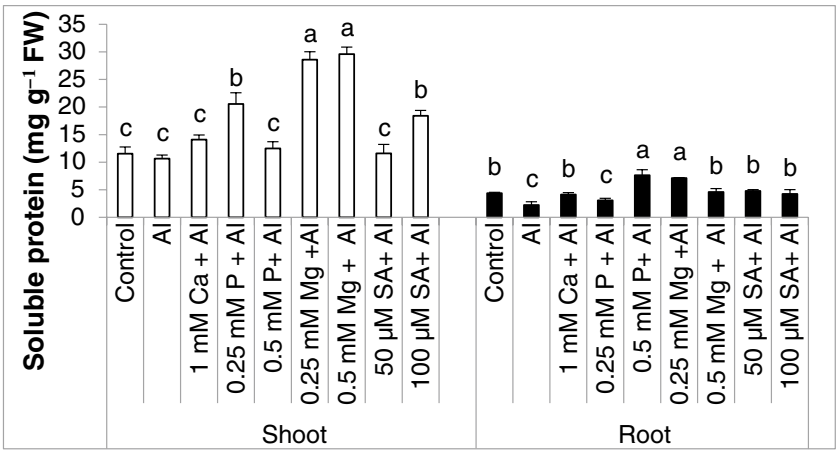

Fig. 5 Effect of treatment on the soluble protein content of shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

normal levels or even higher as compared to control. A 2.4-fold increase in protein was observed with the application of 0.5 mM KH_2PO_4 in the presence of Al.

ROS generation and lipid peroxidation

Lipid peroxidation content in the shoot and root of pea seedlings in the presence of 24 ppm Al in the growing media significantly increased by nearly 78% ($p<0.05$) in the shoot and 110% ($p<0.05$) in the root, respectively compared to no Al (Fig. 6). However, application of 1 mM Ca, 0.5 mM P, 0.25 mM Mg, and 50 µM and 100 µM SA along with Al resulted in an even higher lipid peroxidation than Al treatment alone. However, application of 0.25 mM KH_2PO_4 in the presence of Al reduced lipid peroxidation by 5.7% ($p < 0.05$) with respect to 24 ppm Al treatment alone in pea root.

In the presence of 24 ppm Al for 14 days in sand culture, the level of superoxide was enhanced by nearly 29% ($p<0.05$) and 45% ($p<0.05$) in pea seedlings, respectively compared to the control (Fig. 7). Application of nutrient and SA reduced the production of Al-induced superoxide anion in root and shoot. In Shoot, application of 50 µM SA was best for reducing Al-induced superoxide by 55% ($p<0.05$) followed by 0.5 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.5 mM KH_2PO_4, and 1 mM $CaCl^2$. In the root of pea seedling, 0.25 mM KH_2PO_4 application reduced Al-induced superoxide by 25%.

H_2O_2 production in the shoot was enhanced by 67% ($p<0.05$) and a 3.4-fold ($p<0.05$) in the presence of 24 ppm Al in growing media for 14 days with respect to no Al (Fig. 8). In the shoot of pea seedling, application of 50 µM SA+Al reduced H_2O_2 production by nearly 42% ($p<0.05$) with respect to 24 ppm Al treatment alone. The application of nutrients and SA along with Al inhibits the production of H_2O_2 significantly with respect to 24 ppm Al alone. In The presence of Al, addition of 0.25 mM and 0.5 mM KH_2PO_4 reduced the production of H_2O_2 in the root of pea seedlings by nearly 84% ($p<0.05$) and 72%, respectively compared to 24 ppm Al alone.

Addition of 24 ppm Al in the growing media of pea seedlings for 14 days had nearly 83% ($p<0.05$) enhanced level of hydroxyl free radical in shoot and a 1.2-fold ($p<0.05$)

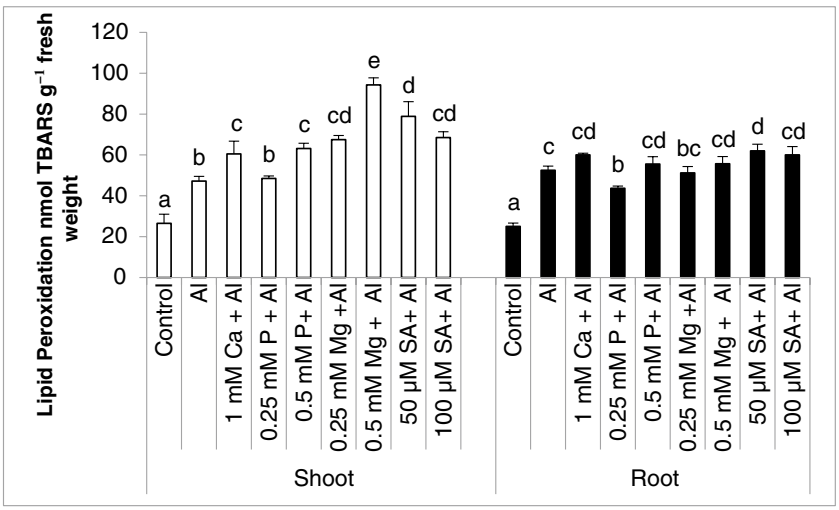

Fig. 6 Effect of treatment on lipid peroxidation in shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

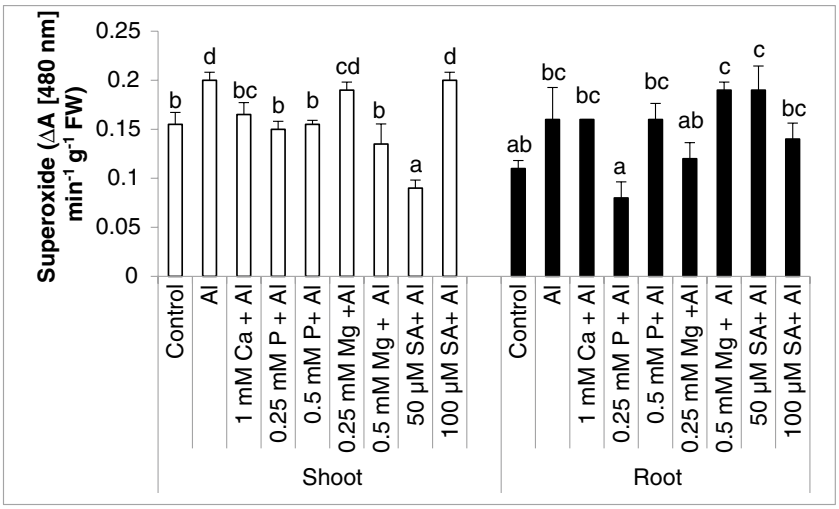

Fig. 7 Effect of treatment on superoxide in shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

increase in root compared to hydroxyl free radical level in control (Fig. 9). In the shoot, only 1 mM $CaCl_2$ was able to reduce Al-induced hydroxyl free radical production by nearly 18%. Root nutrient and SA significantly reduced the production of an Al-induced hydroxyl radical. Application of 100 µM SA reduced Al-induced hydroxyl free radical production by 31% ($p<0.05$) followed by 0.25 mM KH_2PO_4 (29%, $p<0.05$).

Enzymatic antioxidant defense system

Addition of 24 ppm Al resulted in a decrease of nearly 16% ($p<0.05$) and 21% ($p<0.05$) in the catalase activity in shoots and roots of pea seedlings compared to the control (Fig. 10). In pea seedlings applied with 0.5 mM P and 50 µM SA with Al, a significant increase of nearly 20% and 32%, respectively, in catalase activity was observed with Al treatment alone. In the root of pea seedlings, the catalase activity was lower with the application of nutrients and SA along with 24 ppm Al with respect to Al-treated seedlings. Pea seedlings grown for 14 days under 24 ppm Al resulted in nearly 8-fold ($p<0.05$) increased activity of SOD in the shoot and a decrease of 42% ($p<0.05$) in the root compared to the control (Fig. 11). Addition of KH_2PO_4, $MgSO_4$, and SA along with Al reduced the Al-induced SOD activity in the shoot significantly. The highest reduction in the Al-induced SOD activity in the presence of Al was observed with the addition of 0.5 mM $MgSO_4$ in the shoot. In the root of peas, SOD activity was found highest with the application of 0.25 mM KH_2PO_4+24 ppm Al (59.11 U/mg protein). Addition of 24 ppm Al in media increased by nearly 22% ($p<0.05$) and 2.8-fold ($p<0.05$) in the APX activity of shoots and roots of pea seedlings, respectively compared to the control (Fig. 12). Application of 0.25 mM and 0.5 mM KH_2PO_4, 0.25 mM and 0.5 mM $MgSO_4$, and 50 µM and 100 µM SA resulted in a significant decline in Al-induced APX activity in shoots of peas (Fig. 12), whereas in the root of peas, application of 1 mM $CaCl_2$, 0.25 mM KH_2PO_4, 0.25 mM KH_2PO_4, and 0.5 mM and $MgSO_4$ 50 µM SA reduced Al-induced APX significantly**.** Application of

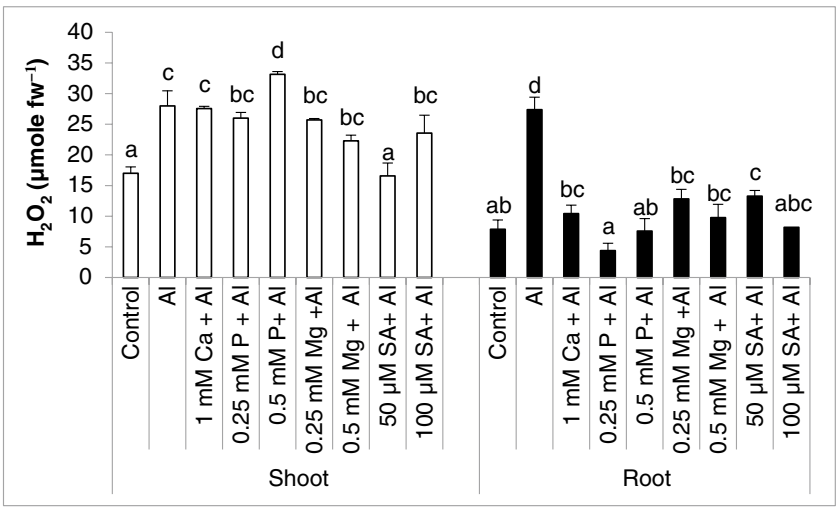

Fig. 8 Effect of treatment on the production of H_2O_2 in shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

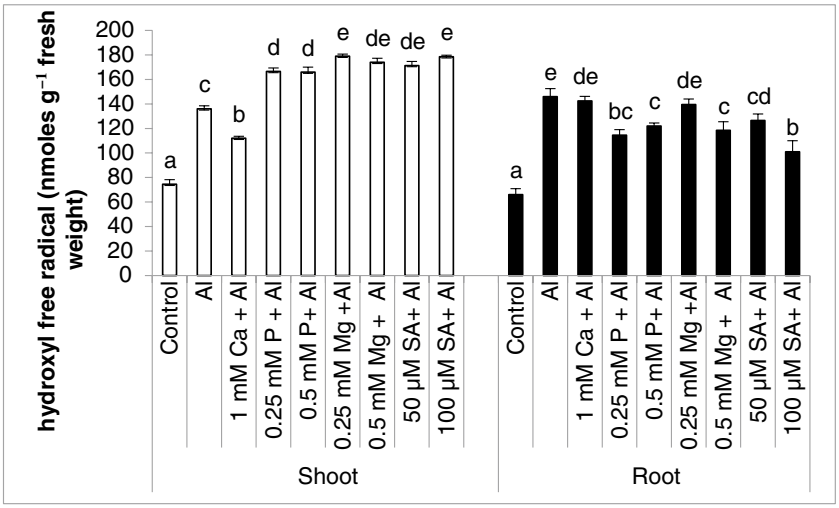

Fig. 9 Effect of treatment on the production of hydroxyl radical in shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

0.5 mM KH_2PO_4+24 ppm Al resulted in the lowest APX activity in the root. Pea seedlings grown for 14 days under 24 ppm Al had a non-significant effect on GPX activity in the shoot and nearly 20% ($p<0.05$) decrease in GPX activity of root compared to no Al (Fig. 13). Application of different treatments could not restore the GPX activity to a normal level.

Non-enzymatic antioxidants

Vitamin C content of pea seedlings grown for 14 days in the presence of 24 ppm Al significantly reduced by nearly 74% ($p<0.05$) in the shoot and nearly 36% ($p<0.05$) in the root compared to vitamin C in the control (Fig. 14). In the presence of 24 ppm Al, a 3.5-fold ($p<0.05$) increase in vitamin C content in the shoot was observed with the application of 0.5 mM KH_2PO_4 (19.04 mg/100 g FW) which was significantly at par with 0.25 mM KH_2PO_4. In the presence of Al application of 0.25 mM $MgSO_4$, an increase of 58% in vitamin C was observed in the root which was at par with 100 µM SA (10.42 mg100 g^{-1} FW) (Fig. 14).

Garden pea var. AP-3 grown for 14 days in the presence of 24 ppm had a nearly 18% ($p<0.05$) decline in the total carotenoid content of leaves (Fig. 15). In the presence of 24 ppm Al, the total carotenoid content of leaves increased nearly 42% ($p<0.05$) with the application of 50 µM SA which was at par with 0.5 mM $MgSO_4$ (0.290 mg g^{-1} FW).

Growing pea seedlings for 14 days in the presence of 24 ppm Al in the media resulted in nearly 12-fold ($p<0.05$) and 24-fold ($p<0.05$) increase in Al content of shoot and root on dry matter basis, respectively compared to the control (Fig. 16). In the presence of 24 ppm Al in media, application of 0.25 mM $MgSO_4$ resulted in the lowest content of Al in the shoot (18.4 µg/g DM) which was at par with 0.25 mM KH_2PO_4 (30.2 µg/g DM) followed by 0.5 mM KH_2PO_4 (80.0 µg/g DM). In the root, lowest content of Al was observed with the application of 0.5 mM KH_2PO_4+24 ppm Al (69.0 µg/g DM).

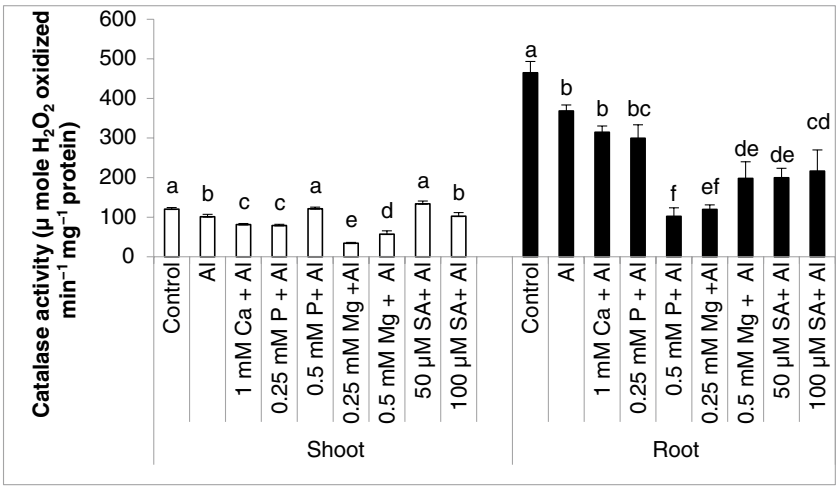

Fig. 10 Effect of treatment on the production of catalase activity in shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

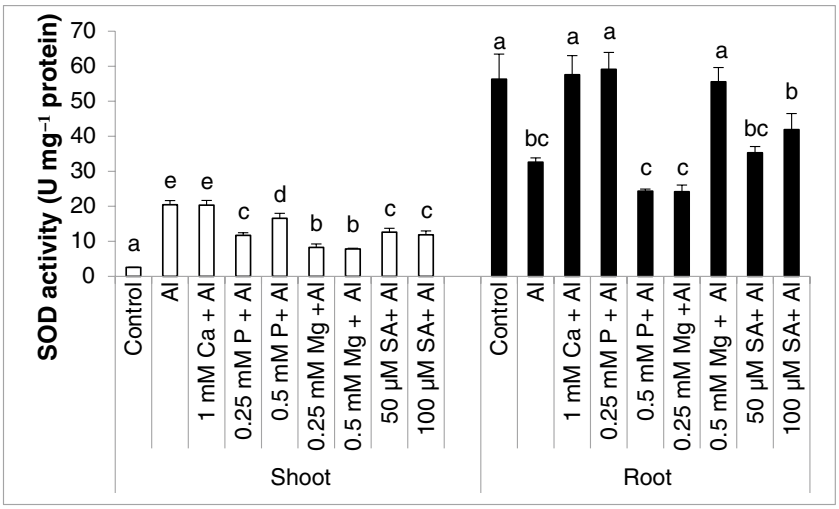

Fig. 11 Effect of treatment on the production of SOD activity in shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

Discussion

The present study revealed that Al in the growth medium resulted in a significant decline in pea seedling growth. Inhibition of plant growth especially root growth is a distinctive characteristic of Al toxicity which is caused due to hindrance in the cell division mechanism resulting in small and brittle roots which can easily break (Panda et al. 2009). We found that the application of Ca, P, Mg, and SA recovered the growth of peas from Al stress. Exogenous application of P was found best for the recovery of root and shoot length than other chemical agents. The application of 0.5 mM KH_2PO_4 and 0.25 mM $MgSO_4$ was better for the recovery of the dry weight of Al-susceptible genotype AP-3. Earlier reports suggested that the incorporation of P in soil fully mitigated the toxic effect of Al in sensitive wheat genotype, the detoxification process of Al by P primarily happens in soil rhizosphere instead of plant tissue (Iqbal, 2014), and adding Mg^{2+} to the growth medium alleviated Al stress in plants (Bose et al. 2011).

Al stress caused a decrease in photosynthetic pigments in pea seedlings. The photosynthetic pigment decrease is caused due to damage of chloroplast and destruction of the primary matter of chlorophyll synthesis. In different stresses, existing chlorophyll in the chloroplast is broken down resulting in the loss of thylakoid structure (Rout et al. 2001; Ghani 2022). With Al in the growing media, the application of Ca, P, Mg, and SA restored the chlorophyll content in pea seedlings. Out of which, 50 µM SA and 0.5 mM Mg addition was better in maintaining chlorophyll pigment in the leaves. Magnesium ion (Mg^{2+}) is a major metal in the chlorophyll, and a substantial quantity is essential for chloroplasts.

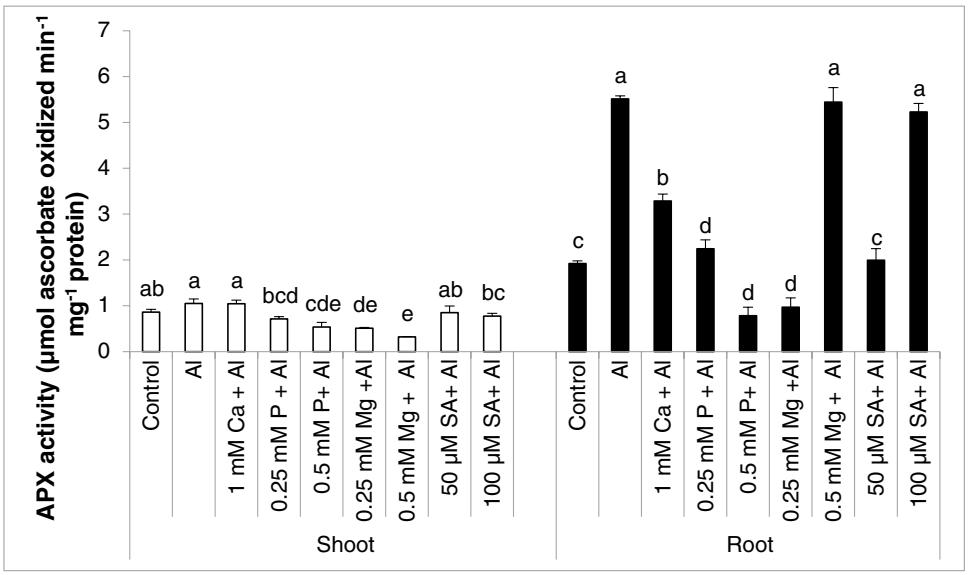

Fig. 12 Effect of treatment on the production of APX activity in shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

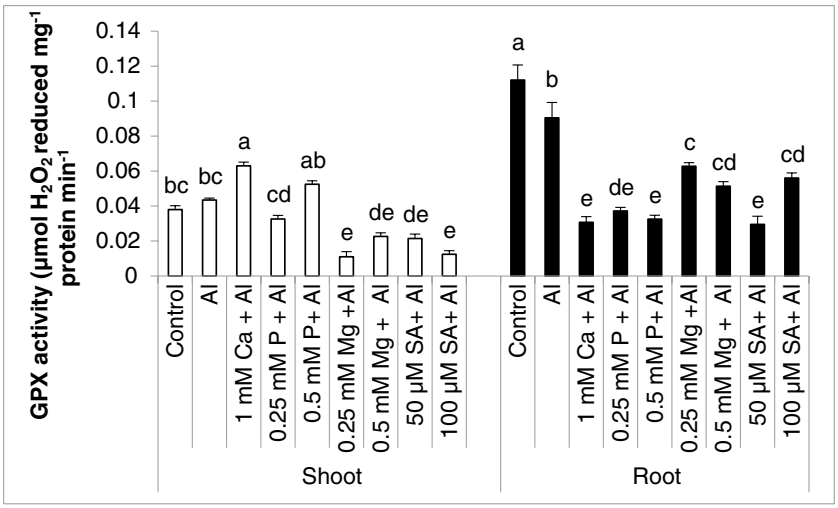

Fig. 13 Effect of treatment on the production of GPX activity in shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

Therefore, the application of $MgSO_4$ alleviated Al stress in several species of plants by maintaining chlorophyll stability (Kinraide et al. 2004). SA plays a significant role in the uptake of nutrients and enhances the growth and yield of plants.

Al treatment caused a substantial drop in the soluble protein content of root in Al-susceptible genotype AP-3. The outcome is in agreement with the findings of Sabat et al. (2016) in mung bean seedlings where Al treatment decreased the protein in the shoot. Hirpa et al. (2015) also reported that Al addition significantly reduced the protein contents of common bean genotypes; a greater reduction was detected for the sensitive genotypes. However, the application of P and Mg in the presence of Al enhanced the soluble protein content in pea seedlings. Protein is a part of various enzymes which play a significant role in the defense mechanism against various types of stress. The increase in soluble protein content along with better growth indicated the ameliorative capacity of P and Mg.

ROS generation and lipid peroxidation

The lipid peroxidation in the shoot/root was high due to the addition of Al in the growth media. However, application of 1 mM Ca, 0.5 mM P, 0.25 mM Mg, and 50 µM and 100 µM SA along with Al resulted in an even higher lipid peroxidation than the Al treatment alone. It is because, solubilizing of the cell wall lipid increases the cell wall permeability for the entry of cations inside the protoplasm. In the pea seedlings grown under Al stress for 14 days, there was an enhanced level of ROS in the shoot/root, and the generation of oxidative stress is a vital part of the manifestation of Al stress in peas (Matsumoto and Motoda, 2013). Superoxide anion production and H_2O_2 got shot up due to Al stress in both shoot and root which led to lipid peroxidation in pea seedlings. Application of Ca, P, Mg, and SA was able to minimize the production of H_2O_2 and superoxide anion with better root elongation depicting an ameliorative effect against Al toxicity. It inhibited the buildup of ROS in the root and shoot

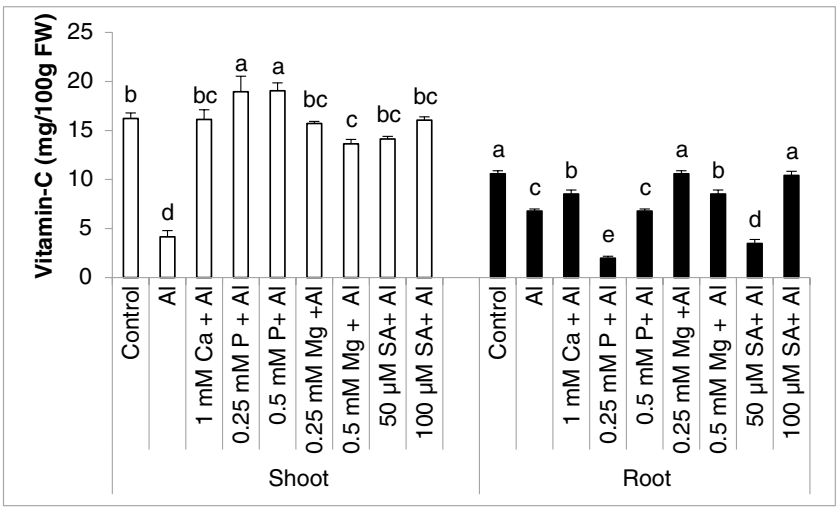

Fig. 14 Effect of treatment on the production of vitamin C in shoot/root. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

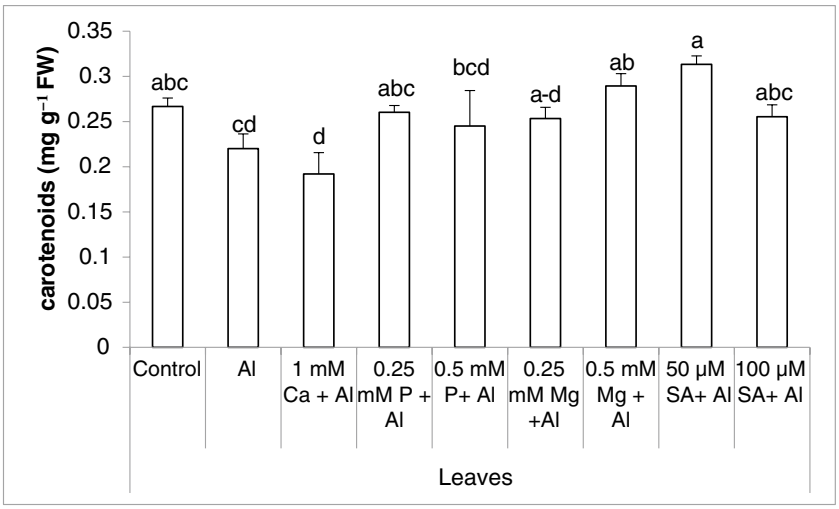

Fig. 15 Effect of treatment on the production of carotenoid content in leaves. Different letters indicate significant differences at $p>0.05$ and the error bar = mean ± SD ($n = 3$)

tissues thus subduing the oxidative damage to pea seedlings and maintained a satisfactory enzymatic and non-enzymatic defense in the pea seeding. Under enzymatic antioxidant, the first role is played by SOD which transforms the superoxide into H_2O_2 followed by catalase and peroxidases which neutralize H_2O_2 in the plant cell (Darko et al. 2004). Al stress leads to oxidative stress due to a higher production of ROS in tomato (Borgo et al. 2020) and rice (Pandey et al. 2013). Among the substance examined, KH_2PO_4 and SA appeared to be superior to $CaCl_2$ and $MgSO_4$ in neutralizing the accumulation of ROS due to Al stress.

Enzymatic defense system

Application of Al increased the production of H_2O_2 and superoxide anion, an elevation in the activity of the enzymatic antioxidant. Among the enzymatic defense system, the level of SOD and APX shot up due to Al stress. SOD is a vital enzyme, and its enhancement is directly related to the conversion of superoxide into H_2O_2 to keep it in check (Pandey et al. 2014; Ghani et al. 2019), signifying that superoxide production may possibly be a reason for an enhanced level of SOD activity. In the root and/or shoot, P and Mg performed better for buffering the SOD activity due to Al-induced damage to the seedling. Less production of superoxide anion was associated with lower SOD production in 0.5 mM Mg+Al- and 0.5 mM P+Al-treated plants as compared to Al treatment alone, suggesting that P and Mg were able to reduce the Al-induced superoxide production in seedlings which led to low SOD activity. In the present study, on Al exposure, among the enzymes involved in the removal of H_2O_2 the activity of CAT and GPX declined, whereas only APX activity got increased. These findings give light that APX activity has a vital role in neutralizing H_2O_2 production

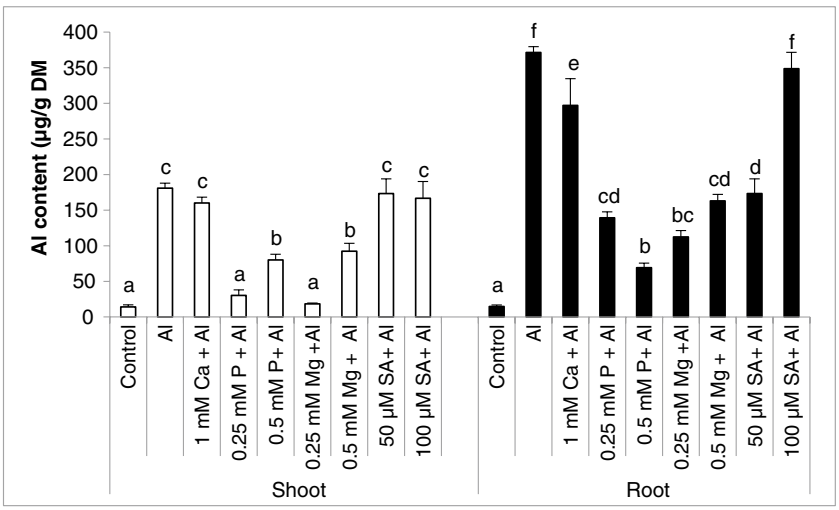

Fig. 16 Effect of treatment on Al content in root and shoot. Different letters indicate significant differences at $p > 0.05$ and the error bar = mean ± SD ($n = 3$)

in stressed pea seedlings. In the previous study on rice, the activity of CAT got reduced due to Al treatment representing that H_2O_2 accumulation inactivated the enzyme (Pandey et al. 2013). The application of Ca, Mg, P, and SA with Al in the growth medium lowered the Al-led activity of APX, and 0.5 mM P and 50 µM SA enhanced the Al-stimulated decline in CAT in the shoot of pea seedlings. This indicated that the chemical agents were able to ameliorate the damage caused by Al stress by buffering the activity of antioxidant enzymes. Peroxidases are considered stress enzymes, and the activity of peroxidases increases under abiotic stress including Al toxicity (Sharma and Dubey 2007). In the present studies, GPX activity decreased due to Al stress, but nutrient and SA could not enhance the level of GPX. This suggests that $CaCl_2$, KH_2PO_4, $MgSO_4$, and SA do not play any role in restoring GPX activity in Al-stressed pea seedlings. It has been reported in rice that SA lowered the Al-induced activity of APX and GPX and imparted a positive effect on the growth and development of plants (Pandey et al. 2013). In several plant species, Mg has been shown to alleviate Al toxicity (Chen and Ma 2013). SA plays an important role in plants under a variety of environmental stresses by retaining the redox state of the antioxidant pool and inducing antioxidative defenses (Song et al. 2011).

Vitamin C (ascorbic acid) and carotenoid are frequently studied non-enzymatic antioxidant compounds and play an important role in the growth and development of plants (Pignocchi and Foyer 2003) and, for the relevance in this research, act as neutralizing agents for ROS generated due to plant under stress (Qian et al. 2014). The concentration of non-enzymatic antioxidants got reduced with Al stress. The application of P and Mg was able to restore the level of vitamin C and carotenoid to normal levels possibly by lowering the accumulation of ROS and better growth.

The decline in the carotenoid content could be due to the enhanced level of ROS and lipid peroxidation which destroys carotenoids in the susceptible variety (Ravi et al. 2011).

Application of Al in the growth media significantly enhanced the content of Al in plant tissue and causes imbalances in the nutrient content in root and shoot. The accumulation of Al in the root was more than the shoot of a pea seedling. Ahn et al. (2002) reported that the root inhibition in squash was related to Al concentration and the period of exposure and damage that occurred in the region with high Al accumulation. With the content of Al by pea roots, the growth of seedlings decreased marked by a reduction in length and dry weight. The Al content in roots was reduced when Ca, P, and Mg were added to the medium. The lowest accumulation of Al in pea seedlings was observed with the application of P in the growth medium. P forms Al-phosphate complexes in solution and makes the Al unavailable in the media thus reducing the content by the plants (Iqbal, 2014). In soybean, it has been shown that both Ca and Mg have a protective effect against Al-induced inhibition of root elongation (Silva *et al*., 2001). It is suggested that the addition of Ca to the growth medium reduces Al activity at the plasma membrane surface of root cells (Silva *et al*., 2001). The addition of Mg^{2+} ions increases the ionic strength of the media (Noble and Sumner, 1988) resulting in a reduction in Al^{3+} saturation on the apoplastic exchange sites.

Conclusion

The Al stress caused a marked reduction in the growth and accumulation of Al along with the buildup of ROS in pea seedlings. The exogenous application of $CaSO_4$, H_2PO_4, $MgSO_4$, and salicylic acid as ameliorating agents for Al stress in pea genotypes indicated restoration of ordinary growth and inhibited the buildup of ROS in the root and shoot tissues thus subduing the oxidative damage and maintaining a satisfactory enzymatic and non-enzymatic defense in the pea seedlings. Application of 0.5 mM H_3PO_4 resulted in 47% recovery of root growth and 60% of shoot growth along with the highest suppression of Al uptake and ROS accumulation. Among the four substances used, amelioration effects of Al toxicity appeared superior at 0.5 mM P treatment. This indicated that 0.05 mM P is more efficient than other nutrients (Mg and Ca) and phytohormone, SA, in the amelioration of Al toxicity.

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Author contributions M. T. Ansari carried out the experiment. Md. Ramjan conceptualized the experiment and methods. Vivek Yadav has done the drafting and editing of the manuscript. M. H. Ansari did the data analysis. M. F. Ali has done the editing of the manuscript. All authors contributed for the preparation of the manuscript.

Declarations

Ethics approval and consent to participate There was no ethical concern in the publication.

Consent for publication All the authors agreed to the publication of the manuscript.

Competing interests The authors declare no competing interests.

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