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Research article

Aluminium stress modulates the osmolytes and enzyme defense system in *Fagopyrum* species

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ABSTRACT

The present investigation describes aluminum-induced changes in the leaves of two buckwheat species using both physiological and biochemical indices. With increasing levels of Al (viz. 100, 200 and 300 µM), the mean length of root, shoot as well as their biomass accumulation decreased linearly with respect to control. Tolerance test of *F. kashmirianum* revealed that it was more tolerant to Al-stress than *F. tataricum* as revealed by higher accumulation of Al in its roots without any significant damage. Translocation factor (TF) values of both species were found to be < 1, indicating more Al is restrained in roots. Total chlorophyll showed a non-significant increase in *F. tataricum* while as decreased in *F. kashmirianum* at 300 µM concentration besides, the carotenoid content exhibited inclined trend in *F. tataricum* and showed a concomitant decrease in *F. kashmirianum*. The anthocyanin level showed a non-significant decline in *F. kashmirianum*. Exposure to different Al-treatments enhances malondialdehyde (MDA), H_2O_2 and membrane stability index (MSI) in both species, with increases being greater in *F. kashmirianum* than *F. tataricum* as also revealed by DAB-mediated in vivo histo-chemical detection method. The osmolyte level in general were elevated in both buckwheat species however, enhancement was more in *F. tataricum* than *F. kashmirianum*. The activities of antioxidant enzymes viz. superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (POD), glutathione reductase (GR), glutathione-S-transferase (GST) were positively correlated with Al-treatment except catalase (CAT) which exhibits a reverse outcome in *F. kashmirianum*. The present investigation could play an essential role to better understand the detoxification mechanisms of Al in plants.

1. Introduction

Acid soils represent about 30–50% of the earth's arable land and in these aluminium (Al) is the most plenteous (ranked third) element that constrains the yield efficiency (Inostroza-Blancheteau et al., 2012). Aluminium being ubiquitous in nature is usually found as alumina-silicates or oxides. These forms are not known to play any particular biological function in plant metabolism. However, decrease in soil pH below 5.0 makes Al available with changed oxidation state thus, causing toxicity to majority of plants (Seguel et al., 2013; Singh et al., 2017). Excessive Al^{3+} concentration poses severe toxicity to plants as it suppress cell elongation and cell division in roots, that in turn leads to enlarged root apices with low or no root-hair development besides, causing hindrances in the nutrient uptake (Kochian et al., 2015).

Several reports revealed that these effects may be caused by Al^{3+} interfering with signal transduction pathways regulating cell growth. Al^{3+} may bind to plasma membrane, distorting ion homeostasis and transport by blocking Ca^{2+}- dependent signaling cascades (Matsumoto, 2000). It might also disrupt the membrane function promoting oxidative stress, which could cause blockade of secondary signaling molecules and affect the organization of cytoskeleton (Kopittke et al., 2015; Singh et al., 2017). Plants growing in acidic soils strategize to limit Al uptake by increasing soil pH by root exudation of organic compounds (viz. malic acid, oxalic acid and phenolics) which prevent Al^{3+} solubilization (Inostroza-Blancheteau et al., 2012; Brunner and Sperisen, 2013; Kochian et al., 2015). Furthermore, antioxidant defense

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machinery such as; superoxide dismutase (SOD), peroxidases, catalase (CAT), glutathione reductase (GR) and non-enzymatic low molecular weight compounds viz. proline, sugars, reduced glutathione, cysteine and other osmolytes is operated in plants in response to heavy metal stress to neutralize the toxicity.

Keeping in view of the above facts, present study was conducted to focus on alternative crops that can resist adverse climatic conditions. Hence, in the current study, we investigated the impact of Al-toxicity on the physiological performance and antioxidative defense in two underutilized buckwheat species.

2. Materials and methods

2.1. Procurement of plant material and experimental design

Healthy and mature seeds of *Fagopyrum tataricum* and *F. kashmirianum* were procured from the Department of Bioresources, University of Kashmir, Srinagar, India). Surface sterilization of seeds were done using 5% sodium hypochlorite (NaOCl) solution (v/v) for 15 min and then washed thoroughly with double distilled water and later the seeds were soaked in sterile distilled water for 1 h. Sowing of seeds was done using plastic pots (8 cm diameter) incorporated with ½ kg of autoclaved acid washed sand. Hoagland's nutrient medium (Hoagland and Arnon, 1950) with pH 4.5 was used for the growth of seedlings. Pots were maintained under controlled conditions (temp. 22.5 ± 1.5 °C at daytime and at 18.5 ± 1.5 °C at nighttime; light intensity $300\,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$ and relative humidity of 60–70%). The aluminium chloride ($AlCl_3$) was prepared in Hoagland nutrient solution and the treatments 0 μM, 100 μM, 200 μM and 300 μM were given for 15 and 30 days after sowing (DAS). Treatments were arranged in a completely randomized block design with 3 replications and the sampling was done at 15 and 30 days after sowing (DAS).

2.2. Estimation of growth indices

Growth indices were determined in terms of root length, shoot length, fresh mass (FM), dry mass (DM). Index of tolerance (TI) were determined followed the Wilkins's equation (Wilkins, 1957).

$TI_{(\%)} = [ML_{Al}/ML_C \times 100]$. where ML_{Al} and ML_C denotes the mean length of longest roots in Al treated and control seedlings, respectively.

2.3. Estimation of biomass and relative water content (RWC)

Estimation of biomass was determined according to Qureshi and expressed as mg/seedling. RWC was determined following the protocol of Chen et al. (2009).

$$RWC_{(\%)} = [(FW - DW)/FW] \times 100$$

2.4. Estimation of aluminium concentration and translocation factor (TF)

Root and shoot samples were oven dried in a hot air oven at 65 °C for 72 h and were then processed by wet ashing following the protocol of Pirzadah et al. (2018). The final volume was adjusted to 50 ml with ultrapure water and filtered. The Al content was analyzed against a reference standard by flame atomic absorption spectrophotometry (Perkin-Elmer Analyst 100, Waltham, MA, USA). The TF was determined followed the Marchiol's equation (Marchiol et al., 2004).

$$TF = [\text{Metal concentration}]_{\text{shoots}}/[\text{Metal concentration}]_{\text{roots}}$$

2.5. Determination of photosynthetic pigment contents

Chlorophyll content was determined by homogenizing 0.2 g leaf material in 10 ml chilled acetone (100%) under dark controlled conditions and measured according to Lichtenthaler (1987). The carotenoid concentration was measured following the equation of Misyura and expressed in mg/g FM. The anthocyanin level was measured according to Mancinelli et al. (1957).

2.6. Al-induced modifications in biomarker

The oxidative stress was measured in terms of MDA content according to Heath and Packer (1968). H_2O_2 was analyzed according to Velikova et al. (2000). Electrolyte leakage or MSI was analyzed followed the protocol of Rodriguez-Hernandez et al. (2013).

2.7. Estimation of osmolytes in Al-induced plants

The concentration of proline was estimated according to Bates et al. (1973) using L-proline as standard. Total soluble sugar was determined following the protocol of Dey (1990). The concentration of reduced glutathione (GSH) was measured according to Anderson (1985). Total soluble protein content was determined following the method of Bradford (1976) using Bovine Serum Albumin (BSA) as standard.

2.8. Enzyme extraction and quantification of enzyme assays

Extraction of the enzyme was done following the protocol of Pirzadah et al. (2018). The superoxide dismutase (SOD) activity was measured according to Bayer and Fridovich (1987). The activity of catalase enzyme (CAT) was calculated following the protocol of Aebi (1984). Peroxidase (POD) activity was measured at 25 °C following the method of Whitaker and Burnherd (1972) using guaiacol as a substrate. The ascorbate peroxidase (APX) activity was quantified following the protocol of Nakano and Asada (1981). The activity of glutathione reductase (GR) was measured according to Cakmak and Marschner (1992). Glutathione-s-transferase (GST) assay was measured following the procedure of Habig and Jacoby (1981).

2.9. Statistical analysis

Experiments were conducted in replicates (N = 3) and the data were represented as mean ± SE. The data were subjected to two-way analysis of variance (ANOVA) and average differences were compared using Tukey's posthoc test to evaluate the significant differences at $p \leq 0.05$ and $p \leq 0.01$ using GraphPad Prism software version 7.01.

3. Results

3.1. Plant growth response

The sensitivities of buckwheat species to Al-stress showed a significant variation and shoots were found less sensitive than roots (Tab. 1; Supplementary file). The shoot length of *F. tataricum* and *F. kashmirianum* were significantly suppressed compared to control and the rate of growth inhibition varied depending upon the Al-concentration used. Shoot growth showed a concomitant decrease with each increment level of Al treatment and the maximum decrease of shoot growth observed was 27.68% and 43.49% respectively in *F. tataricum* and *F. kashmirianum* at 300 μM. Root growth of *F. tataricum* exhibited a concomitant decrease with increase in Al-stress and the maximum reduction observed was 49.63% at 300 μM. However, the *F. kashmirianum* exhibited a reverse in outcome as the root growth increases with each increment of Al-concentration and the maximum increase was found to be 30.68% at 300 μM. Fresh mass of both buckwheat species showed a significant decrease with enhancing Al-treatment and the maximum reduction observed was 58.09% and 58.15% respectively in *F. tataricum* and *F. kashmirianum* at 300 μM. Maximum decline in biomass was found at 300 μM Al-level with respect to control plants, but the reduction was found to be lesser in *F. kashmirianum* compared to *F. tataricum* (Tab. 1;

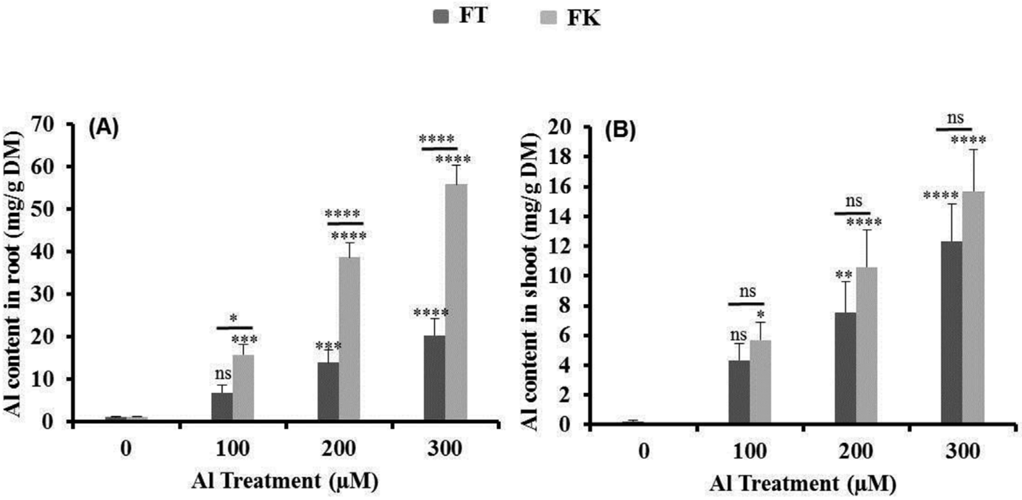

Fig. 1. Aluminium (Al) content (mg/g DM) in roots (A) and shoots (B) of *F. tataricum* (FT) and *F. kashmirianum* (FK) plants grown in absence (0) and presence of 100, 200 and 300 µM Al for 15-days after sowing (DAS). * Different from control; $p \leq 0.05$.

Fig. 2. DAB-mediated tissue profiling for the in vivo detection of H_2O_2 in *F. tataricum* (a) and *F. kashmirianum* (b) leaves.

Supplementary file 1). Further, TI values of both the species varies significantly (Tab. 2; Supplementary file 2) under Al-induced stress. *F. tataricum* exhibits lower TI values than those of *F. kashmirianum* plants. The roots of *F. tataricum* exhibited the highest sensitivity to Al (50.36%). A high percentage of TI values were found in *F. kashmirianum* at 300 µM Al-treatment. Therefore, due to better plant growth according to growth indices results, *F. kashmirianum* was more resistant to Al stress than the *F. tataricum*.

3.2. Aluminium accumulation and TF

The Al-concentration of roots and shoots varied among both buckwheat species and it showed an inclined trend with increasing Al-concentration (Fig. 1a and b). The *F. kashmirianum* exhibited the higher Al-concentration as compared to *F. tataricum*. At 300 µM treatment, Al concentration in shoots of *F. tataricum* and *F. kashmirianum* was found to be 12.32 and 15.7 mg/g DW respectively while correspondingly in roots they were 20.32 and 55.89 mg/g DW. TF values were found to be < 1 in both *Fagopyrum* spp. however; the TF values were found low in case of *F. kashmirianum* in comparison to *F. tataricum*.

3.3. Response of Al-induced stress biomarkers

Electrolyte leakage (EL), an important indicator of membrane integrity was determined after Al-exposure and the results are shown in Tab. 3; Supplementary file. In comparison to control, Al toxicity showed a significant increment of EL in the leaves of *F. tataricum* than *F. kashmirianum*. The increase was more towards the end of the treatment, 6.7 times increase in *F. tataricum* at 300 µM while as only about 2 times increases in *F. kashmirianum*. The relative water content (RWC) in *F. tataricum* increased with increase in Al-concentration and the maximum increment of 94% was observed at 100 µM. However, in *F. kashmirianum*, the RWC decreases with increase in Al-concentration but it was not significant (Tab. 3; Supplementary file). The accumulation of MDA content showed an inclined trend in both *Fagopyrum* species under Al-induced stress. The degree of increment varied for the 100, 200 and 300 µM Al-concentration, with 19%, 58% and 36% in *F. tataricum* respectively and correspondingly 18%, 59% and 113% in *F. kashmirianum* (Tab. 3; Supplementary file). The hydrogen peroxide (H_2O_2) content also increased under Al-stress in both buckwheat species and the maximum concentration was observed in *F. kashmirianum* compared to control. As shown in Tab. 3; (Supplementary file) H_2O_2 content was found to be highest in *F. kashmirianum* with about 2-fold increase at

300 μM Al-stress with respect to control. DAB-mediated tissue profiling for the detection of H_2O_2 also revealed the increased color intensity among both buckwheat species at all Al-concentrations and more intensity was observed in *F. kashmirianum* compared to *F. tataricum* (Fig. 2).

3.4. Estimation of photosynthetic pigments upon Al-induced stress

Chlorophyll and carotenoid contents were declined by increased Al-treatment in *F. kashmirianum* and the maximum reduction was 15% and 17.54% respectively at 300 μM.Overall the decrease in chlorophyll and carotenoid contents were not significant while as, in *F. tataricum* a reverse outcomewas observed as both chlorophyll and carotenoid content increases and the maximum increase observed was 20.87% and 7.19% respectively at 200 μM and 300 μM Al-concentration (Tab. 4; Supplementary file). The anthocyanin level in *F. tataricum* initially decreased by 8.7% at 100 μM Al-stress but at higher levels of Al-treatment it increased up to 32.6% at 300 μM while as it showed a significant decline at all Al-treatments in *F. kashmirianum* and the maximum decline observed was 15.78% at 300 μM (Tab. 4; Supplementary file).

3.5. Al-induced changes in proline, protein, soluble sugar and reduced glutathione

Increase in proline content was directly proportional to the Al-concentration in both *Fagopyrum* species. The maximum increase in proline content was 158% and 140.6% respectively in *F. tataricum* and *F. kashmirianum* at 300 μM Al-stress with respect to control (Fig. 3a). The protein content also increased in a dose-dependent manner and the maximum increment was found to be 81.2% and 57.48% respectively in *F. tataricum* and *F. kashmirianum* at 300 μM, however, the protein content was enhanced more in *F. tataricum* than *F. kashmirianum* (Fig. 3b). Total soluble sugar level exhibited a significant increment in both species except at 300 μM in *F. kashmirianum*; it showed a significant decline by 24.7% (Fig. 3c). Reduced glutathione (GSH) level in *F. tataricum* increased in a dose-dependent manner and the maximum increment found was 67.23% at 200 μM Al-stress while as, in *F. kashmirianum*, GSH level initially decreases up to 200 μM but at 300 μM it significantly increased by 67.73% (Fig. 3d).

3.6. Al-induced modifications in antioxidant enzymes

All antioxidant enzymes viz., SOD, CAT, APX, POD, GR and GST showed remarkable difference among both buckwheat species upon Al-induced stress. SOD activity was found to be higher in *F. tataricum* than in *F. kashmirianum*. Compared to control, 300 μM Al-level resulted in SOD activity of 187% higher in *F. tataricum* and 111.76% in *F. kashmirianum* (Fig. 4a). Catalase (CAT) activity in *F. tataricum* increased in a concentration-dependent manner up to 69.27% at 300 μM while as *F. kashmirianum* exhibited a reverse outcome, as the CAT activity declined to 50.79% at 300 μM (Fig. 4b). Peroxidase (POD) activities also exhibit significant variation among the species (Fig. 4c) and was found higher in *F. tataricum* than *F. kashmirianum*. POD activities increased in concentration-dependent manner in*F.tataricum*upto 91.66% at 300 μM but in *F. kashmirianum* it declined to 3.7% at 300 μM. The level of ascorbate peroxidase (APX) in *Fagopyrum* spp. Increased upon Al-stress and reached a maximum level for *F. tataricum* (70.39%) and *F. kashmirianum* (24.6%) at 300 μM (Fig. 4d). Glutathione reductase (GR) activity was more enhanced in *F. tataricum* than *F. kashmirianum* and the maximum GR activity observed was 148.2% at 300 μM in *F. tataricum* while as the GR activity in *F. kashmirianum* decreased by 53.8% at 300 μM (Fig. 4e). Glutathione-s-transferase (GST) activity in both *Fagopyrum* spp. were

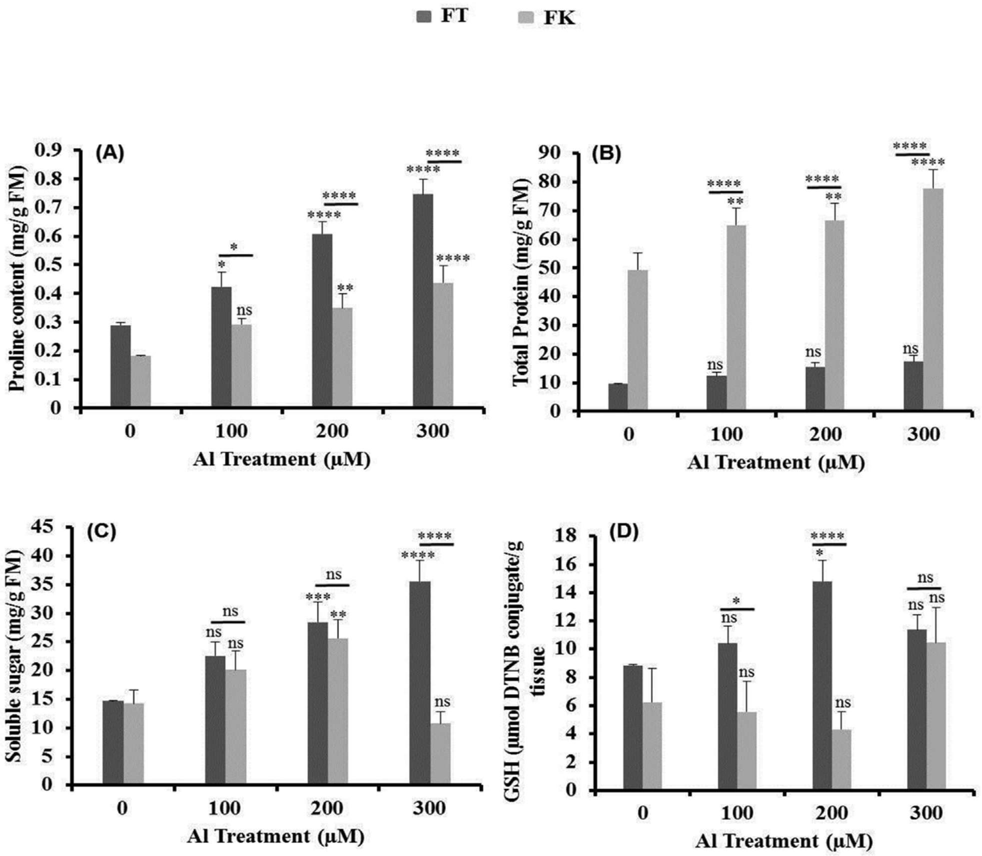

Fig. 3. Effect of different Al treatments on proline content (A), soluble protein (B), total soluble sugar (C) and reduced glutathione content (D) of 15 days old *F. tataricum* (FT) and *F. kashmirianum* (FK) leaves. * Different from control; $p \leq 0.05$.

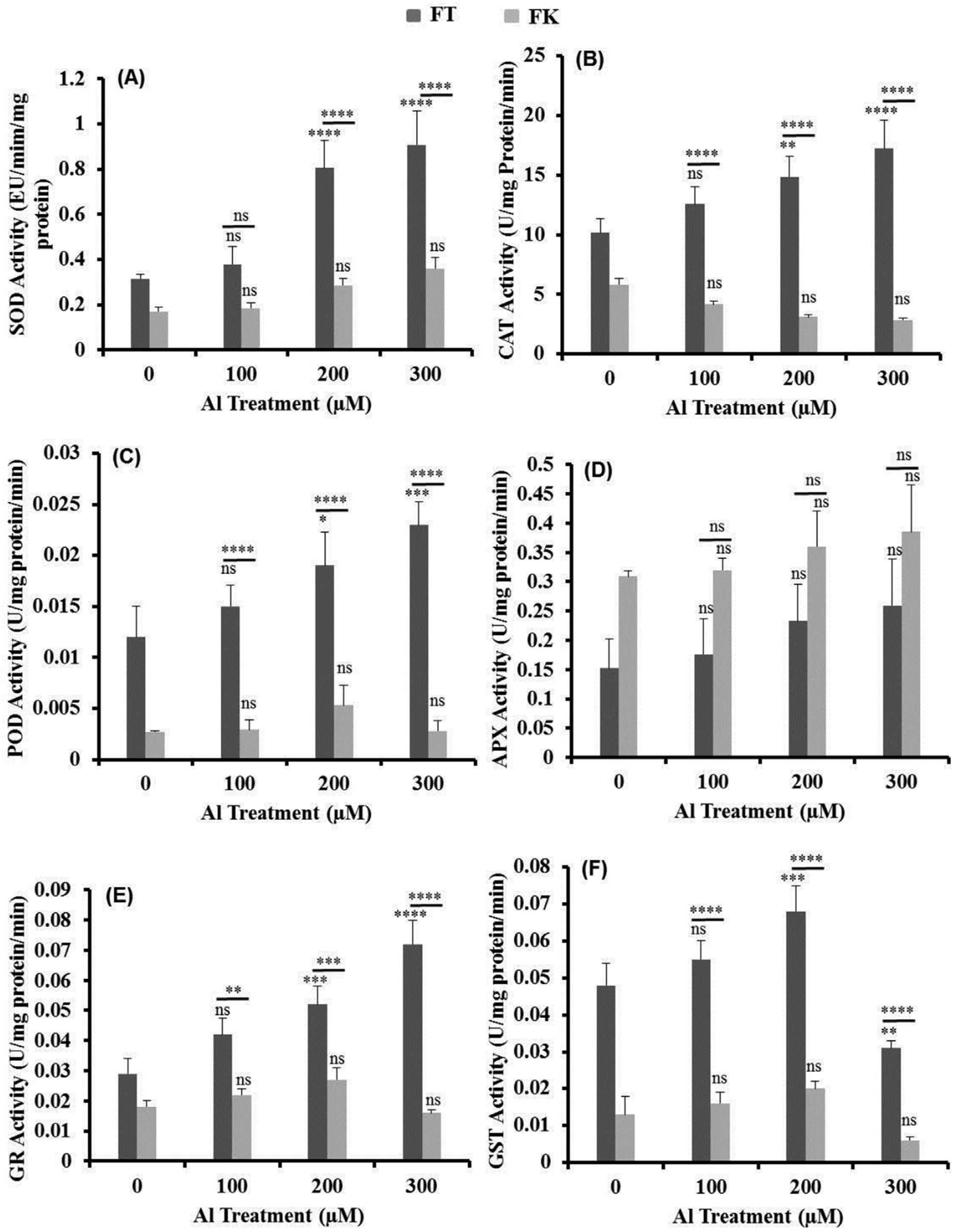

Fig. 4. Effect of different Al treatments on superoxide dismutase: SOD (A), catalase: CAT (B), peroxidase: POD (C), ascorbate peroxidase: APX (D), glutathione reductase: GR (E) and glutathione-s-transferase: GST (F) of 15 days old *F. tataricum* (FT) and *F. kashmirianum* (FK) leaves. *Different from control; $p \leq 0.05$.

found to increase up to 200 μM while as at 300 μM, the GST activity declined by 35.4% and 53.8% respectively in *F. tataricum* and *F. kashmirianum* (Fig. 4f).

3.7. *Al-induced changes in the correlation analysis of various physiological parameters of buckwheat plants*

The correlation analysis among various physiological parameters of buckwheat plants upon Al-induced stress is shown in Fig. 5 (Supplementary file 3). The data revealed that maximum positive correlation was found among BA and RL followed by ACR and ACS, SOD and APX and POD and CAT. Besides, the maximum negative correlation was observed among ACR and RTI followed by ACS and STI, RL and ACR, FM and ACS.

4. Discussion

4.1. *Plant growth response*

Aluminium is ranked third after silicon and oxygen that limits the crop productivity (Singh et al., 2017). The growth response of plants is affected by several factors viz. water conductivity, osmotic potential, cell wall extensibility and threshold turgor and any imbalance in the equilibrium of above factors leads to the decline in plant growth (Mondal et al., 2015; Singh et al., 2017). Present results revealed that different Al-concentration negatively altered plant growth of *F. tataricum* and *F. kashmirianum*, causing significant decline, except a significant increment in the root length of *F. kashmirianum* (Tab. 1; Supplementary file 1). Besides, the biomass accumulation of both species exhibit a non-significant decrease with each increment level of Al stress; however the degree of reduction was lower in *F. kashmirianum*, suggesting its greater Al stress tolerance than *F. tataricum* (Tab. 1; Supplementary file 1).Our results corroborate with the earlier reports of Ouzounidou et al. (1997) and Guo et al. (2018) who proclaimed suppression of shoot and root length of wheat and *citrus sinensis* plants under Cd and Al stress respectively. Gill et al. (2011) also reported that heavy metal tolerance in plants is also dependent on plant genotypes. The decline in the growth parameters under Al-induced stress might be due to the intervention in the elongation and division of meristematic cells (Sivaguru et al., 2013; Blamey et al., 2015). This interference restrains the aquaporins and inhibits water and mineral uptake, thus conditioning stress sensitivity and limiting plant biomass production (Inostroza-Blancheteau et al., 2012). Moreover, greater reduction in root length than shoots could be due to its tendency to accumulate more Al in roots than shoots which in turn suppresses the growth (Singh et al., 2017). However, the increasing root length of *F. kashmirianum* in the current study might be due to the fact that the root apex oozes out some organic acids that causes detoxification of Al either inside the root cells or in its rhizosphere as reported by Ma et al. (1997); Horbowicz et al. (2011) in *F. esculentum*. Besides, the tolerance test also revealed that *F. kashmirianum* was found comparatively tolerant species to Al stress than *F. tataricum* (Tab. 2; Supplementary file 2). Present study suggested that due to the better metal tolerance indices, *Fagopyrum* species have good adaptability capacity and can be exploited by introducing them into varied agro-climatic zones, besides having potential as phytoremediation crop for contaminated areas.

4.2. Aluminium accumulation and TF

Heavy metal uptake and transport varies with plant species and genotype (Gill et al., 2011). Here we have noticed that Al accumulation in roots and shoots of both *Fagopyrum* species increases with increase in Al-content in the substrate. The various levels Al in roots and shoots of *Fagopyrum* spp. even for the same concentrations of exogenous Al treatment reflects the variations in absorption by roots and translocation from root to shoot. The highest Al concentration in roots (Fig. 1a) and intermediate concentration in shoots (Fig. 1b) depicted limited transport. These results clearly suggested that buckwheat plants have the promising potential for adequate accumulation of Al in root with respect to shoots, which corroborates with the previous findings of lead (Pb) stress in *Medicago sativa* (Ghelich et al., 2014); in *Pisum sativum* (Rodriguez et al., 2015); Hg stress in *F. tataricum* (Pirzadah et al., 2018). TF characterizes the plant's capacity to translocate metals from root to shoot (Placek et al., 2016). Al concentration in shoots of both *Fagopyrum* spp. was lower as compared to roots. Therefore, TF values were found to be < 1 (Tab. 2; Supplementary file 2), suggested that huge quantity of Al was confined in roots and lesser amount is translocated to the above-ground parts that are most interesting for phytostabilization (Nouri et al., 2011). Present study revealed that *F. kashmirianum* accumulates more Al concentration and it might be due to the secretion and chelation of Al by means of organic acids viz. oxalic acid or citric acid (Ma et al., 1997). The retention of Al in the root cells might be due to the insolubilization at the root surface or compartmentation in cells avoiding the release to the xylem. It is also reported that the organic acids, phytochelatins and other ligands secreted by roots cells leads to the formation of complex compounds that were found to be important for the retention of heavy metals in the roots (Ma et al., 1997).

4.3. Response of Al-induced stress biomarkers

Al-toxicity affects the plants by causing disturbance in the stress biomarkers such as; lipid peroxidation and enhance H_2O_2 production (Cui et al., 2013). Peroxidation of membrane lipids often leads to disruption of membrane integrity thus causes leakage of electrolytes and therefore EL assay can also be used to estimate stability of membrane under stressful conditions. The present study revealed that EL was more pronounced in *F. kashmirianum* under Al stress as compared to *F. tataricum* (Tab. 3). Moreover, RWC has been reported as one of the biomarker to measure intensity of phytotoxicity in plants exposed to heavy metal stress (Zn and Cr) in *Brassica spp*. and Chinese brake fern (Su et al., 2005). Present results revealed that RWC in *F. tataricum* increases significantly at all Al levels as compared to *F. kashmirianum* (Tab. 3). It might be due to the fact that Al treatments induce stomatal closure triggered over the course of the experiment due to atmospheric carbon fixing activities (Brunet et al., 2008). TBARS is a product of lipid peroxidation and is regarded as an important biomarker of oxidative stress (Sekmen et al., 2012). Our results showed that MDA accumulation gets enhanced more in *F. kashmirianum* under all Al levels (Tab. 3), suggesting that Al-induced stronger peroxidation and caused more oxidative damage to cell membrane. Previous results also reported that TBARS content increased in rice, triticale and wheat when subjected to Al stress emphasizing lipid peroxidation as a signal of Al toxicity in cereals (Liu et al., 2010). As Al accumulation was more in *F. kashmirianum* that could be the major reason for the increased lipid peroxidation and electrolytes leakage. Besides, Al exposure results in the deterioration of antioxidant defense system resulting in production of reactive oxygen species (ROS), which can culminate in the oxidation of membrane lipids (Xu et al., 2012). These data support our results since H_2O_2 content was higher in *F. kashmirianum* than *F. tataricum*, which exhibited a non-significant increment (Tab. 3). The increased content of H_2O_2 in *F. kashmirianum* might be due to its role in signal transduction that induces tolerant responses to Al-stress (Baxter et al., 2014). The data is also supported by the DAB-mediated *in-vivo* tissue profiling (Fig. 3).

4.4. Al-induced changes in photosynthetic pigments

The study of photosynthetic pigments is of paramount importance in stress biology and is often regarded as important biomarkers (Pinheiro et al., 2013). Present study revealed that the chlorophyll, carotenoid and anthocyanin levels in *F. tataricum* initially decreased at low levels of Al toxicity but increased at higher levels of Al treatment (200 and 300 μM) (Tab. 4). This might be because photosynthetic pigment changes may be associated due to its adaptability at low levels of Al induced stress as revealed by earlier reports in *Pelargonium* sp. (KrishnaRaj et al., 2000) and in *Sesbania drummondii* (Ruley et al., 2006). Here we propose that if Al accumulating plant species maintain high pigment concentrations for normal growth and metabolism then it may possess a dynamic hyper-accumulation system for elimination of Al from polluted sites. Besides, the enhancement of accessory pigments in the present study might be due to the reason that these act as shielding agents to protect chlorophyll against ROS by quenching triplet chlorophyll, replacing peroxidation and disruption of chlorophyll membrane. Generally, accumulations of accessory pigments in plants are triggered when subjected to metal stress in order to play an essential role to quench ROS and thus maintains the physiological equilibrium (Chandra et al., 2009). Moreover, the present study also revealed that the pigment content in *F. kashmirianum* does not exhibit any significant change with increasing Al concentrationwhich might be due to its adaptability to combat Al stress by some organic chelators and maintain the pace of photosynthetic rate, chlorophyll content (Inostroza-Blancheteau et al., 2012). Similar findings were reported in *Phyllostachys pubescens* under Cu stress (Chen et al., 2015), in *Oryza sativa* (Mostofa et al., 2015) and in *Vigna radiate* under Hg stress (Mondal et al., 2015).

4.5. Al-induced changes in osmolytes

Proline plays a multi-purpose role in plants such as; nitrogen source under normal conditions; signaling molecule as well as an important osmolyte under stress conditions (Hayat et al., 2012). During our investigation, we found that proline content among both buckwheat plants exhibited a concomitant increment with rise in Al-stress (Fig. 3a). This surge in proline level in response to Al-stress is indicative of a correlation between ROS generation and ROS scavenging by proline. Hajiboland et al. (2013) reported the similar findings in tea leaves and roots exposed to Al-stress, thus suggested an essential role of proline in eliminating free radicals generated during heavy metal oxidative stress. Protein is another important biomarker in stress physiology to measure the intensity of oxidative stress (Plata et al., 2009). In the present investigation, the total protein concentration among both buckwheat plants showed an inclined trend with each level increment in Al content but the increase was more significant in *F. tataricum* compared to *F. kashmirianum* (Fig. 3b). This induction in protein level is possibly due to induction of stress proteins under heavy metal stress or it might be due to the increased activity of some other metal sequestration networks involved in detoxification of toxic metals (Atienzar et al., 2000). Likewise, total soluble sugar level is also associated to impart resistance against heavy metal toxicity (Zhang et al., 2015). Herein, we found that soluble sugar content of *F. tataricum* increased at all levels of Al levels and it might be due to the starch degradation (Fig. 3c). Starch may play a pivotal role in accumulation of soluble sugar in cells. It is reported plants exposed to Al toxicity, accumulate sugars in conjugation with other compatible solutes in order to maintain osmotic balance (Bohnert et al., 1995). In addition to this, these also act as structural components of the cell and signaling molecules to regulate various metabolic pathways (Rosa et al., 2009). However, the soluble sugar content decreased at 300 μM in *F. kashmirianum* and it might be due to the interference in carbohydrate metabolism (Rabie et al., 1992). A decrease in soluble sugar level at high metal concentration was found in a number of earlier studies viz., Zn stress in *Cyamopsis tetragonoloba* (Manivasagaperumal et al., 2011), Cd and Ni stress in *Phaseolus vulgaris* (Aldoobie and Beltagi, 2013). Reduced glutathione (GSH) is an essential osmoprotectant to subside the effects of ROS-induced oxidative stress. Present study revealed that GSH level in *F. tataricum* exhibit an increasing trend with increasing Al level and this might be due to its more demand as a substrate for GSH-metabolizing enzymes and it corroborates with earlier findings in *Oryza sativa* where GSH level was found enhanced under Cu stress (Thounaojam et al., 2012). However, the GSH level in *F. kashmirianum* decreased initially (Fig. 3d) and it might be due to phytochelatin synthesis where GSH acts as a precursor molecule. Previous findings reported the similar reports in *Vigna radiata* under Cd stress (Hossain et al., 2011); As stress in *Luffa* plants (Singh et al., 2015).

4.6. Al-induced changes in antioxidant enzymes

The antioxidant defense system viz., SOD, CAT and peroxidase play a pivotal role in scavenging ROS (Gill and Tuteja, 2010). The first line of defense which includes SOD catalyzes the superoxides ($O^{\cdot -}$) to H_2O_2, that is further catalyzed be CAT and peroxidases. Song et al. (2016) reported that SOD and CAT are important biomarkers to study the plant stress physiology. The present study revealed that SOD activity among both buckwheat species increased with each increment level of Al treatment, however the increase was more prominent in *F. tataricum* compared to *F. kashmirianum* (Fig. 4a). The increased level of SOD enzyme might be either due to the over-production of $O^{\cdot -}$ or over-expression of SOD encoding genes (Feng-tao et al., 2013; Shu et al., 2011). Similar results were reported in *Chlorella vulgaris* under Cd stress (Chenget al., 2016); in *Solanum nigrum* subjected to Cu stress (Fidalgo et al., 2013); in *Dimocarpus logan* under Pb stress (Wang et al., 2016); Hg stress in *F. tataricum* (Pirzadah et al., 2018); Pb stress in *F. kashmirianum* (Hakeem et al., 2019). The byproduct of SOD catalyzing reaction (H_2O_2) at higher concentration is toxic and therefore must be removed and this job is carried out by CAT enzyme in a synergistic action resulting in the formation of H_2O and O_2. CAT and peroxidases could catalyze H_2O_2 into H_2O and O_2, mitigating the oxidative stress caused by H_2O_2. In the present study, the CAT activity in *F. tataricum* showed a concomitant increase with increasing Al treatment (Fig. 4b) and it might be due to increase in its substrate to maintain the steady state level of H_2O_2 as an adaptive mechanisms of the plants (Reddy et al., 2005). However, the CAT activity in *F. kashmirianum* showed a declining trend at each Al treatment and it may be due to the overproduction of ROS by heavy metal toxicity, which in turn can suspend CAT function at higher concentration of Al-levels, apparently by suppressing the enzyme-bound heme group (Sahu et al., 2012; Cheng et al., 2016). Similar findings were observed under Cd stress in *Lemna gibba* (Parlak and Yilmaz, 2013); Pb stress in *Jatropha Curcas* (Shu et al., 2011). APX indirectly catalyzes H_2O_2 to H_2O using ascorbic acid (reducing power) and has more affinity for H_2O_2 than CAT enzyme. Present study revealed that APX activity in both buckwheat species showed an increasing trend with increasing Al treatment (Fig. 4d). The increase in APX activity is generally an adaptive mechanism to combat ROS generated under Al-stress. Similar observations were reported under Cu-toxicity in *Arabidopsis* (Kubo et al., 1995); in *Hibiscus cannabinus* under Cd toxicity (Feng-tao et al., 2013); in *Lactuca sativa* and *Brassica oleracea* under Zn toxicity (Medina et al., 2014). GPX and GST also play an essential role in regulating various pathways to impart heavy metal tolerance to plants (Hossain et al., 2012). Increased levels of GPX and GST impart more resistance to various abiotic stresses including metal stress in hyperaccumulating and transgenics (Roxas et al., 2000). Our results revealed that the enhanced activities of GPX and GST to Al stress suggested that there might be GSH–dependent peroxide scavenging that led to reduce oxidative stress. The reason might also be that the enhanced GST level induces the membrane-protecting role of osmoprotectant by conjugating GSH with various Al-induced electrophiles, thereby shielding them from further damaging the membrane. However, GST at 300 μM in both buckwheat species showed declining trend and it might be due to the inactivation of enzyme by Al stress (Fig. 4f). GR is involved in catalyzing NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). The increase in GR activity among both the buckwheat species in the present investigation corroborate with the earlier findings, depicting its role in generating GSH from GSSG under Al toxicity to maintain GSH/GSSG ratio and total glutathione pool (Foyer and Noctor, 2011; Noctor et al., 2012). The enhanced GR level might be due to *de novo* synthesis of the enzyme protein (Reddy et al., 2005). Results were further analyzed by correlation statistical programme that confirms the positive correlation between Al uptakes by roots and shoots. Moreover, present investigation unravels the most interesting findings, that under high Al stress, significant positive correlation was found among BA with RL, SOD and APX level.

5. Conclusion

The present investigation concluded that Al-induced oxidative stress affects growth indices, photosynthetic pigments, osmolytes, Al-uptake, translocation and physiological attributes of two *Fagopyrum* species differently. However, the inhibition was more dominant for *F. tataricum* than *F. kashmirianum*. Additionally, hyperactivity of antioxidant enzymes also played an essential role in protecting *F. kashmirianum* from Al-toxicity. Our findings revealed the potent Al tolerance in buckwheat, confirming the existence of dynamic detoxification mechanism for quenching ROS by triggering antioxidative defense systems in a synergistic manner. This study forms the basis in establishing the buckwheat as an ideal candidate to rejuvenate soil health from Al-toxicity besides can also be adopted for variable environments.

Authors contribution

Tanveer Bilal Pirzadah and Bisma Malik Inayatullah Tahir and Reiaz Ul Rehman have developed the idea of research, conducted the research and wrote the manuscript.

Khalid Rehman Hakeem and Hesham F. Alharby have analyzed the data, written and critically reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.033.

Disclosure statement

The authors reported no potential conflict of interest.

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