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ALUMINIUM INDUCED OXIDATIVE STRESS AND ANTIOXIDANTS SYSTEM IN TWO BARLEY VARIETIES AND ITS ALLEVIATION THROUGH ASCORBIC ACID AND SALICYLIC ACID SEED PRIMING APPROACH

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ABSTRACT

The phytotoxic effect of various concentrations (2mM-6mM) of Al at acidic pH was investigated in terms of oxidative damage (ROS), molecules of lipid peroxidation (MDA, H_2O_2), non-enzymatic antioxidants (glutathione, flavonoids) and antioxidant enzyme activities (SOD, POD, Catalase) in two Barley varieties RD25522 (Al tolerant) and RD2052 (Al sensitive) and its alleviation was studied using seed priming approach with two PGR's Ascorbic acid (AA) and Salicylic acid (SA). Both AA and SA regulate the plant growth and protect the plant against biotic and abiotic stress due to their antioxidant activity. The experiment was carried out under *in vitro* conditions for six days. ROS content increased significantly with increased concentration of Aluminum. Biophysical parameters like Electrolyte leakage (EL) increased while Relative Leaf Water Content (RLWC) decreased due to increased ROS and also induced lipid peroxidation which was correlated with an increased membrane injury. The antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathion reductase (GR) showed increased activity while pyrogallol peroxidase (POD) and nonenzymatic antioxidant glutathione, flavonoids showed declined content with increased Al concentration. These result showed increased oxidative stress under induced Al phytotoxicity and barley variety RD2552 showed tolerant nature than RD2052 against Al stress.

Abbreviation- AA-Ascorbic Acid, SA-Salicylic Acid, SOD- Superoxide Dismutase, APX- Ascorbate Peroxidase, CAT- Catalase, GR- Glutathione Reductase, POD- Pyrogallol Peroxidase, RLWC- Relative Leaf Water Content, MDA- Malondialdehyde, H_2O_2-Hydrogen Peroxide, GSH- Glutathione

Keywords: Barley, Al toxicity, Ascorbic acid, Salicylic acid, Oxidative damage, Lipid peroxidation

INTRODUCTION

Aluminum (Al) is the third most abundant element in earth crust after oxygen and silicon approximately 8% of its mass.[1] It is never present as free ion in soil though it is tightly bound with oxygen and silicon resulting in formation of nonphytotoxic and insoluble aluminum silicate and oxide forms.[2] It is non essential and harmless in slightly acid or non acidic soil, because toxicity of Al is dependent upon soil acidity and soil acidity is one of the most important factors in soil that constrains crop production in whole world. It is natural process that accelerates by agriculture practices. Soil acidity creates many problems for crop growth due to removal of crucial cations and minerals. It removes important cations Ca^{2+}, Mg^{2+}, K^+, and Na^+ etc. and replace with H_3O and Al^{3+} in soil solution. The primary target of Al toxicity is root. It is reported that the even micromolar concentration of Al can inhibit root growth in many agriculturally important plant species.[3, 4] Active Al increases reactive oxygen species (ROS) production such as superoxide anion (O_2^-), hydroxyl free radical (.OH), hydrogen peroxide (H_2O_2). Excessive ROS generation causes disruption of lipid bilayer, nucleic acid damage and protein denaturation resulting in oxidative damage, physiological disorder and eventually premature senescence and cell death.[5] It has been shown that Al exposure is associated with peroxidative damage of membrane lipids due to the stress-related increase in the production of highly toxic oxygen free radicals.[6] Vitamin C or L-ascorbic acid, or simply ascorbate (the anion of ascorbic acid), is one of simplest and water soluble micronutrient essential for metabolic reactions in all animals and plants. Ascorbate has beneficial influences on various aspects in plants. It also participates in cell signaling modulation in numerous cellular processes including cell division, cell expansion and cell wall growth.[7] Ascorbic acid neutralizes the excess ROS toxic effect by enzymatic as well as non enzymatic detoxification.[8] Salicylic acid act as antioxidant and neutralize the harmful oxide radicals, and also, influences seed germination, cell growth, respiration, stomatal closure, senescence-associated gene expression, responses to abiotic stresses, nodulation in legumes, and fruit yield.[9] This study was based on effect of Al toxicity on two varieties of barley RD2552 and RD2052 analyzed the effect of two plant growth regulators (AA and SA) in mitigating the stress, and assess the biophysical (RLWC and EL), osmolyte (proline), signaling molecule (ROS), lipid peroxidation (MDA and H_2O_2), enzymatic (SOD, APX, CAT, POD and GR) and nonenzymatic antioxidants (GSH and Flavonoids) changes in plants.

MATERIAL AND METHODS

Study area and Plant material

The present work was carried out in Department of Life Sciences, Jaipur National University Jaipur, Rajasthan. Seeds of barley varieties (*Hordeum Vulgare*L.) were collected from Rajasthan Agriculture Research Institute, Durgapura, Jaipur, Rajasthan. This study was targeted to analyze Al toxicity in two barley varieties (RD2052 and RD2552), primed with and without AA and SA. All seeds germinated in glass petri dishes with different concentrations of aluminum (C, 2mM, 4mM and 6mM) in ¼ strength Hoagland solutions at pH4 for six days. After six days of germination analyzed the biophysical parameters, osmolyte (Proline), signaling molecule, lipid peroxidation, enzymatic and nonenzymatic antioxidant by following methods: RLWC,[10] EL,[11] Proline,[12] ROS,[13] MDA,[14] H_2O_2,[15] Glutathione,[16] Flavonoids,[17] SOD,[18] APX,[19] POD,[20] CAT,[21] GR.[22]

Data analysis

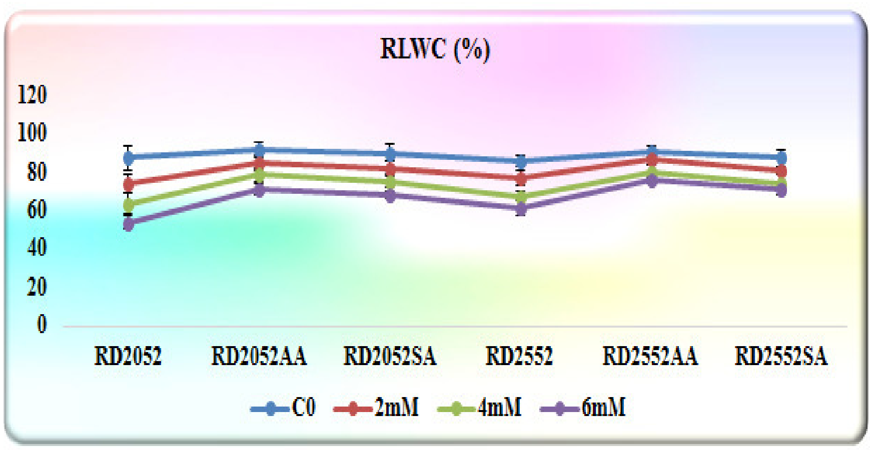
The data were determined by the one way analysis of variance (ANOVA), the design was completely randomized design (CRD). Data analysis was carried out using SPSS software. Vertical bar represent standard error.

RESULTS AND DISCUSSION

The one way analysis of variance (ANOVA) for all data determined that there were significant variation between both varieties ($P<0.01$).Aluminum toxicity decreased the RLWC, the effect increased with Al levels (Graph no.1). The same reduction has been reported in leaves of Sorghum and Mung bean,[23] resulting in loss of turgid and less water availability.[24] In susceptible variety RD2052 the reduction in the RLWC content at 6mM was 38.86% while in tolerant variety RD2552 it decreased by 29.16% over control. Priming with AA and SA improved RLWC (%) in both susceptible and the tolerant varieties over control compared with unprimed. Ekmekçi and Karaman, [25] reported that AA treatment increased the RLWC content and reduction in transpiration rate indicating the efficiency of water uptake and reduction of water loss, which consequently causing increase in leaf water potential. Yalcin and Vardar,[24] reported that SA reduced Al toxicity and maintained reduced RWC content in *Sorghum bicolor* variety. Gondor *et al*., [26] reported in finger millet that supplementation of SA increased concentration of solutes in the cell causing improved water intake, high RWC and maintenance of osmotic balance. Aluminum toxicity increased the EL, the effect increased with Al level (Graph no.2). Surapu *et al*., [27] also reported induced EL content and loss of membrane integrity in tomato cultivars. Tamas *et al*., [28] in barley showed that enhanced ROS generation caused increased electrolyte leakage and decreased cell viability. Similar results on EL were also reported in pea.[29] However, AA and SA significantly reduced membrane damage resulting in decreased EL compared to unprimed barley seedling under Al toxicity. Azooz, [30] reported that ascorbic acid could accelerate cell division and cell enlargement and improved membrane integrity. Krantev *et al*., [31] reported that SA significantly reduced ion leakage by root and maintained cell membrane integrity in maize seedling. Proline content increased (Graph no.3) with increased

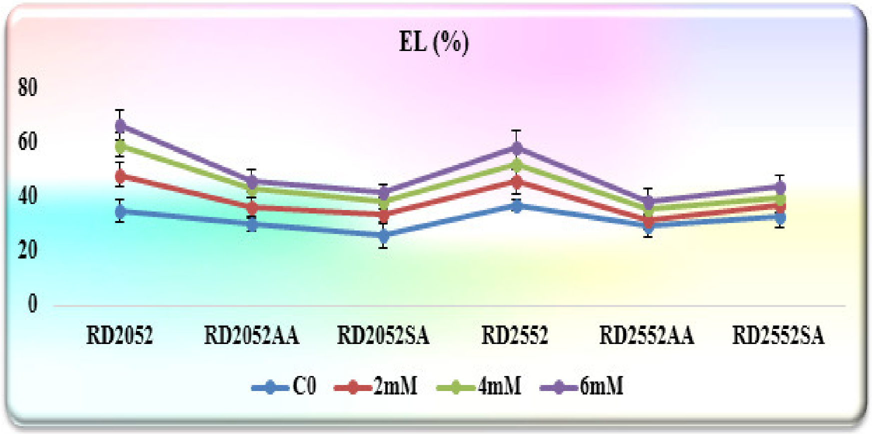
Aluminum concentration. Higher proline content was noticed in RD2052 than RD2552.Proline increase was reported to moderate aluminum tension by its osmolyte and antioxidant characteristic in Sunflower.[32] With AA and SA priming, proline content increased compared with unprimed. Amin *et al*., [33] reported that AA and SA increased the amount of proline in the plants leading to the resistance against water loss, protecting turgidity, reducing the membrane damage and accelerating growth of plants under stress conditions. Azzedine *et al*., [34] reported that application of ascorbic acid was effective to mitigate the adverse effect of salt stress on plant growth of durum wheat due to increased leaf area and enhanced proline accumulation. Misra and Saxena, [35] found that SA alleviated the salt stress of lentil by improving the proline metabolizing system. According to (Graph no.4) results obtained ROS content was increased with increasing Al concentration in both barley varieties. The production of ROS induced by Al is well-known phenomenon.[36, 37] Our results are in support with the findings that prove the subsequent accumulation of total ROS and lipid peroxides. Al is known to promote increased concentration of ROS and changes the redox state of the metabolic system in cells.[38] In experimental result AA and SA play crucial role in scavenging the ROS species. AA and SA showed reduction in ROS content in both varieties compared to unprimed variety. AA can be used by plants for the elimination of excess ROS produced during oxidative stress.[39] ROS species like $O_2^-$and singlet oxygen can be eliminated through AA nonenzymatic defense mechanism.[40] Khan *et al*., [41] reported that both endogenous and exogenous SA was evidenced to play roles in antioxidant metabolism and have a tight control over cellular ROS. The amount of MDA (Graph no.5) increased with the increase in Aluminum stress level, greater lipid peroxidation in RD2052 was observed than RD2552. Present results in agreement with those of Xiao *et al*., [42] who reported damage of the membrane system in Longan leaves under Al stress due to the induction of oxidative damage pertinent to the imbalance of ROS production. Sharma and Dubey, [43] reported that Al toxicity was associated with the Al-induced oxidative stress resultant increased generation of superoxide anion and H_2O_2 and enhanced MDA in rice shoot. Hosseini *et al*., [44] reported that Al toxicity increased the accumulation of lipid peroxidation product, MDA, which is regarded as an indicator of the loss of structural integrity in membranes subjected to heavy meal stress. Supplementation with AA and SA resulted in better recovery of seedling. Shao *et al*., [45] reported that Ascorbic acid protects metabolic processes against H_2O_2 and other toxic derivatives of oxygen affected many enzyme activities, minimize the damage caused by oxidative processes through synergistic function with other antioxidants and stabilize membranes. Salarizdah *et al*., [46] reported that SA led to a significant decrease in the level of lipid peroxidation. Al stress induced a significant increase in hydrogen peroxide (H_2O_2) level in both barley seedlings. According to (Graph no.6) the experimental result in barley seedling under Al toxicity, The RD2052 variety showed significantly increased H_2O_2 content than RD2552 variety with aluminum treatment at 6mM concentration compared to control, but AA and SA treated value was less increased in both varieties compared to unprimed control at 6mM Aluminum. Ascorbic acid can directly scavenge superoxide, hydroxyl radicals and singlet oxygen and reduced H_2O_2 to water via ascorbate peroxidase reaction, and is the major antioxidant that scavenges H_2O_2 produced by ROS and plays important roles in plants under abiotic stress tolerance.[47] Belkhadi *et al*., [48] reported that an increased tolerance of Flax to Cd was attributed to SA-mediated control of H_2O_2 accumulation. GSH level is an important protective mechanism to minimize oxidative damage in plants exposed to metals. It plays a vital role in the antioxidant defense system as well as the glyoxalase system by acting as a substrate or cofactor for certain enzymes. A marked decrease in GSH content with increased Al concentration of the Barley (RD2052 and RD2552) shoot and root was observed under Al stress (6mM) when compared to the controls (Graph no.7). The results are in agreement with Noctor *et al*., [49] and Ezaki *et al*., [50]. Glutathione content significantly maintained in AA, SA treated Shoot and Root of RD 2052 and RD2552 at 6mM Al concentration compared to unprimed barley seedlings. Ascorbic acid and glutathione both are antioxidants that increase in the activity of antioxidant enzymes and scavenging the ROS species, The AA-GSH cycle is a mechanism for removing H_2O_2.[51] Similar observations of SA mediated amelioration of drought stress were reported in mustard and mung bean by improved antioxidant system.[52] SA-application (at 0.5mM) increased the activity of enzymes of AsA-GSH pathway resulted in the increased tolerance of *B. juncea* to salinity stress.[53] Flavonids are one class of secondary metabolites that are also known as

Vitamin P. In our result Flavonoids content (Graph no.8) was also decreased with increased Aluminum concentration, similar result has been found by Zahra *et al*., [54]. Flavonoid was significantly decreased in both verities RD2052 and RD2552. In the same way AA and SA primed seedlings showed maintained flavonoid content compared to unprimed barley seedling at 6mM Al. Major ROS-scavenging enzymes in seedlings include SOD, APX and CAT.[55, 56] The most effective antioxidative enzyme in preventing ROS induced cellular oxidative damage is Superoxide dismutase (SOD) which catalyzes the conversion of O_2^- to H_2O_2, whereas CAT scavenges H_2O_2 and peroxidase uses H_2O_2 for the oxidation of various inorganic and organic substrates.[57] The present studies (Graph no. 9-13) demonstrated significant increase in Catalase, Ascorbate Peroxidase, SOD, Glutathione Reductase activity, while decreased Pyrogallol Peroxidase activities in barley seedlings with increased Aluminum concentration. Increased SOD, Catalase, Ascorbate Peroxidase, Glutathione Reductase activity in RD2052 was more than RD2552, while Pyrogallol Peroxidase activity decreased in RD2052 more than RD2552. Similarly in maize and wheat roots Al treatment also led to the increase of ROS production and simultaneously increased SOD, CAT and APX.[57, 58] Batool *et al*., [59] reported that decreased the activity of POD under salt stress. Divya *et al*., [60] reported decreased POD activity with Al toxicity. Bor *et al*., [61] reported that increased activity of GR is closely related with salt tolerance capacity in sugar beet plant. Supplementation with AA and SA, the enzyme activity gradually changed due to their ameliorative effect. SOD activity gradually decreased on supplement with AA and SA, indicating lower ROS accumulation to scavenge. AA and SA maintain the CAT activity in Al exposed seedling, there by facilitating dismutation of H_2O_2 into non toxic form so as to minimize the ROS induced oxidative stressed. AA acting as a nonenzymatic antioxidant, scavenges the ROS species, and enzymes requires AA as a cofactor in the detoxifying enzymatic processes.[62] AA can directly scavenge superoxide, hydroxyl radicals and singlet oxygen and diminish H_2O_2 to water via ascorbate peroxidase reaction.[62] Hayat *et al*., [63] reported that SA enhanced the activities of antioxidative enzymes and protected the plants against oxidative damage.



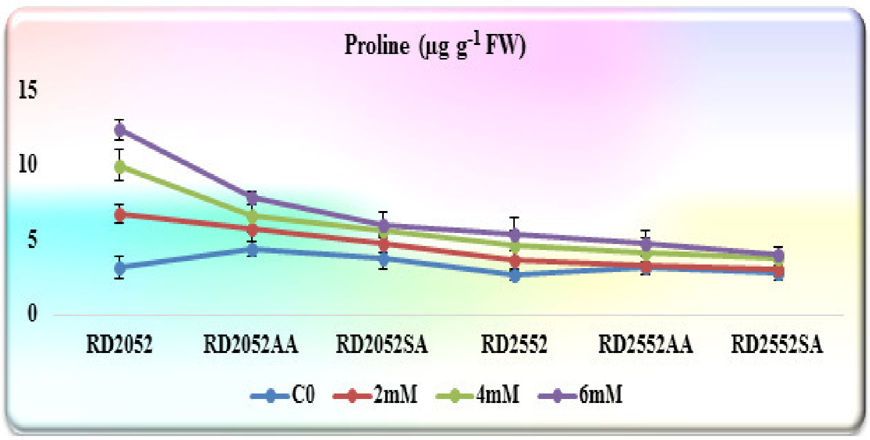
Graph no.1

RLWC (%) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



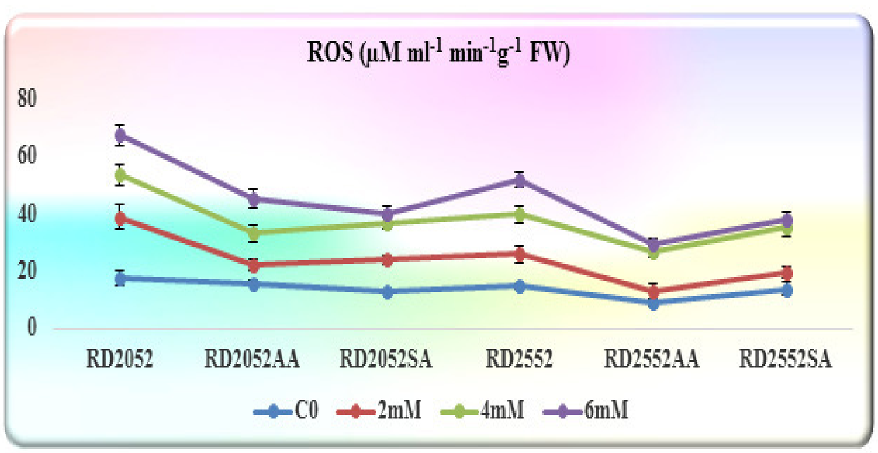
Graph no.2

EL (%) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



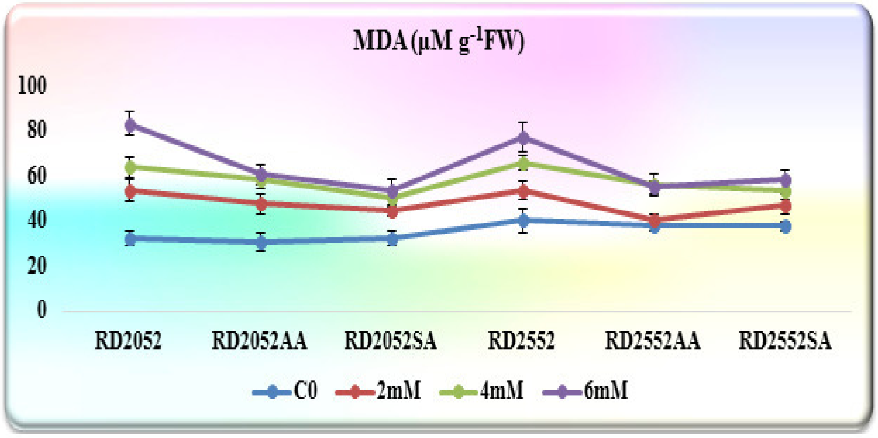
Graph no.3

Proline content (μg g^{-1}FW) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



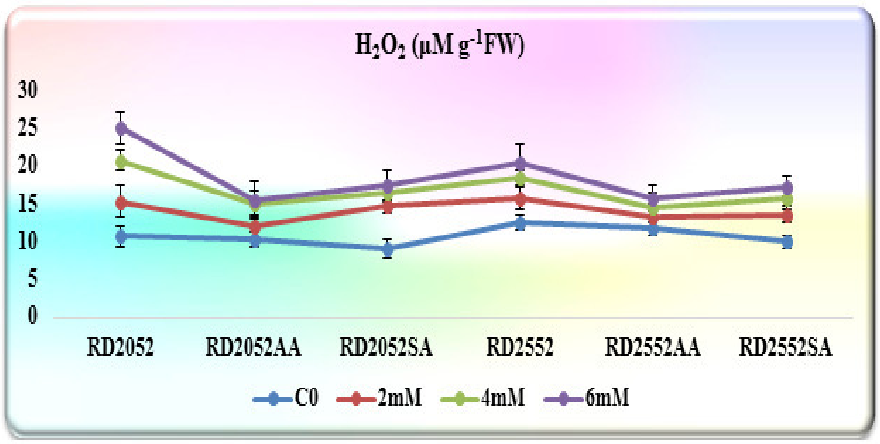
Graph no.4

ROS content (μM ml^{-1} $min^{-1}g^{-1}$ FW) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



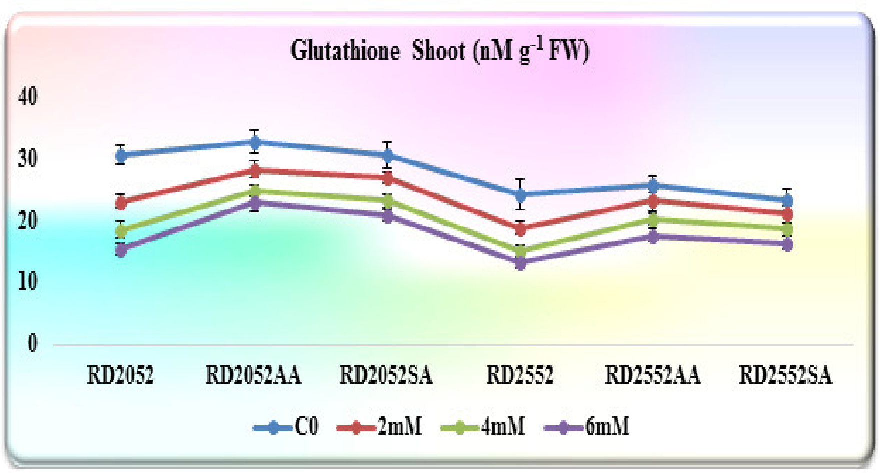
Graph no.5

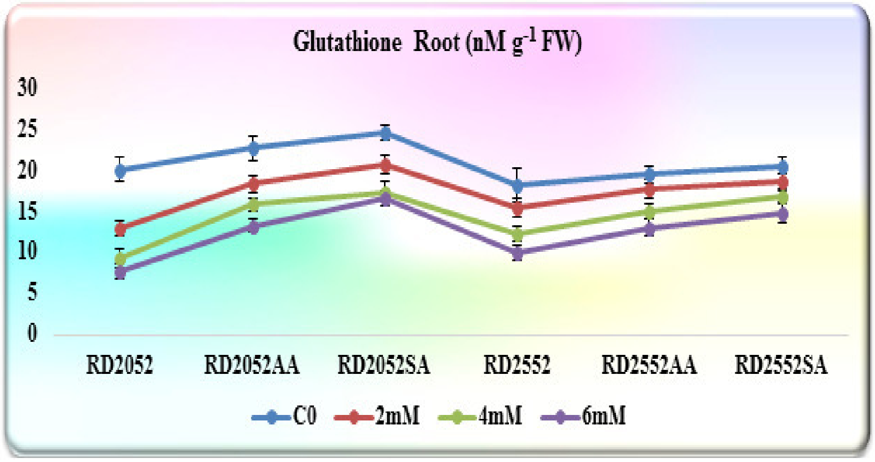
MDA content (µM g^{-1}FW) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



Graph no.6

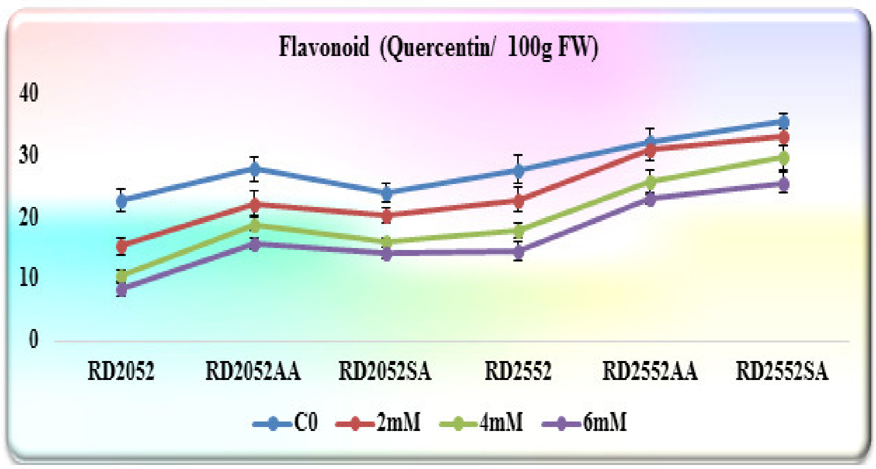
H_2O_2 content (µM g^{-1}FW) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4





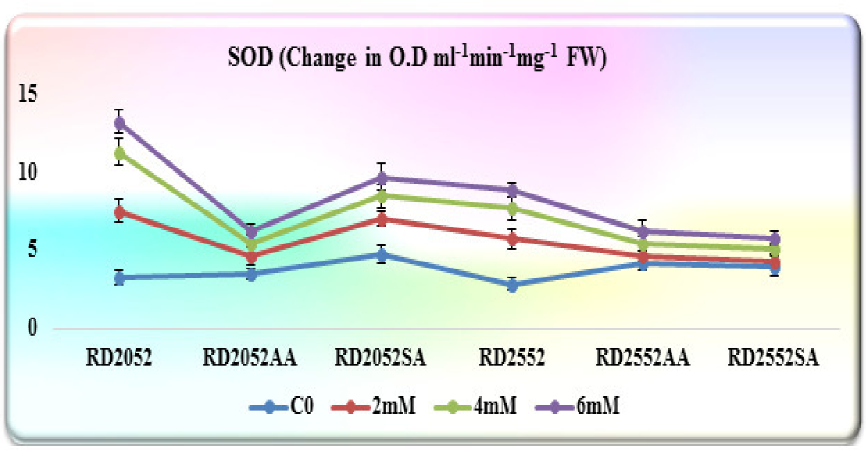
Graph no.7

Glutathione content (nM g^{-1}FW) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



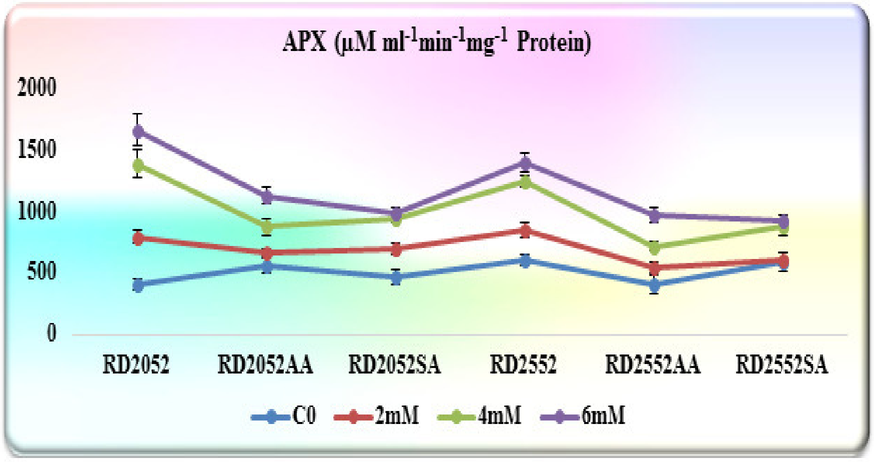
Graph no.8

Flavonoid (Quercentin/ 100g FW) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



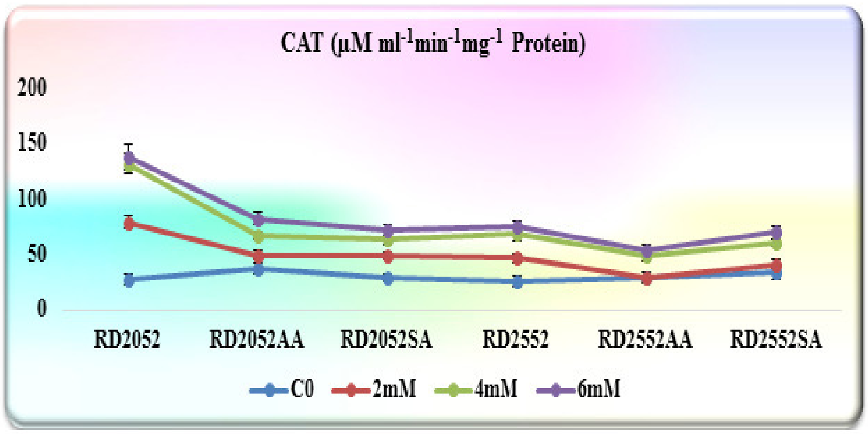
Graph no.9

SOD activity (Change in O.D $ml^{-1}min^{-1}mg^{-1}$ FW) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



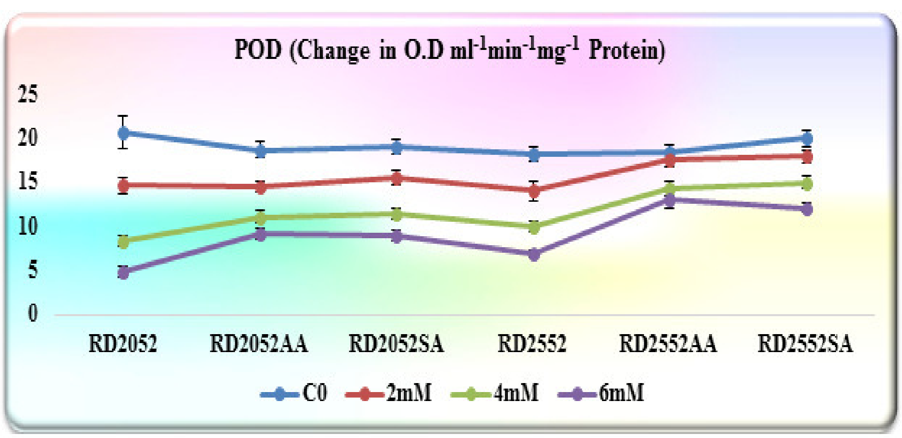
Graph no.10

APX activity (µM $ml^{-1}min^{-1}mg^{-1}$ Protein) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



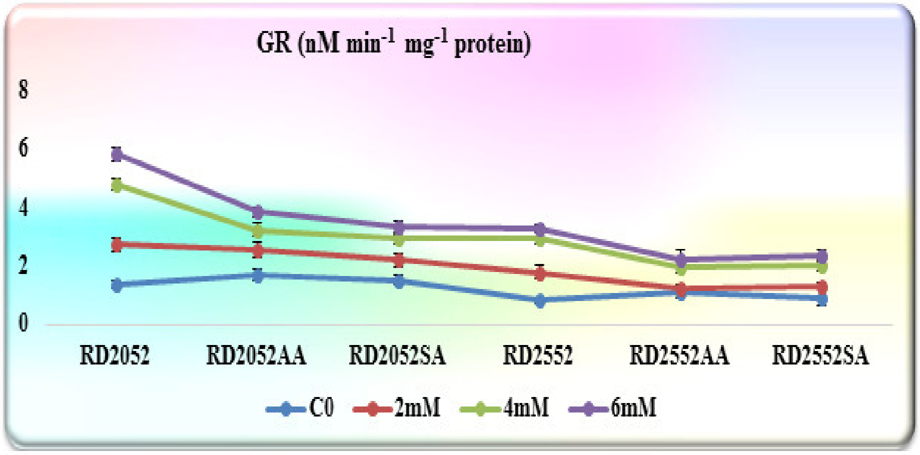
Graph no.11

CAT activity (µM $ml^{-1}min^{-1}mg^{-1}$ Protein) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



Graph no.12

POD (Change in O.D $ml^{-1}min^{-1}mg^{-1}$ Protein) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4



Graph no.13

GR activity (nM min^{-1} mg^{-1} protein) in RD2052 and RD2552 Barley varieties (unprimed and primed with AA and SA) germinated under different concentrations of Aluminum at pH_4

CONCLUSION

The result of the experiment reveled that AA and SA seed priming successfully mitigated the Al toxicity in both barley varieties (RD2052 and RD2552). The antioxidative property of both the PGRs minimized the oxidative damage due to Al toxicity. Seed priming is a convenient and economical approach that can be further used for field experiments. RD2552 proved its tolerant nature by sustaining the harmful effect of Al and performed better than RD2052 against Al stress.

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