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Effect of heavy metal aluminum (AL) on antioxidative enzyme activities and growth and oxidative stress in wheat crop

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Abstract
Heavy metal accumulation is one of the most significant agronomic challenges that poses a serious hazard to food safety. Due to these issues, soil biologists and agronomists have recently voiced concerns regarding heavy metal pollution, which is negatively impacting agroecosystems and crop production. When toxic heavy metals are deposited in excess of permissible limits, they adversely affect the density, composition, and physiological activities of microbiota, as well as the dynamics and fertility of the soil, ultimately resulting in a decrease in wheat production and, via the food chain, human and animal health. In order to preserve the physiological activities of microorganisms, the nutrient pool of soils, and wheat production in an environment that is continuously deteriorating, the metal-induced phytotoxicity issues require immediate and urgent attention. The accumulation of heavy metals is currently one of the most severe environmental issues, not only because many of these metals are toxic to crops, but also because of their potential to harm animals and humans. Metals are non-biodegradable and regarded as significant environmental pollutants, causing cytotoxic, mutagenic, and carcinogenic effects in animals. The accumulation of heavy metals in plants occurs in both roots and aboveground tissue, so there is considerable interest in monitoring the bioavailable reservoir of metals. Wheat is one of the most important commodities and an essential part of the national diet. It provides carbohydrates, proteins, and specific inorganic micronutrients, all of which are essential to human growth. Consumption of grain is secure when the accumulation of metals falls below the allowable thresholds. However, when concentrations exceed the allowable limit, it exerts toxic effects and may cause a variety of human diseases. Historically, the study of heavy metal pollution concentrated on industrialized regions.

Keywords: Heavy metal, aluminum (Al), antioxidative enzyme, growth and oxidative stress, wheat crop

1. Introduction
Wheat (Family: Poaceae, Genus: Triticum) is a globally significant staple commodity. It is an abundant source of carbohydrates for both humans and livestock. The main wheat cultivars grown in India are *Triticum aestivum* (Common bread wheat; hexaploid), Triticum dicoccum (Khapli or Emmer wheat; diploid), and Triticum durum (Durum or Macroni wheat; tetraploid). Wheat cultivated in India's central and western regions is typically tough and nutritious due to its high protein (8-15% in grain and 8-13% in flour) and gluten content. Early stages of wheat growth are sensitive to a number of environmental factors, including light intensities, water availability, temperature, soil pH, and metal toxicity, among others. Temperature fluctuations, improper disposal of chemicals, expanding industrialization, and rising soil acidity are the most significant problems in central India. In addition, the use of synthetic fertilizers has increased the concentration of metals in the soil, reducing the soil's natural fertility and having a negative impact on crop yield.
Temperature and soil pH are significant abiotic factors that influence plant growth and development. Temperature influences germination by affecting the rate of water assimilation and supply of essential nutrients for plant growth and development. While soil pH determines the availability of essential macro- and microelements for plant development, plant growth depends on both. The relationship between temperature and crops is classified as direct, phenological, and physiological. There is a direct correlation between winterkill, water depletion, and vernalization. Phenological relationship classifies the total duration of the growing season, as well as the vegetative and reproductive growth stages.

The physiological relationship is determined by the rates of photosynthesis, respiration, and grain filling. The ambient temperature favors a greater proportion of wheat germination. According to studies conducted, the optimal temperature for wheat germination is between 10 and 15 degrees Celsius. In the Malwa region of Madhya Pradesh, the temperature ranges between 8 and 25 degrees Celsius from late October to early February, which may impact nutrient absorption. On the other hand, the increased use of nitrogen fertilizers, acid rain, irrigation, and the decomposition of soil organic matter are significant contributors to the formation of acid soils. Utilization of ammonium-based nitrogen fertilizers is the primary cause of agriculturally acidified soil. These distinctive acid soils are not restricted to humid regions and are typically found in industrial areas where high-N fertilizers are used. Ammonium sulfate is the most acidifying fertilizer, decreasing the pH of the top 30 centimeters of soil. Numerous plant species have specific physiological requirements for the soil pH in relation to their growth and productivity. Acidic conditions can inhibit plant growth. Under simulated acid rain, visible foliar injuries, increased membrane lipid peroxidation, and cell permeability are observed in wheat, along with decreased chlorophyll, superoxide dismutase (SOD), glutathione reductase (GR), and ascorbic acid (ASA) content.

Heavy metal is a collective term for the group of metals and metalloids with an atomic density greater than 5g/cm3. Aluminum (Al) and other non-essential heavy metals are deleterious to plants even at micromolar concentrations. It leads to an accumulation of reactive oxygen species (ROS), malondialdehyde (MDA), and methylglyoxal (MG) in excess. The accumulation of these reactive species has a negative impact on plant growth and productivity. Some heavy metals, such as nickel (Ni), play an essential role in plant growth when present in trace amounts, but at high concentrations they are toxic to plant growth. Ni functions as a cofactor for a number of metalloenzymes, such as urease, superoxide dismutase, [NiFe]-hydrogenase, etc. (Boer *et al*. 2013) [6]. Hydrogenase and urease perform a crucial role in the nitrogen metabolism. Molybdenum (Mo) on the other hand, is an essential element required for plant metabolism, and its deficiency can negatively impact plant growth. Mo-enzymes are indispensable components of carbon (carbon monoxide dehydrogenase), sulfur (sulfite oxidase), and nitrogen (nitrogenases and nitrate reductase) metabolism. Molybdate is coordinated to produce molybdopterin or metal-containing pterin (MPT) for biological activity, thereby constituting "Moco." Nitrate Reductase (NR), an essential enzyme involved in nitrate assimilation, is composed of a prosthetic group consisting of Moco, heme, and FAD. Mo deficiency caused by a defect in Moco biosynthesis has a detrimental effect on NR activity and is deleterious to plants grown in nitrate-only conditions.

2. Research methodology

Directorate of Wheat Research (DWR), Indian Agriculture Research Institute (IARI), Indore, India, provided the wheat seeds. The seeds were surface sterilized with 0.1% HgCl2 for 1-2 minutes, rinsed with milli Q water, and germinated for three days at 251o C in a petri dish lined with damp filter paper. The seedlings were transferred to a static hydroponic culture in a 50 ml beaker for four days in a growth chamber "Scientech" model SE110 (90 mW/cm2 light intensity, 55% relative humidity, and a 12-hour day/night cycle). To determine the effect of pH and temperature on germinated seedlings, the seedlings were cultivated in a Hoagland nutrient solution diluted to one-fourth strength and subjected to various combinations of pH (4,5,6) and temperature (10°, 20°, 30 °C). The effect of varying pH and temperature on root and shoot tissue was measured in terms of fresh tissue weight and length, total chlorophyll content, total protein, proline content, and In-vivo NR activity. In addition, in order to examine the effect of heavy metals such as Al, Ni, and Mo on selected wheat varieties grown at pH 5 and 20 °C, the germinated seedlings were sprayed with variable concentrations of AlCl3 (0, 25, 50, 100, and 200 M) for four days in a growth chamber.

2.1 Analytical procedure

The treated seedlings were used to evaluate growth in terms of parameters such as the fresh weight of the shoot and root and the length of the shoot and root. For the purpose of analyzing the effect of binary treatment on wheat varieties for 30 days, fresh tissue weight and length/fresh plant weight and length were also recorded.

2.2 Malondialdehyde (MDA) Content

2.2.1 Extraction

Wheat shoot and root tissues were extracted in 1.0 ml of TCA (0.1%w/v) in an ice-cold buffer, and the homogenate was centrifuged at 13,000 g for 15 minutes at 4 °C using the "Sorvall RC 5B Plus."

2.2.2 Estimation

Double the volume of 0.5% thiobarbituric acid in 20% TCA was added to one milliliter of supernatant and incubated in a water bath for 30 minutes, after which the reaction was terminated in a cold bath. The supernatant was centrifuged for 5 minutes at 12,000 rpm and 4 degrees Celsius. Utilizing specific absorbance at 532 nm and non-specific absorbance at 600 nm, the concentration was determined. The level of lipid peroxidation was determined using the extinction coefficient (= 155 mM1 cm1) expressed as M MDA per gram of fresh weight.

2.2.3 H2O2 Content

2.2.3.1 Extraction

Wheat shoot and root tissues were extracted in 1.0 ml of TCA (0.1%w/v) in an ice-cold buffer, and the homogenate was centrifuged at 13,000 g for 15 minutes at 4 degrees Celsius using a "Sorvall RC 5B Plus" centrifuge.

2.2.4 Estimation

To 1 ml of supernatant, 1 milliliter of 10 mM sodium phosphate buffer (pH 7.5) and 2 ml of 1 M potassium iodide were added. At 390 nm, the absorbance of the sample was measured. Calculated from the extinction coefficient (=0.28 M1 cm1), the H2O2 concentration was expressed as M H2O2 g1 FW.

2.2.5 Antioxidative enzyme activity

Root and shoot material was homogenized with 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA in a mortar and pestle at 4 C in a cold chamber. Using the Remi "C-24" chilling centrifuge, the homogenate was centrifuged at 12,000 g for 20 minutes at 4 °C. The supernatant was utilized for various enzyme tests and protein analysis.

2.2.6 Superoxide dismutase
Outlined a procedure for measuring superoxide dismutase activity.

2.2.7 Assay
In a small glass test container, 0.1 ml of enzyme and 60 l of 240 M riboflavin were combined with 1 ml of reaction mixture*. These test tubes were illuminated for 8 minutes at 25 degrees Celsius in a growth chamber. A duplicate solution that was not illuminated served as the void. Simultaneously, a control tube was ran in which the enzyme was replaced with 0.1 ml of buffer. The formation of formazone was measured at a wavelength of 560 nm, and the SOD activity was expressed as units per mg of protein.

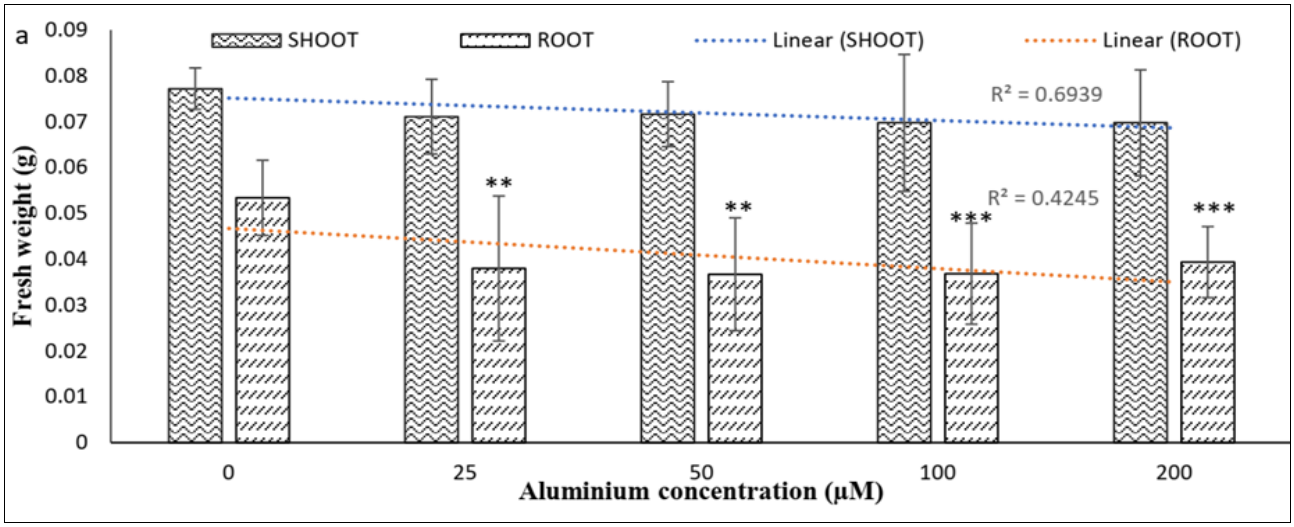
* The reaction mixture consisted of 27 ml of potassium phosphate buffer (50 mM, pH 7.8), 15 ml of 0.2 M L-methionine, 1.0 ml of 1.76 mM nitroblue tetrazolium (NBT), and 0.75 ml of Triton X-100.

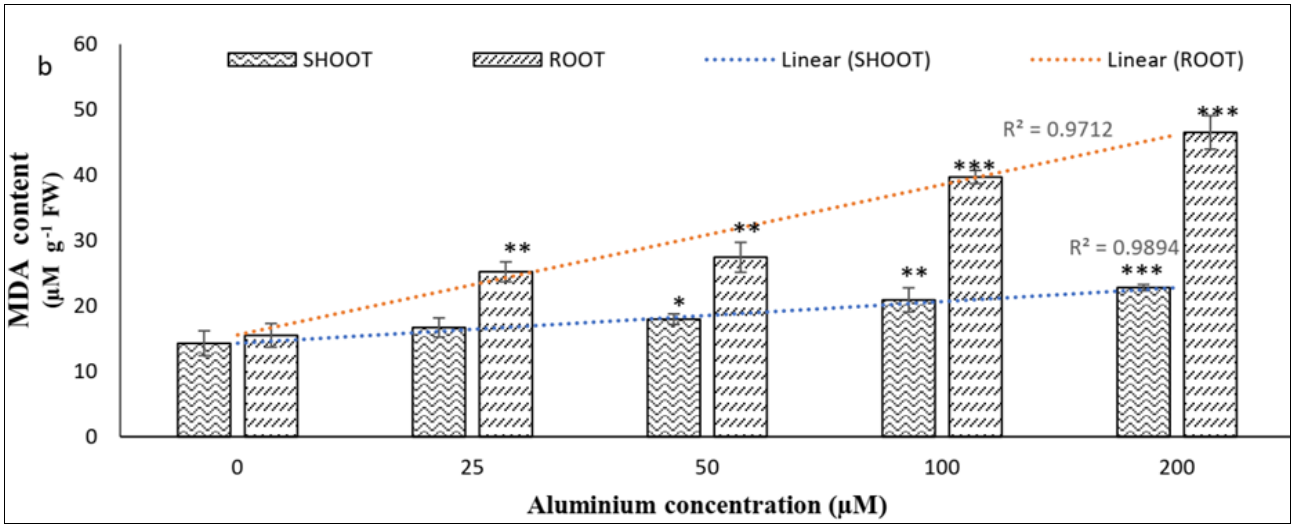
3. Results and Discussion
3.1 Effect of Aluminium, and their interactive effects on *T. durum* varieties
The present study investigated the effect of Al, in terms of antioxidative parameters either alone or in combination i.e. Al50 under acidic condition (pH 5.0) using seedlings of *Triticum durum* var. HD4672.

3.2 *T. durum var.* HD4672
AlCl3 concentrations ranging from 25 to 200 M inhibited the overall growth of wheat seedlings, as measured by fresh weight, significantly in the root but not in the shoot (R2 = 0.694) (Fig. 1a). The level of MDA, an indicator of lipid peroxidation, was greater in the root than in the shoot, with a significant increase at all Al concentrations in the root, but only at and above 50 M in the shoot (Fig. 1b). In addition, a very strong correlation was observed between the root (R2= 0.971) and the shoot (R2= 0.989). The accumulation of H2O2 was greater in the shoot relative to the root, and it increased substantially at all Al concentrations (Fig. 1c, R2=0.970) in the shoot tissue. Only at 100 and 200 M Al did H2O2 concentrations increase in roots, with a correlation coefficient of R2=0.704.

Treatment of seedlings with AlCl3 had a significant effect on the antioxidative enzyme activity of both the root and the shoot, with the former being more active (Fig. 2a-d). The SOD activity decreased considerably in both shoot and root tissues, with a perfect correlation (R2=0.911) only observed in shoot tissue (Fig. 2a). The CAT activity of both tissues significantly increased up to 50 M Al, but then decreased (Fig. 2b). At 100 and 200 M Al, the APX activity in roots decreased substantially, exhibiting a strong correlation with R2 = 0.7010 (Fig. 2c), whereas it was marginally affected in shoots. Exogenous application of Al increased Gu-Pox activity in the root by a significant amount up to 50 M Al, but decreased thereafter, while it had no effect on the shoot (Fig. 2d).

A complex correlation analysis was undertaken between the fresh weight of the tissue and various stress parameters. Reveals a perfect correlation between fresh weight of shoot and SOD (0.973), MDA (-0.845), and H2O2 (-0.907). Demonstrates a robust correlation between SOD (0.800) and MDA (-0.634) and root fresh weight. Among the antioxidative enzymes, a strong correlation was observed between CAT, APX (0.818), and Gu-Pox (0.761) in the shoot, and between CAT, APX (0.849), Gu-Pox (0.804), MDA (-0.609), and H2O2 (-0.919) in the root. Additionally, APX exhibited a correlation with Gu-Pox and a robust correlation with MDA (-0.887) and H2O2 (-0.875).





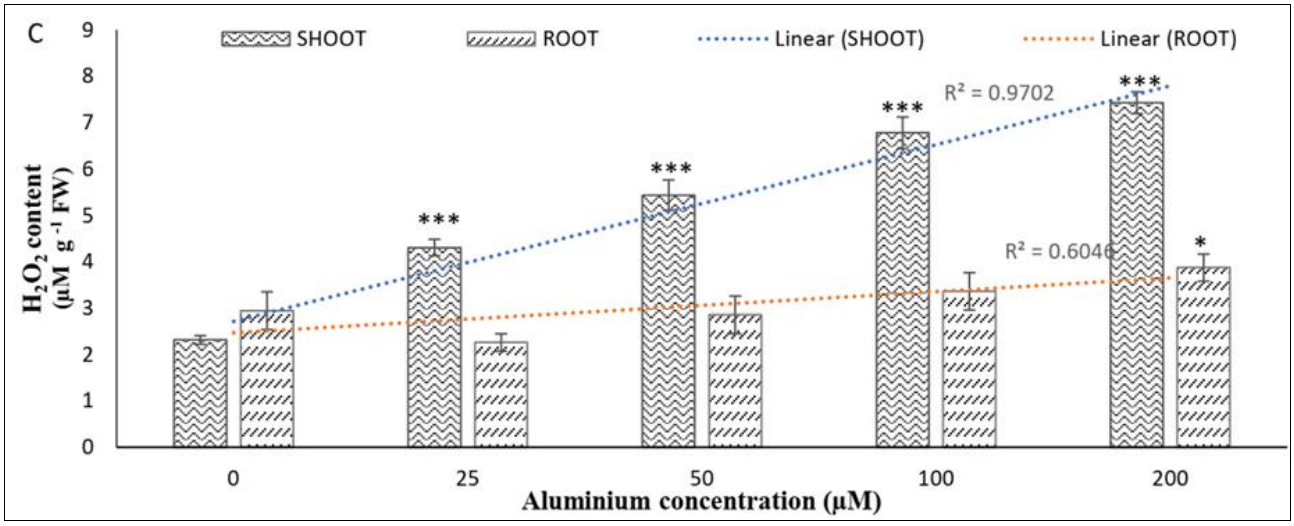


Fig 1: (a to c). Effect of Al on fresh tissue weight, MDA and H2O2 content in *Wheat variety* -HD4672.

Table 1: Compound correlation amongst fresh tissue weight, SOD, CAT, APX, Gu- POX, MDA, H2O2 of Al treated T. *durum* var. HD4672 on Shoot

a) Shoot	SOD	CAT	APX	Gu-Pox	MDA	H2O2
Weight	0.97274	-0.02483	0.215055	0.148279	-0.84453	-0.90734
SOD		-0.20287	0.011174	0.012664	-0.70692	-0.7953
CAT			0.81846	0.761078	-0.47487	-0.30842
APX				0.898066	-0.61102	-0.46369
Gu-POX					-0.43601	-0.27114
MDA						0.981435

Table 2: Compound correlation amongst fresh tissue weight, SOD, CAT, APX, Gu- POX, MDA, H2O2 of Al treated T. *durum* var. HD4672 on Root

b) Root	SOD	CAT	APX	Gu-Pox	MDA	H2O2
Weight	0.800482	-0.22294	0.277934	-0.44879	-0.6349	-0.04232
SOD		0.369367	0.705208	0.084544	-0.95199	-0.57161
CAT			0.849451	0.804311	-0.60986	-0.91938
APX				0.553842	-0.88778	-0.8758
Gu-POX					-0.28763	-0.523
MDA						0.757902

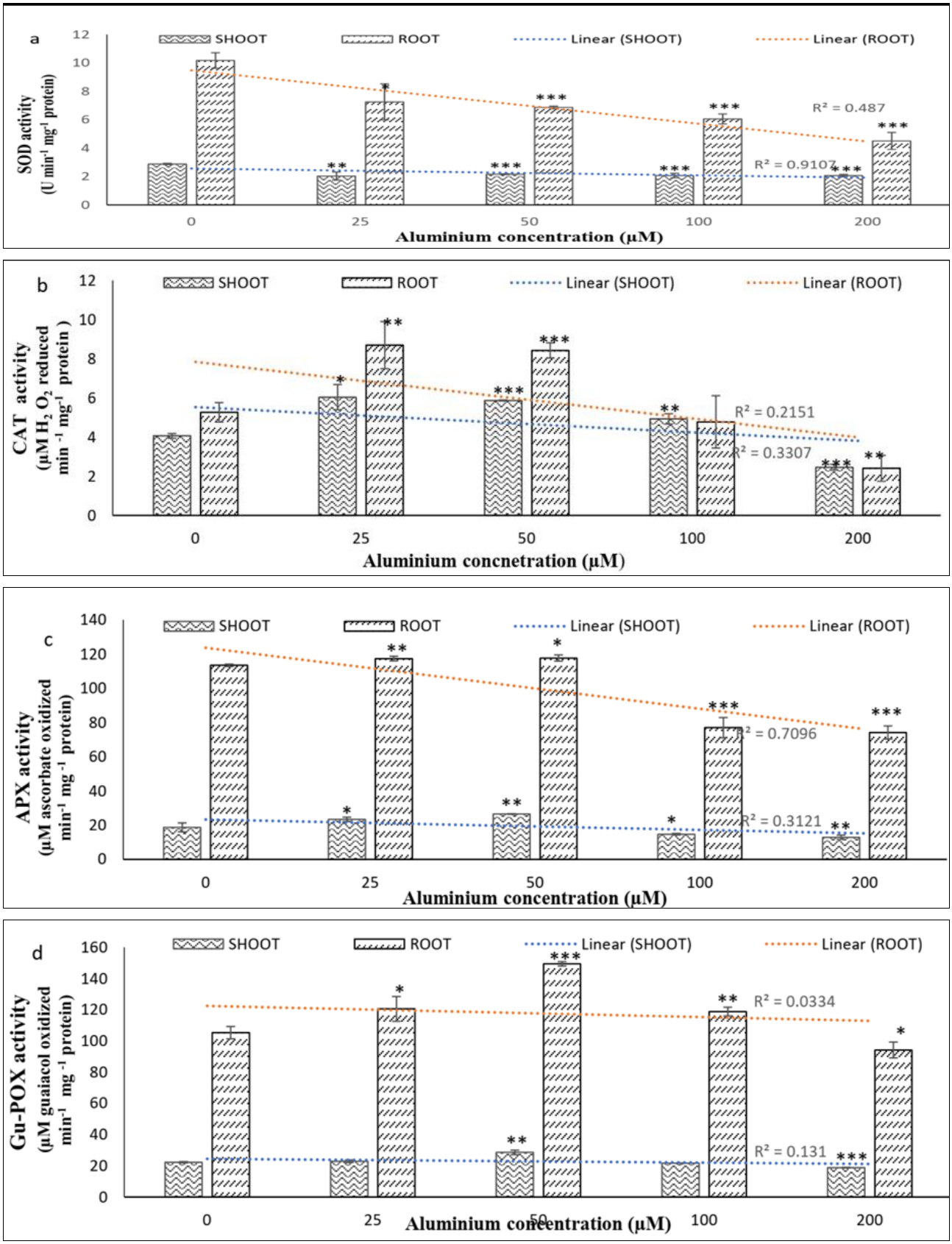


Fig 2: (a to d). Effect of Al on antioxidative enzyme activities in T. *durum* var. HD4672

Aluminium is a non-essential element whose plant toxicity increases as the soil pH falls below 5. Al inhibits root growth under acidic conditions by interfering with root cell expansion and elongation. Al toxicity has a negative impact on root development in wheat (Kinraide and Parker 1987) [18]. T. durum var. HD4672 exhibited decreased growth as measured by fresh weight of shoot and root in this study. Due to Al toxicity, oxidative stress, such as H2O2 production and lipid peroxidation, may contribute to the growth retardation. Different *Triticum aestivum* varieties, including TAM 105 and TAM 202 (Dong *et al*. 2002) [13], Raj 3077 and Raj 4120 (Aggarwal *et al*. 2015b) [14], and Yangami-5 and Jian-864 (Liu *et al*. 2018) [15], have also been reported to have elevated MDA levels. In addition, a

rise in H2O2 concentration has been observed (Xu *et al*. 2012, Aggarwal *et al*. 2015b, Liu *et al*. 2018) [16, 14, 15]. Al treatment increased MDA and H2O2 levels and had a strong correlation in both shoot (R2=0.989; 0.970) and root (R2=0.971; 0.704) tissue. A strong negative correlation was observed between the fresh weight of shoots with H2O2 generation (R=-0.907) and MDA content (R=-0.845) but only for the fresh weight of roots (R= -0.635). This indicated the involvement of oxidative stress during Al toxicity, which appears to primarily involve membrane sensitivity in the root and H2O2 production in the shoot, as higher levels of MDA were observed in the root and H2O2 in the shoot. A strong compound correlation was observed between H2O2 and MDA content in both the shoot (R=0.981) and root (R=0.758). Hossain *et al*. (2005) [17] reported the accumulation of H2O2 in the wheat root in response to Al stress, along with an increased possibility of lipid peroxidation. As MDA content increased above 50 M Al in the shoot and H2O2 accumulation increased after 50 M Al in the root, T. durum var. HD4672 was able to tolerate Al treatments causing membrane injury and peroxide generation up to 50 M in each tissue.

Al supplementation of T. durum var. HI8498 seedlings decreased fresh tissue weight while increasing oxidative stress parameters such as MDA and H2O2 in strong to perfect correlation with Al supplementation. In addition, fresh tissue weight exhibited a strong negative correlation with these stress parameters, indicating that the growth retardation due to Al toxicity results from Al-induced oxidative stress, such as lipid peroxidation and H2O2 production. Different sensitive and tolerant *Triticum aestivum* varieties have also been reported to have elevated MDA levels (Dong *et al*. 2002, Aggarwal *et al*. 2015b, Liu *et al*. 2018) [13, 14, 15]. In addition, an increase in the H2O2 content of wheat varieties has been reported (Xu *et al*. 2012, Aggarwal *et al*. 2015b, Liu *et al*. 2018) [16, 14, 15]. H2O2 is an essential signaling molecule, but it is induced by abiotic or biotic stress. In addition, according to a study conducted by Hossain *et al*. (2005) [17], the accumulation of H2O2 can cause lipid peroxidation. In this study, there was a strong intercorrelation between MDA and H2O2 in both the shoot (0.653) and root (0.794), and a significant increase in H2O2 content was observed at all Al concentrations, but only at 100 and 200 M for MDA. Consequently, it is probable that Al toxicity in T. durum var. HI8498 increases the H2O2 content, leading to lipid peroxidation at higher concentrations.

In T. durum var. HI8737, Al supply decreased growth as measured by fresh weight of shoot and root, and it increased MDA and H2O2 content with a strong correlation in shoot (R2= 0.850; 0.614) and root (R2= 0.923; 0.845) tissue, respectively. There is a strong negative correlation between shoot fresh weight and MDA content (R=- 0.700), and for root fresh weight, correlations were observed for both MDA and H2O2. Thus, indicating that Al has a negative effect on growth as a result of elevated oxidative stress parameters such as MDA and H2O2. In addition, a robust intercorrelation was observed between MDA and H2O2 in both the shoot (R=0.899) and root (R=0.870). Potentially, an increase in H2O2 can increase lipid peroxidation, programmed cell death, and inhibition of root growth elongation (Hussain *et al*. 2005; Xu *et al*. 2011) [16]. The extent of reduction in fresh weight of root was greater than that of shoot, which may be attributable to substantially higher MDA and H2O2 content in root, indicating sensitivity of root tissue of T. durum var. HI8737 to higher Al concentrations.

4. Conclusion

Al stress increases the production of reactive oxygen species (ROS) in plants, which can be mitigated by inducing antioxidative enzymes. Zhang *et al*. (2008) [19] observed an increase in APX and CAT but a decrease in SOD in *Triticum aestivum* L. cv. Yangmai 158, whereas Yanik and Vardar (2018) [20] observed an increase in SOD but a decrease in CAT activity in *Triticum aestivum* L. cv. Demir 2000 when subjected to Al stress. Other investigations investigated the impact of Al on resistant and sensitive wheat varieties. Thus, Xu *et al*. (2012) [16] and Liu *et al*. (2018) [15] found higher CAT and APX activity, but reduced SOD and POD activity in the root apex of the tolerant genotype Jian-864 compared to the sensitive genotype Yangami-5. Aggarwal *et al*. (2015b) [14] observed higher SOD, CAT, and APX in the roots of the Al-resistant (Raj 3077) genotype as compared to the sensitive (Raj 4120) genotype, while higher SOD and APX were observed in the shoots.

The addition of Al to T. durum var. HD4672 led to a progressive decline in SOD activity in both tissues. A compound correlation analysis revealed a perfect positive relationship between SOD and both the fresh weight of the stalk (R=0.973) and the root (R=0.800). This suggests that the overall growth reduction caused by Al toxicity is dependent on SOD's role as a scavenger. Also, the level of stress is affected by SOD activity, as a negative correlation was observed between SOD and oxidative stress parameters such as H2O2 and MDA content in both the shoot and root. In the shoot, H2O2 levels increased while SOD levels decreased substantially. In addition, SOD activity in the shoot is lower than in the root, which may be due to the inactivation of SOD in chloroplasts in the presence of elevated H2O2 levels. SOD also had a strong negative correlation with MDA in shoot, indicating that H2O2 plays a role in boosting MDA and inhibiting SOD activity. The tissue enzymes CAT, APX, and Gu-Pox that convert H2O2 to H2O increased up to 50 M Al. In addition, a strong correlation was observed between these antioxidative enzymes in both tissues, with the exception of APX in the root. CAT and APX in root were correlated with MDA (R=-0.61; -0.888) and H2O2 (R=-0.919; -0.876). While in the shoot, only APX was correlated with MDA (R= -0.611), indicating the participation of CAT and APX in detoxification against Al stress in these tissues. A weak correlation was observed between Gu-Pox and MDA and H2O2, indicating that Gu-Pox may not play a role in detoxification, but its function in cell wall lignification is probable. Although roots are the primary site of Al toxicity, this study observed a lower H2O2 content and higher CAT, APX, and Gu-Pox at least up to 50 M Al in the root relative to the shoot. Therefore, the roots of T. durum var. HD4672 may have a greater antioxidative capacity than the stems, allowing them to tolerate up to 50 M of Al.

5. Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6. Financial Support
Not available

7. References

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