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Antioxidant Responses in Chromium-Stressed Maize as Influenced by Foliar and Root Applications of Fulvic Acid

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Maize (*Zea mays* L.) faces significant challenges to its growth and productivity from heavy metal stress, particularly Chromium (Cr) stress, which induces reactive oxygen species (ROS) generation and damages photosynthetic tissues. This study aimed to investigate the effects of fulvic acid (FA) application, via foliar spray or root irrigation, on mitigating chromium stress in maize by evaluating its impact on antioxidant activity and growth parameters. Two maize varieties, P3939 and 30Y87, were subjected to chromium stress ($CrCl_3 \cdot 6H_2O$) at concentrations of 300 µM and 100 µM for a duration of 5 weeks. The experiment was conducted in a wire house under natural environmental conditions at the Seed Centre, Institute of Botany, University of the Punjab, Lahore, Pakistan. Physiological assessments included electrolyte leakage, chlorophyll pigment content, malondialdehyde (MDA) levels, and activities of antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) in maize leaves. Growth parameters were also monitored. The results revealed that chromium stress significantly reduced chlorophyll content and increased oxidative stress, as evidenced by elevated MDA levels and electrolyte leakage. However, FA application notably mitigated these effects: chlorophyll content improved by 15%, and MDA levels decreased significantly. Irrigation with FA was particularly effective, reducing MDA levels by 40% compared to the 300 µM chromium treatment. Furthermore, while chromium stress enhanced antioxidant enzyme activities, FA application further boosted total soluble protein levels and antioxidant enzyme activities under stress conditions. In conclusion, FA application demonstrates potential in improving maize tolerance to heavy metal stress by enhancing the antioxidant defense system and preserving photosynthetic pigments. These findings highlight FA's promise as a practical strategy for mitigating the negative impacts of chromium stress on maize, promoting sustainable agricultural practices in contaminated environments.

Keywords Antioxidant enzymes, Chromium toxicity, Maize growth, Oxidative damage, Reactive oxygen species, Stress mitigation

Heavy metal contamination is a major problem across the world owing to its negative impacts on plant development, production, and food quality[1]. Heavy metals are introduced into soil by both natural (volcanism and weathering) and human activities, i.e., wastes and chemicals from industries[2]. In Pakistan, heavy metal contamination is primarily driven by industrialization, urbanization, wastewater irrigation, and the use of pesticides. Notably, approximately 600 leather tanneries are operating in Karachi, Kasur, and Sialkot[3]. The excessive release of wastewater and effluents from these tanneries has been identified as a major source of soil and water contamination with heavy metals[4]. Chromium is a widespread heavy metal released by tanning and

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plating industries[5]. Chromium (VI), in particular, is highly toxic due to its high oxidation potential and solubility compared to chromium (III)[6]. Chromium toxicity in plants leads to various harmful effects, including inhibited growth, pigment degradation, suppression of antioxidant enzyme activity (SOD, POD, APX, and CAT), and the generation of reactive oxygen species (ROS), which cause cellular damage. In response, antioxidants like ascorbic acid, glutathione, α-tocopherol, flavonoids, and carotenoids (CAR) become essential, as they play a pivotal role in neutralizing ROS and mitigating oxidative stress[7,8]. Chromium-induced stress disrupts electron transport in the thylakoidal membrane during photosynthesis, leading to oxidative stress and a decrease in photosynthetic capacity[9]. One potential short-term solution to mitigate chromium's adverse effects is the application of soil amendments or foliar sprays containing antioxidant compounds[10].

Humic substances, such as fulvic acid, play an essential role in improving plant growth, enzyme activity, and soil chemistry. Fulvic acid is the acid-soluble fraction of humic substances. These organic compounds have a tendency to form bonds with heavy metals, thereby reducing the detrimental impacts of metal stress on plant development[11]. Fulvic acid is used as natural soil amendment which is formed by biological and chemical decomposition of plants[12]. The major functional groups of fulvic acid include aromatic rings, phenolic hydroxyls and carboxyl groups[13]. Fulvic acid promotes maize growth and photosynthesis while reducing toxic element uptake by forming chemical bonds with heavy metals. Fulvic acid can enhance seed germination and stimulate plant metabolism[14]. It enhances antioxidant enzyme activity, including catalase (CAT) and peroxidase (POD), as well as other enzymes like alkaline phosphatase (e.g., improving phosphate mobilization in plants under stress conditions)[15].Maize (*Zea mays* L.) is one of the most widely cultivated cereal crops globally, playing a crucial role in food security, animal feed, and industrial applications[16]. It is rich in starch, proteins, and fiber, making it crucial for both human and animal nutrition[17]. Due to its economic and nutritional importance, maize is often grown in regions that face environmental challenges, including soil contamination from heavy metals. Chromium, a persistent and toxic heavy metal, can severely affect maize growth and development, leading to reduced yields and compromised nutritional quality[18]. As a staple crop, maize is particularly vulnerable to heavy metal stress, which inhibits its physiological functions, including photosynthesis and antioxidant enzyme activity[19]. Although maize is known to be vulnerable to heavy metal stress, the specific mechanisms by which chromium affects its physiological functions, including photosynthesis and antioxidant enzyme activity, are not fully understood. The objectives of this study were to evaluate the detrimental effects of chromium on maize growth and antioxidant enzyme activity and to assess the potential mitigating role of fulvic acid in counteracting chromium toxicity. While various mitigation strategies have been explored, the role of fulvic acid in enhancing antioxidant defense under chromium stress in maize remains underexplored. The working hypothesis proposes that fulvic acid application enhances maize's antioxidant defense system under chromium stress, offering an innovative approach to improve tolerance and alleviate oxidative damage caused by chromium toxicity.

Materials and methods

Experimental setup and chromium stress with fulvic acid application in maize cultivars P3939 and 30Y87

To evaluate the impact of fulvic acid on two maize cultivars, a pot experiment was conducted at the Seed Centre, Institute of Botany, University of the Punjab, Lahore, Pakistan, in November 2018 utilizing a complete randomized design (CRD) as shown in Fig. 1. Seeds of maize cultivars P3939 and 30Y87 were sourced from Pioneer Seed Company in Sahiwal. The fulvic acid used in the experiment was purchased from Sigma-Aldrich. Pots were grown in a wire house under natural environmental conditions, with regular irrigation twice a week and a foliar spray of N, P, K applied to meet nutritional requirements. The experiment included three replicates for each treatment. Chromium stress was induced using chromium chloride (100 μM[20] and 300 μM[21]) by dissolving the appropriate amounts in distilled water at the 4th leaf stage. After one week of exposure to heavy metal stress, fulvic acid applications via foliar spray (0.5 L/A)[22] and root application (300 L/A) with irrigated water, were randomly allocated to pots to minimize potential bias and environmental variability.

Evaluation of growth parameters

To investigate the effects of fulvic acid on the development of maize cultivars under to chromium stress, several growth parameters (leaf length, leaf breadth, leaf area, plant height, and number of leaves) were evaluated.

Harvesting

After four weeks of applying fulvic acid treatment, maize plants were harvested and carefully divided into shoots and roots. Prior to harvesting, samples were collected from both young and fully matured leaves of each plant. These samples were then subjected to detailed analysis to assess various growth parameters and biochemical indicators. This analysis included measurements of electrolyte leakage, malondialdehyde (MDA) content (a marker of lipid peroxidation), chlorophyll pigment content, total soluble proteins (TSP), and activities of antioxidant enzymes. These assessments provided insights into the physiological responses and biochemical changes induced by the fulvic acid treatment in maize plants.

Determination of chlorophyll content

Chlorophyll content was determined according to the method of Arnon,[23] with slight modifications. For determination of chlorophyll content, leaf 0.1 g of each plant tissue was collected in labelled Eppendorf tubes 1.5 mL and DMSO (Dimethyl sulfoxide.) 1 mL was added. After the Eppendorf were placed in the dark for 48 h, the absorbance of each sample was then measured at 663 nm and 645 nm.

Total Chlorophyll Content = [(0.00802) (D – 663) + (0.0202) (D – 645) (mL of solvent)] / gram fresh weight of the plant.

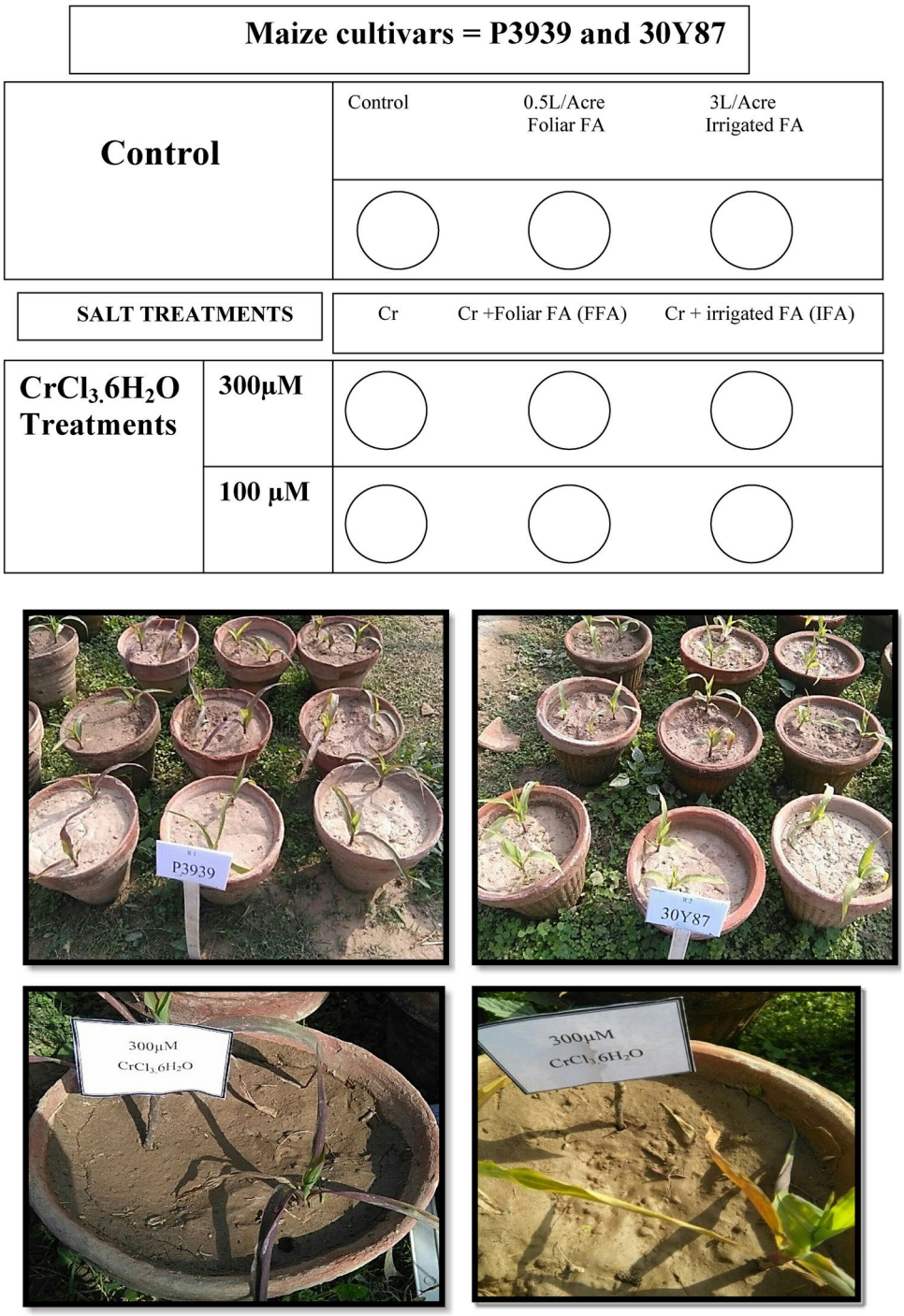

Fig. 1. The experimental layout includes two maize cultivars, P3939 and 30Y87, subjected to different chromium (Cr) stress levels and treatments with fulvic acid. The figure depicts the arrangement of the pots and highlights the visual symptoms in maize plants exposed to Cr, such as leaf discoloration.

Electrolyte-leakage determination

Leaf samples of uniform size were made using a cork borer and put in 20 mL distilled water. These tubes were then shaken for 24 h on an orbital shaker to break the cell membranes. The electrical conductivity (EC_1) was measured after 24 h then autoclaved at 121 °C for 1 h before being put on an orbital shaker for 24 h (120 rpm) to measure electrical conductivity (EC_2)[24]. Electrolyte leakage was calculated by using following formula.

$$\text{Electrolyte leakage} = EC_1/EC_2 \times 100$$

Evaluation of MDA content

MDA content and antioxidant enzyme activity (CAT, APX, and GPX) were measured using the technique of (He et al., 2001). 0.25 g of leaves were crushed in 150 mM phosphate buffer (4 mL) having pH 7.0 and centrifuged at 14,000 rpm for 20 min (4 °C). For MDA content determination, 1 ml of enzyme extract was mixed with 2 ml of a reaction solution containing 20% TCA and 0.5% TBA. After that, the solution was heated in a water bath (95 °C) for 30 min before being centrifuged at 12,000 rpm for 15 min. The absorbance then recorded at 532 and 600 nm [25]

Determination of antioxidant enzymes (CAT, APX, and GPX)

Catalase activity was assessed using a modified version of the technique described by Chance and Maehly[26]. Reaction solution containing pH 7.0 (3 mL), hydrogen peroxide (H_2O_2) 45 mM, and enzyme extract 100 µL were collected. A spectrophotometer was used to measure changes in absorbance of each sample at 240 nm.

The approach of[27] with certain changes was used to determine APX activity. 3 (mL) of the reaction solution 100 mM sodium acetate (pH 5.8), H_2O_2 (5 mM), Ethylenediaminetetraacetic acid (EDTA) 0.003 mM, 10 mM ascorbic acid, and 100 µL extracted solution) was collected and the reaction was started by adding enzyme extract. A spectrophotometer was used to measure changes in absorbance at 290 nm every 10 s for 60 s.

With minor adjustments, GPX activity was assessed using the approach of[26]. 3 mL reaction solution comprising 0.1 mol/L sodium acetate buffer, 0.25% guaiacol, 0.75% H_2O_2, and 50 µL enzyme extract were collected. Spectrophotometer was used to measure changes in absorbance at 460 nm every 10 s for 60 s.

Determination of total soluble protein

The total soluble protein content of the leaf was calculated by adding supernatant 100 µL from extract of plant material then mixed with 3 mL of colour reagent. After 5 min, the absorbance of each sample was measured at 595 nm[28].

Statistical analysis

Using the SAS (Statistical Analysis System) statistical software programme, the data was statistically analyzed using procedure mixed (PROC MIXED) and procedure generalized linear model (PROC GLM) (SAS Institute). The Duncan Multiple range test was used to detect mean differences. Correlation and principal component analyses were performed using RStudio.'

Results

Effects of fulvic acid on growth parameters of maize cultivars

The study investigated the effects of different treatments on the growth parameters of two maize cultivars, P3939 and 30Y87. Significant variations were observed in leaf length, leaf width, leaf area, plant height, and number of leaves across treatments (Table 1). For both cultivars, the control group exhibited intermediate values for most parameters. The application of 100 µM Cr notably reduced leaf length, leaf width, leaf area, and plant height compared to the control, indicating a negative impact on growth. Conversely, treatments combining 100 µM Cr with fulvic acid applied as a foliar spray (FFA, 0.5 L/A) or through the rooting medium (IFA, 300 L/A) showed varying effects. The application of 100 µM Cr + FFA generally mitigated some of the adverse effects associated with chromium exposure, whereas 100 µM Cr + IFA yielded mixed outcomes, occasionally intensifying the

Treatments	Cultivar P3939					Cultivar 30Y87				
	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Plant height (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Plant height (cm)	No. of leaves
Control (0 µM)	23.92 ± 1.12 b	1.55 ± 0.08 b	28.91 ± 2.47 b	33.73 ± 1.86 a-b	6.52 ± 0.33 ab	22.82 ± 1.12 b	1.41 ± 0.08 b	26.91 ± 2.47 b	32.62 ± 1.86 b	6.58 ± 0.33 ab
FFA	28.62 ± 1.12 a	1.77 ± 0.08 a	38.78 ± 2.47 a	35.92 ± 1.86 a	7.12 ± 0.33 a	28.62 ± 1.12 a	1.77 ± 0.08 a	37.78 ± 2.47 a	35.82 ± 1.86 a	7.12 ± 0.33 a
IFA	24.00 ± 1.12 b	1.67 ± 0.08 ab	29.80 ± 2.47 b	34.73 ± 1.86 a	6.70 ± 0.33 ab	23.00 ± 1.12 a-b	1.47 ± 0.08 a-b	28.80 ± 2.47 a-b	33.66 ± 1.86 a-b	6.50 ± 0.33ab
100 µM Cr	17.15 ± 1.46 e	1.15 ± 0.05 d	15.91 ± 1.73 e	23.80 ± 0.64 d	5.90 ± 0.50 b-c-d	16.90 ± 1.56 c-d	1.19 ± 0.09 c-d	15.81 ± 2.67 c	24.35 ± 1.85 d	5.55 ± 0.25 d
100 µM Cr + FFA	21.30 ± 0.92 c	1.30 ± 0.05 b-c	21.82 ± 1.57 c	26.30 ± 1.36 b	6.40 ± 0.50 b	19.90 ± 0.77 b-c	1.30 ± 0.05 b-c	19.52 ± 1.55 b-c	29.50 ± 2.70 c	6.25 ± 0.47 b
100 µM Cr + IFA	19.22 ± 1.78 c-d	1.25 ± 0.08 c	19.66 ± 2.64 c-d	25.02 ± 3.04 b-c	6.25 ± 0.47 b	19.00 ± 1.08 b-c	1.27 ± 0.05 b-c	18.96 ± 1.57 b-c	28.20 ± 5.06 a-c	6.00 ± 0.40 b-c
300 µM Cr	15.45 ± 1.15 e	1.05 ± 0.05 d-e	14.09 ± 1.36 e	20.50 ± 0.98 b-e	5.50 ± 0.70 b-c	15.55 ± 0.87 d	1.10 ± 0.05 d	14.10 ± 1.53 d-e	23.15 ± 1.65 d-e	5.15 ± 0.62 d-e
300 µM Cr + FFA	17.60 ± 2.19 d	1.27 ± 0.05 c-d	19.09 ± 1.81 c-d	24.85 ± 3.63 b-c	5.75 ± 0.25 b-c	16.50 ± 0.54 c-d	1.20 ± 0.08 cd	15.94 ± 1.50 c	25.00 ± 0.99 c-d	5.05 ± 0.47 c
300 µM Cr + IFA	16.65 ± 2.64 d-e	1.23 ± 0.09 d	17.93 ± 3.78 d	22.57 ± 0.73 d	5.61 ± 0.28 d	17.15 ± 1.21 c	1.20 ± 0.08 cd	15.43 ± 2.23 cd	24.65 ± 3.05 c-d	5.00 ± 0.40 c-d

Table 1. Effects of fulvic acid applied as a foliar spray (FFA, 0.5 L/A) and through the rooting medium irrigation (IFA, 300 L/A) on growth parameters of maize cultivars (P3939 and 30Y87). Values represent mean ± standard error (SE), with lowercase letters indicating significant differences among treatments.

harmful impacts of chromium. Overall, these findings highlight the complex interactions between chromium exposure and fulvic acid application methods on the growth metrics of these maize cultivars.

Effects of fulvic acid on chlorophyll content maize cultivars

In both maize cultivar chlorophyll concentration was measured across varying Cr concentrations and foliar application treatments. In cultivar P3939, under normal conditions (0 µM Cr), chlorophyll levels were relatively consistent across treatments: 1.0096 µg/mg FW without FFA, 1.3013 µg/mg FW with FFA, and 1.2113 µg/mg FW with IFA. However, exposure to 100 µM Cr resulted in a decrease in chlorophyll concentration across all treatments: 0.9713 µg/mg FW without FFA, 1.3022 µg/mg FW with FFA, and 0.9992 µg/mg FW with IFA. At 300 µM Cr, chlorophyll concentrations varied further: 1.177 µg/mg FW without FFA, 1.1362 µg/mg FW with FFA, and 1.1399 µg/mg FW with IFA (Fig. 2A). Under control conditions (0 µM Cr), In cultivar 30Y87 chlorophyll levels varied with foliar treatments: 1.0096 µg/mg FW without FFA, 1.3013 µg/mg FW with FFA, and 1.2113 µg/mg FW with IFA. Exposure to 100 µM Cr resulted in a decline in chlorophyll concentration across all treatments: 0.9324 µg/mg FW without FFA, 0.9342 µg/mg FW with FFA, and 0.8633 µg/mg FW with IFA. At 300 µM Cr, chlorophyll concentrations continued to decrease: 0.9747 µg/mg FW without FFA, 1.1332 µg/mg FW with FFA, and 0.8657 µg/mg FW with IFA. These findings indicate that while foliar application of fulvic acid (FFA) generally maintained or slightly elevated chlorophyll levels compared to controls, chromium exposure consistently diminished chlorophyll production (Fig. 2B).

Effects of fulvic acid on oxidative stress (electrolyte leakage and MDA) content maize cultivars

Electrolyte leakage (%)

In maize cultivar P3939, the impact of Cr concentrations on electrolyte leakage percentage was examined across different foliar application treatments. Under normal conditions (0 µM Cr), electrolyte leakage percentages were 84.96% without FFA (fulvic acid), 57.34% with FFA, and 70.67% with IFA (fulvic acid through the rooting medium). As chromium concentration increased to 100 µM, electrolyte leakage decreased to 64.03% without FFA, 56.71% with FFA, and 58.5% with IFA. At the highest Cr level tested (300 µM), electrolyte leakage showed mixed trends: 68.08% without FFA, 55.3% with FFA, and 49.38% with IFA (Fig. 3A).

In maize cultivar 30Y87, electrolyte leakage percentages were examined across varying Cr concentrations and foliar application treatments. At 100 µM Cr, electrolyte leakage increased compared to the control (0 µM

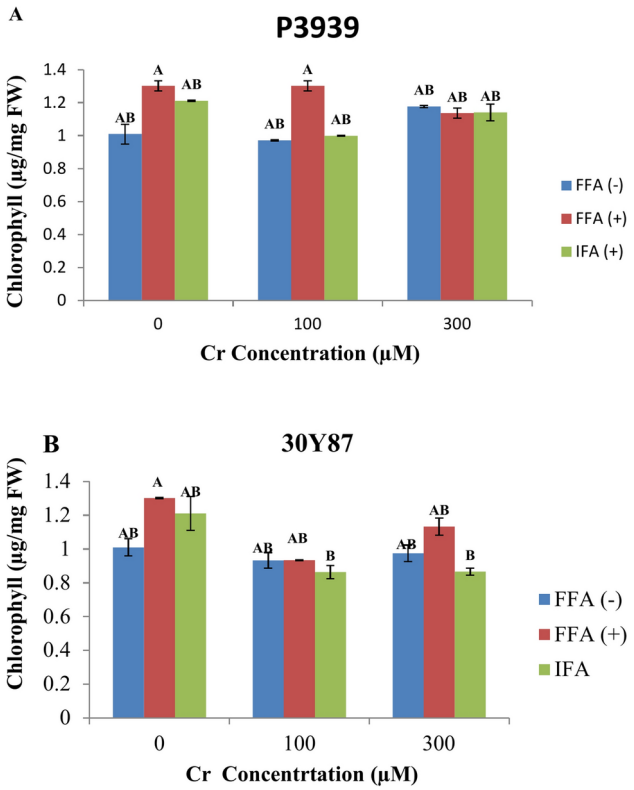

Fig. 2. Effects of fulvic acid applied as a foliar spray (FFA, 0.5 L/A) and through the rooting medium irrigation (IFA, 300 L/A) on Chlorophyll content of maize cultivars P3939 (**A**) and 30Y87 (**B**) under chromium stress. Values represent mean ± standard error (SE), with lowercase letters indicating significant differences among treatments. -FFA indicates no fulvic acid application (control). + FFA represents foliar application of fulvic acid, sprayed on leaves. IFA refers to fulvic acid application through irrigation, delivered via the rooting medium.

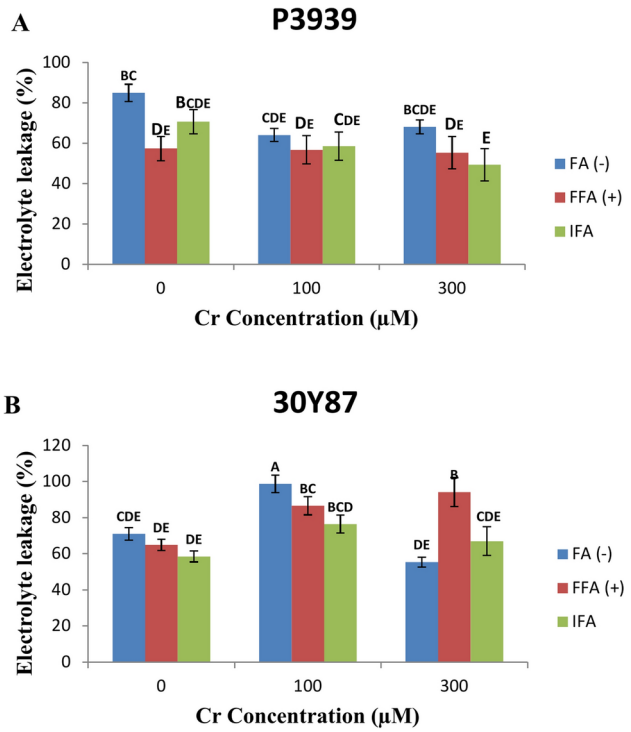

Fig. 3. Effects of fulvic acid applied as a foliar spray (FFA, 0.5 L/A) and through the rooting medium irrigation (IFA, 300 L/A) on Electrolyte leakage (EL) of maize cultivars P3939 (**A**) and 30Y87 (**B**) under chromium stress. Values represent mean ± standard error (SE), with lowercase letters indicating significant differences among treatments. -FFA indicates no fulvic acid application (control). + FFA represents foliar application of fulvic acid, sprayed on leaves. IFA refers to fulvic acid application through irrigation, delivered via the rooting medium.

Cr) across all treatments: FFA (-) increased by 38.97%, FFA (+) by 33.30%, and IFA (+) by 30.68%. However, at 300 µM Cr, the responses varied: FFA (-) showed a decrease in leakage by 21.92%, FFA (+) exhibited an increase by 45.12%, and IFA (+) showed a modest increase by 14.52% (Fig. 3B).

MDA content (nm/g FW)

Maize cultivar P3939 demonstrates distinct changes in MDA (Malondialdehyde) content (nM/g FW) in response to varying concentrations of Cr and fulvic acid (FA). At 0 µM Cr, the MDA content for the cultivar starts at 7.4516 nM/g FW under FA(-) conditions. As the Cr concentration increases to 100 µM, MDA levels show a slight rise to 7.8194 nM/g FW. However, a more notable increase occurs at 300 µM Cr, where MDA content significantly jumps to 14.2065 nM/g FW. These observations indicate that maize cultivar P3939 exhibits a concentration-dependent response to Cr (Fig. 4A). Maize cultivar 30Y87 displays distinct changes in MDA (Malondialdehyde) content (nM/g FW) in response to varying concentrations of chromium (Cr) and different foliar treatments with fulvic acid (FA). Under control conditions (0 µM Cr), the MDA content is recorded at 7.4516 nM/g FW. When exposed to 100 µM Cr without any foliar treatment (FA-), the MDA content increases to 8.3613 nM/g FW. Interestingly, the addition of foliar fulvic acid (FFA+) at the same Cr concentration (100 µM) results in a slight decrease in MDA to 6.4258 nM/g FW, suggesting a potential mitigating effect of FFA against Cr-induced oxidative stress. Further analysis at 300 µM Cr with irrigated fulvic acid (IFA+) shows a rise in MDA content to 6.8129 nM/g FW, indicating a moderate increase compared to the control (Fig. 4B).

Effects of fulvic acid on total soluble protein (TSP) content (mg/g FW)

Maize cultivar P3939 displays varying levels of total soluble protein (TSP) (mg/g) in response to different concentrations of Cr and foliar treatments with fulvic acid (FA). Under normal conditions without Cr (0 µM), TSP levels are 0.95275 mg/g for FA (-), 0.934 mg/g for FFA (+), and 0.963 mg/g for IFA (+). As the Cr concentration increases to 100 µM, there is a noticeable decrease in TSP across all foliar treatments: 0.8555 mg/g for FA (-), 0.877 mg/g for FFA (+), and 0.849 mg/g for IFA (+). This reduction suggests that chromium stress may negatively impact protein synthesis or stability in maize cultivar P3939. At the highest Cr concentration tested (300 µM), TSP levels continue to decrease: 0.848 mg/g for FA (-), 0.86 mg/g for FFA (+), and 0.9125 mg/g for IFA (+) (Fig. 5A). Under normal conditions without Cr (0 µM), the TSP levels are relatively stable across all foliar treatments: 0.95275 mg/g for FA (-), 0.934 mg/g for FFA (+), and 0.963 mg/g for IFA (+). However, as the Cr concentration increases to 100 µM and 300 µM, TSP levels show slight fluctuations among the treatments. At 100 µM Cr, TSP levels increase to 0.9585 mg/g for FA (-), 1.0005 mg/g for FFA (+), and 0.954 mg/g for IFA (+). This suggests a potential response of the cultivar to moderate stress induced by chromium, possibly

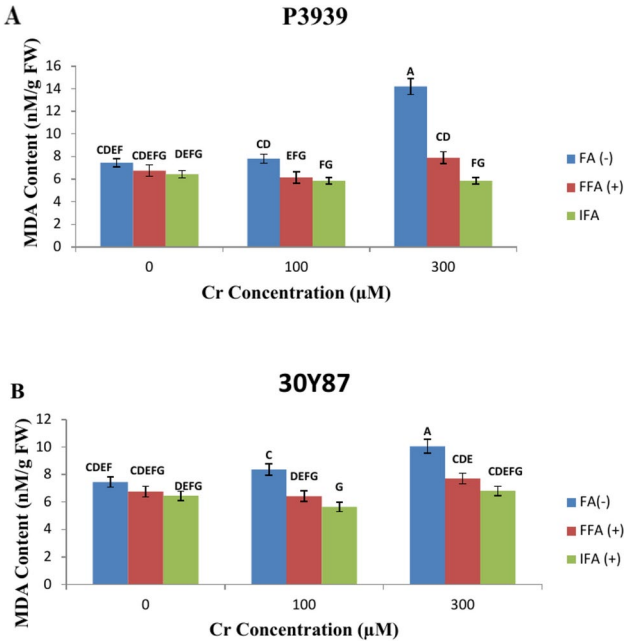

Fig. 4. Effects of fulvic acid applied as a foliar spray (FFA, 0.5 L/A) and through the rooting medium irrigation (IFA, 300 L/A) on MDA content of maize cultivars P3939 (**A**) and 30Y87 (**B**) under chromium stress. Values represent mean ± standard error (SE), with lowercase letters indicating significant differences among treatments. -FFA indicates no fulvic acid application (control). + FFA represents foliar application of fulvic acid, sprayed on leaves. IFA refers to fulvic acid application through irrigation, delivered via the rooting medium.

influencing protein metabolism or synthesis. At 300 µM Cr, TSP levels decrease slightly to 0.933 mg/g for FA (-), 1.005 mg/g for FFA (+), and 0.9465 mg/g for IFA (+), indicating a more pronounced effect of higher chromium concentrations on protein levels in the maize plants (Fig. 5B).

Effects of fulvic acid on antioxidant enzymatic activity under chromium stress

In the results, it was found that fulvic acid played a significant role in modulating antioxidant enzymatic activity in plants under chromium stress conditions. Specifically, enzymes such as catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) were observed to be crucial components of the antioxidant defense system that helped mitigate oxidative damage caused by chromium toxicity.

Catalase activity (units mg^{-1} protein)

Maize cultivar P3939 exhibits varying catalase (CAT) activity (U mg^{-1} protein) in response to different concentrations of Cr and foliar treatments with fulvic acid (FA). Under control conditions without Cr (0 µM), CAT activity levels are measured at 0.006649 U mg^{-1} protein for FA (-), 0.009183 mg^{-1} protein for FFA (+), and 0.009027 mg^{-1} protein for IFA (+). As the Cr concentration increases to 100 µM and 300 µM, changes in CAT activity are observed across all foliar treatments. At 100 µM Cr, CAT activity decreases to 0.003534 mg^{-1} protein for FA (-), 0.006842 U mg^{-1} protein for FFA (+), and 0.007068 U mg^{-1} protein for IFA (+). This reduction suggests a potential suppression of CAT enzyme function under moderate chromium stress conditions. At 300 µM Cr, CAT activity shows further variations: 0.007076 U mg^{-1} protein for FA (-), 0.005585 U mg^{-1} protein for FFA (+), and 0.006243 U mg^{-1} protein for IFA (+) (Fig. 6A). Maize cultivar 30Y87 exhibits varying levels of catalase (CAT) activity (mg-1 protein) in response to different concentrations of chromium (Cr) and foliar treatments with fulvic acid (FA). Under normal conditions without Cr (0 µM), CAT activity levels are recorded at 0.008452 U mg^{-1} protein for FA (-), 0.009154 U mg^{-1} protein for FFA (+), and 0.008106 U mg^{-1} protein for IFA (+). As the Cr concentration increases to 100 µM, changes in CAT activity are observed across the foliar treatments. CAT activity slightly increases to 0.010042 U mg^{-1} protein for FA (-), remains stable at 0.010112 U mg^{-1} protein for FFA (+), and decreases to 0.005225 U mg^{-1} protein for IFA (+). At 300 µM Cr, further variations in CAT activity are noted: 0.013391 U mg^{-1} protein for FA (-), 0.008314 U mg^{-1} protein for FFA (+), and 0.007919 U mg^{-1} protein for IFA (+) (Fig. 6B).

APX activity (units mg^{-1} protein)

Maize cultivar P3939 demonstrates varying ascorbate peroxidase (APX) activity (U mg^{-1} protein) in response to different concentrations of Cr and foliar treatments with fulvic acid (FA). Under normal conditions without Cr (0 µM), APX activity levels are measured at 0.010163 U mg^{-1} protein for FA (-), 0.011059 U mg^{-1} protein for FFA (+), and 0.010007 mg-1 protein for IFA (+). As the Cr concentration increases to 100 µM, APX activity shows fluctuations across the foliar treatments. APX activity increases to 0.015154 mg-1 protein for FA (-), remains

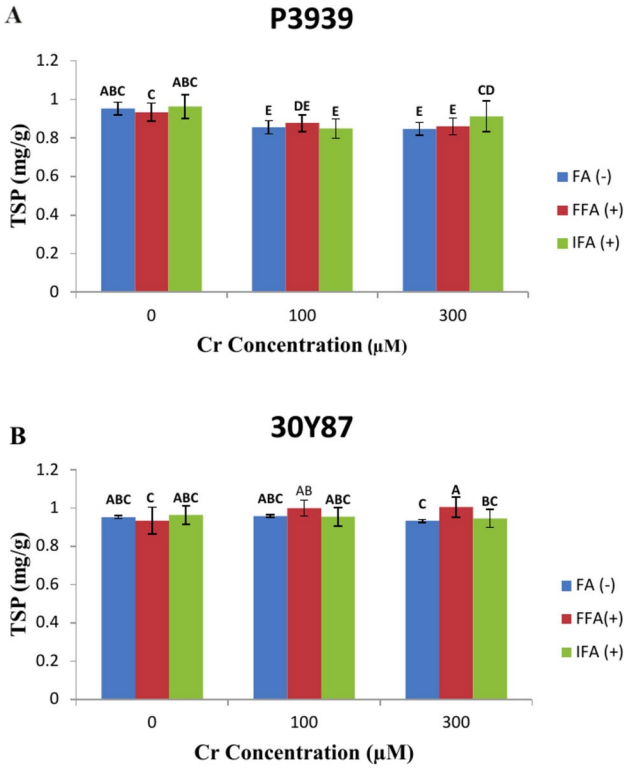

Fig. 5. Effects of fulvic acid applied as a foliar spray (FFA, 0.5 L/A) and through the rooting medium irrigation (IFA, 300 L/A) on Total Soluble protein (TSP) of maize cultivars P3939 (**A**) and 30Y87 (**B**) under chromium stress. Values represent mean ± standard error (SE), with lowercase letters indicating significant differences among treatments. -FFA indicates no fulvic acid application (control). + FFA represents foliar application of fulvic acid, sprayed on leaves. IFA refers to fulvic acid application through irrigation, delivered via the rooting medium.

relatively stable at 0.011348 U mg^{-1} protein for FFA (+), and slightly increases to 0.011529 U mg^{-1} protein for IFA (+). At 300 µM Cr, further variations in APX activity are observed: 0.01151 U mg^{-1} protein for FA (-), 0.011373 U mg^{-1} protein for FFA (+), and 0.015043 U mg^{-1} protein for IFA (+) (Fig. 7A). Maize cultivar 30Y87 displays varying levels of ascorbate peroxidase (APX) activity (U mg^{-1} protein) in response to different concentrations of chromium (Cr) and foliar treatments with fulvic acid (FA). Under control conditions without Cr (0 µM), APX activity levels are relatively consistent: 0.010163 U mg^{-1} protein for FA (-), 0.011059 U mg^{-1} protein for FFA (+), and 0.010007 U mg^{-1} protein for IFA (+). As the Cr concentration increases to 100 µM, APX activity shows minor fluctuations across the foliar treatments. APX activity decreases slightly to 0.010361 U mg^{-1} protein for FA(-), decreases to 0.009479 U mg^{-1} protein for FFA (+), and increases marginally to 0.010184 U mg^{-1} protein for IFA(+).At 300 µM Cr, further variations in APX activity are observed: 0.010944 U mg^{-1} protein for FA(-), 0.011579 U mg^{-1} protein for FFA (+), and 0.010261 U mg^{-1} protein for IFA (+) (Fig. 7B).

GPX activity (units mg^{-1} protein)

Maize cultivar P3939 is evaluated for its response to varying concentrations of Cr, focusing on the enzymatic activity of glutathione peroxidase (GPX). The study examines GPX activity in terms of micromoles (µM) of Cr at concentrations of 0, 100, and 300 µM. Results indicate that at increasing Cr concentrations, GPX activity in P3939 maize cultivar remains within a consistent range, suggesting a robust antioxidative response. The levels of FA (-), FFA (+), and IFA (+) in the cultivar show differential responses to Cr exposure, with fluctuations observed across the treatments. Specifically, FA (-) exhibits a slight increase from 0.005377 to 0.006086 U mg^{-1} protein as Cr levels rise from 0 to 300 µM, while FFA(+) and IFA(+) show more varied responses (Fig. 8A). Maize cultivar 30Y87 was investigated for its response to increasing concentrations of chromium (Cr), focusing on the activity of glutathione peroxidase (GPX) and the levels of FA (-), FFA (+), and IFA (+) in its tissues. The study measured GPX activity in units per milligram of protein across Cr concentrations of 0, 100, and 300 µM. Results indicate varying responses in GPX activity as Cr levels increase, suggesting a nuanced antioxidative capacity in cultivar 30Y87. Specifically, GPX activity fluctuates, with a noticeable decrease from 0.007192 to 0.009094 U mg^{-1} protein as Cr concentration rises from 0 to 300 µM. This variability underscores the cultivar's adaptability to oxidative stress induced by chromium. In terms of FA (-), FFA (+), and IFA (+) levels, cultivar 30Y87 displays differential responses across the Cr treatments. FA (-) levels notably increase from 0.005377 U mg^{-1} protein at 0 µM Cr to 0.009157 U mg^{-1} protein at 300 µM Cr, while FFA (+) and IFA (+) show more complex patterns of change (Fig. 8B).

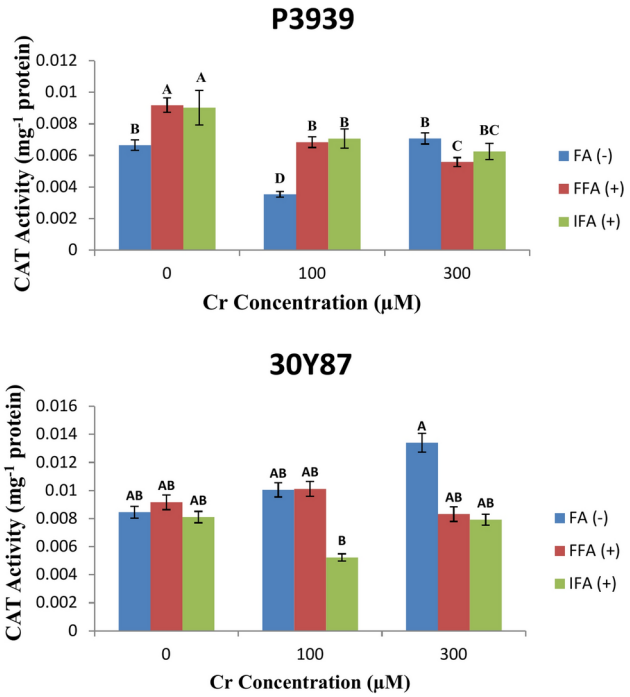

Fig. 6. Effects of fulvic acid applied as a foliar spray (FFA, 0.5 L/A) and through the rooting medium irrigation (IFA, 300 L/A) on Catalase enzyme (CAT) of maize cultivars P3939 (**A**) and 30Y87 (**B**) under chromium stress. Values represent mean ± standard error (SE), with lowercase letters indicating significant differences among treatments. -FFA indicates no fulvic acid application (control). + FFA represents foliar application of fulvic acid, sprayed on leaves. IFA refers to fulvic acid application through irrigation, delivered via the rooting medium.

Principal component analysis (PCA) and correlation analysis

This study employed Principal Component Analysis (PCA) and correlation analysis to assess the biochemical and morphological parameters of maize cultivars under varying levels of Cr stress and application of fulvic acid (FFA) and/or inorganic fulvic acid (IFA) through foliar and irrigated methods (Fig. 9). All parameters were standardized to ensure variables were comparable by scaling to zero mean and unit variance. PC scores were analyzed to identify patterns and relationships among parameters across different experimental treatments. This helped in understanding how Cr stress and fulvic acid applications influenced the biochemical and morphological characteristics of maize cultivars. Pearson correlation coefficients were calculated to determine the strength and direction of linear relationships between pairs of parameters within each experimental group (Fig. 10).

Discussion

In this study, we investigated the role of different modes of fulvic acid application on the antioxidant activity of maize (*Zea mays* L.) subjected to artificial chromium stress. Chromium contamination in agricultural soils is a significant environmental concern, posing detrimental effects on plant growth and productivity[7]. Fulvic acid, known for its chelating properties and ability to enhance plant stress tolerance, was applied through various methods to assess its efficacy in mitigating chromium-induced oxidative stress in maize plants[29]. The antioxidant activity of maize plants serves as a critical indicator of their ability to counteract reactive oxygen species (ROS) accumulation under chromium stress conditions[30]. By examining the effects of different fulvic acid application modes, this study aims to provide insights into optimizing agricultural practices for sustainable crop production in chromium-contaminated soils. A non-significant increase in growth parameters was observed in metal treated plants and Cr treated plants with FA application. However, these non-significant findings may be attributed to the inherent variability among treatments or the stage of plant development at the time of assessment. Our results indicated a reduction in growth parameters in chromium treated plants. These findings are in consistent with work of Arshed et al.[7] who observed the reduction in growth parameters in *Brassica juncea* under chromium stress. Alshegaihi et al.[30] also reported reduction in growth parameters in cooper metal treated plants of wheat. These reductions in growth parameters can be attributed to ultra-structural and physio-chemical alterations in plants exposed to Cr stress[31]. This decrease in growth metrics, such as shoot and root length and biomass, is linked to diminished nutrient uptake[32–34]. Our results indicated a reduction in leaf area under chromium stress. This reduction in leaf area could be linked to restricted water availability impacting leaf expansion, similar to observations under potassium deficiency[35]. Fluoride toxicity in wheat has similarly been shown to reduce leaf area[36,37].

In our experiment, foliar fulvic acid application showed an increase in various growth parameters such as leaf length, width, area, and number of leaves. Our study is consistent with previous studies that demonstrated

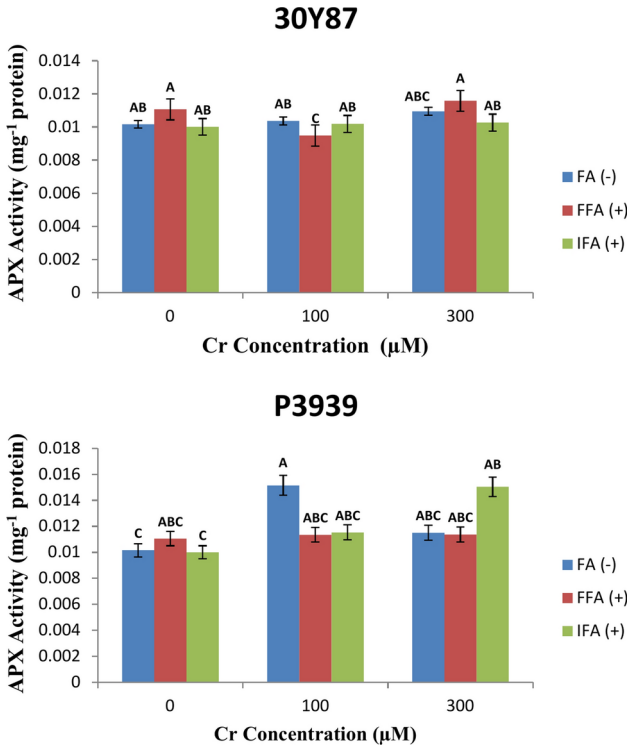

Fig. 7. Effects of fulvic acid applied as a foliar spray (FFA, 0.5 L/A) and through the rooting medium irrigation (IFA, 300 L/A) on Ascorbate enzyme (APX) of maize cultivars P3939 (**A**) and 30Y87 (**B**) under chromium stress. Values represent mean ± standard error (SE), with lowercase letters indicating significant differences among treatments. -FFA indicates no fulvic acid application (control). + FFA represents foliar application of fulvic acid, sprayed on leaves. IFA refers to fulvic acid application through irrigation, delivered via the rooting medium.

the effects of fulvic acid application on plant growth and nutrient content. For instance, one study focused on the effect of foliar application of fulvic acid on plant growth and fruit quality of tomato (*Lycopersicon esculentum* L.). Another study investigated the effects of soil application of fulvic acid on the growth and nutrient content of cucumber (*Cucumis sativus*) plants[38,39]. The capacity of FA to penetrate plant roots and stimulate the expression of genes involved in cell division supports its role in promoting vegetative growth[40].

Chlorophyll content was reduced in plants of both maize cultivars due to chromium stress. This reduction aligns with findings in *Triticum aestivum* under antibiotic stress[41]. Chromium stress results in the production of reactive oxygen species that reacts with pigment-protein complexes and damages the thylakoid membranes of chlorophyll due to the substitution of magnesium with H^+ ions[42]. This biochemical disruption underscores the vulnerability of photosynthetic machinery under heavy metal stress. On the other hand, foliar application of fulvic acid showed an increase in chlorophyll content. For example, 15% increase in chlorophyll content due to foliar application of fulvic acid was observed at 100 µM Cr (1.3022 µg/mg FW) in plants of maize cultivar P3939. Moreover, foliar application of fulvic acid caused a 12% increase in chlorophyll content in maize plants grown under normal conditions. These results are similar to some earlier studies, in which an increase in chlorophyll content by the fulvic acid application has observed in maize plants[43]. This increase suggests that FA mitigates oxidative damage by reducing free Cr ion concentration through adsorption[44].

Electrolyte leakage is an indicator of cellular damage induced by heavy metal stress and other abiotic stresses. Addition of chromium (100 &300 µM) to the growth medium increased the electrolyte leakage in the leaves of both maize cultivars. The rise in leakage is consistent with overproduction of ROS under metal stress, contributing to oxidative damage[45]. However, fulvic acid application (foliar + irrigated) showed reduced electrolyte leakage in chromium treated plants**.** About 16% decrease in electrolyte leakage was observed with IFA application at 300 µM Cr as compared with 300 µM Cr treatment alone. Malondialdehyde content is a reliable indicator of oxidative stress induced by abiotic stresses, heavy metal stress and pathogen attack[46]. A significant increase in MDA content ($P < 0.05$) was observed at 300 µM Cr concentrations in both varieties. This aligns with findings in sunflower and turnip, where Cr-induced oxidative damage elevated MDA levels[47,48] where Cr-induced ROS reacted with lipids, causing membrane disruption and cellular damage[49]. Fulvic acid application showed reduced MDA content in chromium treated plants of both cultivars. However, the application of FA through the soil was found more effective in alleviating oxidative stress by free radical detoxification.

Our results indicate relatively reduced total soluble protein content at 300 µM Cr and 100 µM Cr treatments as compared to control. Previous study also reported a decrease in total soluble protein content under Cr stress[50]. This decline in TSP may also stem from increased Cr uptake interfering with protein stability[51]. On the other

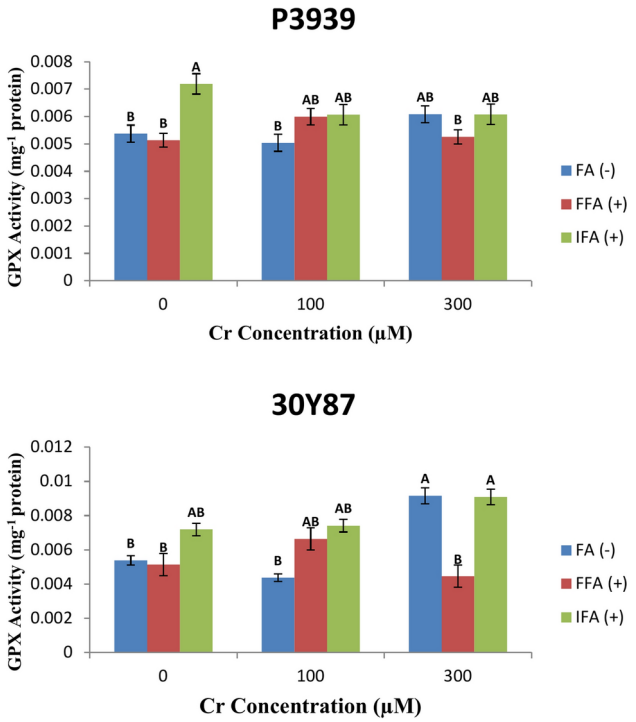

Fig. 8. Effects of fulvic acid applied as a foliar spray (FFA, 0.5 L/A) and through the rooting medium irrigation (IFA, 300 L/A) on glutathione peroxidase (GPX) of maize cultivars P3939 (**A**) and 30Y87 (**B**) under chromium stress. Values represent mean ± standard error (SE), with lowercase letters indicating significant differences among treatments. -FFA indicates no fulvic acid application (control). + FFA represents foliar application of fulvic acid, sprayed on leaves. IFA refers to fulvic acid application through irrigation, delivered via the rooting medium.

hand, application of fulvic acid through rooting medium enhanced the total soluble protein content in metal treated plants. These results are in consistent with work of Askari et al.[52] who also recorded an increase in TSP content in Cr treated wheat plants. Increase in total soluble protein might be due to positive effects of fulvic acid on plant growth and photosynthetic pigments. This improvement underscores the role of FA in supporting protein synthesis and stability under stress. In our study investigating the role of different modes of fulvic acid application on the antioxidant activity of maize under artificial chromium stress, a significant increase in key antioxidant enzymes and compounds was observed. Specifically, levels of catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), and glutathione reductase (GPX) showed marked enhancement. These enzymes play critical roles in mitigating oxidative stress by decomposing ROS, preventing cellular damage, and maintaining cellular homeostasis[53]. Catalase is an important component of antioxidant defense system that is involved in breakdown of hydrogen peroxide generated during photorespiration and fatty acids oxidation[54,55]. Our results indicated no significant increase in CAT activity in chromium treated plants of both cultivars**.** An increase in catalase activity was recorded at a higher concentration of Cr i.e. 300 µM as compared with control and 100 µM Cr. Jabeen et al.[56] also recorded increase in CAT activity in bean plants under Cr stress. The decrease in CAT activity at 100 µM Cr might be due to reason that catalase is iron porphyrin molecule and Cr may react with iron affecting the availability of the active form of iron[57]. Fulvic acid (foliar) application showed an increase in catalase activity at 100 µM Cr concentration. About 33% increase in CAT activity was observed with foliar FA at100µM Cr as compared with 100 µM Cr alone treatment. Peroxidase that breakdown H_2O_2 by using ascorbate as an electron donor is known as ascorbate peroxidase. APX is an essential antioxidant enzyme present in peroxisomes, cytosol and chloroplasts[58]. Our results showed reduced APX activity at a higher concentration of Cr (300 µM). The decrease in APX was also reported in Indian mustard[59], oil seed rape[60] and *Zea mays*[61]. The reason for the decline in APX activity might be that higher Cr toxicity can inhibit the antioxidant defence system responsible for detoxification of ROS. However, foliar application of fulvic acid increased APX activity in chromium stressed plants of cultivar 30Y87**.** These findings are in consistent with work of Wang et al.[22] also recorded an increase in APX activity with fulvic acid application in metal treated plants. About 30% increase in APX activity was observed with foliar FA application at 300 µM Cr treatment. GPX is an antioxidant enzyme that breaks down H_2O_2 by using guaiacol as an electron donor[62]. GPX plays vital role in lignifications; defense and wound healing[63]. The activity of GPX did not change due to chromium stress or exogenous application of fulvic acid**.** An increase in antioxidant enzyme activities (CAT and GPX) under Cr stress is might in response to generation of superoxide radicals by Cr induced blockage of electron transport chain in mitochondria. The decrease in antioxidant enzyme activity (APX) might be due to the inhibitory effects of Cr ions on the enzyme[64]. In our experiment, FA application improved the activity of antioxidant enzymes (CAT and APX)

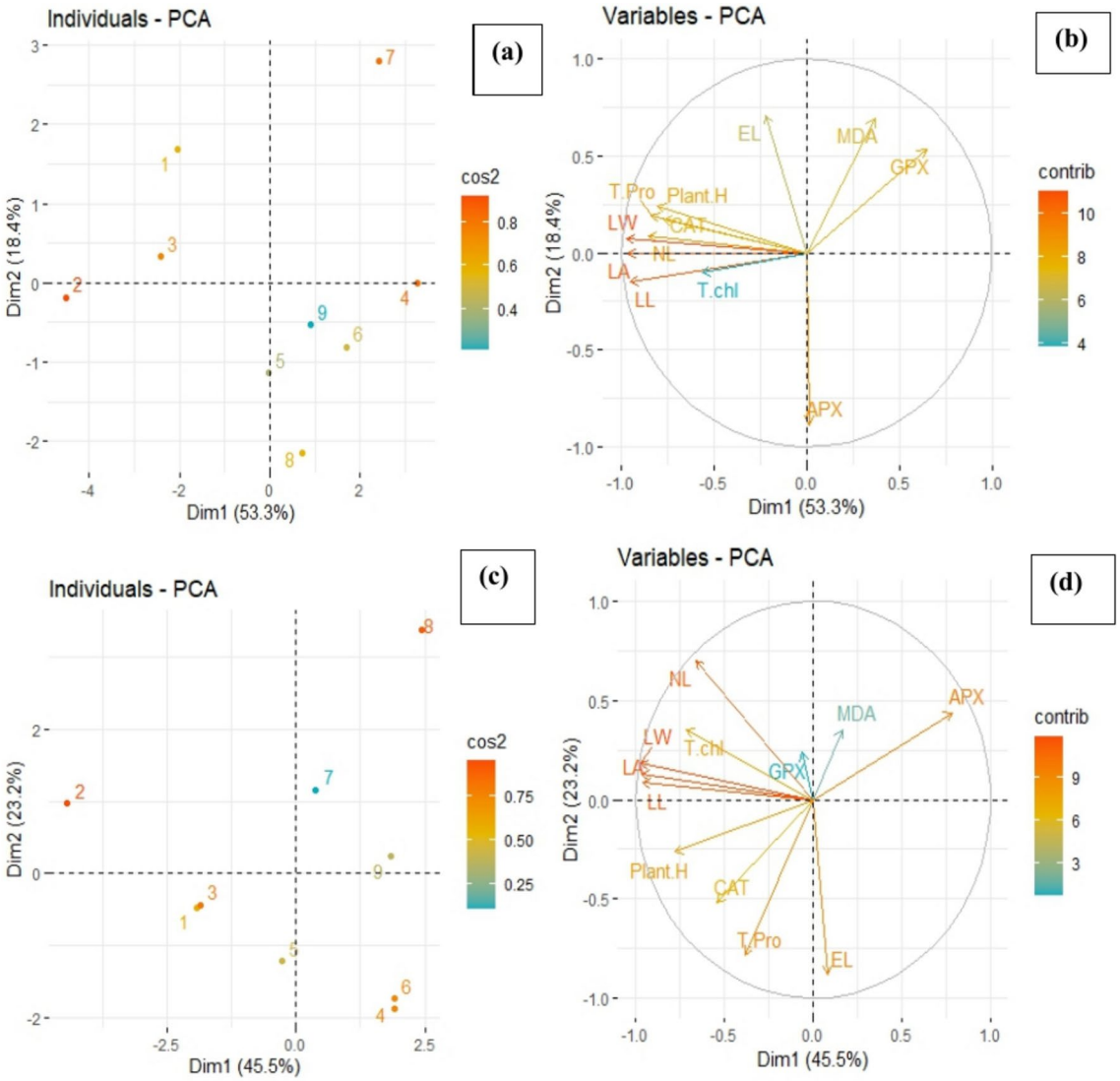

Fig. 9. Score (**a**, **c**) and loading plots (**b**, **d**) of principal component analysis (PCA) on various studied parameters of maize cultivars grown in Cr stressed soil. Score plot represents separation of treatments as (1) Control (without Cr contamination); (2) FFA (3) IFA (4) 100 µM Cr level, 5) 100 µM Cr + FFA 6) 100 µM Cr + IFA, 7) 300 µM Cr, 8) 300 µM Cr + FFA 9) 300 µM Cr + IFA respectively. The abbreviations of parameters are as follows: Plant H: plant height; NL: number of leaves; LW: leaf width; T chl: total chlorophyll; EL: electrolyte leakage; MDA: Lipid peroxidation; T pro: total protein; CAT: catalase; APX: Ascorbate peroxidase GPX: Guaiacol peroxidases.

under chromium stress. It might be due to reason that FA acts as a free radical scavenger and antioxidant. FA has the ability to react with both positive and negatively charges unpaired electrons and hence detoxify the free radicals might be due to enhanced uptake of nutrients[65].

Conclusion

Chromium toxicity adversely affects antioxidant enzyme activities, photosynthetic pigments, and overall plant growth. Fulvic acid application (irrigated) led to an increase in chlorophyll content and total soluble proteins, although these results were statistically non-significant. Additionally, fulvic acid reduced malondialdehyde (MDA) content and electrolyte leakage in chromium-treated plants. Foliar fulvic acid application significantly enhanced the activities of antioxidant enzymes, particularly catalase (CAT) and ascorbate peroxidase (APX), while it did not affect the activity of guaiacol peroxidase in metal-treated plants. The increased activities of CAT and APX suggest that foliar fulvic acid application may improve plant resilience against chromium stress by effectively scavenging reactive oxygen species (ROS). The potential mechanism of fulvic acid's action in this context involves enhancing antioxidant enzyme activities, which helps mitigate oxidative stress caused by chromium exposure. Although chromium stress resulted in reduced growth parameters, foliar and root irrigated fulvic acid application had a positive impact on various growth measures. We recommend fulvic acid application

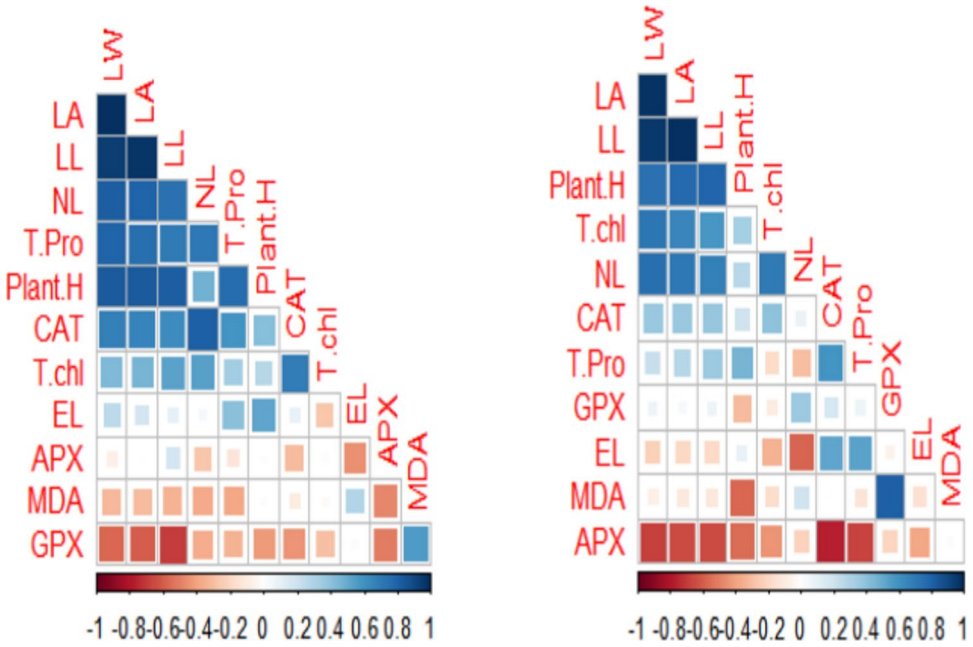

Fig. 10. Correlation between growth and physiological parameters of maize cultivars grown in Cr stressed soil.

as a more effective approach for mitigating chromium toxicity, given its significant impact on the antioxidant defense system. Future research should explore the long-term effects of foliar versus irrigated fulvic acid, as well as its potential to alleviate other types of abiotic stress.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

Conceptualization, F.I., A.Z., and A.K.; methodology, F.I.; software, A.S., and M.Z.A.; validation and formal analysis, A.S., and F.I.; resources, I.D., and P.V.P.; data curation, U.Z.; writing—original draft preparation, F.I., A.Z., A.S., U.Z., and M.Z.A,; writing—review and editing, I.D., P.V.P., and W.S.; supervision, A.Z. All authors have read and agreed to the published version of the manuscript.

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Declarations

Ethics approval and consent to participate

All methods were performed in accordance with the relevant guidelines and regulations. We have obtained permission to collect plant material and seedlings.

Competing interests

The authors declare no competing interests.

Additional information

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