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VANILLIC ACID ALLEVIATES CHROMIUM TOXICITY IN SPINACH (*SPINACIA OLERACEA* L.) AND LETTUCE (*LACTUCA SATIVA* L.) PLANTS IRRIGATED WITH DIFFERENT LEVELS OF TANNERY WASTEWATER

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Abstract

Pollution is one of the largest threats to the planet. Human activity is the primary source of environmental contamination. Untreated tannery wastewater releases chromium (Cr) into streams, which is toxic to aquatic life and humans. The goal of this study is to find out how spinach (*Spinacia oleracea* L) and lettuce (*Lactuca sativa* L) are affected by tannery effluent and how vanillic acid (VA) helps plants that are irrigated with this effluent deal with Cr stress. In this study, three different VA concentrations (0, 25, and 50 μM) and three different tannery wastewater levels (0, 50, and 100%) were used. The findings show a considerable reduction in the morphological and physiological indices of both of these plants when they were irrigated with tannery effluent. On little-researched plants, chromium stress caused significant oxidative stress and decreased the plant's antioxidant enzyme system. Significant concentrations of Cr were discovered in the roots of plants that received tannery effluent irrigation. The use of VA might lessen the negative consequences of Cr stress. By boosting the activity of antioxidant enzymes and reducing Cr-induced oxidative stress, vanillic acid dramatically promotes plant growth and chlorophyll content. It also enhances gas exchange characteristics and photosynthetic rate. Furthermore, the use of VA dramatically lowers Cr uptake and translocation in both plants. Vanillic acid is an effective heavy metal stress reliever for other crops.

Key words: *Spinacia oleracea* L., *Lactuca sativa* L., Vanillic acid, Tannery wastewater irrigation, Oxidative stress.

Introduction

Globally, heavy metal contamination is a prevalent issue. Because of its widespread release into the environment and carcinogenic conse- quences, one of the most hazardous metals among those that damage the environment is copper (Cr) (Ashraf *et al.,* 2018). In Pakistan, the primary source of Cr contamination is wastewater discharged from tanneries put into water channels without treatment (Qadir *et al.,* 2008). Untreated discharge of tannery wastewater has enormous potential to contaminate both water and soil.

Chromium is regarded as the most hazardous pollutant for both biota and humans (Ashraf *et al.,* 2018; Patel *et al.,* 2021). While poor farmers all over the globe frequently irrigate their fields with tannery effluent, this practice has a major detrimental effect on plant growth and crop productivity when the soil is contaminated with an excessive amount of Cr (Roohi *et al.,* 2016). Worldwide, public health and food safety issues are raised when tannery effluent contaminated with chromium is utilised to irrigate different food crops (Younas *et al.,* 2022). Due to the rapid growth of tannery industries, soil and water contamination with Cr is becoming critically important to understand in order to adopt its remedial measures (Zhao *et al.,* 2017).

Chromium may have caused stress on plants in addition to its harmful effects on people (Hussain *et al.,* 2018). Plant height, root length, biomass, and yield were found to be drastically decreased by Cr stress (Gill *et al.,* 2015; Alam *et al.,* 2021). Plant life was dependent on the photosynthetic apparatus; plant mortality resulted from a reduction in photosynthetic pigments and transpiration rate caused by Cr stress. The PSI and PS II photosystems' effectiveness is decreased by chromium stress. Additionally, it lowers the amount of proline and biomass accumulation in plants (Ayyaz *et al.,* 2021). When exposed to Cr stress, plants suffer severe oxidative stress and cell membrane damage (Gill *et al.,* 2014; Singh & Prasad, 2019). Antioxidant enzymes were part of a plant's natural defensive mechanism against oxidative stress (Mathur *et al.,* 2016). Cr's toxicity led to plant death since it interfered with the plants' antioxidant system (Khan *et al.,* 2013). Reactive oxygen species (ROS) are produced in excess by plants whose antioxidant defense system is dysregulated. This can have detrimental effects on the plants' growth and development as well as their resilience to hazardous settings (Dvořák *et al.,* 2021). Plants are sessile creatures, hence they can't protect themselves from toxins in the environment like Cr. Rather, they depend on several biological processes that enable them to tolerate the harmful effects of metals. The effectiveness of various biological mechanisms in plants, such as metal avoidance (Betti *et al.,* 2021), excretion (Zhang *et al.,* 2021), exclusion (Liu *et al.,* 2022), metal chelation (Yu *et al.,* 2019), metal binding (Samuel *et al.,* 2021), and separating different types of heavy metals (Huang *et al.,* 2021), has been demonstrated in a number of recent scientific reports.

The most adaptable mechanism employed by plants to withstand heavy metal stress is their well-balanced antioxidant defence system, which includes both non-enzymatic (glutathione (GSH), ascorbic acid (AsA), total flavonoids, tocopherol, carotenoids, and phenolic compounds) and enzymatic (catalase (CAT), glutathione S-transferase (GST), superoxide dismutase (SOD), glutathione peroxidase (GPX), and ascorbate peroxidase (APX)) antioxidants that scavenge high ROS production in plants (Garcia *et al.,* 2020; Hasanuzzaman *et al.,* 2020). Numerous phytoprotectants have been discovered recently, such as osmoprotectants like proline and glycine betaine (Ali *et al., 2020*; Hayat *et al.,* 2021), phytohormones like salicylic acid, abscisic acid, jasmonic acid, and gibberellic acid (Imran *et al.,* 2021; Kamran *et al.,* 2021; Ahmad *et al.,* 2021), micronutrients like zinc and iron (Elazab *et al.,* 2017), antioxidants like glutathione and ascorbic acid (Qadir *et al.,* 2008; Zhou *et al.,* 2021), trace elements like silicon and selenium (Gheshlaghpour *et al.,* 2021; Zhou *et al.,* 2021), and secondary metabolites (Kumar *et al.,* 2018). Such plant-beneficial phytoprotectants are thought to be an economical and environmentally responsible method of effectively immobilising heavy metals in polluted soils and reducing their uptake by plants.

One such phytochemical substance that is essential to plants' ability to survive under adverse circumstances is vanillic acid (VA). According to Hegab & Ghareib (2010), it functions as an antioxidant, antifungal, and anti-mutagen agent in plants. Plant development is regulated by vanillic acid (VA) (Garcia *et al.,* 2020). Exogenous administration of VA was found to improve seedling development, proline and chlorophyll contents, and antioxidant activities in rice and tomato plants growing in environments with limited water supply (Villango *et al.,* 2016; Nguyen *et al*., 2018). Applying VA topically improves rice growth in Cd-stressed environments by increasing antioxidant enzymes and nutrient absorption while lowering Cd uptake and mobility within the plant. According to Bhuyan *et al*., (2020), Applying VA to rice increases antioxidant enzymes and photosynthetic pigments, according to another study. According to Xuan & Khang (2018), a high VA content function as a powerful antioxidant, but a low dosage also encourages tomato development. Report show that VA improved plants' resistance to drought (Nguyen *et al*., 2018). More research is required to determine the effectiveness of VA application in developing plants subjected to heavy metal stress because there aren't many studies that explain the role of exogenous VA administration in plants under metal stress (Villango *et al.,* 2016).

Spinacia oleracea and *Lactuca sativa* are the two most consumed and widely cultivated crops around the world. The leaves of *Spinacia oleracea* have higher nutritional values than the other leafy vegetables consumed around the world. Different nutritionists around the world recommend the consumption of *Spinacia oleracea* in food as it provides plentiful iron, minerals, and nutrients (Swain *et al.,* 2021). *Lactuca sativa* is also a vital source of different fibres, vitamin C, and iron. It contains health-beneficial bioactive compounds (Ketnawa *et al.,* 2020). Like other vegetables, both of these crops are globally cultivated in contaminated soils and are thereby subjected to different pollutants that are greatly associated with the industrialization and urbanisation processes. A high concentration of heavy metals in the edible parts of these leafy vegetables is the main cause of human contact with heavy metal toxicity (Gao *et al.,* 2020). Hence, the use of an effective and simple approach is indispensable for the improvement of the safety and quality of crops (Nguyen *et al*., 2018). To the best of the available literature and knowledge, there are very few studies available about the communicating effect of vanillic acid in combating Cr stress in plants, especially *Lactuca sativa* and *Spinacia oleracea*. Therefore, it is important to find out if vanillic acid may improve these two green vegetables' ability to operate when under Cr stress. The present study thus hypothesised that application of vanillic acid (VA) would efficiently mitigate the oxidative stress and improve the rate of photosynthesis, coupled with enhanced growth of both Lactuca sativa and Spinacia oleracea planted exposed to Cr stress by strengthening the antioxidant defence mechanism.

Material and Methods

Environment for seed, soil, and growth: In Government College University Faisalabad's botanical area (31.4161° N, 73.0700° E), the experiment below was conducted. The healthy lettuce seeds came from the Ayoub Agriculture Research Institute (AARI) in Faisalabad, Pakistan (*Lactuca sativa*) and spinach (*Spinacia oleracea* L.) plants (31.4041°N, 73.0487°E). Numerous studies pertaining to Cr-stressed soil have made substantial use of *Spinacia oleracea* L. and *Lactuca sativa* (Zaheer *et al.,* 2020a; Hussain *et al.,* 2021; Christou *et al.,* 2021). After that, they were sterilized in H_2O_2 (3%) for 15 minutes to prevent bacterial and fungal infections. Following that, they were rinsed 10 times with distilled water. 5 kilogram of soil that had been sieved to a size of 2 mm was placed inside plastic pots to hold the seeds of these two plants.

Soil sample collection and analysis: The soil used in this experiment was collected from the agriculture fields of the University of Agriculture, Faisalabad, Punjab, Pakistan. Samples of the soil were collected from the surface (0–200 mm) using a scoop shovel, dried in the air, and sifted through a 2mm sift for analysis and pot culturing. Soil chemical and physical properties were determined, such as soil texture (silt clay), pH (7.67), Zn (0.35 mg kg^{-1}), electrical conductivity (6.61 dS $^{m-1}$), SAR (24 mmol L^{-1}), Ca^{2+} + Mg^{+2} (18.7 meq L-1), available phosphorous (2.58 mg kg^{-1}), and available Cr (0.02 mg kg^{-1}). Five morphologically homogenised seedlings were chosen for additional experimentation after 10 days of germination. Three triplicates of each treatment were used in the carefully planned placement of all the experimental pots using a completely randomised design (CRD). Fertilisers, including phosphate and potassium sulphate (SOP), were used in accordance to help alleviate the macronutrient shortage.

Treatments: Six trials total, three duplicates of each treatment, make up the experimental research. Weeds were physically removed from experimental pots and randomly rotated on a regular basis over the course of the experimental inquiry. Here is a detailed explanation of the experimental design.

T1(Cr 0% +VA 0 µM), T2(Cr 0% + VA 25 µM) T3(Cr 0% + VA 50 µM), T4(Cr 50% + VA 0 µM), T5(Cr 50% + VA 25 µM), T6(Cr 50% + VA 50 µM), T7(Cr 100% + VA 0 µM), T8(Cr 100% + VA 25 µM), T9(Cr 100% + VA 50 µM).

Plant harvesting and analysis: The two plants were taken, following a 10-week (70-day) period of successive treatments. With the use of tissue paper, the plants were meticulously cleaned after being gently cleansed with distilled water. Both of these plants' roots, stems, and leaves were carefully separated. The following growth-related measurements were taken: plant height, root/stem length, leaf area, and the total number of leaves per plant. A scale was used to measure the lengths of the roots and shoots. Prior to additional analysis, the fresh biomass of these two plants was also assessed, and they were oven dried for 72 hours at 70 degrees Celsius.

Photosynthetic pigment: The tallest fully developed leaf was chosen to analyze photosynthetic pigments after 70 days of treatment. 85% acetone was used to remove the pigments at 4°C in the dark. A spectrophotometer (Halo DB-20/DB-20S, Dynamica Company, London, UK) was used for analysis after the extract had been centrifuged for 12 minutes at 4000 rpm (Metzner *et al.*, 1965). The formulas used to calculate the photosynthetic pigment concentration were presented by Lichtenthaler in 1987.

Determination of antioxidant enzymes: The second completely expanded leaf was chosen for analysis of the CAT (catalase), POD (peroxide dismutase), and SOD (superoxide dismutase) activities after 55 days of treatment. Using the Zhang (1992) approach, the activities of SOD and POD were ascertained. Using Aebi (1984) approach, the activity of CAT was ascertained.

Evaluation of MDA and H_2O_2 contents: The MDA content was determined using the Heath & Packer (1968) technique, with some modifications made by Zhang & Kirham (1994) and Dhindsa *et al*., (1981). The technique developed by Jana & Choudhuri (1981) to measure the H_2O_2 content.

Determination of Cr concentration: Using the approach outlined in (Ketnawa *et al.,* 2020), the total Cr content in the roots, stems, and leaves of these two plants was thoroughly assessed. Each plant provided a total of 0.5g of dried material, which was crushed into an extremely fine powder and burned for 12 hours at a temperature of 1000 ºC in a muffle furnace. The next day, acid digestion (HNO3:HClO4 at a ratio of 5:1, v/v) was used to create the ash. After materials were thoroughly filtered many times, a clear extract was obtained. To create a suitable solution of 50 ml in the experimental volumetric flask, samples were added together with distilled water. In order to accurately estimate the Cr levels in plant biomass, samples were examined using an atomic absorption spectrophotometer (AAS) (novA A400 Analytik Jena, Germany).

Statistical analysis

The study's data is all the average of three replicates. Using the statistical software SPSS version 16.0 (SPSS, Chicago, IL), a significant difference between the various treatments was statistically calculated by analysis of variance (ANOVA) and Tukey's test to identify significant differences between the treatment means.

Results

Growth of plants and biomass: When treated with tannery effluent, Spinacia oleracea and Lactuca sativa plants' height, number of leaves per plant, root length, fresh weight, and dry weight all decreased. Cr stress negatively affects the growth of both *Spinacia oleracea* and *Lactuca sativa*. For Spinacia oleracea and Lactuca sativa, the greatest loss in plant height was 53% and 61%, respectively, comparable to the control treatment, at the maximum concentration (100%) of tannery effluent. Irrigating 100% treated tannery effluent resulted in a 57% and 47% increase in root length for Lactuca sativa and Spinacia oleracea, respectively. Fresh root weight of *Spinacia oleracea* and *Lactuca sativa* decreased by 56 and 65 percent, respectively under 100% level of Cr stress. Application of vanillic acid (VA) increases the plant growth attributes of both plants under Cr stress as depicted in Figures 1 and 2. With the application of (VA 50 M) under 100% Cr stress, Spinacia oleracea and Lactuca sativa showed the greatest increases in Plant height is determined by (29, 30%), the number of leaves (39, 73%), the length of the roots (41, 28%), the area of the leaves (31, 18%), the fresh root weight (24, 22%), the fresh leaf weight (8, 30%), the fresh root weight (21, 25%), and the fresh leaf weight (65, 44%). The plants treated with 100% tannery wastewater showed maximum suppression in all other attributes. Applications of VA substantially recovered the plants from stress and increased biomass and growth of plants. However, high of VA's concentration has proven more beneficial than the lower one.

Contents of chlorophyll and gas exchange parameters: According to Fig. 3, the effects of chromium stress on Lactuca sativa and Spinacia oleracea plants' contents of gas exchange, chlorophyll, and carotenoid characteristics are all greatly reduced. The highest degree of Cr application (100% tannery effluent) resulted in the greatest loss in chlorophyll levels and gas exchange parameters. However, the Application of VA (VA 0µM, 25µM, 50 µM) considerably improve the chlorophyll contents, carotenoids, and gas exchange parameter of both *Spinacia oleracea* and *Lactuca sativa*. The detrimental effects of Cr toxicity were greatly reduced and these qualities were enhanced by the administration of VA. Vanillic acid's levels (VA 0µM, 25µM, 50µM) improved the chlorophyll content and gas exchange attributes, nevertheless, the maximum level of VA (50µM) showed much better performance in both plants. Application of VA (50µM) showed maximum increase in carotenoid contents by (34, 43%) at 50% Cr level and (77, 28%) at 100% Cr level and enhanced the total chlorophyll contents by (38, 32%) at 100% Cr level in *Spinacia oleracea* and *Lactuca sativa* respectively. The addition of Vanillic acid (VA) as 25µM, 50µM improved the gas exchange attributes in contrast to the treatment without VA application (VA 0µM,). Application of vanillic acid (50µM) maximum increased the net photosynthesis by 55, 22%, stomatal conductance by 43, 32%, water use efficiency by 56, 22%, and transpiration rate by 64, 25% in *Spinacia oleracea* and *Lactuca sativa* plants respectively under 100% Cr stress.

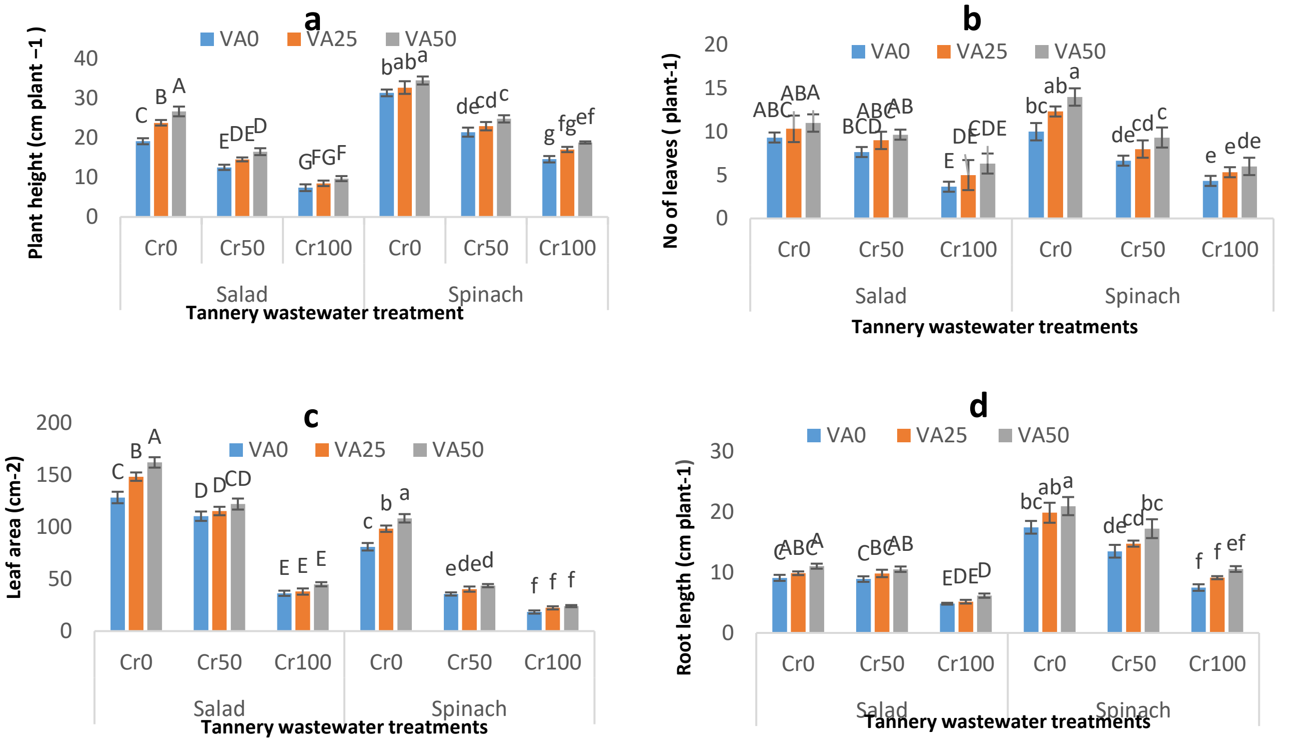

Fig. 1. Effects of spraying vanillic acid (VA) on S. lettuce and spinach plant height, leaf area, number of leaves, and root length at different tannery effluent concentrations. The averages of the three replicates and the standard deviation (SD; n = 3) are displayed as the results. The highest significant deviation (HSD) (P0.05) in a one-way ANOVA was employed to analyze mean differences. Lowercase characters in the error bars show a significant difference between the treatments. From zero (no wastewater irrigation) to one hundred (full wastewater irrigation), the plastic filter's relative brightness was measured.

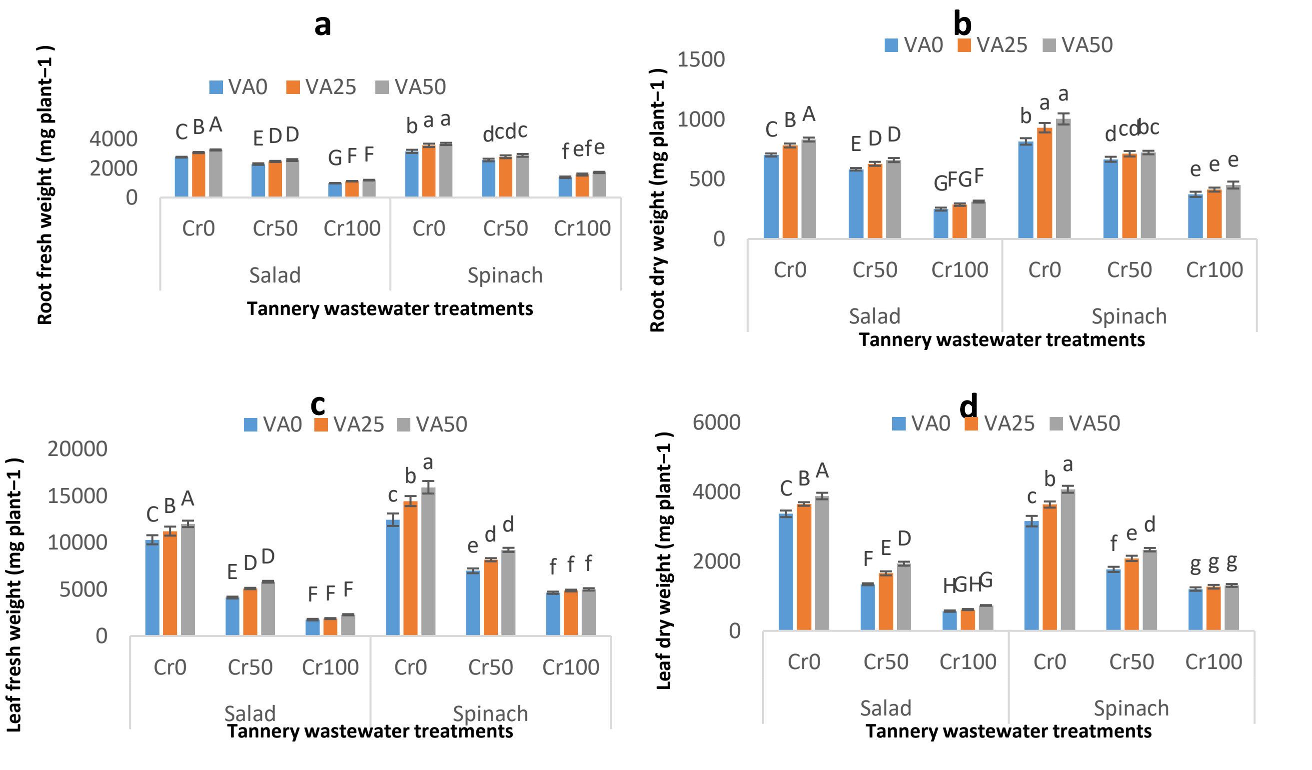

Fig. 2. When spinach and salad plants are treated with vanillic acid (VA), variations in the concentrations of tannery effluent have an effect on the fresh, dry, fresh, and dry weights of the roots. The averages of the three replicates and the standard deviation (SD; n = 3) are displayed as the results. Following a one-way ANOVA, HSD was used to analyze mean differences (P 0.05). On the error bars, different lowercase letters stand in for significant variations across the treatments. The employed plastic filter's relative brightness was 0 when there was no wastewater irrigation.

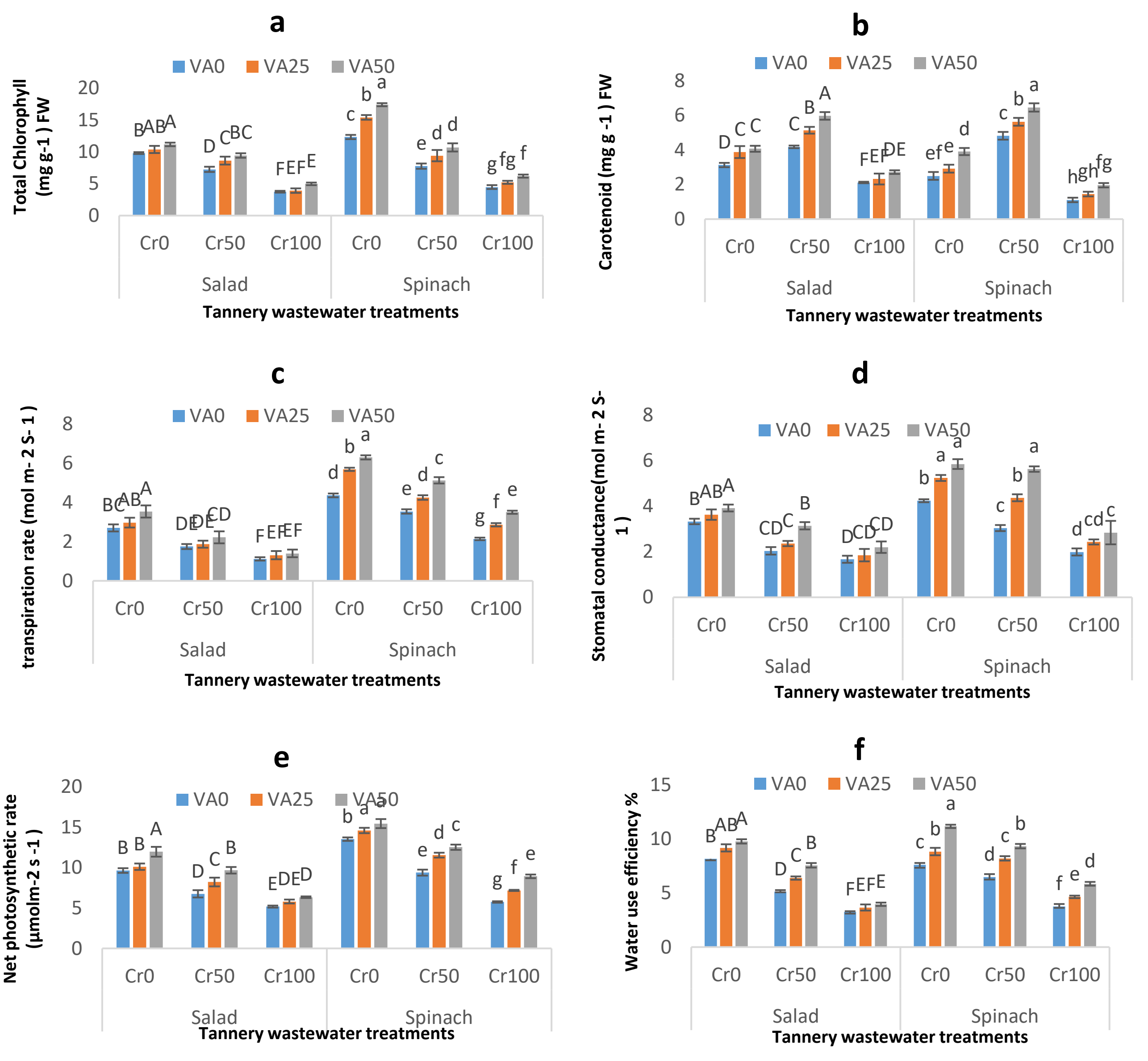

Fig. 3. The amount of chlorophyll (a), carotenoid (b), transpiration rate (c), stomatal conductance (d), net photosynthesis (e), and water consumption efficiency (f) varies on spinach and salad plants treated with vanillic acid (VA) dependent on the quantity of tannery wastewater sprayed on the plants. The averages of the three replicates and the standard deviation (SD; n = 3) are displayed as the results. Following a one-way ANOVA, HSD was used to analyze mean differences (P 0.05). On the error bars, different lowercase letters stand in for significant variations across the treatments. The plants' relative radiances range from zero (no irrigation with wastewater) to fifty (fifty percent irrigation with wastewater) to one hundred (100 percent irrigation with wastewater).

Electrolyte leakage, MDA, and H_2O_2: At different concentrations of tannery wastewater (0%, 50%, and 100%), as well as with and without the application of various vanillic acid (VA 0M, 25M, and 50M), the levels of hydrogen peroxide (H2O2), malondialdehyde (MDA), and electrolyte leakage (EL) in soil were rigorously determined. The current study's findings showed that in both *Spinacia oleracea* and *Lactuca sativa* plants, increasing the concentration of Cr (0%, 50%, and 100%) in the soil dramatically increases the levels of malondialdehyde (MDA), hydrogen peroxide (H2O2), and electrolyte leakage (EL). *Lactuca sativa's* roots and *Spinacia oleracea* showed a maximum increase of (EL) of 58% and 96%, respectively, vs the plants created using the control treatment. While Spinacia oleracea and Lactuca sativa leaves showed increases in EL content of 88% and 45%, respectively. Maximum increase observed in the contents of malondialdehyde (MDA) was 39% and 51% in roots and 72, 65% were detected in leaves of *Spinacia oleracea* and *Lactuca sativa* respectively. Whereas, a maximum increase of 42% and 43% in roots and 55% and 94% in leaves was observed for the hydrogen peroxide (H_2O_2) in both *Spinacia oleracea* and *Lactuca sativa* plants respectively. However, the application of Vanillic acid (VA 0µM, 25µM, 50µM), by minimizing the amount of electrolyte loss, Spinacia oleracea and Lactuca sativa's roots and leaves experienced less oxidative stress, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in both compared to plants that were cultivated without the use of vanillic acid as given in Fig. 4. Electrolyte leakage

(EL) in the roots and leaves of Spinacia oleracea and Lactuca sativa plants, respectively, was dramatically decreased as compared to plants produced with 100% tannery wastewater treatment by exogenous application of vanillic acid (VA 0 M, 25 M, 50 M). Contents of malondialdehyde (MDA) were reduced by 23% and 15% in roots and 17 and 18% in leaves of the *Spinacia oleracea* and *Lactuca sativa* plants. Similarly, maximum reductions of 14% and 19% in roots and 12% and 28% were observed in the leaves of *Spinacia oleracea* and *Lactuca sativa* plants respectively.

Anti-oxidant enzymes activities: In the current study, an increase in MDA, EL, and H2O2 concentrations in roots and shoots under acute Cr stress (100% Cr level) results in severe oxidative stress and a decrease in the activity of antioxidant enzymes. When compared to the control treatment, all Cr concentrations dramatically decreased the activity of the antioxidant enzymes POD, CAT, SOD, and APX. The reduction was more apparent at a higher level of Cr stress (100% Cr level). In contrast, the addition of VA at concentrations of 0µM, 25µM, and 50µM, the plant's roots and leaves, increasing the activity of these enzymes, with VA 50µM being more effective than VA 25µM. At 50% and 100% Cr treatment doses, both VA levels considerably enhance the activities of antioxidant enzymes (Figs. 5, 6). *Spinacia oleracea* and *Lactuca sativa* at 100% Cr level showed increases in SOD activities in their roots and leaves of 35%, 15%, 31%, and 32%, respectively, with application of VA 50µM. POD activities were increased in *Lactuca sativa* and *Spinacia oleracea* roots and leaves by 22%, 12%, 30%, and 32%, respectively. Similar to this, when VA 50µM under 100% Cr level was applied to the roots and leaves of *Spinacia oleracea* and *Lactuca sativa* plants, respectively, APX activities improved by 54%, 58%, and 22 and 43%, while CAT activities rose by 26%, 31%, and 36% and 24% in the roots and leaves, respectively.

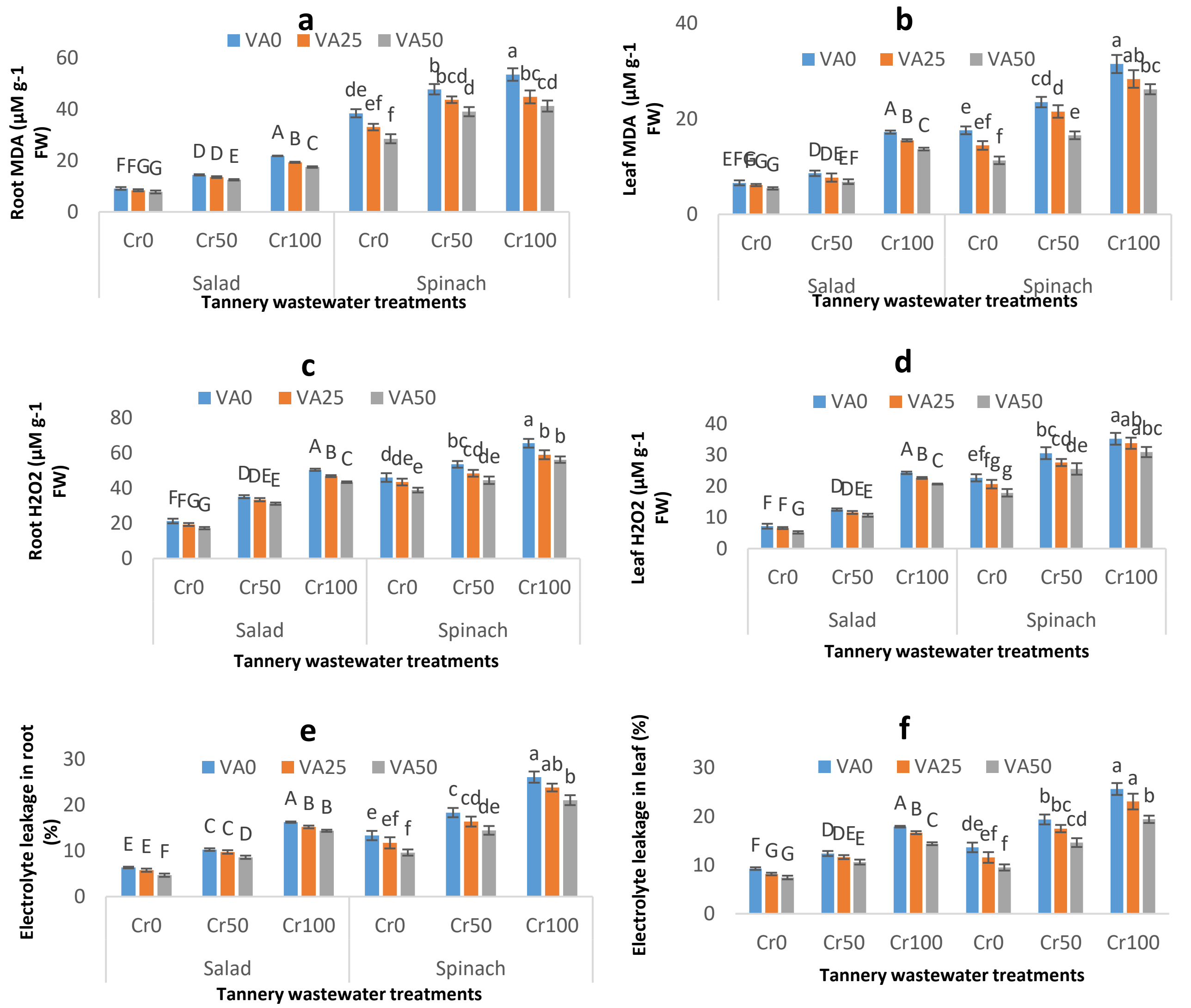

Fig. 4. The following outcomes are seen when lettuce and spinach plants are treated with vanillic acid (VA): The levels of MDA in the roots, MDA in the leaves, H2O2 in the roots, H2O2 in the leaves, and EL in the roots and EL in the leaves are shown in (a), (b), (c), (d), (e), and (f), respectively. Averages of three replicates are used to calculate the values, together with the standard deviation (SD; n = 3). HSD (P 0.05) was used to analyze mean differences after a one-way ANOVA. A substantial difference between the treatments is shown by different lower case letters on the error bars.

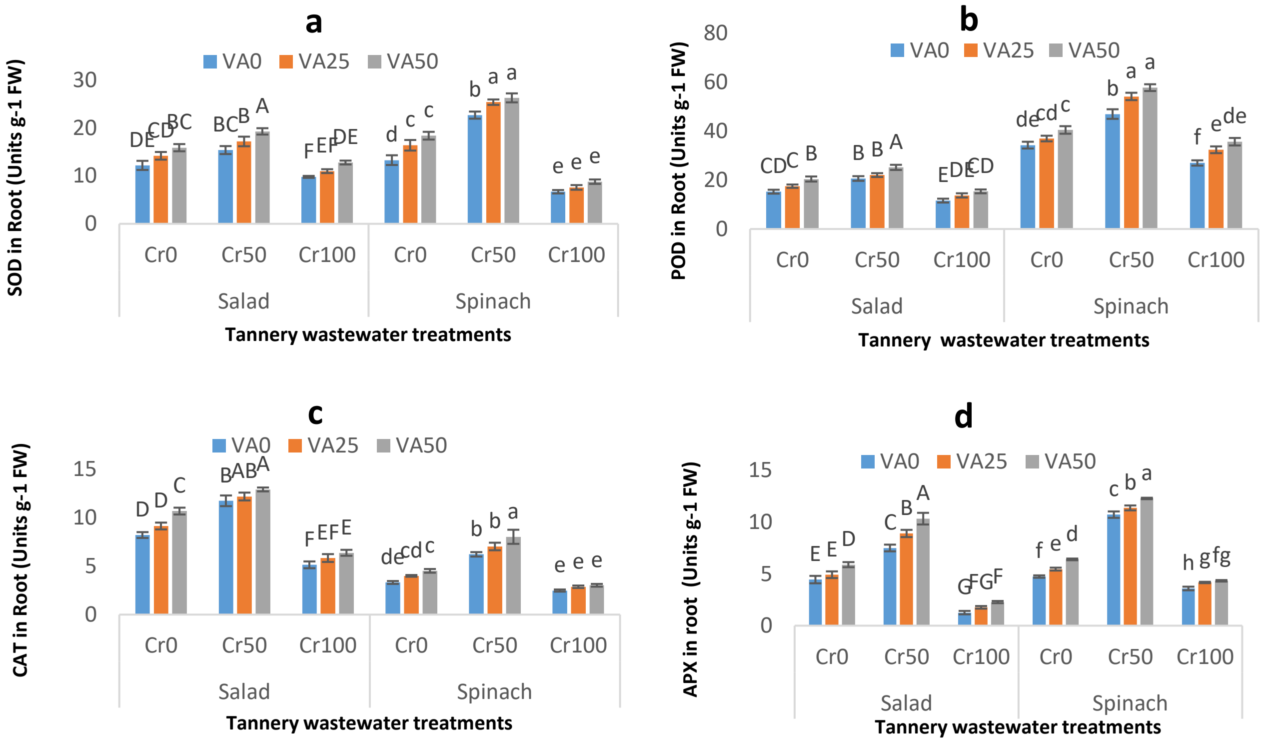

Fig. 5. SOD, POD, CAT, and APX levels in the roots of lettuce and spinach plants that have received vanillic acid (VA) treatment as a result of varied tannery effluent concentrations. The standard deviation (SD; n = 3) and the average of three replicates are used to illustrate values. A one-way ANOVA (P 0.05) was followed by an HSD to assess mean differences. Different lowercase letters signify a significant variation in the treatments across the error bars. The relative radiances of the used plastic filters are 0 (without irrigation with wastewater), 50 (with 50% irrigation with wastewater), and 100 (with 100% irrigation with wastewater).

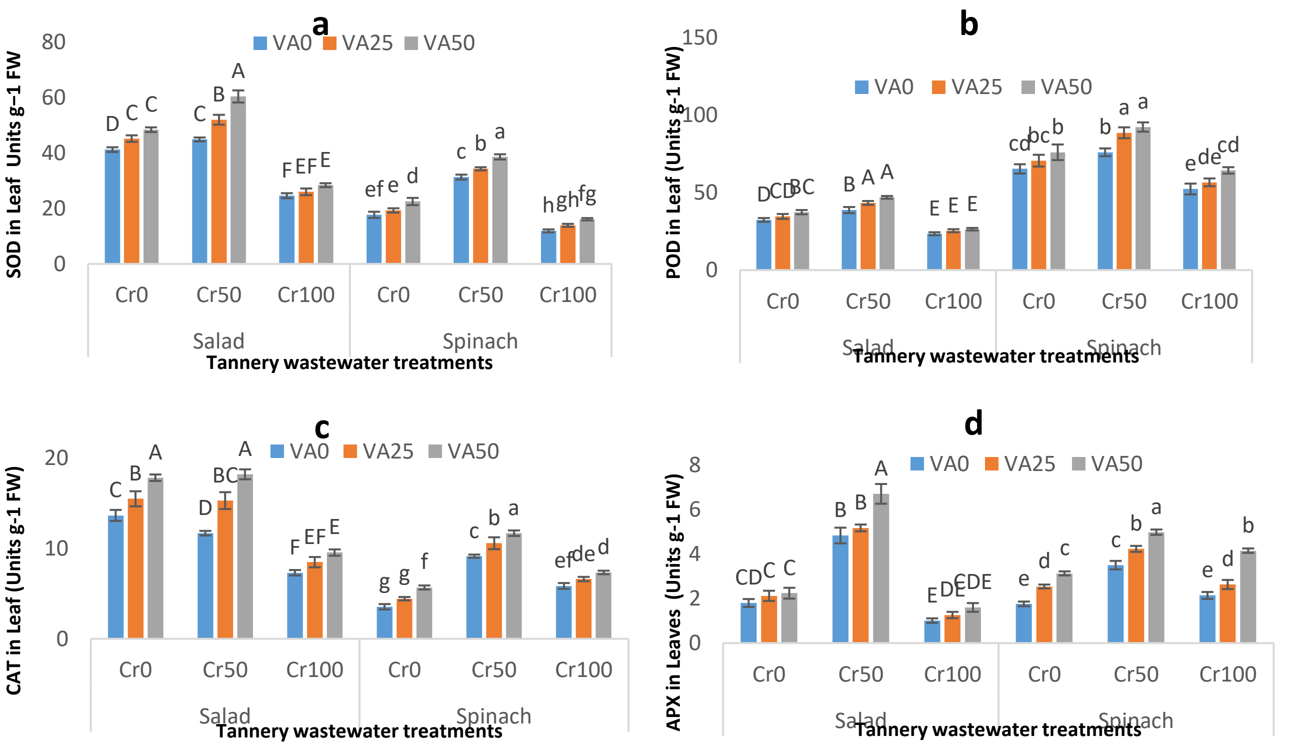

Fig. 6. Different tannery effluent concentrations have an impact on the levels of SOD, POD, CAT, and APX in the leaves of lettuce and spinach plants that have been treated with vanillic acid (VA). The standard deviation (SD; n = 3) and the average of three replicates are used to display values. A one-way ANOVA (P 0.05) was followed by an HSD to assess mean differences. Different lowercase letters signify a significant variation in the treatments across the error bars. Relative radiances of used plastic filters were 0 (without irrigation with wastewater), 50 (with 50% irrigation), and 100 (100% irrigation).

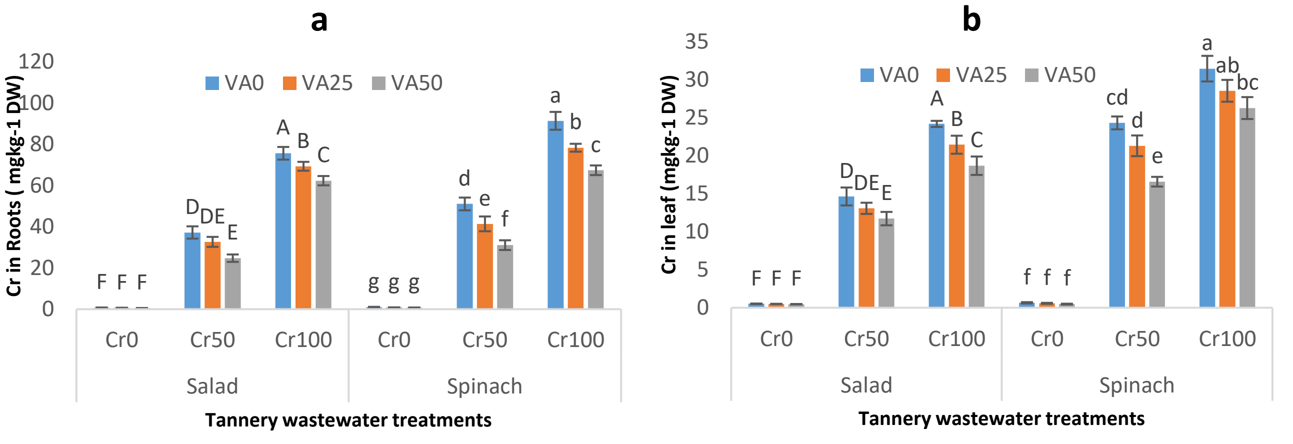

Fig. 7. Effect of various tannery wastewater concentrations on the uptake/accumulation of Cr contents in the roots (a) and Cr contents in the shoots (b) when vanillic acid (VA) is sprayed on lettuce and spinach plants. The standard deviation (SD; n = 3) and the average of three replicates are used to display values. A one-way ANOVA (P 0.05) was followed by an HSD to assess mean differences. Different lowercase letters signify a significant variation in the treatments across the error bars. Relative radiances of used plastic filters were 0 (without irrigation with wastewater), 50 (with 50% irrigation), and 100 (100% irrigation).

Cr Concentration: Plants from the genus Spinacia oleracea and the genus Lactuca sativa have much higher Cr concentrations in their roots and leaves. When tannery effluent was present in soil at several concentrations (0%, 50%, and 100%), it caused an increase in the amount of Cr that accumulated in the roots and leaves of Lactuca sativa and Spinacia oleracea (Fig. 7). Results indicated that roots showed a maximum accumulation of Cr than shoots of the plants. Exogenous application of Vanillic acid enhanced the growth of the plants under Cr stress which improved the plant’s ability to survive under severe stress of chromium. Vanillic acid (VA 0M, 25M, 50M) application reduces the amount of Cr in the plants' roots and leaves as compared to the Cr treatments alone. The amount of Cr in the roots of Spinacia oleracea and Lactuca sativa fell by 17%, 20% at 50% Cr level, and 13% and 14% at 100% Cr level when VA (50 M) was applied. Similarly, Cr concentration in leaves of *Spinacia oleracea* and *Lactuca sativa* was reduced by 22%, 20 at 50% Cr level and 24% and 23% at 100% Cr level respectively. Generally, VA (50µM) found more effective than (VA 25µM) in mitigation of translocation of Cr from a plant's roots to its leaves.

Discussion

The goal of the current study was to ascertain the impact of tanneries' effluent on many physiological, morphological, and biochemical characteristics of *Spinacia oleracea* and *Lactuca sativa*. The new study also examined how well plants absorbed Cr. Figures 1 and 2 demonstrate that chromium treatment significantly decreases (P 0.05) plant growth and biomass as soil Cr concentration rises. *Brassica napus* L., *Zea mays*, and *Ricinus communis* L. are three examples of plants where chromium has been linked to reduced growth (Zaheer *et al.*, 2022; Vishnupradeep *et al.*, 2022a). Since Cr stress significantly affects the nutrients in plants' homeostasis, the enormous loss in plant development may be related to the restriction in the absorption of vital nutrients in the root zone (Pradas-del-Real *et al.*, 2013). The current study's findings showed that the excessive buildup of Cr in the root zone caused damage to the root tip cells, which is what caused both of these plants' roots to become shorter (Ali *et al.*, 2013). Similarly, the subsequent reduction in shoot length is attributed to the ultrastructure damage of mesophyll cells in leaves, which eventually leads to a decline in shoot growth (Gill *et al.*, 2015). However, exogenous application of vanillic acid (VA) considerably improves the growth and biomass of both plants by effectively mitigating Cr stress. Numerous studies that show the beneficial impact of exogenously applied VA in helping plants recover from abiotic stress are consistent with our findings.

It was previously documented that VA regulates photosynthesis and nutrient uptake in stressed plants. Therefore, VA improved both of these plants' growth and biomass under Cr stress in our investigation.

Chromium stress considerably declines the photosynthetic pigment, water use efficiency, and gas exchange attributes (Figure 3). Photosynthesis is indispensable for appropriate plant growth and development (Muhammad *et al.*, 2021). The production of photosynthetic pigments is inhibited by chromium stress. Chl a, Chl b, and total chlorophyll are photosynthetic pigments that are essential to plant life. They also play a crucial role in the transmission and absorption of light energy. Photosynthesis triggers many metabolic processes in plants by stimulating the transformation of light energy into chemical energy (Demmig-Adams *et al.*, 2018). Application of tannery wastewater in this investigation resulted in a considerable decline of photosynthetic pigments. It may happen as protochlorophyllid reductase reduces the biosynthesis of chlorophyll, which is the main reason for the subsequent decrease in photosynthetic pigment under Cr stress (Zhao *et al.*, 2017). Another explanation for this phenomenon is that the Cr in plant cells damaged the chloroplast structure and decreased the intake of minerals necessary for the production of chlorophyll (Gill *et al.*, 2015). This consequent reduction in photosynthetic pigments was previously reported in different crops, including castor bean, canola (Ayyaz *et al.*, 2021), Solanum lycopersicum (Gupta & Seth, 2021), rice (Basit *et al.*, 2021), and Zea mays (Vishnupradeep *et al.*, 2022b). An increase in the concentration of water use efficiency, gas exchange attributes, and photosynthetic pigment were observed in both *Spinacia oleracea* and *Lactuca sativa* facing Cr stress with the application of vanillic acid (VA 0µM, 25µM, 50µM). The VA application increased the photosynthetic pigments in plants facing Cr stress. Different authors support the findings of this study (Parvin *et al.*, 2020; Ketnawa *et al.*, 2020). That might be because VA increased the antioxidative mechanism in cells and prevented chloroplast damage (Singh *et al.*, 2019).

Both of the understudied plants in this study experienced significant oxidative stress as a result of the increased production of reactive oxygen species (ROS) and electrolyte leakage under Cr stress (Fig. 4). Chromium led to the overproduction of ROS, which caused oxidative damage to a variety of biomolecules, including proteins, RNA, DNA, and pigments. Numerous plants, including rapeseed (Zaheer *et al.*, 2020b), wheat (Askari *et al.*, 2021), okra (Ashraf *et al.*, 2021), and rice (Basit *et al.*, 2022), were proven to have Higher quantities of oxidative stress brought on by chromium, hydrogen peroxide (H2O2), electrolyte leakage (EL), malondialdehyde (MDA), and EL are seen. A considerable imbalance between the production of ROS and the activity of antioxidant enzymes involved in the scavenging of ROS may be the cause of the extensive membrane damage in both of these plants. Activities for SOD, POD, APX, and CAT have steadily grown, indicating that the plant has improved its defences against Cr-induced oxidative stress. This could occur as a result of Cr reducing the function of antioxidant enzymes and causing cells to produce more reactive oxygen species (Chen & Murata, 2011). Reduced EL, MDA, and H_2O_2 levels were seen in *Spinacia oleracea* and *Lactuca sativa* plants after VA treatment (Fig. 4). According to reports, when rice plants were lacking in water, VA decreased the amount of MDA in the plants (Parvin *et al.*, 2020). Therefore, our study has demonstrated that applying VA helps boost plants' antioxidant defence systems and reduce Cr translocation.

According to a variety of prior research studies, Cr stress causes changes in the synthesis and storage of enzymatic antioxidant enzymes in plants, which limits the activity of these enzymes (Wakeel *et al.*, 2020; Malik *et al.*, 2021). Important enzymes in this work, such as SOD, POD, and CAT activities, were decreased when tannery effluent was applied to *Lactuca sativa* and *Spinacia oleracea* plants (Fig. 5). Numerous plants, including *Zea mays* and *Triticum aestivum*, have been demonstrated to have decreased antioxidant enzyme activity under severe Cr stress (Malik *et al.*, 2021; Lei *et al.*, 2021). When large concentrations of Cr accumulated in plant cells, the activity of these antioxidant enzymes decreased sequentially, destroying the mitochondrial structure. depress the antioxidant machinery of cells (Gill *et al.*, 2014). At increasing levels of Cr stress, the activity of these enzymes was considerably decreased (100% Cr). The exogenous application of VA boosts the activity of antioxidant enzymes in both stressed and control plants. When VA is applied, these enzymes' activity is increased in the roots and shoots of both plants; VA 50 M is more effective than VA 0 M and VA 25 M. The activities of the enzymes are marginally improved by VA at both 50% and 100% Cr levels. The study's findings are consistent with those of other research investigations (Parvin *et al.*, 2020; Demmig-Adams *et al.*, 2018). By successfully reducing excessive ROS, exogenous administration of VA controlled oxidation and reduced structural damage to mitochondria.

The findings of the study indicate that the concentrations of Cr absorption and accumulation increase with the amount of Cr in the medium. The contents of Cr in these plants increased considerably with tannery wastewater treatment (Fig. 6). Numerous writers have reported that plants' Cr contents rose when they were irrigated with effluent from tanneries (Maqbool *et al.*, 2018). Because chromium is harmful to plants, it greatly disrupts agricultural productivity and metabolic processes. Increased Cr absorption limits the spectrum of powerful nutrients that plants can properly take, which causes abnormalities in normal development. This is because photosynthesis is interrupted, and there is ultrastructural damage (Farid *et al.*, 2019). But compared to shoots, roots had greater Cr concentrations. Previous research on barley and wheat that were published also showed a similar pattern (Ali *et al.*, 2015; Hussain *et al.*, 2018). Because roots had direct contact with soil-borne Cr or with precipitated Cr in the form of macromolecules, roots accumulated Cr far more than shoots did (Ali *et al.*, 2015). *Spinacia oleracea* and *Lactuca sativa* roots and shoots have reduced Cr concentrations after applying VA (Fig 6). Previous research has shown that applying VA to rice plants reduced their Cd level as well (Demmig-Adams *et al.*, 2018). According to Giri (2011) and Nardi *et al.*, (2002), exogenous administration of VA enhanced the absorption of nutrients, which in turn limited the uptake of Cr or reduced structural damages to root tips, resulting in low uptake of Cr in plants.

Conclusion

Under Cr stress, the morphology, physiology, and biochemistry of *Spinacia oleracea* and *Lactuca sativa* were significantly improved by the exogenous administration of vanillic acid (VA). Vanillic acid application lowered Cr levels in each of these research plants. Exogenously administered VA boosted the biomass, growth, and chlorophyll content of the plants by increasing the activity of important antioxidant enzymes. Our results demonstrated the potential advantages of exogenous VA administration in minimizing the effects of heavy metal stress in spinach (*Spinacia oleracea*) and lettuce (*Lactuca sativa*), as well as maybe in other plants. To completely comprehend the mechanism behind plants' arbitrated resistance to heavy metal stress brought on by vanillic acid, further study is nonetheless required.

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