sustainability

MDPI

Article

Iron–Lysine Mediated Alleviation of Chromium Toxicity in Spinach (*Spinacia oleracea* L.) Plants in Relation to Morpho-Physiological Traits and Iron Uptake When Irrigated with Tannery Wastewater

Ihsan Elahi Zaheer [1,†], Shafaqat Ali [1,2,*], Muhammad Hamzah Saleem [3,*,†], Iqra Noor [4], Mohamed A. El-Esawi [5], Kashif Hayat [6,7], Muhammad Rizwan [1], Zohaib Abbas [1], Mohamed A. El-Sheikh [8], Mohammed Nasser Alyemeni [8] and Leonard Wijaya [8]

1 Department of Environmental Sciences and Engineering, Government College University, Allama Iqbal Road, Faisalabad 38000, Pakistan; ihsankhanlashari@gmail.com (I.E.Z.); mrizwan@gcuf.edu.pk (M.R.); zohaib.abbas83@gmail.com (Z.A.)
2 Department of Biological Sciences and Technology, China Medical University, Taichung 40402, Taiwan
3 MOA Key Laboratory of Crop Ecophysiology and Farming System in the Middle Reaches of the Yangtze River, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
4 Key Laboratory of Horticultural Plant Biology–Ministry of Education, College of Horticulture and Forest Sciences, Huazhong Agricultural University, Wuhan 430070, China; iqranoor@webmail.hzau.edu.cn
5 Botany Department, Faculty of Science, Tanta University, Tanta 31527, Egypt; mohamed.elesawi@science.tanta.edu.eg
6 School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China; khayat97@sjtu.edu.cn
7 Key Laboratory of Urban Agriculture, Ministry of Agriculture and Rural Affairs, Shanghai 200240, China
8 Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia; melsheikh@ksu.edu.sa (M.A.E.-S.); mnyemeni@ksu.edu.sa (M.N.A.); leon077@gmail.com (L.W.)
* Correspondence: shafaqataligill@gcuf.edu.pk (S.A.); saleemhamza312@webmail.hzau.edu.cn (M.H.S.)
† These authors contributed equally to this work.

Received: 29 June 2020; Accepted: 16 August 2020; Published: 18 August 2020

Abstract: Chromium (Cr) is among the most widespread toxic trace elements found in agricultural soils due to various anthropogenic activities. However, the role of micronutrient-amino chelates on reducing Cr toxicity in crop plants was recently introduced. In the current experiment, the exogenous application of micronutrients [iron (Fe)] chelated with amino acid [lysine (lys)] was examined, using an in vivo approach that involved plant growth and biomass, photosynthetic pigments and gaseous exchange parameters, oxidative stress indicators and antioxidant response. The uptake and accumulation of Fe and Cr were determined under different levels of tannery wastewater (33, 66, 100%) used along with the exogenous supplementation of Fe-lys (5 mM) to *Spinacia oleracea* plants. Results revealed that tannery wastewater in the soil decreased plant growth and growth-related attributes, photosynthetic apparatus and Fe contents in different parts of the plants. In contrast, the addition of different levels of tannery wastewater to the soil significantly increased the contents of malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and electrolyte leakage (EL), which induced oxidative damage in the roots and leaves of *S. oleracea* plants. However, *S. oleracea* plants increased the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX), which scavenge the over-production of reactive oxygen species (ROS). Cr toxicity can be overcome by the supplementation of Fe-lys, which significantly increased plant growth and biomass, improved photosynthetic machinery and increased the activities of different antioxidative enzymes, even in the plants grown under different levels of tannery wastewater in the soil. Furthermore, the supplementation of Fe-lys increased the contents of essential nutrients (Fe) and decreased the contents of Cr in all plant parts compared to the plants cultivated in tannery wastewater without application of Fe-lys. In conclusion, the application of Fe-lys is an innovative approach to mitigate Cr stress in spinach plants, which not only increased plant growth and biomass but also decreased the Cr contents in different plant organs.

Keywords: heavy metals; leafy green vegetable; micronutrients chelation; plant growth; photosynthesis; oxidative stress

1. Introduction

Heavy metals are omnipresent pollutants of the environment, and their unnecessary level in arable soil can pose serious threats not only to normal plant growth and development, but also to human health [1–4]. Agricultural land uses 70% of tannery wastewater due to the shortage of fresh water extremely prominent in different regions around the world. In addition, the irrigation of agricultural land with industrial wastewater in the world's arid and semi-arid regions has become a common practice that is dangerous to plant growth and becomes toxic to human health when it enters the food chain [5–7]. Normally, agricultural land has a low concentration of heavy metals, but the application of different fertilizers and irrigation with tannery wastewater increased the amount of heavy metals in soil [8–10]. It was also noticed that tannery wastewater contains a low level of heavy metals, but the plant contains much higher contents of wastewater due to metal accumulation [11,12]. In some developing countries, such as Pakistan, aquifers were pooped from industrial sources into the water bodies, which were then mixed in canals used for field irrigation [6,13]. Therefore, this wastewater needs to be investigated, and some artificial methods should be used to help impart crop yield and productivity. Among various heavy metals, chromium (Cr) is an extremely noxious metal to living organisms and has negative effects reported in humans [14], animals [15], plants [16] and micro-organisms [17]. Cr is a major toxic element discharged into the environment through various industries, such as tanning, electroplating, manufacturing of pigments, production of nuclear weapons and corrosion control [18–20]. This extensive industrial use of chromium composites and their subsequent releases, without prior treatment, into the surrounding environment contaminates the entire ecosystem and can lead to catastrophic health risks [21,22].

Plants take up Cr from the soil along with other essential micronutrients. Excess Cr is highly toxic and detrimental to plant growth and development [23,24]. Therefore, plants take up high Cr content from the soil, resulting in a variability of photosynthetic apparatus [25], oxidative stress [11] and decreased plant nutrient uptake [5]. Moreover, Cr enters the human body through the ingestion of Cr-contaminated food, which can also lead to lung cancer, ulcer and hepatic irritation [14,26]. However, previously reported studies investigating the toxic effect of Cr stress on plant growth and composition have been performed [20,21,27–29]. Therefore, accumulation of Cr by plants and eventually its amount in foodstuffs must be eradicated. However, vegetables such as spinach (*Spinacia oleracea* L.) showed more tolerance to Cr stress when irrigated on Cr-contaminated soil [5,11,30]. *Spinacia oleracea* is a worldwide cultivated vegetable crop because of its relative high growth rates, its increased production of biomass and its use of heavy metals and other important soil nutrients [31,32]. Furthermore, *S. oleracea* has been extensively investigated on the basis of these distinctive features to analyze its growth performance and stress responses to various heavy metals [11,33]. Wastewater irrigation, however, is a common practice in different regions of the world where water shortages are extremely prominent and irrigation puts tremendous pressure on crop quality and yield [6,11,34]. It was also shown in various studies that tannery wastewater irrigation resulted in unwanted Cr accumulation in different plants, which eventually restricts plant growth and development [5,11,35,36]. Similarly, Maqbool et al. [11] also studied the similar type of decline in growth, biomass and photosynthesis in *S. oleracea* treated with wastewater effluents from a tannery.

Recently, various techniques and methodologies have been developed to reduce the heavy metal stress in plants [37–40]. In the past few years, a new method has been adopted to emphasize the use of amino acids as chelates to reduce toxic heavy metals [14,26,41]. Souri [42] has previously investigated the role of iron (Fe) chelated fertilizer with the amino acid lysine in improving crop growth and yield. Because Fe is an essential micronutrient for proper plant growth and development, it plays a crucial role in plant metabolism in enzymatic and metabolic processes [1,43,44]. Conversely, Fe deficiency significantly alters the morpho-physiological characteristics of plants and results in stunned plant growth and biomass [45]. Nevertheless, the application of Fe complex with lysine (lys) greatly reduces the toxicity induced by heavy metals stress. Fe complex with lys significantly reduced the Cd stress by improving rice growth, photosynthesis and the antioxidant system [45]. Large amounts of heavy metals in plant organs seriously affected the quality of their vegetative characters. In order to overcome this drawback, the current study examined the exogenous impact of Fe-lys on plant growth and biomass by decreasing the consumption of Cr in plant organs grown in soil irrigated with wastewater tanning.

2. Materials and Methods

2.1. Collection and Analysis of Wastewater and Soil

Samples of soil used to perform the current experimental study were collected from the Department of Botany, University of Punjab Lahore, Pakistan (31.4015° N, 74.3070° E), from an average depth of 0–15 cm. The 2 mm sieve was used to separate mud and debris from the dried soil intentionally. A comprehensive analysis of organic soil content was performed following the reported procedures [46]. For determining the precise size of the soil elements, a hydrometer was used [47]. Likewise, electrical conductivity (EC), sodium adsorption ratio (SAR) and soil ions were also carefully measured following the reported method [48]. Soil sampling was carried out with the help of ammonium bicarbonate diethylenetriamine penta acetic acid (AB-DTPA) solution for the sufficient estimation of extractable trace components [49,50]. The physicochemical characteristics of the soil under study are given in Table 1. The tannery wastewater used for the pot experiment was collected from the tannery industries based in Kasur, Punjab, Pakistan. The physicochemical properties of the tannery wastewater used in the present study were estimated according to the set protocols [51]. Comprehensive details of major characteristics of the tannery wastewater used in this experiment are presented in Table 2. The same soil with the tannery wastewater was used in our previous study, by Zaheer et al. [5].

Table 1. Physicochemical properties of the loam soil used in the pot experiment.

Texture	Clay Loam
Silt	11.9
Clay	23.4
pH	7.1
ECE (dS m^{-1})	3.86
Cation exchange capacity (CEC) (cmol kg^{-1})	4.78
Soluble CO_3^{-2} (mmol L^{-1})	0.85
Soluble HCO_3 (mmol L^{-1})	3.45
Soluble Cl^- (mmol L^{-1})	5.91
Soluble Ca^{2+} + Mg^{2+} (mmol L^{-1})	14.93
Organic matter (%)	0.52
Ni (mg kg^{-1})	0.21
Cu (mg kg^{-1})	0.39
Zn (mg kg^{-1})	0.64
Cr (mg kg^{-1})	0.10

Table 2. Characteristics of the tannery wastewater used for irrigation of the soil used in the pot experiment.

Parameters	Values	Permissible Limits **
EC (dS m^{-1})	1.41	<1.5
SAR (mmol $L^{-1})^{1/2}$	4.02	<7.5
RSC (mmol c L^{-1})	2.24	<2.0
Ni (mg L^{-1})	0.09	0.20
Cd (mg L^{-1})	0.04	0.01
Pb (mg L^{-1})	1.24	5.0
Co (mg L^{-1})	0.02	0.05
Cr (mg L^{-1})	4.03	0.10
Zn (mg L^{-1})	1.95	2.00

** Ayers and Westcot [52].

2.2. Pot Experiment and Treatments

This study was conducted in a botanical garden under a glasshouse environment, at the Department of Botany of the University of Punjab Lahore 54,000, Pakistan. Healthy and mature seeds of spinach (*Spinacia oleracea* L.) were collected from Ayyub Agriculture Research Institute Faisalabad, Pakistan. *S. oleracea* seeds have been carefully sowed in experimental pots filled with approximately 5 kg of soil after diligent washing with H_2O_2 (10%) (to avoid fungal or bacterial infection) and rising with deionized water. Five seeds were sowed in each pot. After thinning, three seedlings remained in each pot. After 2 weeks of seed germination, foliar application of Fe-lys (5 mg·L^{-1}), along with various levels of tannery wastewater (33, 66 and 100%), were gently applied. A hand sprayer was used for the exogenous supplementation of Fe and Fe-lys to the *S. oleracea* plants. A total volume of 2 L of Fe-lys and 1 L of Fe-lys was used in the whole experiment in each treatment, and every treatment was replicated five times, with three plants in each pot. Under the control conditions of the glasshouse, tannery wastewater was added in the pots, and all pots were given 0, 33, 66 and 100% wastewater on the basis of the treatments, when needed with other intercultural operations such as weeding. Moreover, with the addition of tannery wastewater (at least) once a week, we also sprayed Zn-lys regularly, on the basis of demand, on whole plant parts. In order to maintain an optimum amount of micronutrients in plant organs, specific amounts of fertilizers in the form of phosphate and potassium sulphate were also applied, as previously described [45]. The pots used in this study (under the glasshouse environment) were rotated regularly in order to avoid environmental effects on the plants.

2.3. Plant Harvesting

Plants of *S. oleracea* were carefully rooted-up after 30 days of experimental treatment (precisely after 60 days of germination) and washed gently with the help of distilled water to eliminate the aerial dust and deposition. All the harvested plants were divided into two parts, i.e., roots and shoots to study different biological traits. Plant height was measured straightway after the harvesting using a measuring scale, and the number of leaves per plant was also counted. The harvested plant samples were attentively washed with the help of de-ionized water, followed by oven-drying at 70 °C for three days, and were ground in a stainless steel mill and passed through a 0.1 mm nylon sieve for further investigation.

2.4. Determination of Photosynthetic Pigments and Gaseous Exchange Parameters

A certain weight of plant leaf samples was crushed and kept in the tubes containing 85% acetone (*v*/*v*). The tubes were placed in the dark for the extraction of pigments for 24 h. The tubes were then centrifuged at 4000× *g* at 4 °C for 10 min. The supernatant was used to measure absorbance at 470, 647 and 664.5 nm by using a spectrophotometer (Halo DB-20/ DB-20S, UK). Chlorophyll contents were measured as recommended by Lichtenthaler [53]. Net photosynthesis, stomatal conductance, transpiration rate and water use efficiency were measured from three different plants in each treatment group. The measurements were conducted between 9 am and 11 am on days with clear sky with a portable IRGA (Infra-Red Gas Analyzer, Hoddesdon, UK).

2.5. Determination of Malondialdehyde (MDA), Hydogen Peroxide (H_2O_2) and Electrolyte Leakage (EL)

The degree of lipid peroxidation was evaluated as malondialdehyde (MDA) content. Briefly, 0.1 g of frozen leaves were ground at 4 °C in a mortar with 25 mL of 50 mM phosphate buffer solution (pH 7.8) containing 1% polyethene pyrrole. The homogenate was centrifuged at 10,000× *g* at 4 °C for 15 min. The mixtures were heated at 100 °C for 15–30 min and then quickly cooled in an ice bath. The absorbance of the supernatant was recorded using a spectrophotometer (xMark™ microplate absorbance spectrophotometer; Bio-Rad, United States) at wavelengths of 532, 600 and 450 nm. Lipid peroxidation was expressed as l mol g^{-1} using the following formula: 6.45 (A532−A600)−0.56 A450. Lipid peroxidation was measured using a method previously published by Heath and Packer [54].

To estimate the H_2O_2 content of plant tissues (root and leaf), 3 mL of sample extract was mixed with 1 mL of 0.1% titanium sulfate in 20% (*v*/*v*) H_2SO_4 and centrifuged at 6000 g for 15 min. The yellow color intensity was evaluated at 410 nm. The H_2O_2 level was computed by an extinction coefficient of 0.28 $mmol^{-1}$ cm^{-1}.

The stress-induced electrolyte leakage (EL) of uppermost stretched leaves was determined by Dionisio-Sese and Tobita's [55] method. The leaves were cut into minor slices (5 mm length) and placed in test tubes containing 8 mL distilled water. These tubes were incubated and transferred into a water bath for 2 h prior to measuring the initial electrical conductivity (EC_1). The samples were autoclaved at 121 °C for 20 min, and then cooled down to 25 °C before measuring the final electrical conductivity (EC_2). Electrolyte leakage was measured using a pH/conductivity meter (model 720, INCO-LAB Company, Kuwait) and calculated as:

$$EL = (EC_1/EC_2) \times 100$$

2.6. Determination of Superoxidase (SOD), Peroxidase (POD), Catalase (CAT) and Ascorbate Peroxidase (APX) Activity

To evaluate enzyme activities, fresh leaves (0.5 g) were homogenized in liquid nitrogen and 5 mL of 50 mmol sodium phosphate buffer (pH 7.0) including 0.5 mmol ethylenediaminetetraacetic acid (EDTA) and 0.15 mol NaCl. The homogenate was centrifuged at 12,000× *g* for 10 min at 4 °C, and the supernatant was used for the measurement of SOD and POD activities. SOD activity was assayed in a 3 mL reaction mixture containing 50 mM sodium phosphate buffer (pH 7), 56 mM nitro blue tetrazolium, 1.17 mM riboflavin, 10 mM methionine and 100 µL enzyme extract. Finally, the sample was measured using a spectrophotometer (xMark™ microplate absorbance spectrophotometer; Bio-Rad). Enzyme activity was measured using a method by Chen and Pan [56] and expressed as U g^{-1} FW.

POD activity in the leaves was estimated using the method of Sakharov and Ardila [57] using guaiacol as the substrate. A reaction mixture (3 mL) containing 0.05 mL of enzyme extract, 2.75 mL of 50 mM phosphate buffer (pH 7.0), 0.1 mL of 1% H_2O_2 and 0.1 mL of 4% guaiacol solution was prepared. Increases in the absorbance at 470 nm because of guaiacol oxidation were recorded for 2 min. One unit of enzyme activity was defined as the amount of the enzyme.

Catalase activity was analyzed according to Aebi [58]. The assay mixture (3.0 mL) was comprised of 100 µL enzyme extract, 100 µL H_2O_2 (300 mM) and 2.8 mL of 50 mM phosphate buffer with 2 mM ETDA (pH 7.0). The CAT activity was measured from the decline in absorbance at 240 nm as a result of H_2O_2 loss (ε = 39.4 mM^{-1} cm^{-1}).

Ascorbate peroxidase activity was measured according to Nakano and Asada [59]. The mixture containing 100 µL enzyme extract, 100 µL ascorbate (7.5 mM), 100 µL H_2O_2 (300 mM) and 2.7 mL 25 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0) was used for measuring APX activity.

The oxidation pattern of ascorbate was estimated from the variations in wavelength at 290 nm (ε = 2.8 mM^{-1} cm^{-1}).

2.7. Determination of Iron (Fe) and Cromium (Cr) Contents from the Plants

The digestion of plant samples was carried out through the di-acid (HNO_3-$HClO_4$) method. Dry samples (0.5 g) of both roots and leaves were added to a flask containing 10 mL of HNO3-HClO4 (3:1, v:v), and the mixture was kept overnight. After that, 5 mL HNO_3 was added, and the samples were completely digested by placing them on a hot plate as described by Rehman et al. [60]. The concentration of Fe and Cr in the roots and leaves of the plants was measured using an atomic absorption spectrophotometer.

2.8. Statistical Analysis

The normality of data was analyzed using IBM SPSS software (Version 21.0. Armonk, NY, USA: IBM Corp) through a multivariate post hoc test, followed by a Duncan's test in order to determine the interaction among significant values. One-way analysis of variance (ANOVA) was used to assess the significance of the variations of Cr among the different plant parts, followed by highest significant deviation (HSD) tests. Where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. The analysis showed that the data in this study were almost normally distributed. Thus, the mean difference between the treatments was deemed significant at $p \leq 0.05$ between the treatments. The graphical presentation was carried out using SigmaPlot 12 (Systat Software Inc., San Jose, CA 95110, USA). The Pearson correlation coefficients and heatmap between the measured variables of *S. oleracea* were also calculated. The plots of principal component analysis on rapeseed parameters were carried out using the Rstudio software (4.3.1) (Rstudio PBC, Boston, MA, USA).

3. Results

3.1. Effect of Foliar Application of Fe-lys on Plant Growth and Biomass under Different Levels of Tannery Wastewater

Alterations in different morphological traits in *S. oleracea* plants were observed when grown in tannery wastewater. Data regarding different morphological parameters of *S. oleracea* plants are presented in Figures 1 and 2. According to the results, the toxic contents of Cr in the wastewater caused a significant decrease in all the morphological traits of *S. oleracea* plants studied in this study (Figures 1 and 2). Compared to the control, a maximum decrease (significantly, $p < 0.05$) in plant height, number of leaves, leaf area, root length, root fresh weight, root dry weight, leaf fresh weight and leaf dry weight by 52.8, 47.5, 79.7, 48.2, 56.7, 55.0, 67.7 and 68.8%, respectively, was recorded in the plants grown in 100% addition of tannery wastewater without the application of Fe-lys. Consequently, the exogenous supplementation of Fe-lys is helpful to the plants, increasing all the morphological parameters both in a tannery wastewater environment alone and treated with wastewater in the soil. At all levels of tannery wastewater (33%, 66% and 100%), the application of Fe-lys improved all morphological traits, while at 100% tannery wastewater treatment the above mentioned morphological traits increased by 18.1, 34.1, 26.3, 28.0, 15.0, 18.0, 23.2 and 12.7%, respectively, when the exogenous foliar application of Fe-lys was applied.

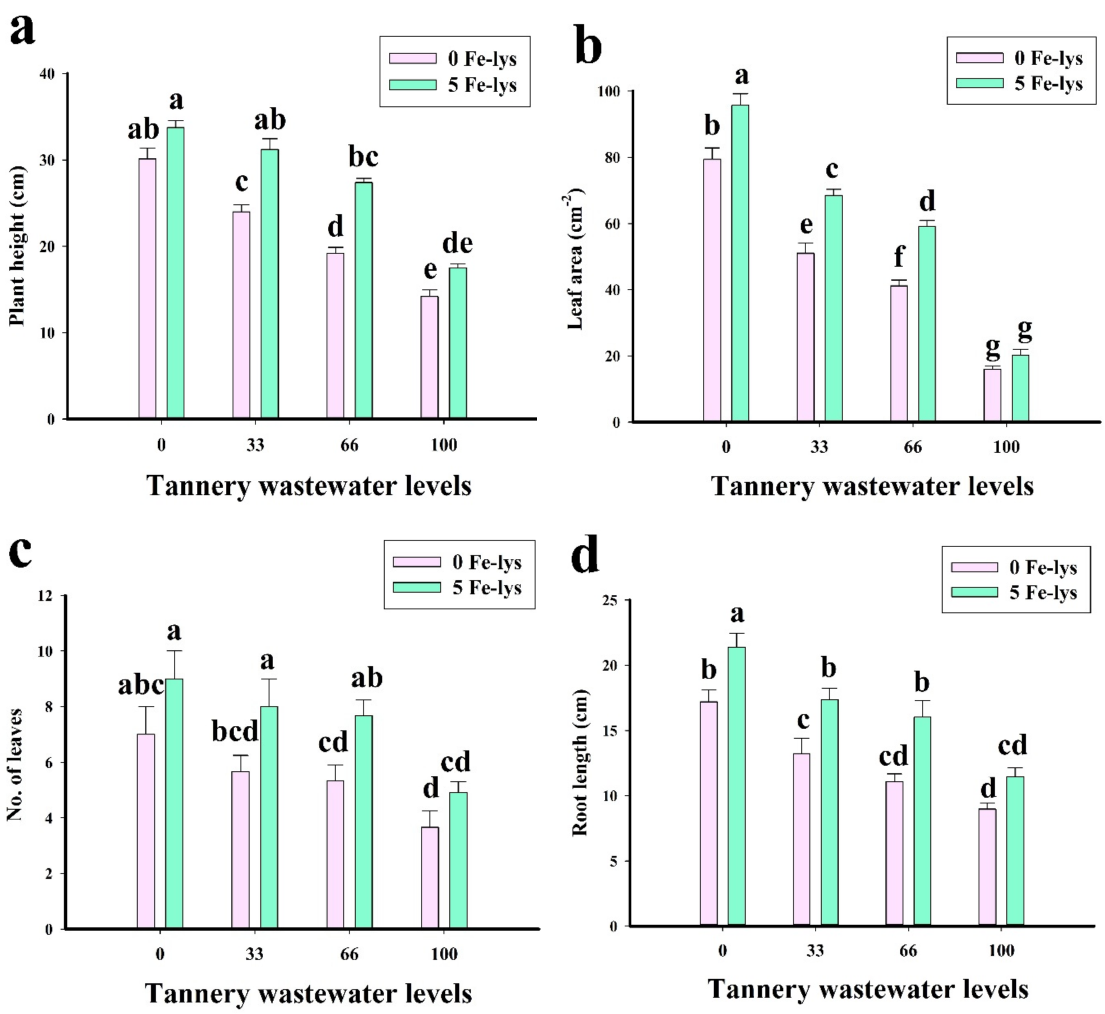

Figure 1. Effect of different levels of tannery wastewater on plant height (**a**), leaf area (**b**), number of leaves (**c**) and root length (**d**) under the application of iron-lysine to *S. oleracea* plants. Values are demonstrated as means of three replicates along with standard deviation (SD; n = 3). One-way ANOVA was performed, and mean differences were tested by highest significant deviation (HSD) ($p < 0.05$). Different lowercase letters on the error bars indicate a significant difference between the treatments. Relative radiance of plastic filter used: 0 (without irrigation with wastewater), 33 (33% irrigation with wastewater), 66 (66% irrigation with wastewater) and 100 (100% irrigation with wastewater).

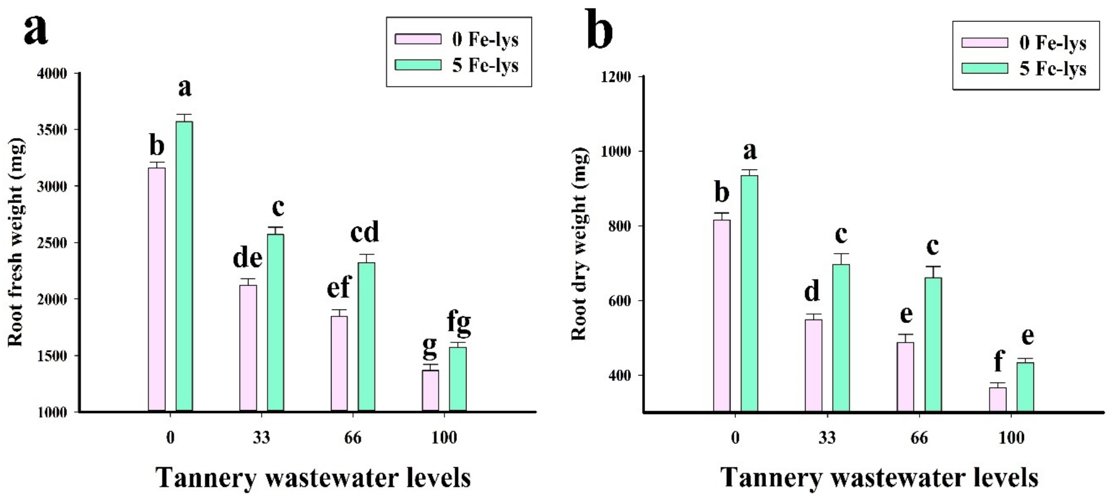

Figure 2. *Cont.*

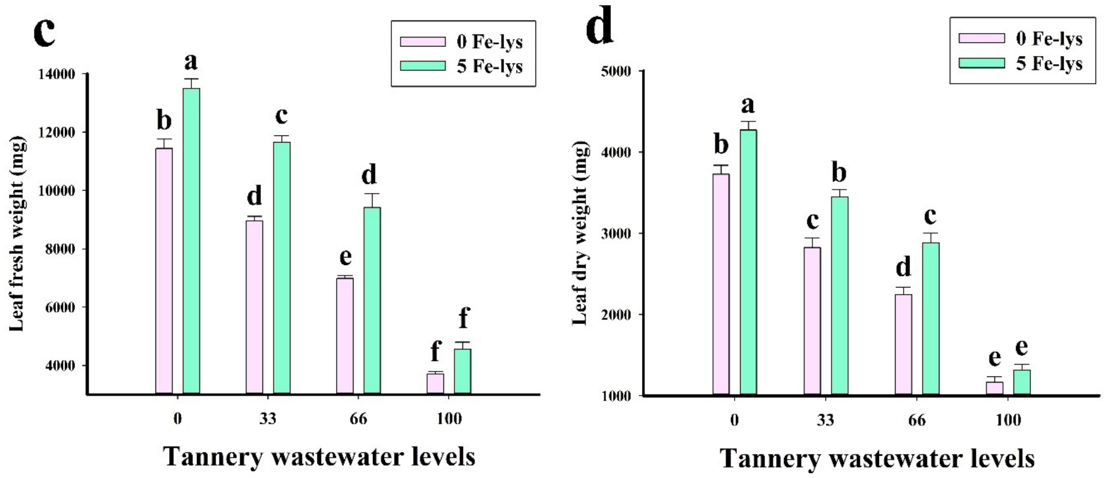

Figure 2. Effect of different levels of tannery wastewater on root fresh weight (**a**), root dry weight (**b**), leaf fresh weight (**c**) and leaf dry weight (**d**) under the application of iron-lysine to *S. oleracea* plants. Values are demonstrated as means of three replicates along with standard deviation (SD; n = 3). One-way ANOVA was performed, and mean differences were tested by HSD ($p < 0.05$). Different lowercase letters on the error bars indicate significant difference between the treatments. Relative radiance of plastic filter used: 0 (without irrigation with wastewater), 33 (33% irrigation with wastewater), 66 (66% irrigation with wastewater) and 100 (100% irrigation with wastewater).

3.2. Effect of Foliar Application of Fe-lys on Chlorophyll Contents and Gaseous Exchange Attributes under Different Levels of Tannery Wastewater

Photosynthetic pigments, along with different gaseous exchange parameters, were significantly decreased in *S. oleracea* plants when growing in soil having a large amount of tannery wastewater. The increasing level of tannery wastewater (33, 66 and 100%) in soil reduced the photosynthetic pigments and gaseous exchange parameters of *S. oleracea* plants when compared to control treatment. The data regarding these parameters are presented in Figure 3. Figure 3 shows that the maximum contents of chl, carot, Tr, Gs, Pn and Wi in the plants grown under 100% addition of tannery wastewater in the soil decreased (significantly, $p < 0.05$) by 43.1, 28.4, 35.7, 30.1, 71.1 and 41.8%, respectively, compared to the control treatment. Although the exogenous supplementation of Fe-lys increased (significantly, $p < 0.05$) chl, carot, Tr, Gs, Pn and Wi by 16.4, 22.5, 4.5, 34.5, 6.9 and 89.0%, respectively, in the plants grown under 100% addition of tannery wastewater in the soil without supplementation of Fe-lys, compared to the plants grown under 100% addition of tannery wastewater in the soil with the supplementation of Fe-lys. Moreover, it was also noticed that the application of Fe-lys also increased the photosynthetic pigments and gaseous exchange parameters without the addition of different levels of tannery wastewater in the soil.

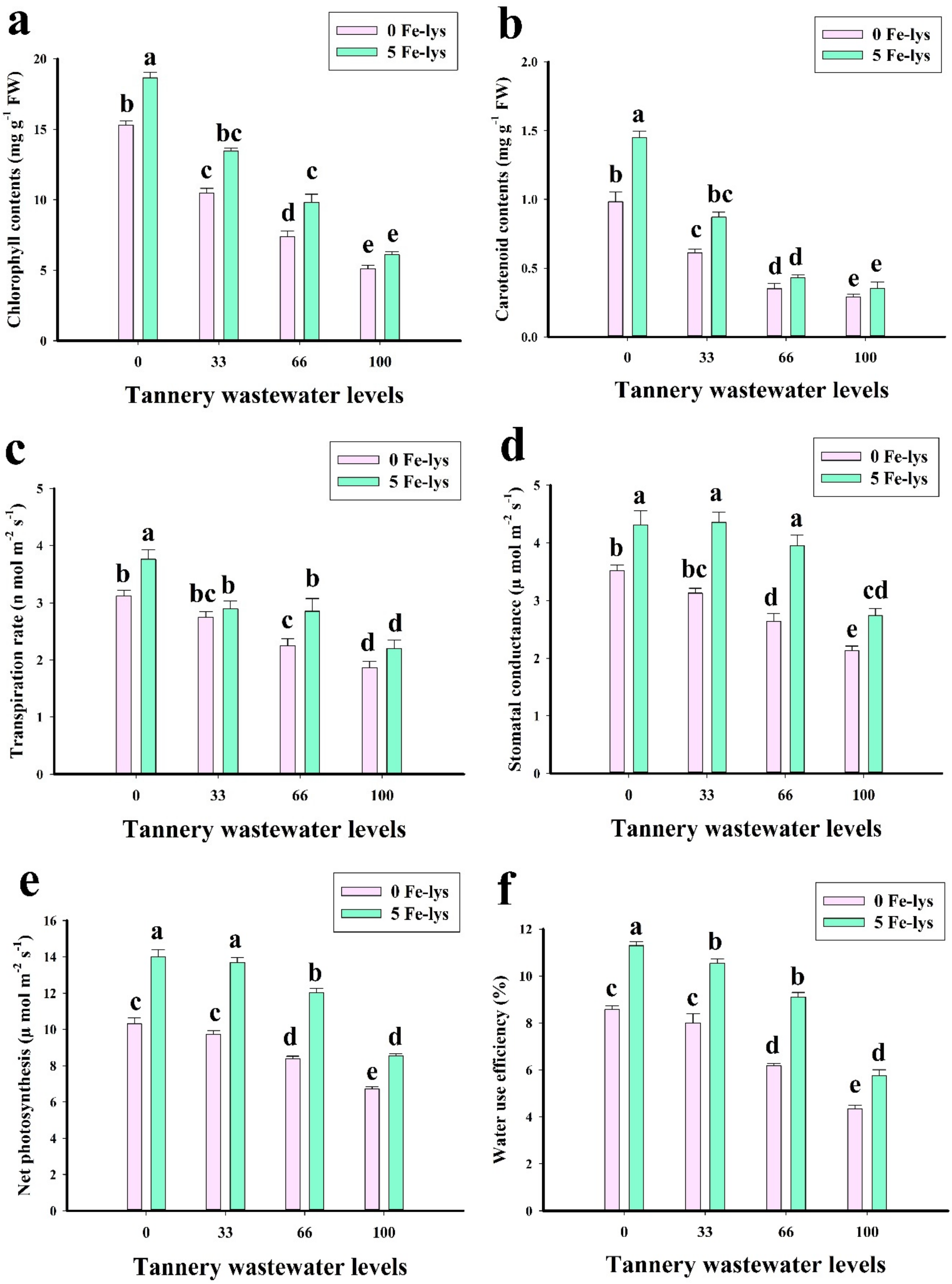

Figure 3. Effect of different levels of tannery wastewater on chlorophyll contents (**a**), carotenoid contents (**b**), transpiration rate (**c**), stomatal conductance (**d**), net photosynthesis (**e**) and water use efficiency (**f**) under the application of iron-lysine to *S. oleracea* plants. Values are demonstrated as means of three replicates along with standard deviation (SD; n = 3). One-way ANOVA was performed, and mean differences were tested by HSD ($p < 0.05$). Different lowercase letters on the error bars indicate significant difference between the treatments. Relative radiance of plastic filter used: 0 (without irrigation with wastewater), 33 (33% irrigation with wastewater), 66 (66% irrigation with wastewater) and 100 (100% irrigation with wastewater).

3.3. Effect of Foliar Application of Fe-lys on Oxidative Stress and Antioxidant Response under Different Levels of Tannery Wastewater

The increased contents of MDA, H_2O_2 and EL (%) indicate that the high contents of metal in the soil used caused oxidative damage in *S. oleracea* plants. Similarly, the activities of various antioxidative enzymes also increased with the addition of tannery wastewater in the soil, when compared to the control treatment. The data regarding oxidative stress and antioxidant response in the roots and leaves of *S. oleracea* plants are presented in Figures 4–6, respectively. Compared to the control treatment, maximum contents of MDA, H_2O_2 initiation and EL (%) were shown in the plants grown in 100% addition of tannery wastewater in the soil, which increased 3.9, 1.8 and 3.6 times, respectively, in the roots and 2.6, 3.4 and 3.2 times, respectively, in the leaves. However, the exogenous application of Fe-lys decreased the contents of MDA, H_2O_2 initiation and EL (%) in the roots as well as in the leaves of *S. oleracea* plants. Similarly, the activities of antioxidants increased with the increase of tannery wastewater in the soil. Compared to the plants grown in the control treatment, the maximum activities of SOD, POD, CAT and APX increased (significantly, $p < 0.05$) by 18.9, 21.6, 29.3 and 36.4%, respectively, in the roots and 28.0, 21.6, 35.6 and 25.2%, respectively, in the leaves of the plants grown in 100% addition of tannery wastewater without the application of Fe-lys. Compared with the plants grown in wastewater treatments, the application of Fe-lys further enhanced the activities of antioxidants in the roots as well as in the leaves or even in the plants grown without the addition of tannery wastewater in the soil (Figures 5 and 6).

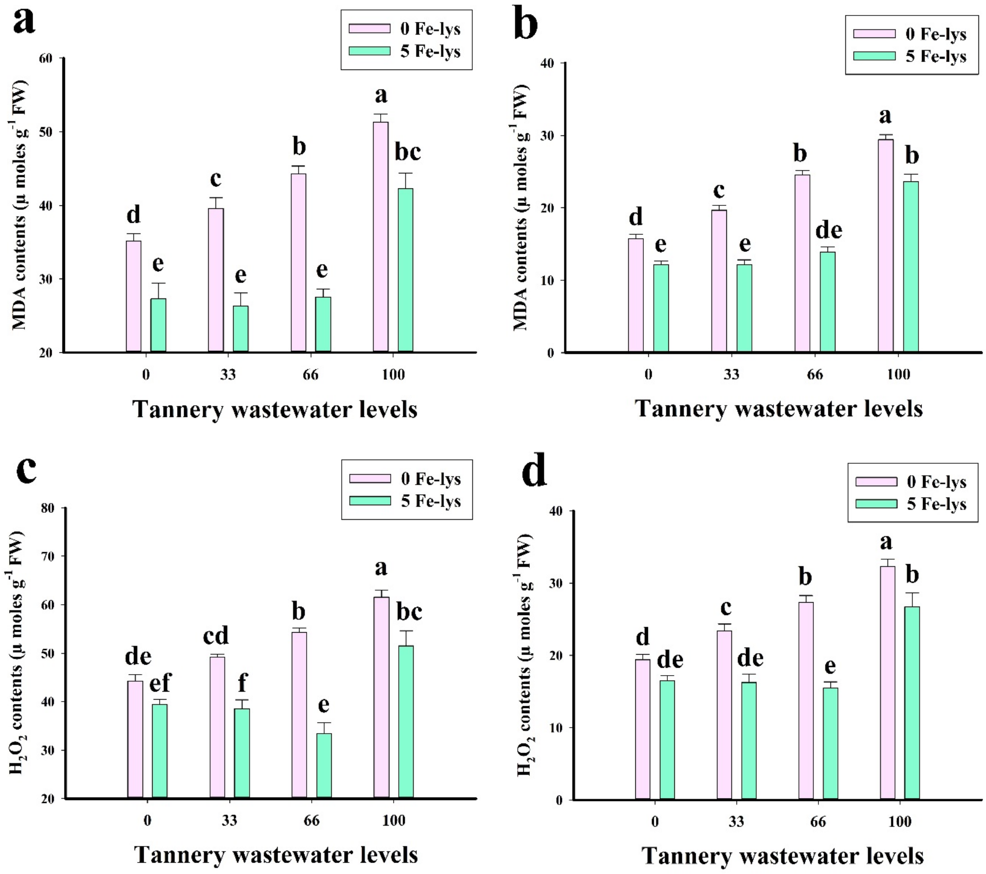

Figure 4. *Cont.*

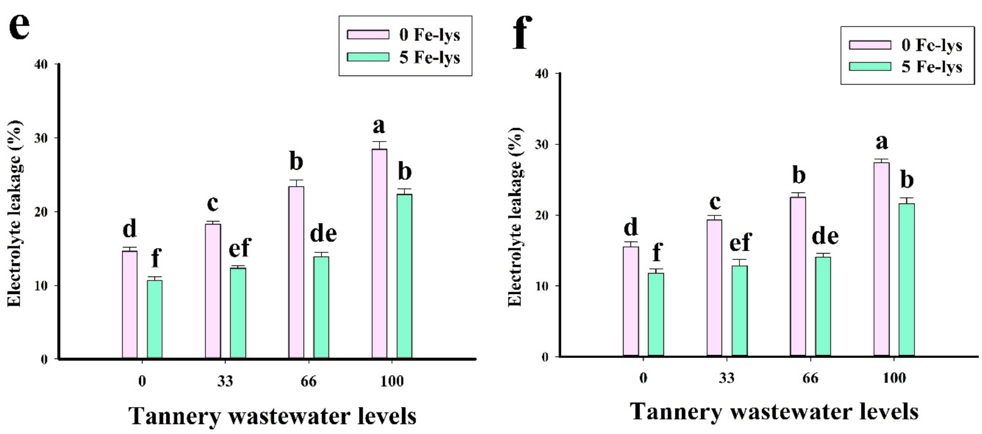

Figure 4. Effect of different levels of tannery wastewater on MDA contents in the roots (**a**), MDA contents in the leaves (**b**), H_2O_2 contents in the roots (**c**), H_2O_2 contents in the leaves (**d**), EL percentage in the roots (**e**) and EL percentage in the leaves (**f**) under the application of iron-lysine to *S. oleracea* plants. Values are demonstrated as means of three replicates along with standard deviation (SD; n = 3). One-way ANOVA was performed, and mean differences were tested by HSD ($p < 0.05$). Different lowercase letters on the error bars indicate significant difference between the treatments. Relative radiance of plastic filter used: 0 (without irrigation with wastewater), 33 (33% irrigation with wastewater), 66 (66% irrigation with wastewater) and 100 (100% irrigation with wastewater).

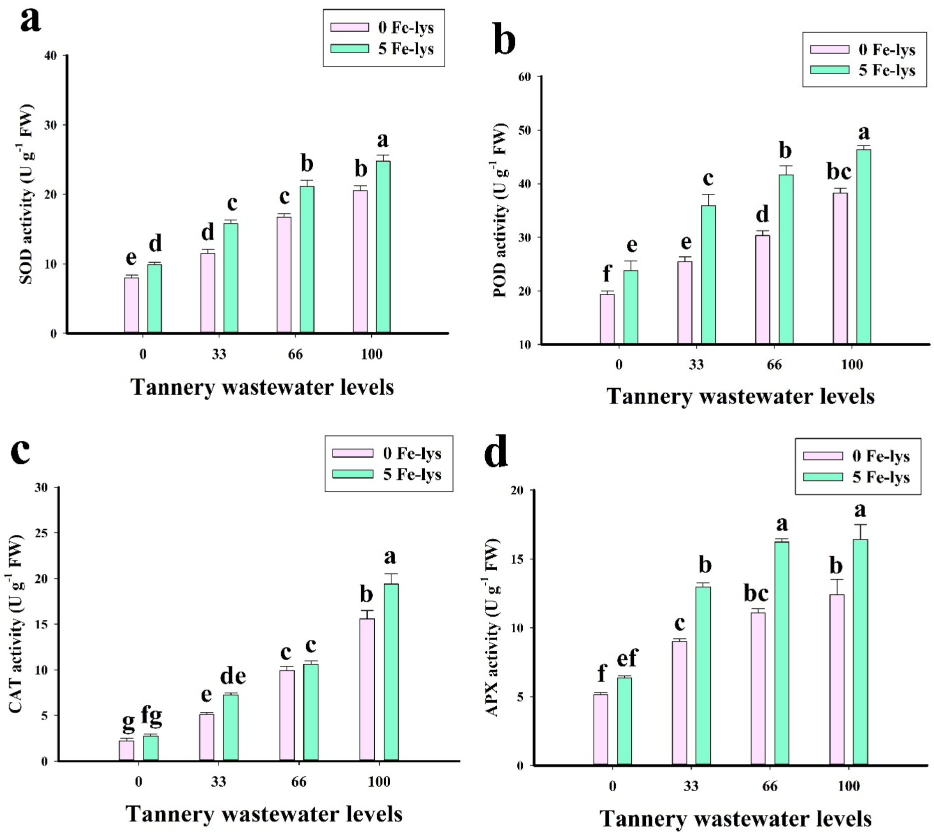

Figure 5. Effect of different levels of tannery wastewater on SOD (**a**), POD (**b**), CAT (**c**) and APX (**d**) in the roots, under the application of iron-lysine to *S. oleracea* plants. Values are demonstrated as means of three replicates along with standard deviation (SD; n = 3). One-way ANOVA was performed, and mean differences were tested by HSD ($p < 0.05$). Different lowercase letters on the error bars indicate significant difference between the treatments. Relative radiance of plastic filter used: 0 (without irrigation with wastewater), 33 (33% irrigation with wastewater), 66 (66% irrigation with wastewater) and 100 (100% irrigation with wastewater).

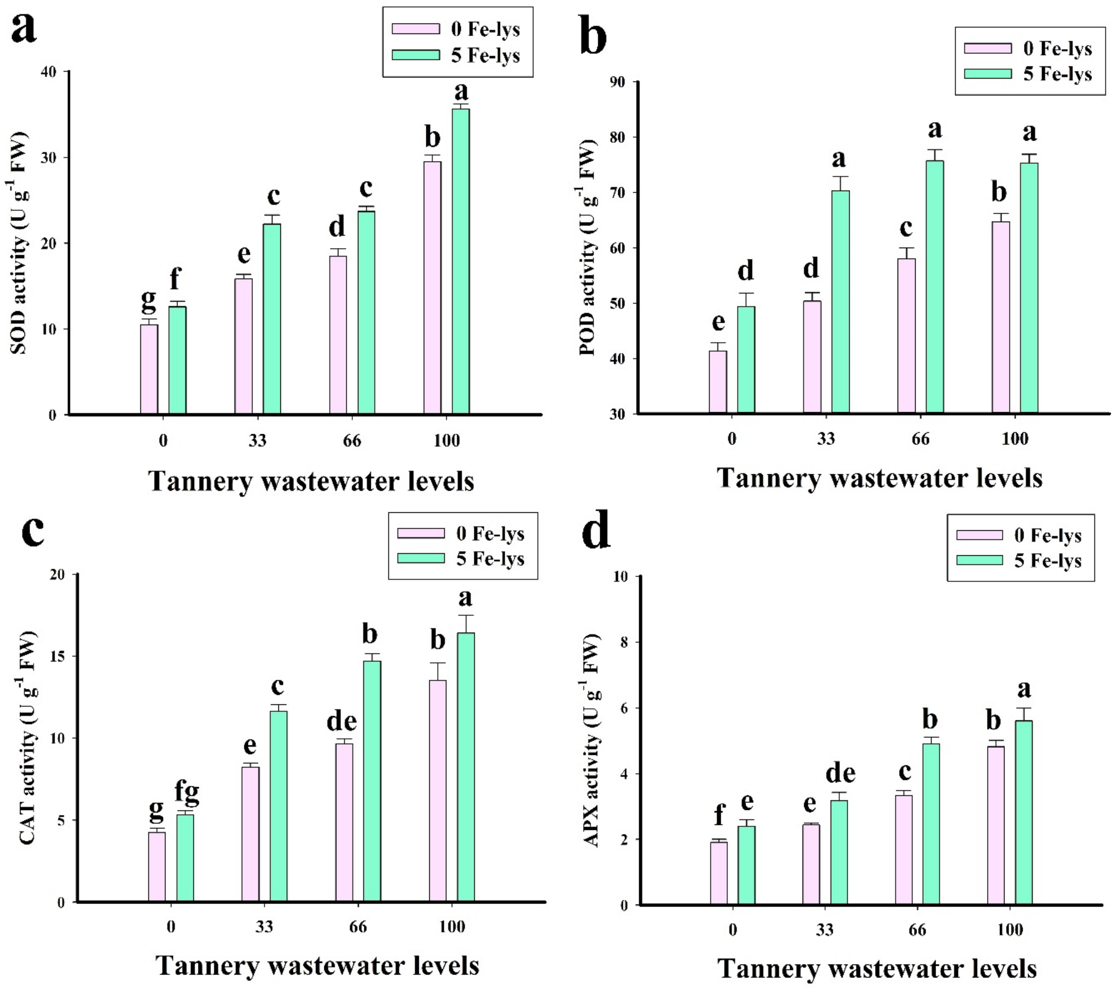

Figure 6. Effect of different levels of tannery wastewater on SOD (**a**), POD (**b**), CAT (**c**) and APX (**d**) in the leaves, under the application of iron-lysine to *S. oleracea* plants. Values are demonstrated as means of three replicates along with standard deviation (SD; n = 3). One-way ANOVA was performed, and mean differences were tested by HSD ($p < 0.05$). Different lowercase letters on the error bars indicate significant difference between the treatments. Relative radiance of plastic filter used: 0 (without irrigation with wastewater), 33 (33% irrigation with wastewater), 66 (66% irrigation with wastewater) and 100 (100% irrigation with wastewater).

3.4. Effect of Foliar Application of Fe-lys on the Uptake and Accumulation of Cr and Fe under Different Levels of Tannery Wastewater

The results of the Cr and Fe uptake in the roots and shoots of *S. oleracea* plants were also determined in this study (Figure 7). The data suggested that increasing levels of wastewater in the soil caused a significant increase in Cr uptake in all organs of the plants. At the same time, a further addition of wastewater caused a significant decrease in Fe contents in all organs of the *S. oleracea* plants (Figure 7). According to the results, maximum contents of Cr were observed in the plants grown in 100% addition of tannery wastewater in the soil, which were 118 mg kg^{-1} in the roots and 33.5 mg kg^{-1} in the shoots of *S. oleracea* plants. In contrast, Fe content was found to be in its maximum level in the control treatment (201 mg kg^{-1} in roots and 132 mg kg^{-1} in the shoots). At the same time, a further addition of wastewater in the soil significantly decreased Fe contents in all organs of *S. oleracea* plants. The exogenous application of Fe-lys decreased Cr contents and increased Fe contents in all the treatments of wastewater compared to the treatments without application of Fe-lys and grown in tannery wastewater. However, we also noticed that the application of Fe-lys decreased Cr contents and increased Fe contents in the control treatment or the plants grown without addition of tannery wastewater in the soil.

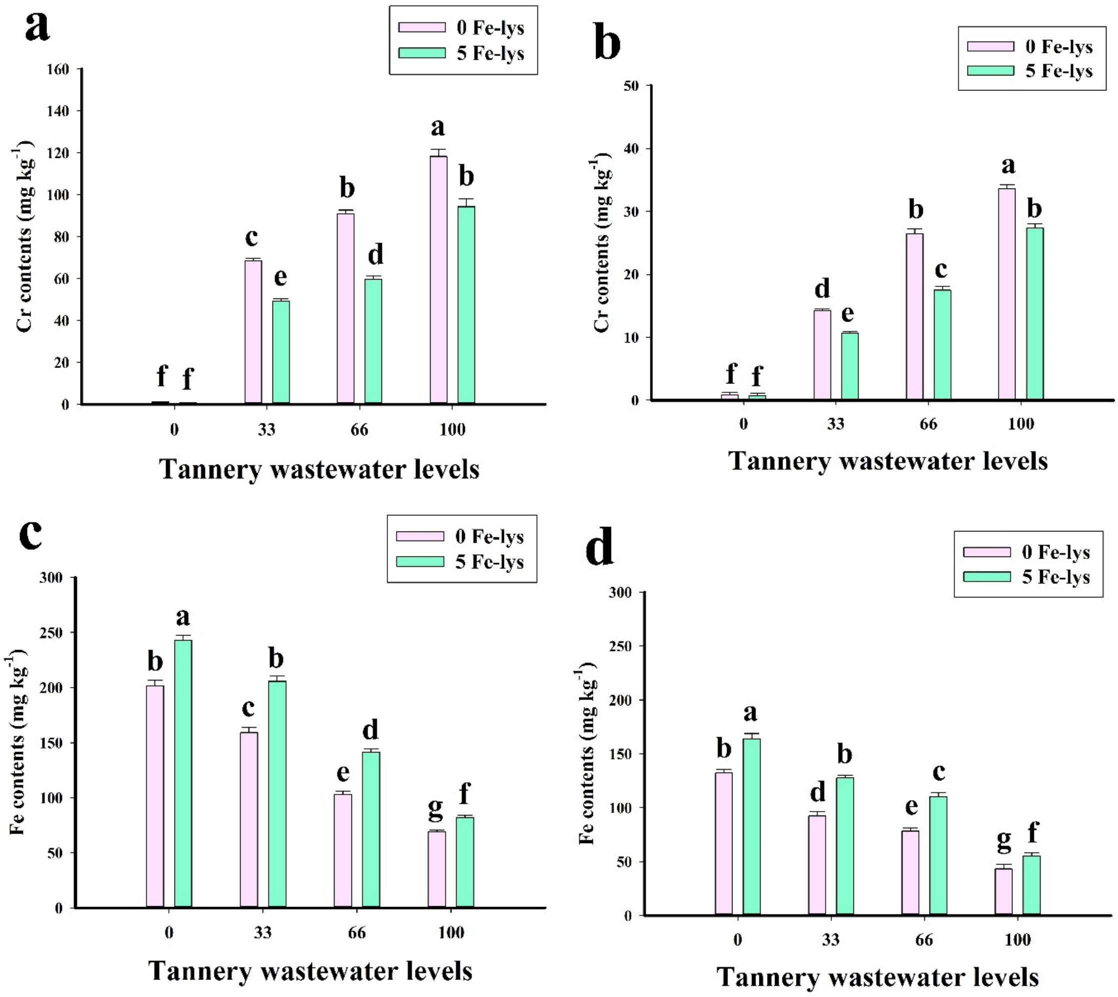

Figure 7. Effect of different levels of tannery wastewater on the uptake/accumulation of Cr contents in the roots (**a**), Cr contents in the shoots (**b**), Fe contents in the roots (**c**) and Fe contents in the shoots (**d**) under the application of iron-lysine to *S. oleracea* plants. Values are demonstrated as means of three replicates along with standard deviation (SD; n = 3). One-way ANOVA was performed, and mean differences were tested by HSD ($p < 0.05$). Different lowercase letters on the error bars indicate significant difference between the treatments. Relative radiance of plastic filter used: 0 (without irrigation with wastewater), 33 (33% irrigation with wastewater), 66 (66% irrigation with wastewater) and 100 (100% irrigation with wastewater).

3.5. Relationship between Morpho-Physiological Attributes and Cr Uptake in Different Parts of the Plants

The Pearson correlation analysis was carried out to quantify the relationship between Cr uptake/accumulation and different morphological and physiological attributes of *S. oleracea* plants (Figure 8). Cr concentration in the roots was positively correlated with Cr concentration in the shoots, malondialdehyde contents in the roots and leaves, hydrogen peroxide contents in the roots and leaves and electrolyte leakage in the roots and leaves of *S. oleracea* plants while negatively correlated with plant height, fresh biomass of roots and leaves, dry biomass of roots and leaves, root length, number of leaves, leaf area, total chlorophyll contents, carotenoid contents, transpiration rate, stomatal conductance, net photosynthesis, water use efficiency, superoxide dismutase activity in the roots and leaves, peroxidase activity in the roots and leaves, catalase activity in the roots and leaves, ascorbate peroxidase activity in the roots and leaves and iron contents in the roots and shoots of *S. oleracea*.

Similarly, Cr concentration in the shoots was positively correlated with Cr concentration in the roots, oxidative stress indicators while negative correlated with all other morpho physiological traits and Fe uptake. This correlation reflected the close connection between Cr uptake and growth in *S. oleracea*.

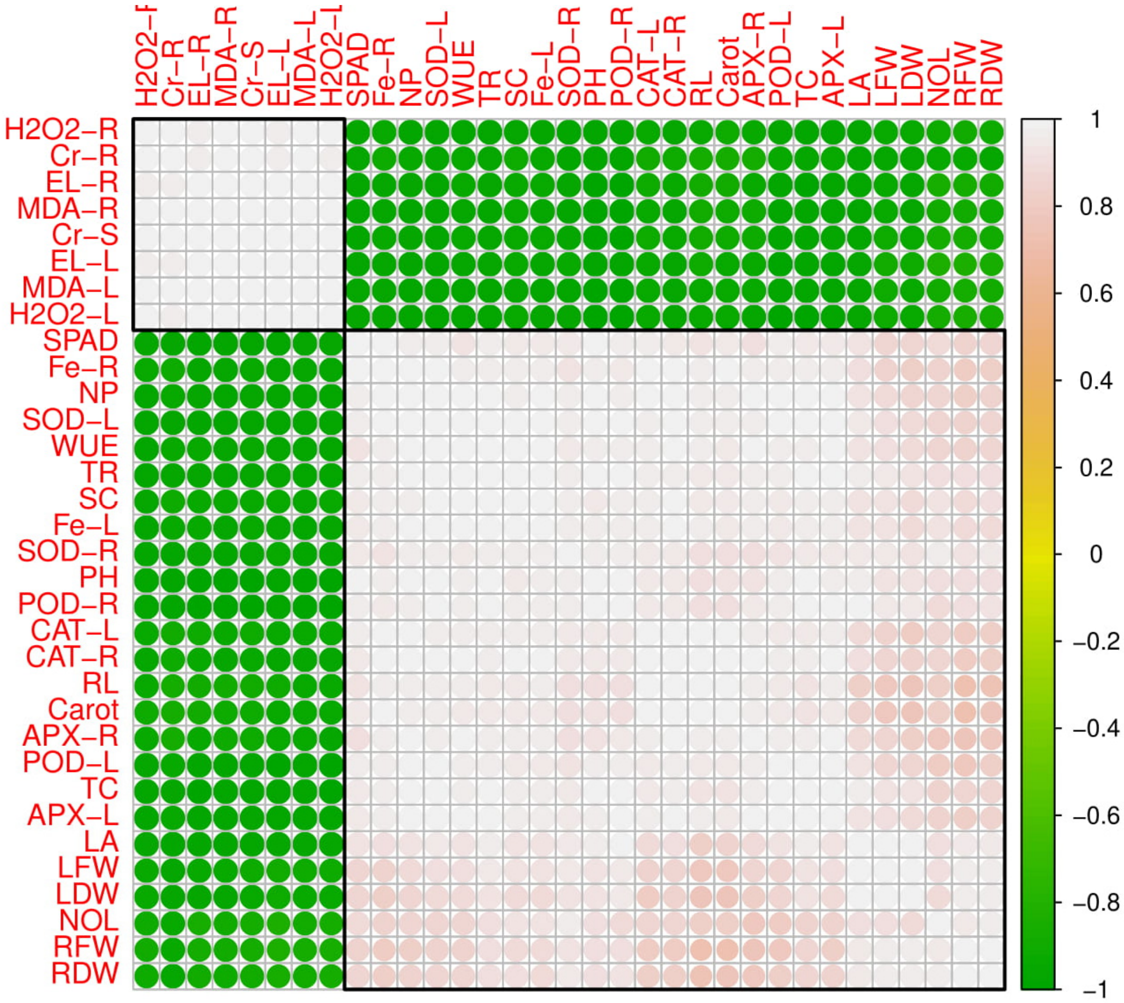

Figure 8. Correlation between different growth and physiological attributes with Cr uptake and accumulation in *S. oleraceas* plants. H2O2-R (H_2O_2 initiation in roots), CR-R (Cr contents in roots), EL-R (electrolyte leakage in roots), MDA-R (MDA contents in roots), CR-S (Cr contents in shoots), EL-L (electrolyte leakage in leaves), MDA-L (MDA contents in leaves), H2O2-L (H_2O_2 initiation in leaves), SPAD (SPAD values), Fe-R (iron contents in roots), NP (net photosynthesis), SOD-L (SOD activity in leaves), WUE (water use efficiency), TR (transpiration rate), SC (stomatal conductance), Fe-L (iron contents in leaves), SOD-R (SOD activity in roots), PH (plant height), POD-R (POD activity in roots), CAT-L (CAT activity in leaves), CAT-R (CAT activity in roots), RL (root length), Carot (carotenoid contents), APX-R (APX activity in roots), POD-L (POD activity in leaves), TC (total chlorophyll contents), APX-L (APX activity in leaves), LA (leaf area), LFW (leaves fresh weight), LDW (leaves dry weight), NOL (number of leaves), RFW (root fresh weight), RDW (root dry weight).

3.6. Principal Component Analysis

The score and loading plots of principal component analysis (PCA) to evaluate the effects of various levels of levels of tannery wastewater with the foliar application of Fe lys on some important studied attributes of *S. oleracea* plants are given in Figure 9. Among all the principal components, first two components i.e. PC1 (Dim 1) and PC2 (Dim 2) exhibited maximum contribution and accounted for 94.8% of the total variance in the database (Figure 9). Of which, PC1 contributed 74.2%, while PC2 contributed 20.6%, accordingly. All of the 8 treatments were distributed successfully by first two principal components. This distribution of treatments gave a clear indication that increasing level of tannery wastewater had significant effects on studied attributes of *S. oleracea* s compared to control. The tannery wastewater treatments were displaced control (1), indicating that higher Cr concentration imposed hazardous impacts on growth and ecophysiology of *S. oleracea* plants. The first group of variables with which PC1 is positively correlated include CAT, EL and Cr contents in the leaves. While, a significant negative correlation of PC1 variables was found with the variables aligned with SOD activity in the roots, Fe contents in the leaves, plant height, leaf area and carotenoid contents in the leaves.

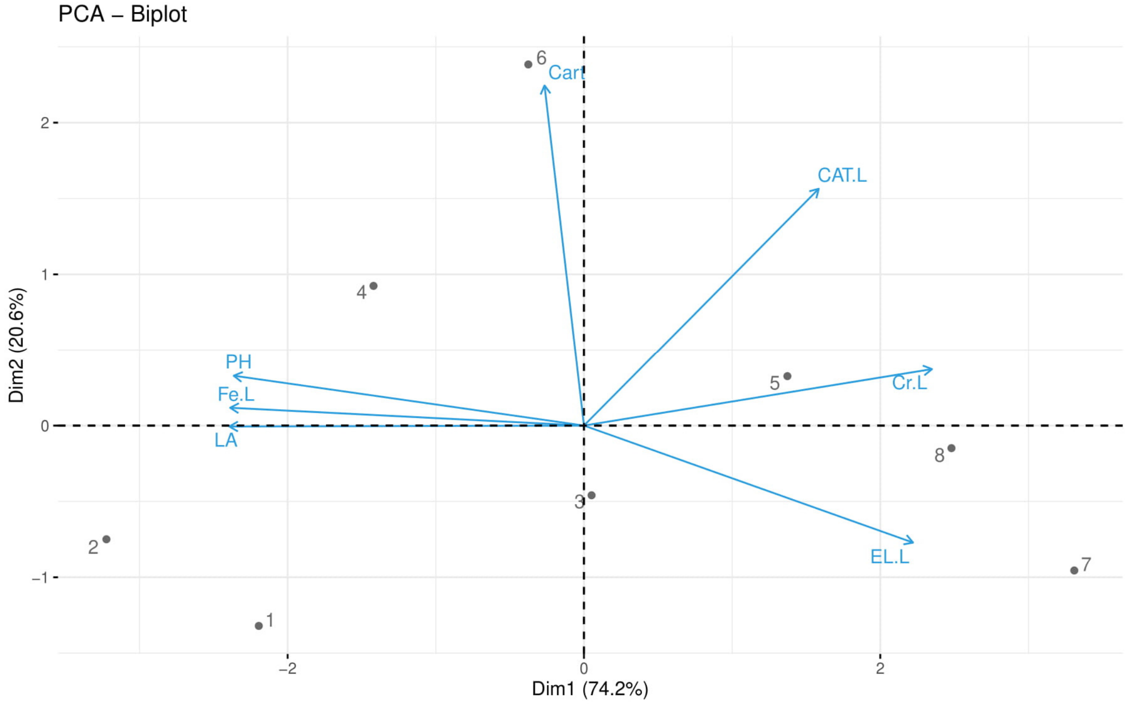

Figure 9. Score and loading plots of principal component analysis (PCA) on different studied attributes of *S. oleracea* plants grown in tannery wastewater soil. Score plot represents the separation of treatments: (1) Cr 0%, Fe-lys 0 mg L^{-1} (2) Cr 0%, Fe-lys 10 mg L^{-1} (3) Cr 33%, Fe-lys 0 mg L^{-1} (4) Cr 33%, Fe -lys 10 mg L^{-1} (5) Cr 66%, Fe-lys 0 mg L^{-1} (6) Cr 66%, Fe -lys 10 mg L^{-1} (7) Cr 100%, Fe-lys 0 mg L^{-1} (8) Cr 100%, Fe -lys 10 mg L^{-1}. The abbreviations are as follows: PH (plant height), Fe-L (Fe contents in the shoots), LA (leaf area), carot (carotenoid contents), CAT.L (CAT activity in the leaves), CR.L (Cr contents in leaves) and EL.L (electrolyte leakage in the leaves).

4. Discussion

High levels of heavy metals in arable land, due to the existence of industrial effluents and industrial waste, pose a serious ecological threat to plants having various important antioxidants [61–65]. The addition of wastewater seriously affects plant growth and biomass [1,6], due to the toxic effects of heavy metals in the soil [66–68]. In the current study, increasing levels of wastewater (33, 66 and 100%) in soil displayed inhibitory effects on all morphological traits of the *S. oleracea* plants, by declining different parameters of plant growth and biomass when compared to the control treatment (Figures 2 and 3). We also elucidated that the application of Fe-lys increased plant growth and biomass even in the toxic levels of Cr in soil (Figures 2 and 3). Several studies showed that the addition of wastewater in the soil directly influenced the plants' composition and caused their significant reduction ($p < 0.05$) [11,34]. This is because Cr is the most toxic heavy metal, causes a delay in seed germination [25] and ultimately decreases plant growth and biomass [16,21,27]. However, the application of Fe-lys ameliorates Cr toxicity in the *S. oleracea* plants by Fe fortification. A similar trend was recorded by Bashir et al. [45], who studied *Oryza sative* growth under toxic levels of Cd and reported that the foliar spray of Fe-lys ameliorates Cd toxicity by decreasing its contents and increasing plant growth and biomass, compared to plants grown in the absence of Fe-lys. In the present study, we also noticed that the application of Fe-lys increased plant growth and biomass in the plants which are not grown in the tannery wastewater soil (Figures 2 and 3). This is because Fe-lys enhances protein properties (metabolic function and stock of amino acid function in plants), increases the photosynthetic processes for creating a healthy plant and provides a substantial growth in a short time in the stress condition or even in the plants grown in a normal soil [45].

Photosynthetic traits are important biological parameters when studying environmental stress response in plants [65,69–71]. In the current study, photosynthetic pigments and gaseous exchange traits were affected significantly ($p < 0.05$) by the addition of different levels of tannery wastewater (33, 66 and 100%) in the soil (Figure 3), while under toxic concentrations of Cr in soil, the photosynthetic machinery was improved by the exogenous application of Fe-lys to Cr-stressed plants (Figure 3). The decrease in chlorophyll contents and gaseous exchange traits under Cr stress is the common response in plants, when they are subjected to the Cr-stressed environment [5,20,72]. Several experiments showed that the photosynthetic machinery was severely affected under a toxic concentration of Cr in soil [17,21,30]. Micronutrients chelated with amino acids increased photosynthetic pigments, compared to plants grown without the application of micronutrient chelation with amino acids [42,73]. In a Cr stress environment, the increase in photosynthetic pigments and gaseous exchange traits might be due to the increase in nutrient uptake and decrease in Cr contents by the plants [74,75].

In addition to the decrease in plant growth and composition, redox imbalance is another toxic effect of Cr in the plants [11,21,26]. Previously, the phytotoxicity of Cr caused oxidative stress in the plants through the generation of extra reactive oxygen species (ROS) [24,72]. However, plants have well-organized enzymatic and non-enzymatic antioxidants which scavenge ROS production [16,76,77]. Toxic levels of Cr in the soil led to oxidative damage to the membranous bounded organelles in *Oryza sative* [27], *Brassica napus* [78] and *Helianthus annuus* [25]. The increasing contents of MDA, initiation of H_2O_2 and EL (%) suggested that the phytotoxicity of Cr may damage cellular organelles and alter the function of the plasma membrane, which ultimately decreases plant growth and biomass, as shown in *Helianthus annuus* by Farid et al. [79]. Cr toxicity also induced oxidative damage by increasing the contents of MDA, H_2O_2 and EL (%) in the roots and leaves of *S. oleracea* plants (Figure 4), and increased the activities of various antioxidants studied by increasing the addition of tannery wastewater in the soil (Figures 5 and 6). Increasing the activities of various antioxidants suggests that the plant has a better defense system, which can scavenge ROS generation [80–82]. Furthermore, foliar applications of Fe-lys to the Cr-stressed plants further enhanced the activities of various antioxidants studied in this experiment, compared to plants grown in the absence of Fe-lys (Figures 5 and 6). Moreover, lower oxidative stress indicators in the plants treated with Fe-lys might be due to the decreased Cr contents and increased Fe contents in the different plant organs (Figure 7). It is well known that the exogenous supplementation of Fe-lys decreases oxidative stress in plants [74,75]. Previously, the foliar spray of Fe-lys decreased the oxidative stress in *Oryza sative* by increasing the activities of various antioxidants, compared to plants grown in the absence of Fe-lys [45].

In the current study, the increasing levels of wastewater (33, 66 and 100%) in the soil increased Cr contents and decreased Fe contents in all organs of *S. oleracea* plants (Figure 7). However, the exogenous supplementation of Fe-lys decreased Cr contents and increased the essential micronutrient (Fe) in all organs of *S. oleracea* plants (Figure 7). In many previous studies, it was also observed that the increasing levels of Cr in the soil caused a significant ($p < 0.05$) increase in Cr contents in all organs of the plants [5,11,19,21]. The decrease in essential nutrients such as Fe might be linked with the decrease in the nutrient uptake by the plants, which might be due to excess contents of Cr in the soil [18,19,24,41]. It is also well documented that Cr is extremely toxic for plants and disturbs metabolic processes, which ultimately decreases the quantity of essential nutrients in plant organs and thus affects plant growth and yield [16,72,83]. Previously, Zaheer et al. [5] also reported the same trend, showing that various levels of Cr in the soil also enhance Cr contents in different plant parts and reduce essential nutrients due to excess contents of Cr in plant tissues. However, the availability and toxicity of Cr can be overcome by the exogenous application of micronutrients chelated with amino acids [42,75]. The present study also demonstrated that the exogenous supplementation of Fe-lys decreased Cr contents and increased Fe contents in various parts of *S. oleracea* plants, compared to plants grown in the absence of Fe-lys (Figure 7). The reduced Cr concentration in all organs of *S. oleracea* plants might be due to the higher Fe contents in the plants, as Cr and Fe showed antagonistic effects reported previously by Danish et al. [29]. However, the reduction in Cr contents might also be due to the increase in Fe contents in different organs of the plants (Figure 7). Moreover, under flooded conditions, Fe^{2+} is the main form of Fe in the soil, which may compete with Cr at the root surface during plant uptake [11]. Bashir et al. [45] reported that foliar spray of Fe-lys increased the Fe contents and decreased the heavy metal (Cd) contents in all parts of rice (*Oryza sative*) plants, compared to plants grown in the absence of Fe-lys. It was also reported that both Fe and Cr compete in an environment with an excess of Cr, while the external application of Fe-lys helps the plants to uptake large amounts of Fe and decrease the uptake of Cr by the plant organs [45]. Taken together, the application of Fe-lys helps the plants to maintain their growth and composition and also promotes the uptake of Fe contents and decrease Cr contents in plants grown in an environment with an excess of Cr.

5. Conclusions

The irrigation of green leafy vegetable crops, such as *S. oleracea*, with tannery wastewater is dangerous because the latter may contain toxic pollutants (i.e., Cr) which significantly affect the plants' growth and yield. The phytotoxicity of Cr also decreases plant photosynthetic pigments and induces oxidative damage due to the overproduction of ROS in internal tissues. Moreover, a toxic level of Cr in the soil decreases the essential nutrients (i.e., Fe) in the plant organs, which are essential for the normal growth and development of the plant's body. The phytotoxicity of Cr can be overcome by the exogenous application of Fe-lys, which increases not only the morphological traits but also the activities of various antioxidants, which helps in mitigating oxidative stress in the plants. Moreover, the supplementation of Fe-lys increases the amino acids in the plant organs which restrict the movement of toxic heavy metals and reduce their availability for the plant. Hence, the application of Fe-lys under a heavy metal stress environment is a safer option to get the maximum yield from green leafy vegetable crops, to fulfill the market demand for the vegetables.

Author Contributions: Conceptualization, S.A., M.H.S., K.H. and Z.A.; data curation, M.H.S., I.N. and M.R.; formal analysis, M.A.E.-E., K.H. and M.N.A.; funding acquisition, I.E.Z., M.R., Z.A., M.A.E.-S. and M.N.A.; investigation, I.E.Z., I.N., M.N.A., M.A.E.-E. and L.W.; methodology, M.R., M.A.E.-S., M.A.E.-E., M.N.A. and L.W.; project administration, M.A.E.-S. and L.W.; resources, M.H.S., M.A.E.-E., K.H., M.R. and L.W.; software, I.E.Z., M.H.S., I.N., M.A.E.-E., K.H., Z.A., and L.W.; supervision, S.A. and M.A.E.-E.; validation, I.E.Z.; visualization, S.A. and K.H.; writing—original draft, M.H.S., I.N., Z.A. and M.A.E.-E.; writing—review & editing, S.A., M.A.E.-E. and M.H.S. All authors have read and agreed to the published version of the manuscript.

Funding: The authors would like to express their sincere appreciation to the Researchers Supporting Project Number (RSP-2020/180), King Saud University, Riyadh, Saudi Arabia. The authors are deeply indebted to the Government College University, Faisalabad, Pakistan, for its support.

Acknowledgments: The authors would like to express their sincere appreciation to the Researchers Supporting Project Number (RSP-2020/180), King Saud University, Riyadh, Saudi Arabia. The authors are deeply indebted to the Government College University, Faisalabad, Pakistan, for its support.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hashem, I.A.; Abbas, A.Y.; Abd El-Hamed, A.E.-N.H.; Salem, H.M.S.; El-hosseiny, O.E.M.; Abdel-Salam, M.A.; Saleem, M.H.; Zhou, W.; Hu, R. Potential of rice straw biochar, sulfur and ryegrass (*Lolium perenne* L.) in remediating soil contaminated with nickel through irrigation with untreated wastewater. *Peer J.* **2020**, *8*, e9267. [CrossRef] [PubMed]

2. Rehman, M.; Liu, L.; Bashir, S.; Saleem, M.H.; Chen, C.; Peng, D.; Siddique, K.H. Influence of rice straw biochar on growth, antioxidant capacity and copper uptake in ramie (*Boehmeria nivea* L.) grown as forage in aged copper-contaminated soil. *Plant. Physiol. Biochem.* **2019**, *138*, 121–129. [CrossRef] [PubMed]
3. Saleem, M.; Ali, S.; Rehman, M.; Rana, M.; Rizwan, M.; Kamran, M.; Imran, M.; Riaz, M.; Hussein, M.; Elkelish, A. Influence of phosphorus on copper phytoextraction via modulating cellular organelles in two jute (*Corchorus capsularis* L.) varieties grown in a copper mining soil of Hubei Province, China. *Chemosphere* **2020**, *248*, 126023. [CrossRef] [PubMed]
4. ŞENKAL, B.C.; USKUTOĞLU, T.; CESUR, C.; ÖZAVCI, V.; DOĞAN, H. Determination of essential oil components, mineral matter, and heavy metal content of *Salvia virgata* Jacq. grown in culture conditions. *Turk. J. Agric. For.* **2019**, *43*, 395–404. [CrossRef]
5. Zaheer, I.E.; Ali, S.; Rizwan, M.; Abbas, Z.; Bukhari, S.A.H.; Wijaya, L.; Alyemeni, M.N.; Ahmad, P. Zinc-lysine prevents chromium-induced morphological, photosynthetic, and oxidative alterations in spinach irrigated with tannery wastewater. *Environ. Sci. Pollut. Res.* **2019**, *26*, 28951–28961. [CrossRef] [PubMed]
6. Kamran, M.; Malik, Z.; Parveen, A.; Huang, L.; Riaz, M.; Bashir, S.; Mustafa, A.; Abbasi, G.H.; Xue, B.; Ali, U. Ameliorative Effects of Biochar on Rapeseed (*Brassica napus* L.) Growth and Heavy Metal Immobilization in Soil Irrigated with Untreated Wastewater. *J. Plant. Growth Regul.* **2020**, *39*, 266–281. [CrossRef]
7. Zhou, Q.; Li, X.; Lin, Y.; Yang, C.; Tang, W.; Wu, S.; Li, D.; Lou, W. Effects of copper ions on removal of nutrients from swine wastewater and on release of dissolved organic matter in duckweed systems. *Water Res.* **2019**, *158*, 171–181.
8. Rana, M.S.; Hu, C.X.; Shaaban, M.; Imran, M.; Afzal, J.; Moussa, M.G.; Elyamine, A.M.; Bhantana, P.; Saleem, M.H.; Syaifudin, M. Soil phosphorus transformation characteristics in response to molybdenum supply in leguminous crops. *J. Environ. Manag.* **2020**, *268*, 110610. [CrossRef]
9. Saleem, M.H.; Ali, S.; Hussain, S.; Kamran, M.; Chattha, M.S.; Ahmad, S.; Aqeel, M.; Rizwan, M.; Aljarba, N.H.; Alkahtani, S. Flax (*Linum usitatissimum* L.): A Potential Candidate for Phytoremediation? Biological and Economical Points of View. *Plants* **2020**, *9*, 496. [CrossRef]
10. Saleem, M.H.; Ali, S.; Rehman, M.; Hasanuzzaman, M.; Rizwan, M.; Irshad, S.; Shafiq, F.; Iqbal, M.; Alharbi, B.M.; Alnusaire, T.S. Jute: A Potential Candidate for Phytoremediation of Metals—A Review. *Plants* **2020**, *9*, 258. [CrossRef]
11. Maqbool, A.; Ali, S.; Rizwan, M.; Ishaque, W.; Rasool, N.; ur Rehman, M.Z.; Bashir, A.; Abid, M.; Wu, L. Management of tannery wastewater for improving growth attributes and reducing chromium uptake in spinach through citric acid application. *Environ. Sci. Pollut. Res.* **2018**, *25*, 10848–10856. [CrossRef] [PubMed]
12. Tai, Y.; Yang, Y.; Li, Z.; Yang, Y.; Wang, J.; Zhuang, P.; Zou, B. Phytoextraction of 55-year-old wastewater-irrigated soil in a Zn–Pb mine district: Effect of plant species and chelators. *Environ. Technol.* **2018**, *39*, 2138–2150. [CrossRef] [PubMed]
13. Zia, M.H.; Watts, M.J.; Niaz, A.; Middleton, D.R.; Kim, A.W. Health risk assessment of potentially harmful elements and dietary minerals from vegetables irrigated with untreated wastewater, Pakistan. *Environ. Geochem. Health* **2017**, *39*, 707–728. [CrossRef] [PubMed]
14. Rizwan, M.; Ali, S.; Hussain, A.; Ali, Q.; Shakoor, M.B.; Zia-ur-Rehman, M.; Farid, M.; Asma, M. Effect of zinc-lysine on growth, yield and cadmium uptake in wheat (*Triticum aestivum* L.) and health risk assessment. *Chemosphere* **2017**, *187*, 35–42. [CrossRef] [PubMed]
15. El-Demerdash, F.M.; Jebur, A.B.; Nasr, H.M.; Hamid, H.M. Modulatory effect of Turnera diffusa against testicular toxicity induced by fenitrothion and/or hexavalent chromium in rats. *Environ. Toxicol.* **2019**, *34*, 330–339. [CrossRef] [PubMed]
16. Singh, H.P.; Mahajan, P.; Kaur, S.; Batish, D.R.; Kohli, R.K. Chromium toxicity and tolerance in plants. *Environ. Chem. Lett.* **2013**, *11*, 229–254. [CrossRef]
17. Tang, R.; Li, X.; Mo, Y.; Ma, Y.; Ding, C.; Wang, J.; Zhang, T.; Wang, X. Toxic responses of metabolites, organelles and gut microorganisms of Eisenia fetida in a soil with chromium contamination. *Environ. Pollut.* **2019**, *251*, 910–920. [CrossRef]
18. Gill, R.A.; Ali, B.; Cui, P.; Shen, E.; Farooq, M.A.; Islam, F.; Ali, S.; Mao, B.; Zhou, W. Comparative transcriptome profiling of two Brassica napus cultivars under chromium toxicity and its alleviation by reduced glutathione. *BMC Genom.* **2016**, *17*, 885. [CrossRef]

19. Riaz, M.; Yasmeen, T.; Arif, M.S.; Ashraf, M.A.; Hussain, Q.; Shahzad, S.M.; Rizwan, M.; Mehmood, M.W.; Zia, A.; Mian, I.A. Variations in morphological and physiological traits of wheat regulated by chromium species in long-term tannery effluent irrigated soils. *Chemosphere* **2019**, *222*, 891–903. [CrossRef]
20. Ali, S.; Chaudhary, A.; Rizwan, M.; Anwar, H.T.; Adrees, M.; Farid, M.; Irshad, M.K.; Hayat, T.; Anjum, S.A. Alleviation of chromium toxicity by glycinebetaine is related to elevated antioxidant enzymes and suppressed chromium uptake and oxidative stress in wheat (*Triticum aestivum* L.). *Environ. Sci. Pollut. Res.* **2015**, *22*, 10669–10678. [CrossRef]
21. Li, L.; Zhang, K.; Gill, R.A.; Islam, F.; Farooq, M.A.; Wang, J.; Zhou, W. Ecotoxicological and Interactive Effects of Copper and Chromium on Physiochemical, Ultrastructural, and Molecular Profiling in *Brassica napus* L. *BioMed Res. Int.* **2018**, *2018*, 17. [CrossRef] [PubMed]
22. Sallah-Ud-Din, R.; Farid, M.; Saeed, R.; Ali, S.; Rizwan, M.; Tauqeer, H.M.; Bukhari, S.A.H. Citric acid enhanced the antioxidant defense system and chromium uptake by *Lemna minor* L. grown in hydroponics under Cr stress. *Environ. Sci. Pollut. Res.* **2017**, *24*, 17669–17678. [CrossRef] [PubMed]
23. Medda, S.; Mondal, N.K. Chromium toxicity and ultrastructural deformation of Cicer arietinum with special reference of root elongation and coleoptile growth. *Ann. Agrar. Sci.* **2017**, *15*, 396–401. [CrossRef]
24. Farid, M.; Ali, S.; Rizwan, M.; Ali, Q.; Saeed, R.; Nasir, T.; Abbasi, G.H.; Rehmani, M.I.A.; Ata-Ul-Karim, S.T.; Bukhari, S.A.H. Phyto-management of chromium contaminated soils through sunflower under exogenously applied 5-aminolevulinic acid. *Ecotoxicol. Environ. Saf.* **2018**, *151*, 255–265. [CrossRef] [PubMed]
25. Farid, M.; Ali, S.; Rizwan, M.; Ali, Q.; Abbas, F.; Bukhari, S.A.H.; Saeed, R.; Wu, L. Citric acid assisted phytoextraction of chromium by sunflower; morpho-physiological and biochemical alterations in plants. *Ecotoxicol. Environ. Saf.* **2017**, *145*, 90–102. [CrossRef] [PubMed]
26. Hussain, A.; Ali, S.; Rizwan, M.; ur Rehman, M.Z.; Hameed, A.; Hafeez, F.; Alamri, S.A.; Alyemeni, M.N.; Wijaya, L. Role of zinc–lysine on growth and chromium uptake in rice plants under Cr stress. *J. Plant Growth Regul.* **2018**, *37*, 1413–1422. [CrossRef]
27. Yu, X.-Z.; Lu, C.-J.; Li, Y.-H. Role of cytochrome c in modulating chromium-induced oxidative stress in Oryza sativa. *Environ. Sci. Pollut. Res.* **2018**, *25*, 27639–27649. [CrossRef]
28. Yu, X.-Z.; Gu, J.-D. The role of EDTA in phytoextraction of hexavalent and trivalent chromium by two willow trees. *Ecotoxicology* **2008**, *17*, 143–152. [CrossRef]
29. Danish, S.; Kiran, S.; Fahad, S.; Ahmad, N.; Ali, M.A.; Tahir, F.A.; Rasheed, M.K.; Shahzad, K.; Li, X.; Wang, D. Alleviation of chromium toxicity in maize by Fe fortification and chromium tolerant ACC deaminase producing plant growth promoting rhizobacteria. *Ecotoxicol. Environ. Saf.* **2019**, *185*, 109706. [CrossRef]
30. Danish, S.; Tahir, F.; Rasheed, M.; Ahmad, N.; Ali, M.; Kiran, S.; Younis, U.; Irshad, I.; Butt, B. Comparative effect of foliar application of Fe and banana peel biochar addition in spinach for alleviation of chromium (IV) toxicity. *Open Agric* **2019**, *4*, 381–390. [CrossRef]
31. Jiraungkoorskul, W. Review of neuro-nutrition used as anti-alzheimer plant, spinach, *Spinacia oleracea*. *Pharmacogn. Rev.* **2016**, *10*, 105. [CrossRef] [PubMed]
32. Agarwal, A.; Gupta, S.D.; Barman, M.; Mitra, A. Photosynthetic apparatus plays a central role in photosensitive physiological acclimations affecting spinach (*Spinacia oleracea* L.) growth in response to blue and red photon flux ratios. *Environ. Exp. Bot.* **2018**, *156*, 170–182. [CrossRef]
33. Jabeen, M.; Akram, N.A.; Aziz, M.A.; Aniqa, A. Assessment of Biochemical Changes in Spinach (*Spinacea oleracea* L.) Subjected to Varying Water Regimes. *Sains Malays.* **2019**, *48*, 533–541. [CrossRef]
34. Ahmed, D.A.; Slima, D.F. Heavy metal accumulation by *Corchorus olitorius* L. irrigated with wastewater. *Environ. Sci. Pollut. Res.* **2018**, *25*, 14996–15005. [CrossRef]
35. Sangeetha, V.; Sharavanan, P. Use of tannery effluent for irrigation: An evaluative study on the response of Sorghum plants its growth and biochemical characteristics. *J. Appl. Adv. Res.* **2018**, *3*, 135–138. [CrossRef]
36. Ali, S.; Zeng, F.; Qiu, L.; Zhang, G. The effect of chromium and aluminum on growth, root morphology, photosynthetic parameters and transpiration of the two barley cultivars. *Biol. Plant.* **2011**, *55*, 291–296. [CrossRef]
37. Saleem, M.H.; Kamran, M.; Zhou, Y.; Parveen, A.; Rehman, M.; Ahmar, S.; Malik, Z.; Mustafa, A.; Anjum, R.M.A.; Wang, B. Appraising growth, oxidative stress and copper phytoextraction potential of flax (*Linum usitatissimum* L.) grown in soil differentially spiked with copper. *J. Environ. Manag.* **2020**, *257*, 109994. [CrossRef]

38. Saleem, M.H.; Fahad, S.; Rehman, M.; Saud, S.; Jamal, Y.; Khan, S.; Liu, L. Morpho-physiological traits, biochemical response and phytoextraction potential of short-term copper stress on kenaf (*Hibiscus cannabinus* L.) seedlings. *Peer J.* **2020**, *8*, e8321. [CrossRef]
39. Parveen, A.; Saleem, M.H.; Kamran, M.; Haider, M.Z.; Chen, J.-T.; Malik, Z.; Rana, M.S.; Hassan, A.; Hur, G.; Javed, M.T. Effect of Citric Acid on Growth, Ecophysiology, Chloroplast Ultrastructure, and Phytoremediation Potential of Jute (*Corchorus capsularis* L.) Seedlings Exposed to Copper Stress. *Biomolecules* **2020**, *10*, 592. [CrossRef]
40. Saleem, M.H.; Ali, S.; Rehman, M.; Rizwan, M.; Kamran, M.; Mohamed, I.A.; Bamagoos, A.A.; Alharby, H.F.; Hakeem, K.R.; Liu, L. Individual and combined application of EDTA and citric acid assisted phytoextraction of copper using jute (*Corchorus capsularis* L.) seedlings. *Environ. Technol. Innov.* **2020**, *19*, 100895. [CrossRef]
41. Gill, R.A.; Ali, B.; Islam, F.; Farooq, M.A.; Gill, M.B.; Mwamba, T.M.; Zhou, W. Physiological and molecular analyses of black and yellow seeded Brassica napus regulated by 5-aminolivulinic acid under chromium stress. *Plant Physiol. Biochem.* **2015**, *94*, 130–143. [CrossRef] [PubMed]
42. Souri, M.K. Aminochelate fertilizers: The new approach to the old problem; a review. *Open Agric.* **2016**, *1*, 118–123. [CrossRef]
43. Rout, G.R.; Sahoo, S. Role of iron in plant growth and metabolism. *Rev. Agric. Sci.* **2015**, *3*, 1–24. [CrossRef]
44. Zhao, Y.; Zhang, C.; Wang, C.; Huang, Y.; Liu, Z. Increasing phosphate inhibits cadmium uptake in plants and promotes synthesis of amino acids in grains of rice. *Environ. Pollut.* **2019**, *257*, 113496. [CrossRef]
45. Bashir, A.; Rizwan, M.; Ali, S.; ur Rehman, M.Z.; Ishaque, W.; Riaz, M.A.; Maqbool, A. Effect of foliar-applied iron complexed with lysine on growth and cadmium (Cd) uptake in rice under Cd stress. *Environ. Sci. Pollut. Res.* **2018**, *25*, 20691–20699. [CrossRef]
46. Walkley, A.; Black, I.A. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil Sci.* **1934**, *37*, 29–38. [CrossRef]
47. Bouyoucos, G.J. Hydrometer method improved for making particle size analyses of soils 1. *Agron. J.* **1962**, *54*, 464–465. [CrossRef]
48. Page, A. *Methods of Soil Analysis. Part. 2. Chemical and Microbiological Properties*; American Society of Agronomy, Soil Science Society of America: Madison, WI, USA, 1965.
49. Amacher, M.C. Nickel, cadmium, and lead. *Methods Soil Anal. Part 3 Chem. Methods* **1996**, *5*, 739–768.
50. Soltanpour, P. Use of ammonium bicarbonate DTPA soil test to evaluate elemental availability and toxicity. *Commun. Soil Sci. Plant. Anal.* **1985**, *16*, 323–338. [CrossRef]
51. Apha, A. *WPCF, Standard Methods for the Examination of Water and Wastewater*; American Public Health Association/American Water Works Association/Water Environment Federation: Washington DC, USA, 1995.
52. Ayers, R.; Westcot, D. FAO Irrigation and Drainage Paper 29 Rev. 1. In *Water Quality for Agriculture*; Food and Agriculture Organization of the United Nations: Roma, Italy, 1985; Volume 15, p. 2016.
53. Lichtenthaler, H.K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 1987; Volume 148, pp. 350–382.
54. Heath, R.L.; Packer, L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **1968**, *125*, 189–198. [CrossRef]
55. Dionisio-Sese, M.L.; Tobita, S. Antioxidant responses of rice seedlings to salinity stress. *Plant Sci.* **1998**, *135*, 1–9. [CrossRef]
56. Chen, C.-N.; Pan, S.-M. Assay of superoxide dismutase activity by combining electrophoresis and densitometry. *Bot. Bull. Acad. Sin.* **1996**, *37*, 107–111.
57. Sakharov, I.Y.; Ardila, G.B. Variations of peroxidase activity in cocoa (*Theobroma cacao* L.) beans during their ripening, fermentation and drying. *Food Chem.* **1999**, *65*, 51–54. [CrossRef]
58. Aebi, H. Catalase in vitro. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 1984; Volume 105, pp. 121–126.
59. Nakano, Y.; Asada, K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* **1981**, *22*, 867–880.
60. Rehman, M.Z.-u.; Rizwan, M.; Ghafoor, A.; Naeem, A.; Ali, S.; Sabir, M.; Qayyum, M.F. Effect of inorganic amendments for in situ stabilization of cadmium in contaminated soils and its phyto-availability to wheat and rice under rotation. *Environ. Sci. Pollut. Res.* **2015**, *22*, 16897–16906. [CrossRef]

61. Saleem, M.H.; Ali, S.; Seleiman, M.F.; Rizwan, M.; Rehman, M.; Akram, N.A.; Liu, L.; Alotaibi, M.; Al-Ashkar, I.; Mubushar, M. Assessing the Correlations between Different Traits in Copper-Sensitive and Copper-Resistant Varieties of Jute (*Corchorus capsularis* L.). *Plants* **2019**, *8*, 545. [CrossRef]
62. El-Esawi, M.A.; Elkelish, A.; Elansary, H.O.; Ali, H.M.; Elshikh, M.; Witczak, J.; Ahmad, M. Genetic transformation and hairy root induction enhance the antioxidant potential of *Lactuca serriola* L. *Oxid. Med. Cell. Longev.* **2017**, *2017*, 5604746. [CrossRef]
63. El-Esawi, M.A. Genetic diversity and evolution of Brassica genetic resources: From morphology to novel genomic technologies—A review. *Plant. Genet. Resour.* **2017**, *15*, 388–399. [CrossRef]
64. El-Esawi, M.A.; Germaine, K.; Bourke, P.; Malone, R. AFLP analysis of genetic diversity and phylogenetic relationships of *Brassica oleracea* in Ireland. *C. R. Biol.* **2016**, *339*, 163–170. [CrossRef]
65. Saleem, M.H.; Fahad, S.; Khan, S.U.; Din, M.; Ullah, A.; Sabagh, A.E.L.; Hossain, A.; Llanes, A.; Liu, L. Copper-induced oxidative stress, initiation of antioxidants and phytoremediation potential of flax (*Linum usitatissimum* L.) seedlings grown under the mixing of two different soils of China. *Environ. Sci. Pollut. Res.* **2020**, *27*, 5211–5221. [CrossRef] [PubMed]
66. Saleem, M.H.; Ali, S.; Kamran, M.; Iqbal, N.; Azeem, M.; Tariq Javed, M.; Ali, Q.; Zulqurnain Haider, M.; Irshad, S.; Rizwan, M. Ethylenediaminetetraacetic Acid (EDTA) Mitigates the Toxic Effect of Excessive Copper Concentrations on Growth, Gaseous Exchange and Chloroplast Ultrastructure of *Corchorus capsularis* L. and Improves Copper Accumulation Capabilities. *Plants* **2020**, *9*, 756. [CrossRef] [PubMed]
67. Saleem, M.H.; Fahad, S.; Adnan, M.; Ali, M.; Rana, M.S.; Kamran, M.; Ali, Q.; Hashem, I.A.; Bhantana, P.; Ali, M.; et al. Foliar application of gibberellic acid endorsed phytoextraction of copper and alleviates oxidative stress in jute (*Corchorus capsularis* L.) plant grown in highly copper-contaminated soil of China. *Environ. Sci. Pollut. Res.* **2020**. [CrossRef] [PubMed]
68. Adhikari, A.; Adhikari, S.; Ghosh, S.; Azahar, I.; Shaw, A.K.; Roy, D.; Roy, S.; Saha, S.; Hossain, Z. Imbalance of redox homeostasis and antioxidant defense status in maize under chromium (VI) stress. *Environ. Exp. Bot.* **2020**, *169*, 103873. [CrossRef]
69. El-Esawi, M.A.; Alaraidh, I.A.; Alsahli, A.A.; Ali, H.M.; Alayafi, A.A.; Witczak, J.; Ahmad, M. Genetic variation and alleviation of salinity stress in barley (*Hordeum vulgare* L.). *Molecules* **2018**, *23*, 2488. [CrossRef] [PubMed]
70. El-Esawi, M.A.; Al-Ghamdi, A.A.; Ali, H.M.; Alayafi, A.A.; Witczak, J.; Ahmad, M. Analysis of Genetic Variation and Enhancement of Salt Tolerance in French Pea (*Pisum Sativum* L.). *Int. J. Mol. Sci.* **2018**, *19*, 2433. [CrossRef]
71. El-Esawi, M.A.; Alayafi, A.A. Overexpression of rice *Rab7* gene improves drought and heat tolerance and increases grain yield in rice (*Oryza sativa* L.). *Genes* **2019**, *10*, 56. [CrossRef]
72. Sehrish, A.K.; Aziz, R.; Hussain, M.M.; Rafiq, M.T.; Rizwan, M.; Muhammad, N.; Rafiq, M.K.; Sehar, A.; ud Din, J.; Al-Wabel, M.I. Effect of poultry litter biochar on chromium (Cr) bioavailability and accumulation in spinach (*Spinacia oleracea*) grown in Cr-polluted soil. *Arab. J. Geosci.* **2019**, *12*, 57. [CrossRef]
73. Sadak, M.S.; Abdelhamid, M.T. Influence of amino acids mixture application on some biochemical aspects, antioxidant enzymes and endogenous polyamines of Vicia faba plant grown under seawater salinity stress. *Gesunde Pflanz.* **2015**, *67*, 119–129. [CrossRef]
74. Ghasemi, S.; Khoshgoftarmanesh, A.H.; Hadadzadeh, H.; Jafari, M. Synthesis of iron-amino acid chelates and evaluation of their efficacy as iron source and growth stimulator for tomato in nutrient solution culture. *J. Plant Growth Regul.* **2012**, *31*, 498–508. [CrossRef]
75. Ghasemi, S.; Khoshgoftarmanesh, A.H.; Afyuni, M.; Hadadzadeh, H. Iron (II)–amino acid chelates alleviate salt-stress induced oxidative damages on tomato grown in nutrient solution culture. *Sci. Hortic.* **2014**, *165*, 91–98. [CrossRef]
76. El-Esawi, M.A.; Alaraidh, I.A.; Alsahli, A.A.; Alzahrani, S.M.; Ali, H.M.; Alayafi, A.A.; Ahmad, M. *Serratia liquefaciens* KM4 Improves Salt Stress Tolerance in Maize by Regulating Redox Potential, Ion Homeostasis, Leaf Gas Exchange and Stress-Related Gene Expression. *Int. J. Mol. Sci.* **2018**, *19*, 3310. [CrossRef] [PubMed]
77. El-Esawi, M.A.; Al-Ghamdi, A.A.; Ali, H.M.; Ahmad, M. Overexpression of *AtWRKY30* Transcription Factor Enhances Heat and Drought Stress Tolerance in Wheat (*Triticum aestivum* L.). *Genes* **2019**, *10*, 163. [CrossRef] [PubMed]

78. Nafees, M.; Ali, S.; Naveed, M.; Rizwan, M. Efficiency of biogas slurry and Burkholderia phytofirmans PsJN to improve growth, physiology, and antioxidant activity of *Brassica napus* L. in chromium-contaminated soil. *Environ. Sci. Pollut. Res.* **2018**, *25*, 6387–6397. [CrossRef]
79. Farid, M.; Ali, S.; Saeed, R.; Rizwan, M.; Bukhari, S.A.H.; Abbasi, G.H.; Hussain, A.; Ali, B.; Zamir, M.S.I.; Ahmad, I. Combined application of citric acid and 5-aminolevulinic acid improved biomass, photosynthesis and gas exchange attributes of sunflower (*Helianthus annuus* L.) grown on chromium contaminated soil. *Int. J. Phytoremediation* **2019**, *21*, 1–8. [CrossRef]
80. Elkelish, A.A.; Soliman, M.H.; Alhaithloul, H.A.; El-Esawi, M.A. Selenium protects wheat seedlings against salt stress-mediated oxidative damage by up-regulating antioxidants and osmolytes metabolism. *Plant. Physiol. Biochem.* **2019**, *137*, 144–153. [CrossRef]
81. El-Esawi, M.A.; Al-Ghamdi, A.A.; Ali, H.M.; Alayafi, A.A. *Azospirillum lipoferum* FK1 confers improved salt tolerance in chickpea (*Cicer arietinum* L.) by modulating osmolytes, antioxidant machinery and stress-related genes expression. *Environ. Exp. Bot.* **2019**, *159*, 55–65. [CrossRef]
82. Vwioko, E.; Adinkwu, O.; El-Esawi, M.A. Comparative Physiological, Biochemical and Genetic Responses to Prolonged Waterlogging Stress in Okra and Maize Given Exogenous Ethylene Priming. *Front. Physiol.* **2017**, *8*, 632. [CrossRef]
83. Shahid, M.; Shamshad, S.; Rafiq, M.; Khalid, S.; Bibi, I.; Niazi, N.K.; Dumat, C.; Rashid, M.I. Chromium speciation, bioavailability, uptake, toxicity and detoxification in soil-plant system: A review. *Chemosphere* **2017**, *178*, 513–533. [CrossRef]