Journal of Plant Stress Physiology 2026, 12: 69-76
https://doi.org/10.25081/jpsp.2026.v12.9752

Research Article

Zinc oxide nanoparticles alleviate chromium stress in two spinach (*Spinacia oleracea* L.) varieties through physiological and biochemical responses

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(Received: October 05, 2025; Revised: September 28, 2026; Accepted: October 04, 2026; Published: October 21, 2026)
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Abstract

Chromium (Cr) toxicity can impair growth, photosynthetic pigments and cellular redox balance in leafy vegetables, while the efficacy of foliar zinc oxide nanoparticles (ZnO-NPs) under Cr stress remains insufficiently characterized in spinach. Therefore, two spinach varieties, Desi palak and Lahori palak, were evaluated under two Cr stress levels (50 and 100 mM) with foliar ZnO-NP applications of 300 and 600 ppm. Plants were grown under controlled greenhouse conditions in a completely randomized design using 54 pots and were assessed for biomass and root/shoot growth, chlorophyll a and b, carotenoids, total chlorophyll, chlorophyll a/b ratio, total soluble protein, H_2O_2, MDA, and antioxidant enzymes including SOD, POD and CAT. Cr stress reduced biomass and photosynthetic pigment contents, whereas ZnO-NP application improved most growth and pigment responses relative to the corresponding Cr-stressed plants. The biochemical responses also varied with Cr level and ZnO-NP treatment, indicating changes in oxidative stress and antioxidant defense. Overall, foliar ZnO-NPs, particularly 600 ppm within the tested range, improved several measured physiological and biochemical attributes under Cr stress. Because plant-tissue Cr concentration was not quantified in the available experimental record, the present study does not claim Cr immobilization or reduced Cr uptake.

Keywords: Antioxidant defense, Chromium stress, Physiological responses, *Spinacia oleracea* L., Zinc oxide nanoparticles

Introduction

Spinach (*Spinacia oleracea* L.) is an important leafy vegetable valued for its nutritional and antioxidant properties. Its rapid vegetative growth and edible leaves also make spinach relevant to studies of metal exposure in vegetable production systems. Genotypic differences in spinach growth and antioxidant capacity have been reported, supporting the use of contrasting varieties when evaluating environmental stress (Howard *et al.,* 2002; Morelock & Correll, 2008; Roberts & Moreau, 2016; Huda-Faujan *et al.,* 2023).

Chromium is an important environmental contaminant associated with industrial activities including tanning and electroplating. In agricultural systems, Cr can enter plants through contaminated soil or irrigation water and can disrupt mineral nutrition, photosynthesis, growth and cellular redox homeostasis (Jobby *et al.,* 2018; Sharma *et al.,* 2020). Spinach is relevant because leafy tissues can accumulate toxic metals and contaminated irrigation water may increase dietary exposure (Anwar *et al.,* 2016; Maqbool *et al.,* 2018; Seyfferth *et al.,* 2024). Previous work in spinach has shown that increasing Cr exposure can reduce biomass and pigment production and alter oxidative metabolism (Ma *et al.,* 2022). Cr toxicity is closely associated with oxidative stress. Excess Cr can increase reactive oxygen species (ROS), disturb antioxidant metabolism, impair photosynthetic processes and ultimately reduce plant growth (Mathur *et al.,* 2016; Mahajan *et al.,* 2019; Ulhassan *et al.,* 2019). Measurements of H_2O_2, MDA, SOD, POD and CAT, together with photosynthetic pigments and biomass, provide complementary indicators of this responses. Zinc is an essential micronutrient involved in enzyme activity, protein synthesis, membrane stability and photosynthetic processes. Nanoparticulate Zn can improve nutrient delivery because its physicochemical properties differ from bulk forms, although plant responses are strongly concentration-dependent (Seleiman *et al.,* 2020a; Rajput *et al.,* 2021; Zulfiqar *et al.,* 2019). Beneficial effects of ZnO-NPs on growth and antioxidant responses have been reported in chickpea and other crops (Burman *et al.,* 2013; Pokhrel & Dubey, 2013; Verma *et al.,* 2018).

The concentration of ZnO-NPs is critical because low or moderate doses may be beneficial whereas excessive exposure can become phytotoxic (Rajput *et al.,* 2021). The 300 and 600 ppm foliar concentrations used here provide two application levels for testing whether increasing ZnO-NP supply improves responses to Cr stress. Related work has demonstrated nanoparticle effects on biomass, photosynthetic characteristics and antioxidant capacity, including in spinach (Jeyasubramanian *et al.,* 2016; Zaheer *et al.,* 2020a). Although nanoparticles have been studied as nanofertilizers and stress-mitigation agents, evidence specifically addressing foliar ZnO-NP application in spinach under defined Cr stress levels remains limited. The objectives were to determine (i) how 50 and 100 mM

Cr affect growth and photosynthetic traits in two spinach varieties, (ii) how 300 and 600 ppm foliar ZnO-NPs modify these responses, and (iii) whether the treatments alter oxidative-stress indicators and antioxidant enzyme activities.

Materials and methods

Experimental site and plant material

Seeds of two spinach varieties, Desi palak and Lahori palak, were obtained from the Ayub Agriculture Research Institute (AARI). The experiment was conducted in a greenhouse of the Department of Entomology, University of Agriculture Faisalabad, under controlled conditions. Fifty-four pots were arranged in a completely randomized design (CRD), comprising two varieties and nine treatment conditions: control, 50 mM Cr, 100 mM Cr, 300 ppm ZnO-NPs, 600 ppm ZnO-NPs, 50 mM Cr + 300 ppm ZnO-NPs, 50 mM Cr + 600 ppm ZnO-NPs, 100 mM Cr + 300 ppm ZnO-NPs and 100 mM Cr + 600 ppm ZnO-NPs.

Growth and biomass measurements

Plants were washed and blotted dry. Whole-plant fresh weight was recorded, roots and shoots were then separated and weighed. Samples were oven-dried for 72 h before dry weight was recorded. Root and shoot lengths were measured on the corresponding samples.

Physiological and biochemical analyses

Photosynthetic pigments

Chlorophyll a, chlorophyll b and carotenoids were determined spectrophotometrically. Leaf samples from 25-day-old plants were extracted in 80% acetone overnight at 4 °C. After centrifugation (10,000 × g for 5 min), absorbance was measured at 645, 663 and 480 nm using a Hitachi-U2001 spectrophotometer (Tokyo, Japan).

Hydrogen peroxide (H_2O_2)

H_2O_2 was quantified using a trichloroacetic acid-based method. Fresh tissue was homogenized in 0.1% TCA and centrifuged; the supernatant was mixed with potassium phosphate buffer and potassium iodide, and absorbance was measured at 390 nm.

Enzyme extraction and total soluble protein

Fresh tissue (0.5 g) was homogenized in 10 mL chilled 50 mM phosphate buffer (pH 7.8) and centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant was used for total soluble protein and antioxidant enzyme assays.

Superoxide dismutase (SOD)

SOD activity was assayed from inhibition of photochemical reduction of nitro blue tetrazolium (NBT). The reaction mixture contained NBT, riboflavin, methionine, EDTA and phosphate buffer. Absorbance was measured at 560 nm after illumination; one unit of SOD was defined as the amount causing 50% inhibition of NBT photoreduction.

Peroxidase (POD)

POD activity was determined using the guaiacol oxidation method. The reaction mixture contained enzyme extract, phosphate buffer, guaiacol and H_2O_2, and absorbance at 470 nm was recorded at 20-s intervals.

Catalase (CAT)

CAT activity was determined from the decline in absorbance at 240 nm during H_2O_2 decomposition. The reaction mixture contained phosphate buffer, H_2O_2 and enzyme extract, and readings were taken at 20-s intervals. Activity was expressed relative to protein content.

Malondialdehyde (MDA)

MDA was determined using a thiobarbituric-acid-reactive substances procedure. Leaf tissue (1 g) was homogenized in 0.1% TCA, centrifuged, and the supernatant was reacted with thiobarbituric acid in TCA. After heating at 95 °C for 50 min and cooling, absorbance was recorded at 532 and 600 nm.

Results

Plant growth and biomass

Cr stress reduced growth traits in both spinach varieties. ANOVA showed significant treatment effects for root fresh weight, shoot fresh weight, root dry weight, shoot dry weight, root length and shoot length; variety effects and several variety × treatment interactions were also significant (Tables 1-17 & Figures 1-17). Across the treatment means shown in the figures, Cr exposure generally reduced growth relative to the control, whereas foliar ZnO-NP application improved growth under Cr stress, with 600 ppm generally producing the stronger response within the tested range.

Plants were washed and blotted dry. Whole-plant fresh weight was recorded, roots and shoots were then separated and weighed. Samples were oven-dried for 72 h before dry weight was recorded. Root and shoot lengths were measured on the corresponding samples.

Discussion

The present experiment demonstrates that Cr exposure altered growth, photosynthetic pigments and oxidative-stress-related biochemical traits in both spinach varieties. The decline in biomass and root/shoot length under Cr treatment agrees with reports that Cr disrupts nutrient acquisition, photosynthesis and cellular metabolism (Ertani *et al.,* 2017; Sharma *et al.,* 2020; Ma *et al.,* 2022). The significant treatment effects for major growth traits

Table 1: ANOVA (fresh root weight) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	0.474140741	0.4741407	12.375465	0.0012**
Treatment	8	4.802014815	0.6002519	15.667069	0.0000***
V*T	8	0.657859259	0.0822324	2.1463338	0.0564^{ns}
Error	36	1.379266667	0.038313		
Total	53	7.313281481			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 2: ANOVA (fresh weight of shoot) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	4.407265352	4.4072654	88.956491	0.0000***
Treatment	8	10.990278	1.3737848	27.728549	0.0000***
V*T	8	2.086819481	0.2608524	5.2650602	0.0002***
Error	36	1.783586	0.0495441		
Total	53	19.26794883			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 3: ANOVA (root dry weight) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	0.094585185	0.0945852	13.311441	0.0008***
Treatment	8	1.82997037	0.2287463	32.192598	0.0000***
V*T	8	0.075148148	0.0093935	1.3219964	0.2642^{ns}
Error	36	0.0071056	0.2558		
Total	53	2.255503704			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 4: ANOVA (shoot dry weight) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	3.942001852	3.9420019	4719.9135	0.0000***
Treatment	8	2.585133333	0.3231417	386.9102	0.0000***
V*T	8	0.587481481	0.0734352	87.926829	0.0000***
Error	36	0.030066667	8.3519e^{-4}		
Total	53	7.144683333			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 5: ANOVA (root length) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	18.75140817	18.751408	825.56763	0.0000***
Treatment	8	50.96271015	6.3703388	280.4667	0.0000***
V*T	8	1.746762	0.2183452	9.6130792	0.0000***
Error	36	0.817680667	0.0227134		
Total	53	72.27856098			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 6: ANOVA (shoot length) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	16.42311852	16.423119	280.80818	0.0000***
Treatment	8	194.2348037	24.27935	415.13676	0.0000***
V*T	8	3.919914815	0.4899894	8.378008	0.0000***
Error	36	2.105466667	0.0584852		
Total	53	216.6833037			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

indicate a measurable stress response under the controlled conditions.

Table 7: ANOVA (Chlorophyll a) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	2.081918685	2.0819187	79.482826	0.0000***
Treatment	8	25.69413393	3.2117667	122.6178	0.0000***
V*T	8	1.206669481	0.1508337	5.7584802	0.0001***
Error	36	0.942959333	0.0261933		
Total	53	29.925681429			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 8: ANOVA (Chlorophyll b) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	0.135	0.135	8.4728033	0.0061**
Treatment	8	1.141059259	0.1426324	8.9518247	0.0000***
V*T	8	0.119866667	0.0149833	0.9403766	0.4963^{ns}
Error	36	0.5736	0.0159333		
Total	53	1.969525926			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 9: ANOVA (Carotenoid) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	0.115740741	0.1157407	203.58306	0.0000***
Treatment	8	0.6247	0.0780875	137.35261	0.0000***
V*T	8	0.006625926	8.2824	1.4568404	0.2074^{ns}
Error	36	0.020466667	5.6852		
Total	53	0.767533333			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 10: ANOVA for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	3.277218685	3.2772187	73.620885	0.0000***
Treatment	8	37.18744504	4.6484306	104.42439	0.0000***
V*T	8	1.855502815	0.2319379	5.2103541	0.0002***
Error	36	1.602532667	0.0445148		
Total	53	43.9226992			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 11: ANOVA (Chlorophyll a/b ratio) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	0.003675029	0.003675	0.1276008	0.7230^{ns}
Treatment	8	1.329886138	0.1662358	5.7718783	0.0001***
V*T	8	6.645777804	0.8307222	28.843537	0.0000***
Error	36	1.036835384	0.028801		
Total	53	9.016174355			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Improvement in growth following foliar ZnO-NP application suggests that Zn nutrition may partially offset the physiological consequences of Cr stress. Zinc contributes to enzyme function, membrane stability and photosynthetic metabolism, while nanoparticle formulations can modify nutrient delivery (Suganya *et al.,* 2020; Seleiman *et al.,* 2020b). Previous studies report beneficial ZnO-NP effects on growth and antioxidant systems (Burman *et al.,* 2013; Pokhrel & Dubey, 2013; Rajput *et al.,* 2021). The present results support physiological stress alleviation, but not Cr immobilization, because tissue Cr was not quantified.

Table 12: ANOVA (catalase activity) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

S0V	DF	SS	MS	F	P
Variety	1	0.411847099	0.4118471	115.1847	0.0000***
Treatment	8	1.683310655	0.2104138	58.848187	0.0000***
V*T	8	0.171387017	0.0214234	5.9916541	0.0001***
Error	36	0.12871931	0.0035755		
Total	53	2.395264081			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 13: ANOVA (POD) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	84494.5036	84494.504	80.432063	0.0000***
Treatment	8	1253921.775	156740.22	149.20425	0.0000***
V*T	8	12634.52327	1579.3154	1.5033829	0.1905^{ns}
Error	36	37818.27826	1050.5077		
Total	53	1388869.08			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 14: ANOVA (TSP) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	90488.07012	90488.07	87.760604	0.0000***
Treatment	8	1389857.32	173732.17	168.49558	0.0000***
V*T	8	7624.370561	953.04632	0.9243199	0.5083^{ns}
Error	36	37118.82522	1031.0785		
Total	53	1525088.586			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 15: ANOVA (H_2O_2) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	16960.02489	16960.025	23.443214	0.0000***
Treatment	8	801580.5264	100197.57	138.49938	0.0000***
V*T	8	3452.474698	431.55934	0.5965285	0.7742^{ns}
Error	36	26044.24847	723.45135		
Total	53	848037.2745			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 16: ANOVA (SOD) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	0.745094826	0.7450948	64.719707	0.0000***
Treatment	8	7.494201812	0.9367752	81.369265	0.0000***
V*T	8	0.167294266	0.0209118	1.8164191	0.1060^{ns}
Error	36	0.414455117	0.0115126		
Total	53	8.821046021			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

Table 17: ANOVA (Malondialdehyde content) for the corresponding trait in two *S. oleracea* varieties under Cr and foliar ZnO-NP treatments

SOV	DF	SS	MS	F	P
Variety	1	54.46872274	54.468723	69.171233	0.0000***
Treatment	8	1501.855089	187.73189	238.40555	0.0000***
V*T	8	8.407940182	1.0509925	1.3346825	0.2583^{ns}
Error	36	28.34811428	0.7874476		
Total	53	1593.079866			

'*'-p≤0.05, '**'-p≤0.01, '***'- p≤0.001, ns-non-significant

The reduction in chlorophyll a, chlorophyll b, carotenoids and total chlorophyll under Cr stress indicates

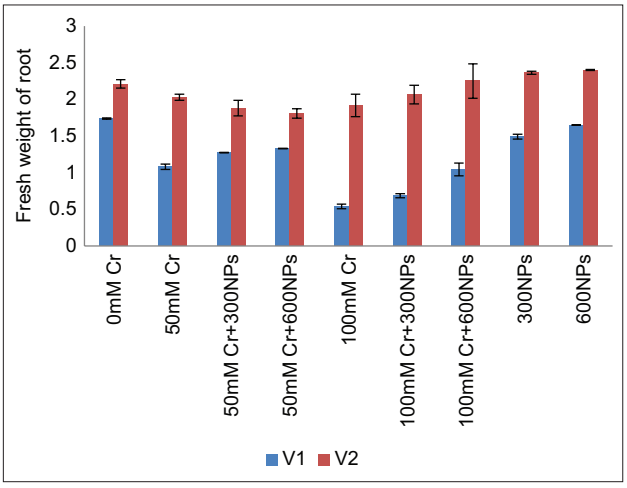


Figure 1: Measured trait (fresh weight of root) in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

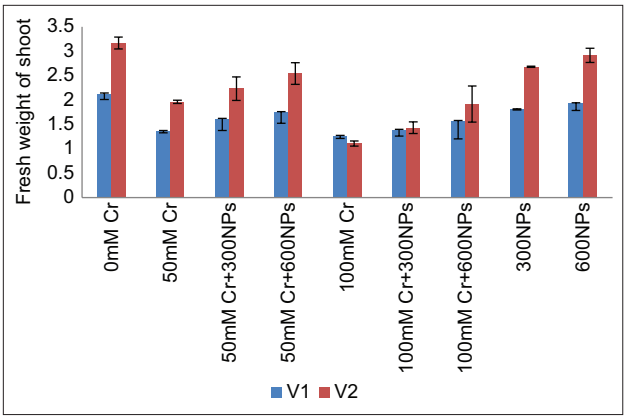


Figure 2: Measured trait (fresh weight of shoot) in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

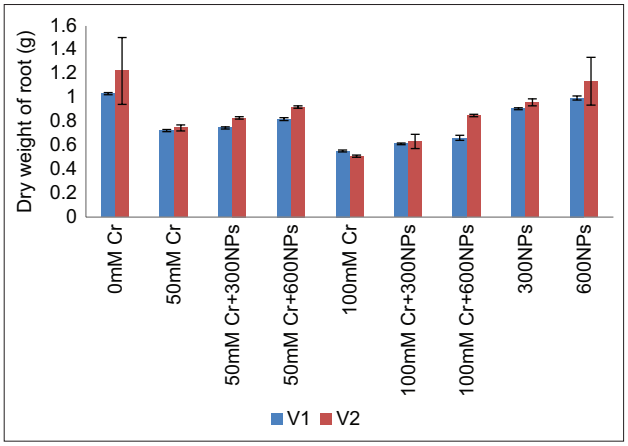


Figure 3: Measured trait (root dry weight) in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

impairment of photosynthetic performance. Similar responses have been reported in wheat and spinach (Mathur *et al.,* 2016; Ma *et al.,* 2022). ZnO-NP treatment improved pigment values relative to the corresponding stressed plants, suggesting improved physiological performance. Significant variety and treatment effects for several pigment traits also indicate genotype-dependent responses.

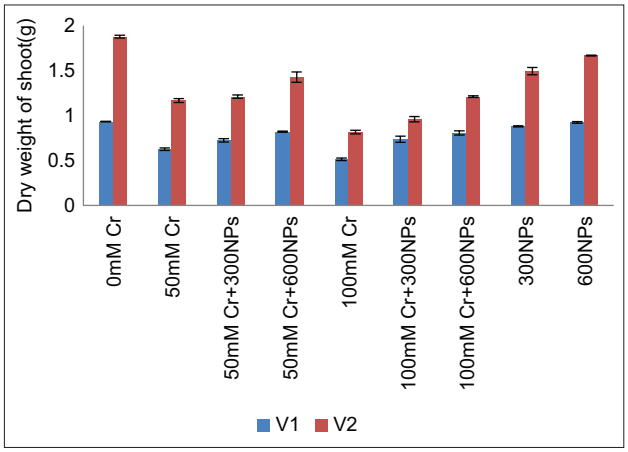


Figure 4: Shoot dry weight (g) in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

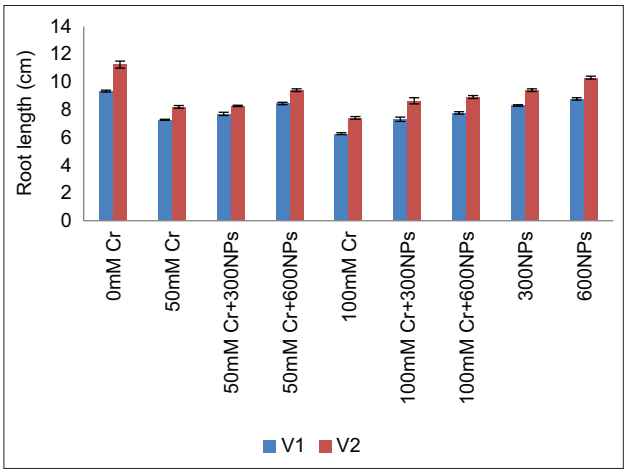


Figure 5: Root length (cm) in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

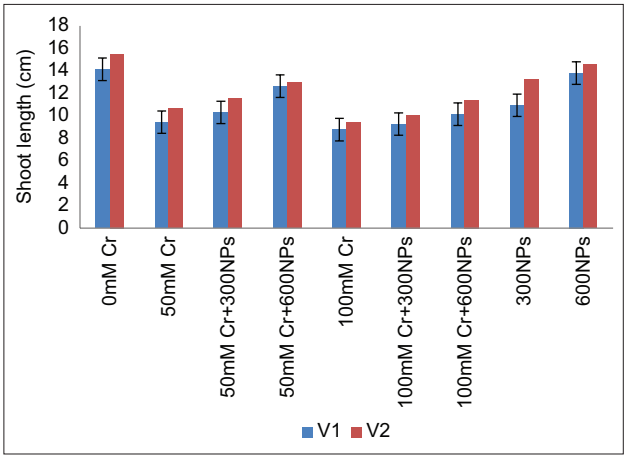


Figure 6: Measured trait (shoot length) in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments.

The antioxidant measurements provide additional evidence of treatment-dependent changes in redox balance. Cr stress altered H_2O_2 and MDA and affected SOD, POD and CAT, while ZnO-NP application shifted several variables toward the values observed in less-stressed plants. Such responses are consistent with the role of antioxidant enzymes in controlling ROS during metal stress (Hasanuzzaman *et al.,* 2011; Mahajan *et al.,* 2019).

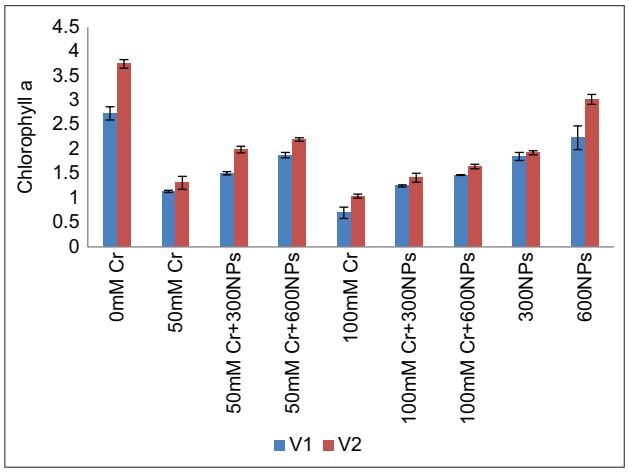


Figure 7: Chlorophyll a content in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

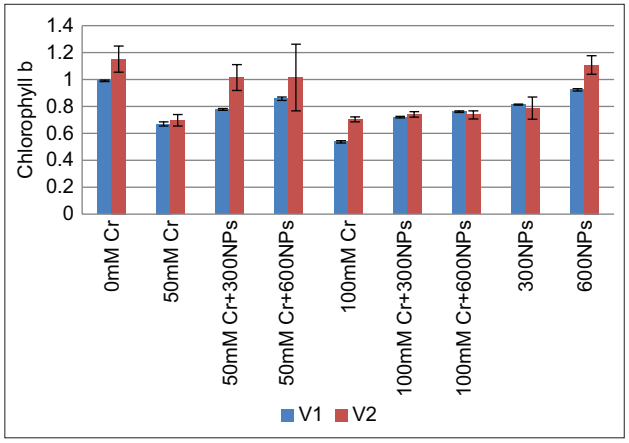


Figure 8: Chlorophyll b content in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

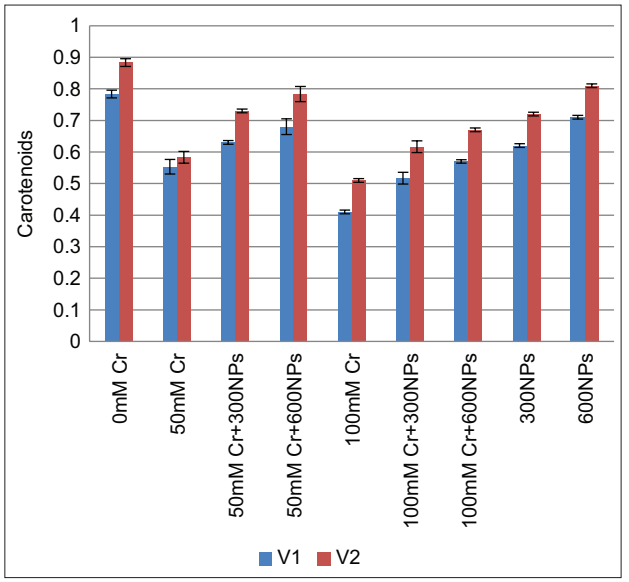


Figure 9: Carotenoid content in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

The 600 ppm treatment generally showed the stronger recovery among the tested concentrations. Because tissue Cr concentration was not measured, these data support improved oxidative balance rather than a direct mechanism of Cr immobilization.

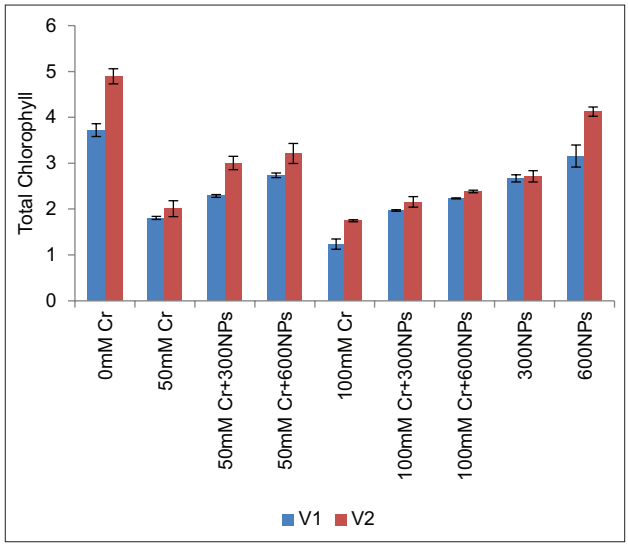


Figure 10: Total chlorophyll content in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

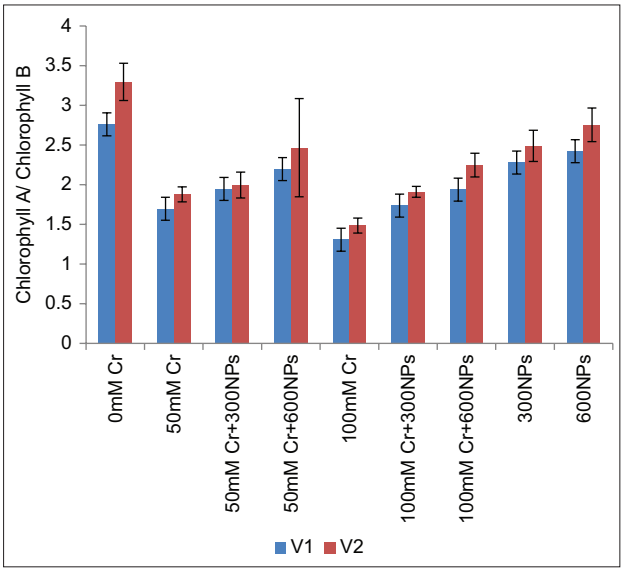


Figure 11: Chlorophyll a/b ratio in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

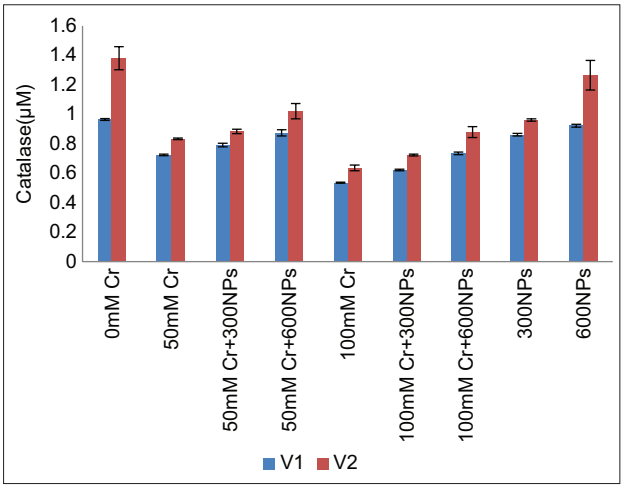


Figure 12: Catalase activity in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

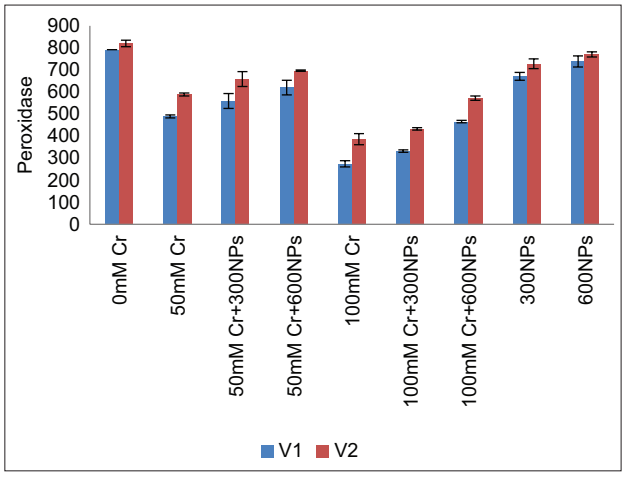


Figure 13: Peroxidase activity in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

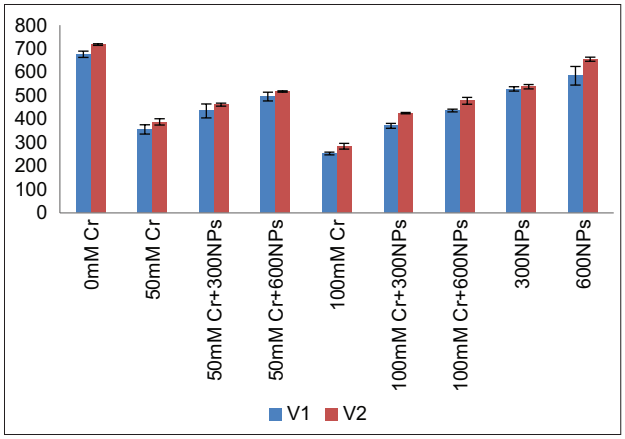


Figure 14: Total soluble protein in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

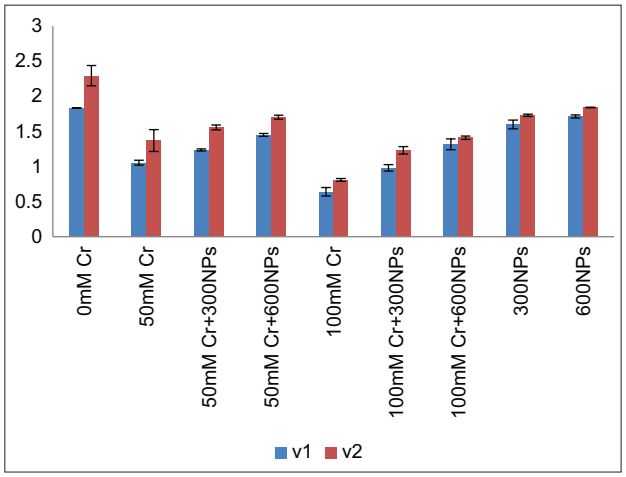


Figure 15: H_2O_2 content in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

Spinach is a food crop, so stress management should be evaluated together with food-safety considerations. Previous studies have explored nutrient-mediated and phytoremediation approaches to metal stress (Ullah *et al.,* 2015; Maqbool *et al.,* 2018; Zaheer *et al.,* 2020b). The

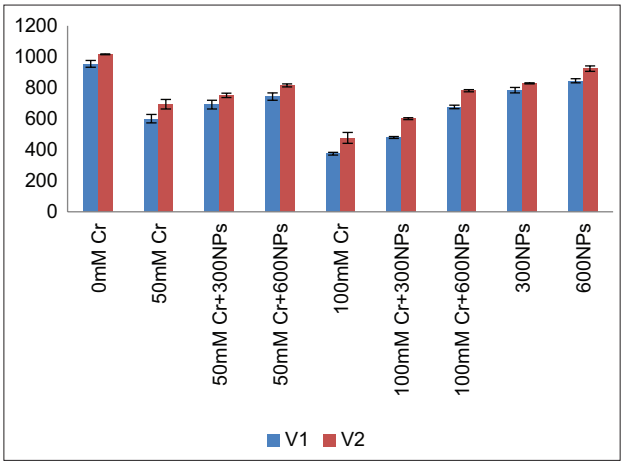


Figure 16: Superoxide dismutase activity in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

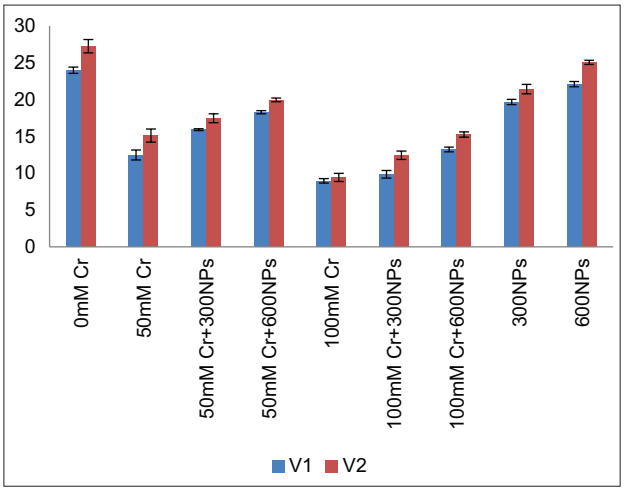


Figure 17: Malondialdehyde content in two *S. oleracea* varieties under Cr stress and foliar ZnO-NP treatments

present findings support further investigation of foliar ZnO-NPs, but future experiments should quantify Cr concentrations in roots and shoots, Cr translocation factors, Zn accumulation and yield before any claim of reduced Cr uptake or immobilization is made.

The varietal differences observed here are also important for future work. Genotype, nutrient status and nanoparticle type can modify stress responses (Jeyasubramanian *et al.,* 2016; Zaheer *et al.,* 2020a). Further trials should optimize ZnO-NP dose and application frequency, verify nanoparticle characterization, and assess yield and food-safety traits under agronomically realistic Cr exposure.

Conclusion

Chromium stress reduced growth and photosynthetic pigment traits and altered antioxidant and oxidative-stress indicators in both spinach varieties. Foliar ZnO-NP application, particularly 600 ppm within the tested range, improved several measured traits under Cr stress, indicating potential for physiological stress alleviation. Lahori palak generally showed stronger performance under the tested conditions. The study does not demonstrate Cr immobilization or reduced Cr uptake because plant-tissue Cr was not quantified. Future studies should directly measure root and shoot Cr, translocation, Zn accumulation, nanoparticle characteristics, yield and food-safety responses before recommending the treatment for contaminated soils or edible production systems.

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