Plant Diversity of Central Asia *OPEN ACCESS*

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Research Article

Serine-mediated alleviation of chromium-induced phytotoxicity of two wheat cultivars: a multivariate and dose–response statistical assessment

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For editorial use
Academic Editor: Komiljon Sh. Tojibaev

Received: 02.03.2026

Revised: 18.04.2026

Accepted: 02.09.2026

Published: 03.09.2026

Citation: Asad, F., Ali, B., Dilawar, N., Abdullah, & Javed, A. (2026). Serine-mediated alleviation of chromium-induced phytotoxicity of two wheat cultivars: A multivariate and dose–response statistical assessment. *Plant Diversity of Central Asia, 5*: 1–18

https://doi.org/10.54981/PDCA/vol5_iss1/a1

Abstract: Chromium (Cr) contamination can impair wheat growth and metabolism, while serine may modulate plant stress responses. This study analyzed a pot experiment with two wheat cultivars, Akbar-19 and Fakhar-e-Bhakkar, exposed to 0, 30, 60 and 90 ppm Cr, with or without serine co-application. Morphological and biochemical responses were evaluated using three-way ANOVA, linear mixed-effects models, four-parameter log-logistic dose-response models, principal component analysis (PCA) and multivariate analysis of variance (MANOVA). Chromium caused a clear dose-dependent decline in growth and biochemical traits. Significant Cr × Serine interactions for total chlorophyll, protein, proline and soluble sugar ($p < 0.01$) showed that mitigation by serine was not uniform across doses. Mixed-effects models confirmed significant negative associations between Cr dose and all tested responses ($p < 0.01$). In Akbar-19, serine shifted estimated EC50 values to higher concentrations for several endpoints, whereas patterns were less consistent in Fakhar-e-Bhakkar and some fits were poorly constrained. The first two PCA axes explained 74.7% of biochemical variation, and MANOVA detected significant effects of cultivar and Cr dose on the joint biochemical profile ($p < 0.001$). Overall, serine co-application was associated with partial, dose- and cultivar-dependent alleviation of Cr phytotoxicity rather than complete reversal. Because some higher-level analyses used reconstructed replicate data and the experiment lacked a serine-only treatment, these findings require confirmation with original replicate-level datasets.

Keywords: chromium stress; *Triticum aestivum*; serine; three-way ANOVA; linear mixed-effects model; dose-response modelling; principal component analysis; MANOVA

1. Introduction

Heavy-metal contamination of agricultural soils in developing countries has been steadily increasing due to the combination of rapid industrialization, heavy burden of agricultural activities, and unregulated mining (Gill, 2014; Zenk, 1996). Heavy metals are non-degradable, meaning that once they enter into the soil–water system, they do not get degraded but are redistributed between soil particles, pore water, and living tissues (Asad et al., 2021; Babel & del Mundo Dacera, 2006; Singh, 2007). Irrigation water, wastewater effluents and phosphate fertilizers are all known sources of metal contamination, and the peri-urban location of croplands near industrial corridors, as well as the proximity of effluents from wastewater treatment plants, is a significant contributor to this problem with soils carrying high loads of cadmium, arsenic, mercury, lead and chromium, not only affecting productivity but also the food chain that depends on it (Gill, 2014; Sherene, 2010). The chromium (Cr) element is one of the most significant of these contaminants due to its dual toxicity and its importance to the major industrial processes. It is mainly released into the environment by the discharge from tannery processes, electroplating baths, chromite ore processing and metal finishing processes and exists in the soil mainly as the trivalent (Cr^{3+}) or hexavalent (Cr^{6+}) oxidation state (Avudainayagam et al., 2003; Beukes et al., 2017). Hexavalent chromium is significantly more soluble, mobile and biologically reactive than the trivalent form and it is considered as a priority environmental contaminant because it readily penetrates cell walls and causes oxidative damage within plant tissue (Avudainayagam et al., 2003; Cary et al., 1977; Dilawar et al., 2021).

The phytotoxic effect of chromium has been reported in various crop species and the overall pattern of the effect is remarkably consistent although the magnitude of the effect may differ from species to species. At high Cr concentrations, the germination is delayed or even stopped, the growth of roots is more strongly inhibited than that of shoots, reflecting the fact that the root is the part of the plant that contacts and accumulates the metal, and, after a threshold concentration that varies among the investigated genotypes, chlorosis, necrosis, and reduction in biomass production are observed (Goyal et al., 2020; Shahid et al., 2015; Shahid et al., 2023; Shaikh et al., 2013). At the biochemical level, chromium supply negatively affects the biosynthesis of chlorophyll as well as the activity of the Hill reaction, denatures structural and enzymatic proteins and disrupts the hormonal signalling network involved in growth under stress and have been reported in wheat (*Triticum aestivum* L.) by several separate groups using different cultivars and exposure conditions (Datta et al., 2011; Mathur et al., 2016; Shahid et al., 2026; Sharma et al., 1995). However, serine is not an essential amino acid but serves as a metabolic hub known for its role in the synthesis of glycine, cysteine, one-carbon (folate) metabolism, as a carbon skeleton for phospholipid and sphingolipid biosynthesis, in nucleotide biosynthesis, and as a precursor for the antioxidant tripeptide glutathione (De Koning et al., 2003; Kalhan & Hanson, 2012; Reeds, 2000). The supplementation with serine has a mechanistically plausible pathway to ameliorate stress as the pathway for the biosynthesis of this amino acid is conserved in bacteria, animals, and plants and because several of the products of this pathway are critical for the plant's antioxidant defense against heavy-metal induced oxidative stress, it is not just an empirical addition (Ros et al., 2013).

This study was thus designed to: (i) characterize the dose-dependent effect of Cr on the vegetative growth of two locally grown varieties of wheat, Akbar-19 and Fakhar-e-Bhakkar; (ii) assess the effectiveness of serine when added exogenously in suppressing this growth inhibition over various Cr concentrations; and (iii) evaluate whether serine was effective in improving the underlying biochemical status of the plant (phytohormone, protein and osmolyte content, soluble sugar reserves and photosynthetic pigment pool). In addition to multivariate statistical methods, the present study also offers biological information about the differential sensitivity of two wheat cultivars to chromium toxicity and the impact of exogenous serine on a variety of morphological and biochemical aspects. The results suggest that the apparent protective effect of serine is dose- and cultivar-dependent, meaning that mitigation does not appear to be uniform across physiological processes. These observations help to elucidate wheat response to chromium stress at the phenotypic level and also to offer a statistical approach to assessing the effect of treatment under a variety of biological endpoints.

2. Materials and Methods

2.1. Experimental site

The pot experiment was carried out in Department of Botany, Bacha Khan University, Charsadda (Lat.). 34°8'43.08" N, Long. 71°43'50.880" E; Alt. 282 m) in November 2023.

2.2. Plant material, treatments and design

The seeds of two wheat varieties, Akbar-19 and Fakhar-e-Bhakkar, were sown in completely randomized pot arrangement (10–12 seeds/pot with 3 pot replicates/treatment). Seedlings were treated with Cr alone at 30, 60 and 90 ppm, and with Cr with serine at the same concentrations (30, 60 and 90 ppm) with an untreated control (irrigated with tap water). The seven treatment groups used in the results reported below are summarized in Table 1.

Table 1. Treatment structure used in the pot experiment (n = 3 pot replicates per treatment).

Group	Treatment	Cr (ppm)	Serine (ppm)
1	Control	0	0
2	Cr 30 ppm	30	0
3	Cr 60 ppm	60	0
4	Cr 90 ppm	90	0
5	Cr 30 + Ser 30	30	30
6	Cr 60 + Ser 60	60	60
7	Cr 90 + Ser 90	90	90

Morphological parameters (root number, root length, shoot length, leaf number, fresh/dry biomass of root, shoot and leaf tissue) were measured 21 days after germination.

2.3. Biochemical assays

Photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids) were determined following Arnon (1949), leaf protein content was measured following Lowry et al. (1951), free proline was measured following Bates et al. (1973) and total soluble sugars was measured following the phenol–sulphuric acid method of DuBois et al. (1956). The quantities of endogenous phytohormones (gibberellic acid, indole-3-acetic acid and salicylic acid) were spectrophotometrically determined following the methodology used in the original thesis.

2.4. Statistical analysis

This report uses statistical modelling all in Python 3 (pandas, NumPy, SciPy, statsmodels, scikit-learn). Four analytical frameworks, each complementary, were used: Three-way Analysis of Variance (Type II sum of squares) with variety (Akbar-19, Fakhar-e-Bhakkar), Cr dose (0, 30, 60, 90 ppm, treated as a categorical factor) and Serine (present/absent) as fixed factors was fitted separately for eight representative response variables (root length, root fresh biomass, shoot length, leaf number, total chlorophyll, protein, proline, sugar). Linear mixed-effects models (LMM) with restricted maximum likelihood (REML) fits, using Cr dose (continuous) and Serine (binary) as fixed effects, a Cr-dose × Serine interaction term, a Variety fixed covariate, and a Variety random-intercept grouping factor to remove any baseline variation between varieties.

A four-parameter log-logistic function (Response = Bottom + (Top - Bottom) / [1 + (dose/EC50)^Hill]) was fitted by nonlinear least squares separately to the dose series for each variety for each response variable, giving an EC50 as an index of relative sensitivity, as the concentration of Cr giving a half-maximal effect.

Using Principal Component Analysis (PCA) on standardized variables, multivariate analysis of ten variables (biochemical profile namely chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, protein, proline, sugar, gibberellic acid, indole-3-acetic acid, and salicylic acid) was carried out and the simultaneous effect of Variety, Cr dose and Serine on the full biochemical profile tested using Multivariate Analysis of Variance (MANOVA; Wilks' Lambda, Pillai's Trace, Hotelling–Lawley Trace and Roy's Greatest Root). Throughout, the significance level was defined as $\alpha = 0.05$.

A number of variables lacked original replicate-level data, so secondary statistical analyses (three-way ANOVA, linear mixed-effects models, PCA, MANOVA and dose-response modelling) were conducted with reconstructed datasets based on the reported treatment means, standard deviations and sample sizes. The following reconstructed datasets is used to capture the reported summary statistics but not the full biological variability and covariance structure. Thus, the inference from the statistics derived in this way should be viewed as an approximate re-analysis, not a substitute for analyses using original data from biological replicates.

3. Results

3.1. Morphological responses to chromium and serine

Root and shoot growth in both wheat varieties was obviously decreased by chromium exposure, and this decrease was dose-dependent; the exposure to chromium and co-exposure to serine resulted in a significant partial reduction of this decrease at low-to-moderate concentrations of chromium. Table 2 shows the four main root system traits observed for Akbar-19 while Table 3 shows the same for Fakhar-e-Bhakkar.

Table 2. Root system morphological parameters (Mean ± SD, n = 3) of the Akbar-19 treated with Cr and Cr+Serine. Means with the same letter superscript are not significantly different from each other (Tukey HSD; $p > 0.05$), means with different letter superscripts are significantly different ($p < 0.05$).

Treatment	Root number	Root length (cm)	Root fresh biomass (g)	Root dry biomass (g)
Control	4.33a ± 0.53	8.65a ± 0.17	4.02a ± 0.10	2.25a ± 0.00
Cr 30 ppm	3.22a ± 0.86	4.43a ± 0.85	3.06b ± 0.02	2.14b ± 0.03
Cr 60 ppm	3.29a ± 0.13	8.42a ± 0.08	2.03c ± 0.01	1.49d ± 0.02
Cr 90 ppm	2.48b ± 0.55	5.87a ± 3.85	1.00d ± 0.02	1.00e ± 0.02
Cr30+Ser30	4.04a ± 0.04	6.69a ± 1.16	3.00b ± 0.01	2.02c ± 0.02
Cr60+Ser60	4.06a ± 0.06	5.38a ± 0.32	2.04c ± 0.05	1.02e ± 0.01
Cr90+Ser90	3.78a ± 0.33	5.70a ± 0.06	1.00d ± 0.02	0.90f ± 0.00

Table 3. Root-system morphological parameters mean ± SD (n = 3) of Fakhar-e-Bhakkar treated with Cr and Cr+Serine. In a column, means are not significantly different from each other (Tukey HSD: $p > 0.05$); means are significantly different ($p < 0.05$).

Treatment	Root number	Root length (cm)	Root fresh biomass (g)	Root dry biomass (g)
Control	4.51a ± 0.51	9.17a ± 1.40	0.34a ± 0.01	0.16a ± 0.01
Cr 30 ppm	2.99a ± 0.48	6.56b ± 1.18	0.28a ± 0.01	0.13a ± 0.02
Cr 60 ppm	3.00a ± 0.26	7.33a ± 0.39	0.24a ± 0.01	0.13a ± 0.03
Cr 90 ppm	1.61b ± 0.96	5.03b ± 0.61	0.19b ± 0.11	0.09b ± 0.01
Cr30+Ser30	3.55a ± 1.11	7.15a ± 0.43	0.28a ± 0.04	0.17a ± 0.04
Cr60+Ser60	4.30a ± 0.18	6.41b ± 0.11	0.32a ± 0.00	0.17a ± 0.01
Cr90+Ser90	3.97a ± 0.06	5.98b ± 1.09	0.22b ± 0.01	0.11a ± 0.00

The root fresh and dry biomass of Akbar-19 was separated into 4 to 6 Tukey groups under the 7 treatments, with control and Cr30+Ser30 grouped in the top tier and Cr90 and Cr90+Ser90 in the lowest tier, which were not statistically different from one another, suggesting a graded, dose-resolved reduction as Cr concentration increased, with serine no longer statistically significantly benefiting at the highest concentration of Cr (Table 2). The two parameters of root number and root length were comparatively less sensitive, most treatments sharing the same letter 'a', and only Cr90 being significantly reduced.

Root number was also significantly different only between Cr90 and all other treatments (letter 'b' versus 'a'; Table 3), but root length showed two significantly different tiers, with the treatment groups Cr-only and Cr+Serine showing the same tier at each dose (e.g., Cr30 'b' versus Cr60+Ser60 'b' versus Control 'a'); this indicated that serine's benefit, if present, was concentration-specific rather than consistent across the dose range. For root fresh biomass, there was a significant reduction in root fresh biomass only at 90 ppm Cr (letter 'b') and no significant difference between all other treatments and the control (no serine and Cr), suggesting that for this cultivar, root fresh biomass is comparatively resistant to Cr up to 60 ppm of Cr regardless of serine co-treatment.

In both varieties, root length decreased by 44-47% at 90 ppm Cr, compared to control, and root fresh biomass decreased by 75-45%. These features were restored by some extent by the co-application of serine, especially at 30 ppm, though the ameliorating effect was reduced at higher concentrations of Cr, as in the original thesis. These and other important morphological responses are shown graphically in Figure 1-4, and error bars are standard deviations (SD) of ± 1.

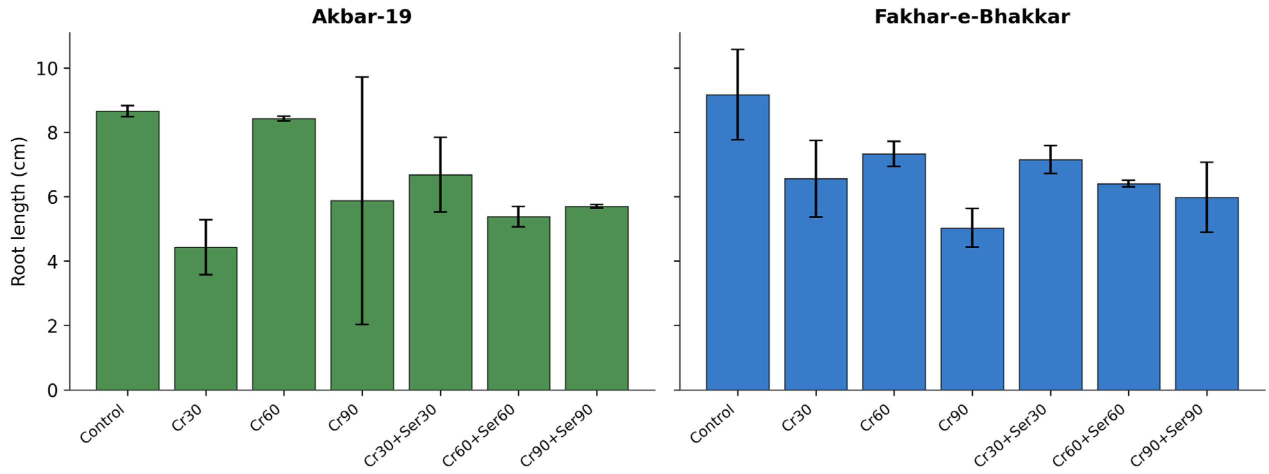

Figure 1. The results of root length (cm) of Akbar-19 and Fakhar-e-Bhakkar under seven treatments (Mean ± SD, n = 3).

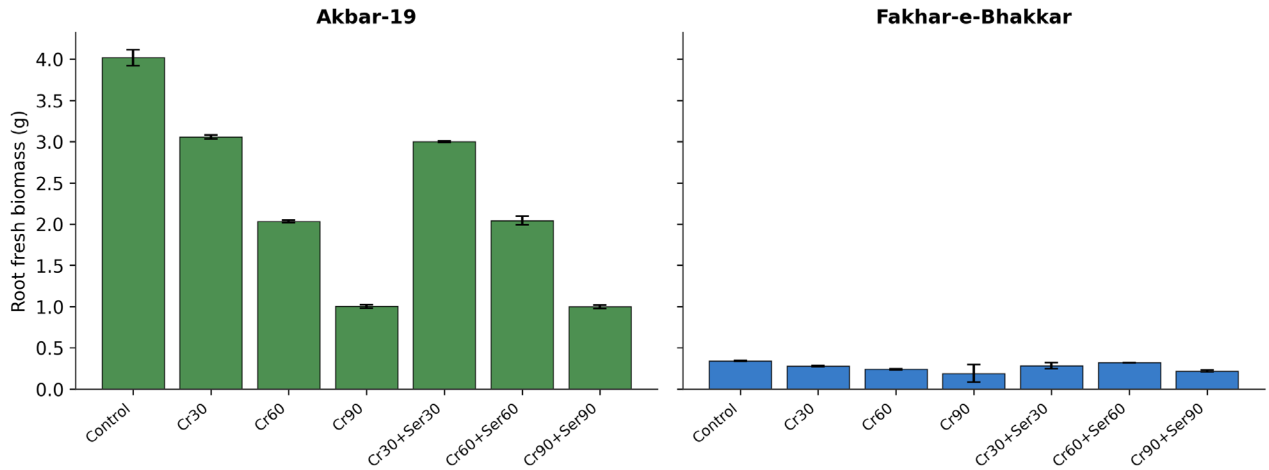

Figure 2. Root fresh biomass (g) of Akbar-19 and Fakhar-e-Bhakkar of the seven treatments (mean ± SD, n = 3).

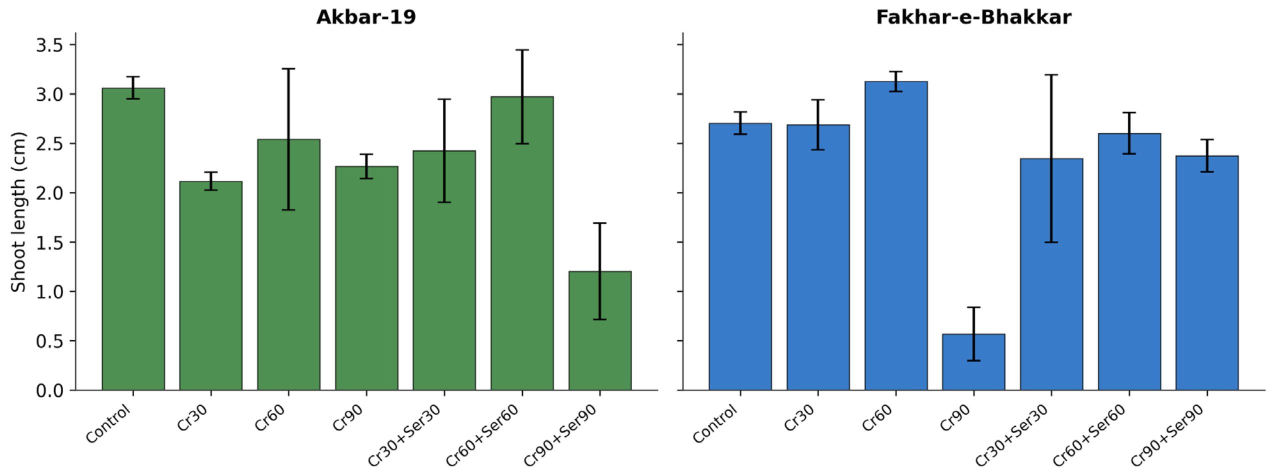

Figure 3. Shoot length (cm) of Akbar-19 and Fakhar-e-Bhakkar under seven treatments (mean ± SD; n = 3).

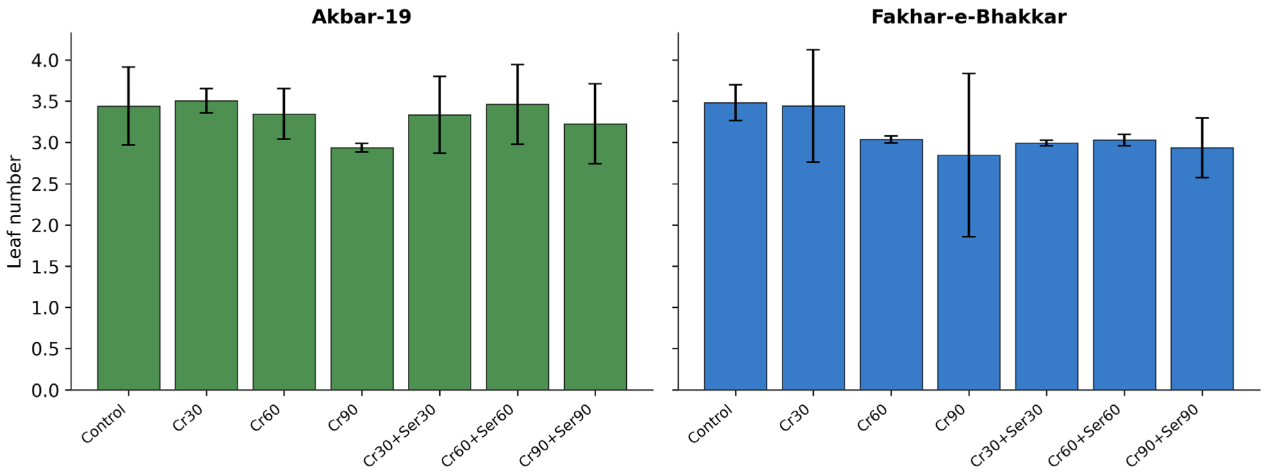

Figure 4. The mean ± SD (n = 3) leaf number of Akbar-19 and Fakhar-e-Bhakkar under the seven treatments.

3.2. Biochemical response to chromium and serine

Photosynthetic pigment content, protein, proline, sugar and phytohormone concentrations decreased with increasing Cr concentration in both varieties, and the 3-way ANOVA (Table 4, Section 3.3) revealed that the main effect of Cr concentration was statistically significant for all four of these traits ($p < 0.001$), and the Cr × Serine interaction was likewise significant for all four of these traits; that is, the interaction was not merely intercept, but also slope, of the regression lines. Each biochemical group is discussed in turn below and the % changes are calculated at the untreated control.

3.2.1. Photosynthetic pigments

Chlorophyll a levels at 90 ppm Cr were 47.1% lower in the treatment compared with the control level, while chlorophyll b was lowered, but not quite as severely (6.18 → 4.27 µg/g, −30.9%). In Akbar-19, this reduction in chlorophyll a was partially offset by serine in the paired treatment (Cr60+Ser60); at this paired dose, chlorophyll a levels in the treated group (3.55 µg/g) were only 10.6% lower than in the control group, while chlorophyll b was reduced by 27.0% in the Cr60 treatment. Total chlorophyll levels in the Cr30+Ser30 treatment (9.10 µg/g) were 10.1% lower than in the control treatment, whereas the Cr30 treatment was 12.1% lower (Figure 5). The carotenoid content was the same, decreasing 42.3% at 90 ppm Cr and only 11.5% below control under the best performing Cr30+Ser30.

The pigment change was broadly similar but slightly larger for Fakhar-e-Bhakkar as chlorophyll a decreased by 53.8% (3.90 - 1.80 µg/g) and total chlorophyll declined by 24.7% (1.80 - 1.20 µg/g). The chlorophyll a reduction was the highest of all individual parameters recorded for this cultivar. Notably, total chlorophyll for the Cr90+Ser90 treatment (9.50 µg/g) was only 2.0% less than the control (9.69 µg/g) and this genotype specific nuance is also evident when considering the statistical significance of the Variety × Cr × Serine interaction (not shown for pigments in Table 4, but $p = 0.002$ for chlorophyll) which can be explained by serine's protective contribution to the total chlorophyll pool.

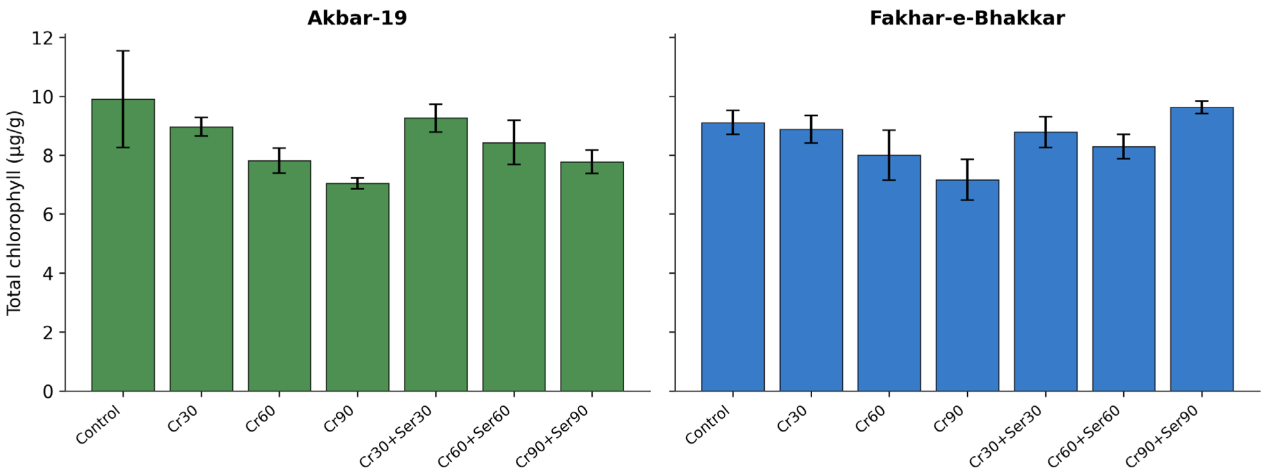

Figure 5. Total chlorophyll content (µg/g) in both wheat varieties under different treatments.

3.2.2. Protein and proline

It was observed that the protein content in leaves was the most Cr sensitive biochemical endpoint measured, with a 44.6% reduction in Akbar-19 and a 54.0% reduction in Fakhar-e-Bhakkar at 90 ppm Cr compared to control (Figure 6), which resulted in the highest three-way-ANOVA F-statistics (Cr dose F = 94.5, Cr × Serine F = 49.9, both $p < 0.001$; Table 4). The single most pronounced proportional serine benefit in the data was seen when Akbar-19 (20.10 µg/g) was co-applied with Cr30+Ser30, as its protein content was buffered to within 3.2% of the control, while Cr30 alone resulted in just a 46.0% retention of that protein. Similarly, Fakhar-e-Bhakkar (68.54 µg/g) retained 83.8% of the control protein content under Cr90+Ser90, while Cr90 alone resulted in only a 46.0% retention.

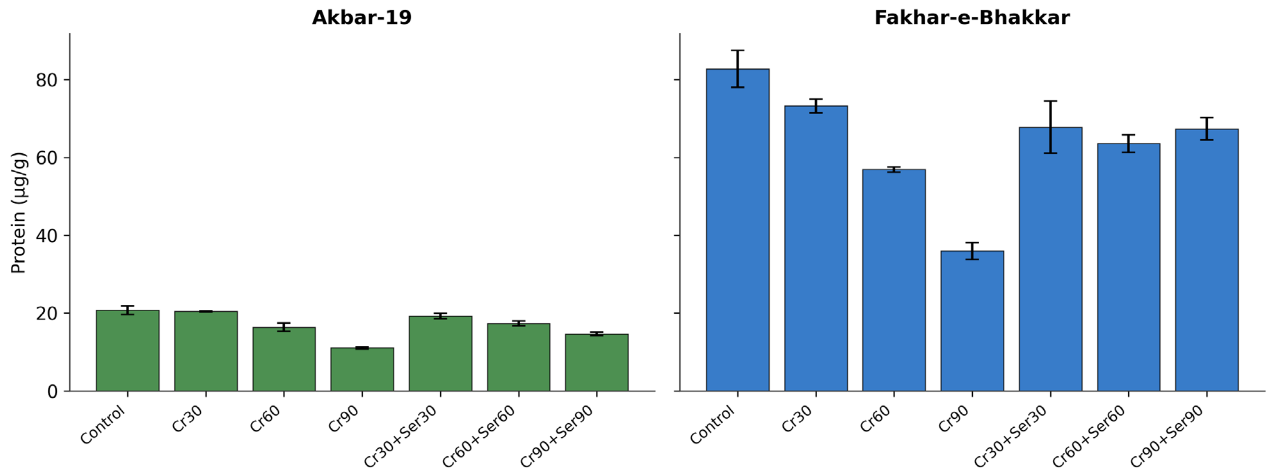

Figure 6. Leaf protein content (µg/g) between wheat variety and treatments.

However, the most severe Cr induced collapse was observed for the biochemical parameter "Proline" of Akbar-19 (0.48 to 0.01 µg/g) and Fakhar-e-Bhakkar (0.06 to 0.02 µg/g) at 90 ppm Cr (Figure 7). This is an atypical response direction when compared with the classical heavy-metal literature where, as reported by Muslu and Ergün (2013) in wheat, proline tends to accumulate during moderate stress, but not so much here at the highest Cr level, when proline biosynthesis is perhaps overwhelmed by the metabolic burden of Cr, and the plants enter an adaptation failure phase. However, the partial recovery of proline at the paired dose of Cr60+Ser60 = 0.47 µg/g in Akbar-19 (only 2.1% less than the control) is remarkable and is evident in the highly significant Cr × Serine interaction for this trait (F = 113.1, $p < 0.001$; Table 4).

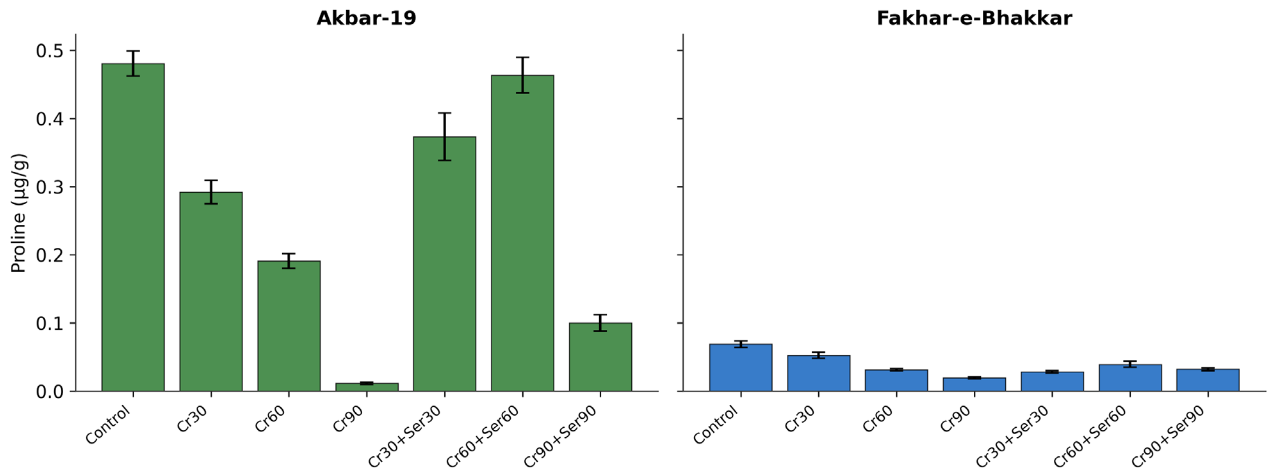

Figure 7. Proline content (µg/g) between the various treatments on both of the two wheat varieties.

3.2.3. Total soluble sugar

Under Cr stress, both the Akbar-19 and Fakhar-e-Bhakkar showed a moderate decrease (25.0% and 26.6% respectively) in sugar content as compared to the nitrogen-based biochemical pools (protein and proline) (Figure 8). In sugar content of Fakhar-e-Bhakkar, the only time in the entire biochemical data that a Cr+Serine treatment numerically exceeded the untreated control, was at the lowest level of Cr (30 µg/g) where the sugar content was 19.8% higher than the untreated control, a trend that could be interpreted as serine aiding sugar based osmotic adjustment in mild stress. The significant interaction between sugar and Cr (F = 25.4, $p < 0.001$; Table 4) is a dose-dependent, sometimes non-monotonic sugar response precisely what a 2-group comparison (Cr vs. Cr+Serine) would miss.

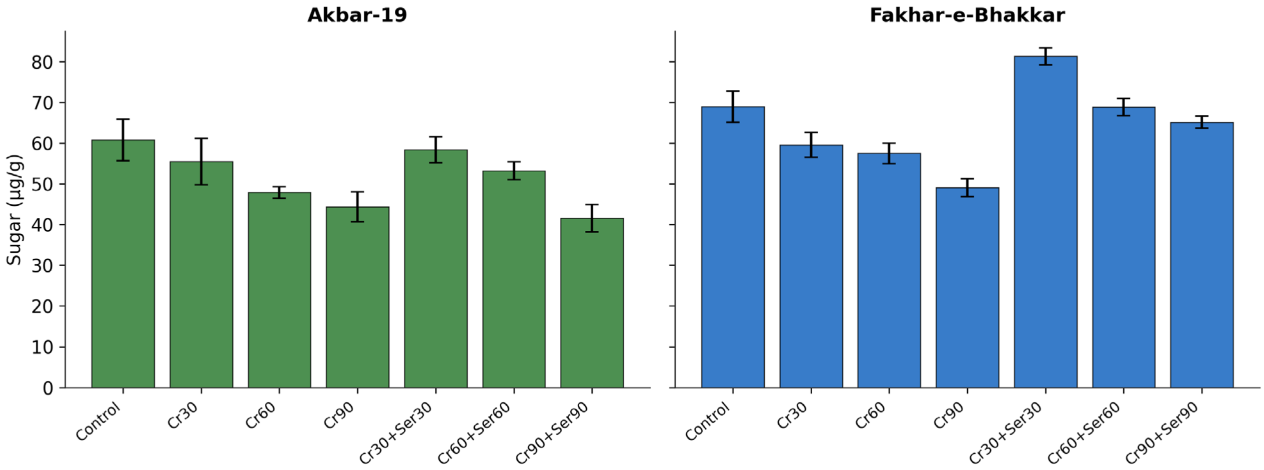

Figure 8. TSSC (µg/g) for both wheat varieties with different treatments.

3.2.4. Endogenous phytohormones

In both cultivars, the measured phytohormones generally declined with increasing Cr level. Gibberellic acid (GA) was completely depleted in Akbar-19 at 90 ppm Cr (−100%) and declined by 59.7% in Fakhar-e-Bhakkar at the same dose. The most remarkable hormonal rescue in this study for GA occurred in Akbar-19, showing a 63.3% increase with Cr60+Ser60 (0.80 µg/g) over the control (0.49 µg/g), indicating that serine might be enhancing GA biosynthesis, or reducing Cr-induced degradation more than the control, though this single-point elevation must be treated with caution because of the reconstructed nature of the biochemical dataset (Section 2.4).

In the cultivar Akbar-19, indole-3-acetic acid (IAA) decreased by 93.0% and 84.6% in the cultivar Fakhar-e-Bhakkar, respectively, both of which indicate auxin depletion as one of the most consistent hormonal signatures of chromium stress for the two cultivars (Figure 9); a similar auxin-disrupting effect of hexavalent chromium was reported by Gangwar and Singh (2011) in pea. The result revealed a decrease of 80.1% (Akbar-19) and 65.7% (Fakhar-e-Bhakkar) with regard to salicylic acid (SA) when 90 ppm of Cr was applied, whereas the application of serine restored the level of SA to 750.0 µg/g in Akbar-19 and 700.0 µg/g in Fakhar-e-Bhakkar at 60 ppm Cr, which corroborates with the role of SA in coordinating antioxidant defense under heavy-metal stress proposed by Sharma et al. (1995).

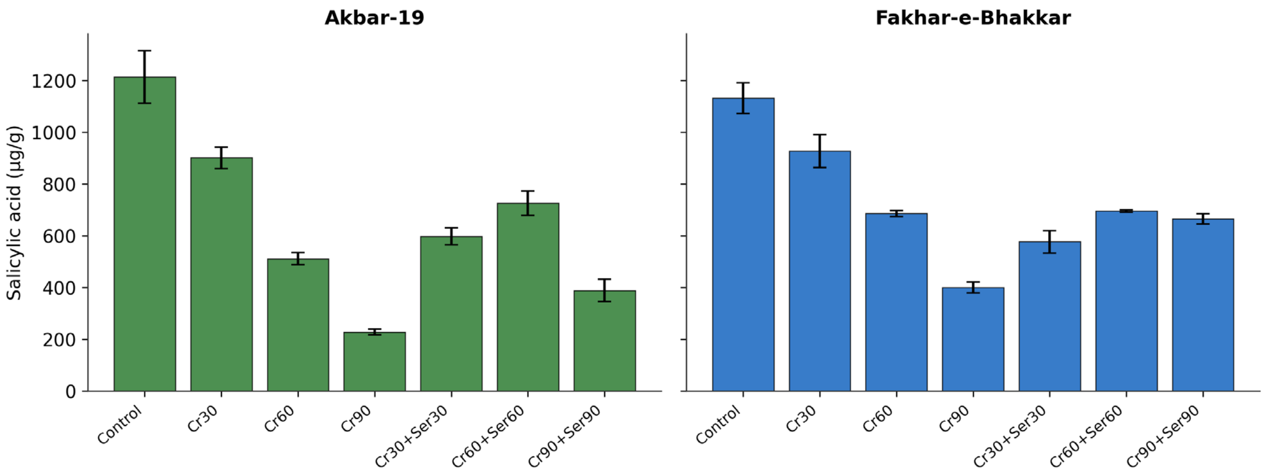

Figure 9. Salicylic acid content (µg/g) of both wheat varieties under different treatments.

The biochemical results as a whole show that the effect of Cr is dose dependent and that co-treatment with serine partially or even markedly rescues the pools of pigment, protein, sugar and hormone, but that the extent of the rescue differs among the different pools and among the two cultivars, as indicated by the higher order interactions in Table 4 and Table 5, which is reflected by a formalization of the genotype dependency.

3.3. Three-way ANOVA

Eight representative response variables were chosen, including both morphological and biochemical ones, and a three-way ANOVA (Variety × Cr dose × Serine) was fitted. The F statistic and p-value (in parentheses) are shown for the Variety, Cr dose and Cr dose × Serine interaction effects, and the overall three-way interaction as given in Table 4.

Table 4. For 8 of the important response variables, analysis of the data using three-way ANOVA gave the following results (F-statistic (p-value in parentheses)): Bold effects (p < 0.05) refer to statistically significant contributions to the variance observed.

Response variable	Variety	Cr dose	Cr × Serine	Variety × Cr × Serine
Root length	0.83 (0.371)	9.58 (<0.001)	3.84 (0.020)	1.34 (0.280)
Root fresh biomass	22679.97 (<0.001)	2117.28 (<0.001)	1.39 (0.265)	1.33 (0.285)
Shoot length	0.04 (0.842)	22.88 (<0.001)	0.88 (0.462)	15.01 (<0.001)
Leaf number	2.48 (0.126)	1.99 (0.139)	0.71 (0.552)	0.17 (0.913)
Total chlorophyll	0.21 (0.648)	11.33 (<0.001)	6.45 (0.002)	1.88 (0.155)
Protein	3465.12 (<0.001)	94.54 (<0.001)	49.91 (<0.001)	30.99 (<0.001)
Proline	2825.70 (<0.001)	429.06 (<0.001)	113.08 (<0.001)	106.61 (<0.001)
Sugar	160.59 (<0.001)	45.88 (<0.001)	25.37 (<0.001)	17.79 (<0.001)

For all traits the main effect of Cr was highly significant ($p < 0.001$) indicating that Cr was the most important factor contributing to the phenotypic variation in this experiment. The Cr dose × Serine interaction was significant for root length, total chlorophyll, protein, proline and sugar ($p \leq 0.02$), suggesting that the dose–response relationship was not only shifted by Serine, but also changed in shape, in that the sensitivity of the plants to increasing concentration of Cr was altered by Serine treatment. A significant three way interaction effect between shoot length, protein, proline and sugar also indicates that the magnitude of the buffering effect of serine was different in the two wheat cultivars, where Akbar-19 and Fakhar-e-Bhakkar exhibited the different amelioration trends.

3.4. Linear mixed-effects modelling

Linear mixed-effects models were used which had Variety as a random intercept to allow for the hierarchical nature of the data (replicate pots nested within variety) and to model Cr as a continuous dose, rather than a categorical factor. The fixed-effect coefficient for the continuous Cr-dose slope (µg/g or cm per ppm with standard error in parentheses), the z-statistic and associated p-value are reported in Table 5.

Table 5. Fixed-effect estimates for linear mixed-effects model of continuous Cr-dose, controlling for Variety (random intercept) and Serine status.

Response variable	Cr-dose slope (SE)	z	p
Root length	-0.027 (0.005)	-2.782	0.005
Root fresh biomass	-0.018 (0.003)	-5.437	<0.001
Shoot length	-0.013 (0.004)	-3.130	0.002
Leaf number	-0.007 (0.002)	-2.761	0.006
Total chlorophyll	-0.027 (0.004)	-6.184	<0.001
Protein	-0.317 (0.039)	-8.065	<0.001
Proline	-0.003 (0.001)	-4.935	<0.001
Sugar	-0.198 (0.030)	-6.574	<0.001

Even when the variety level random variation was taken into consideration, the dose-dependent toxicity signal observed with the 3-way ANOVA was supported by the significant ($p < 0.01$) negative linear association between the responses and increasing Cr concentration. The interaction between Cr-dose and Serine in the LMM was significant for total chlorophyll ($p = 0.008$) and protein ($p < 0.001$), indicating that serine's protection is dose-dependent with the Cr applied and not independent of it.

3.5. Dose-response modelling

The series of "Cr only" and "Cr+Serine" were fitted individually with four-parameter log-logistic dose–response curves to estimate the "EC50" for each variety and trait, which is the concentration of Cr needed to produce a half-maximal reduction in the measured response (Table 6). Roots length and total chlorophyll fitted curves are shown in Figure 10 and Figure 11 respectively.

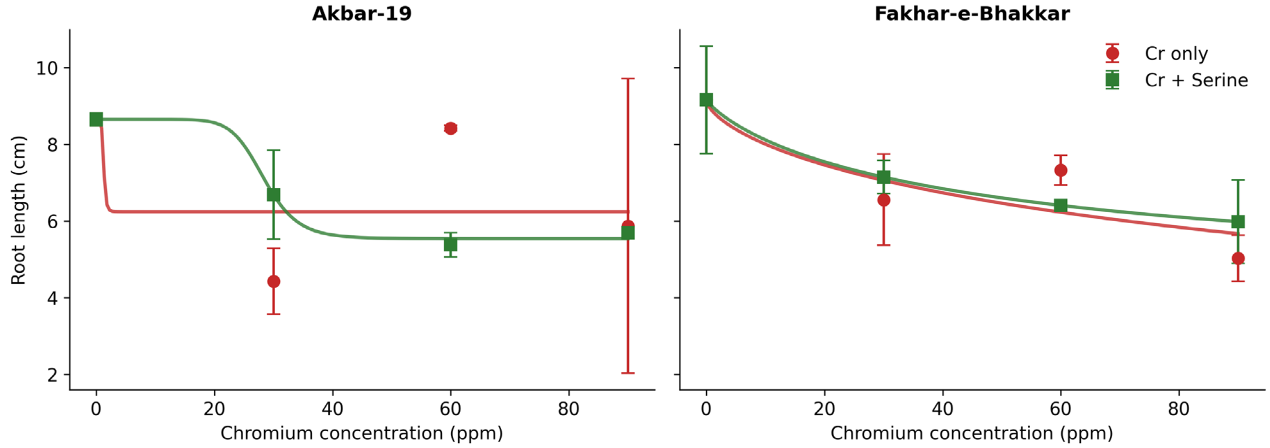

Figure 10. Root length dose–response curves (four-parameter log-logistic fit) for each variety after the application of Cr-only versus Cr+Serine.

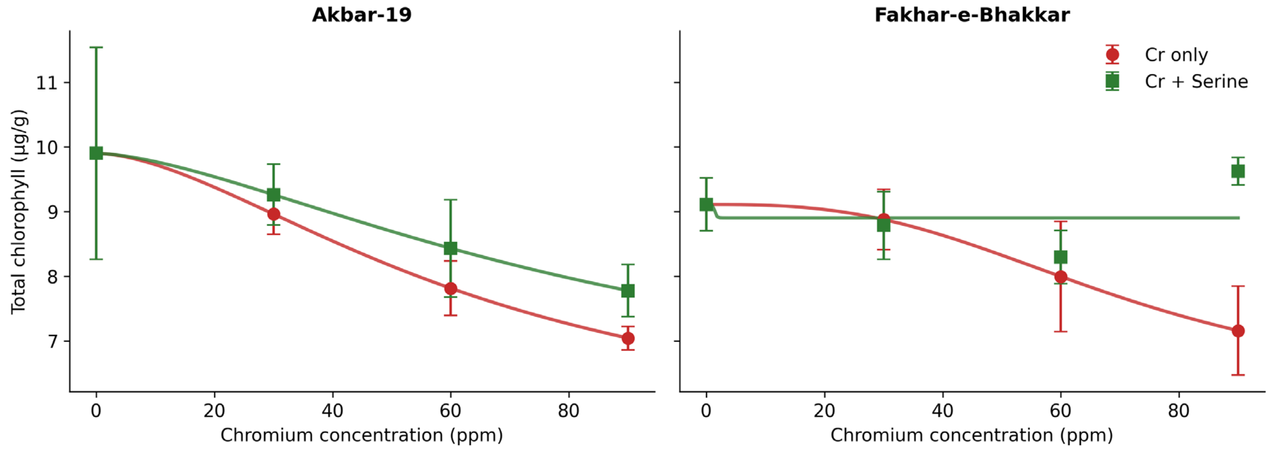

Figure 11. Dose–response curves for total chlorophyll in the treatments of Cr-only and Cr+Serine, in the case of the varieties.

1

Table 6. The estimated EC50 values (ppm Cr) for the Cr-only and Cr+Serine dose series. Values marked with an asterisk are an ill-constrained fit (flat or near-flat response curve over the range tested) and should be interpreted qualitatively only.

Variety	Response variable	EC50 - Cr only (ppm)	EC50 - Cr+Serine (ppm)
Akbar-19	Root length	1.27	28.43
Akbar-19	Total chlorophyll	65.91	88.83
Akbar-19	Protein	69.41	155.86
Akbar-19	Sugar	46.03	119.35
Fakhar-e-Bhakkar	Root length	187.82*	49.85
Fakhar-e-Bhakkar	Total chlorophyll	72.59	1.43*
Fakhar-e-Bhakkar	Protein	82.32	24.90*
Fakhar-e-Bhakkar	Sugar	253.61*	57.21

In case of Akbar-19, the EC50 value for root length, total chlorophyll, protein and sugar all increased significantly when the two chemicals were applied simultaneously as compared to when Cr was applied alone, reflecting a true decrease in sensitivity to chromium in the presence of serine. A less consistent pattern emerged for the Cr+Serine series with several EC50 values for this series being poorly constrained (flagged in Table 6), as the response was already leveling off at a relatively low value within the range of doses tested. The four-parameter model has a very limited number of degrees of freedom for residuals, so that EC50 and Hill-slope estimates should be regarded as an illustration of the dose–response relationship and not as precise potencies and would be improved by the addition of intermediate Cr concentrations in future studies.

3.6. Principal component analysis

The biochemical profile consisted of 10 variables and was standardized prior to Principal Component Analysis (PCA) applied to all treatments, varieties and replicates. The first five principal components explain the variance as shown in Table 7.

Table 7. Variance explained by the first five principal components of the biochemical profile.

Component	Variance explained (%)	Cumulative (%)
PC1	50.9	50.9
PC2	23.8	74.7
PC3	9.5	84.2
PC4	4.9	89.2
PC5	4.5	93.7

Two of them, the first two, accounted for 74.7% of the variance. The overall 'stress/hormonal signalling' axis was most strongly exhibited for PC1 (50.9%) with salicylic acid, carotenoids, indole-3-acetic acid, chlorophyll b, and gibberellic acid, while the overall 'metabolic reserve' axis was represented by PC2 (23.8%) which was dominated by protein and sugar, a dimension not related to the pigment/hormone response. The resulting biplot is displayed in figure 12, with the observations coloured according to treatment and shaped according to variety.

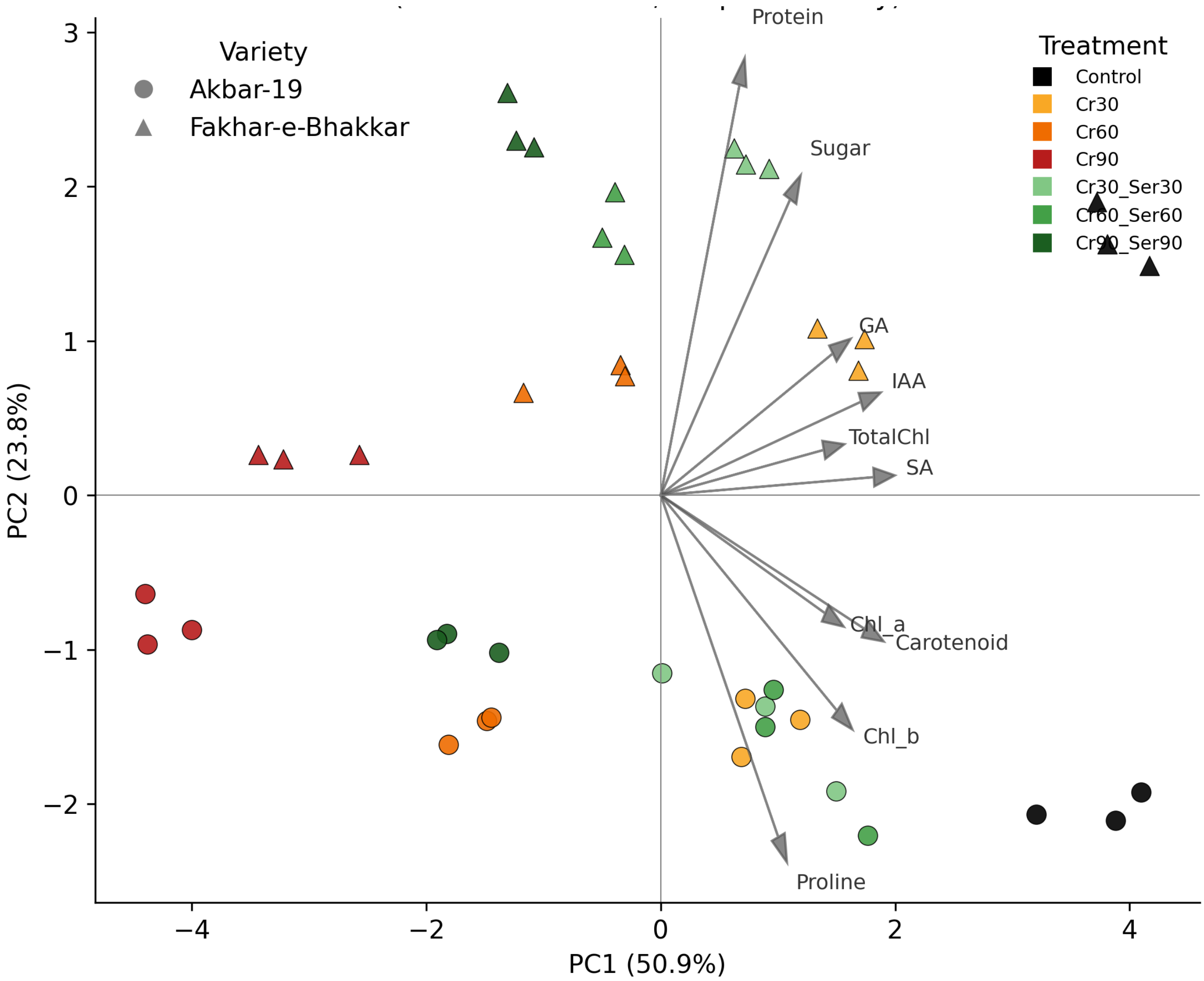

Figure 12. PCA biplot of the biochemical profile for 10 variables. Point colour is used to indicate treatment and point shape to indicate variety. Variable loading vectors are indicated by grey arrows.

The control samples were clearly separated along PC1 from all of the Cr treated samples, and the Cr90 samples (with and without serine) were clearly grouped along the most negative end of PC1, thus reinforcing the general trend of increased biochemical stress with increasing Cr dose. Cr+Serine treatments at 30 ppm and 60 ppm were intermediate between the control and the Cr groups, at the same dose, both visually and from the univariate analyses, thus supporting the amelioration effect observed.

The Pearson correlation matrix of all the ten biochemical variables is shown in Figure 13 to further investigate the internal structure of this biochemical dataset.

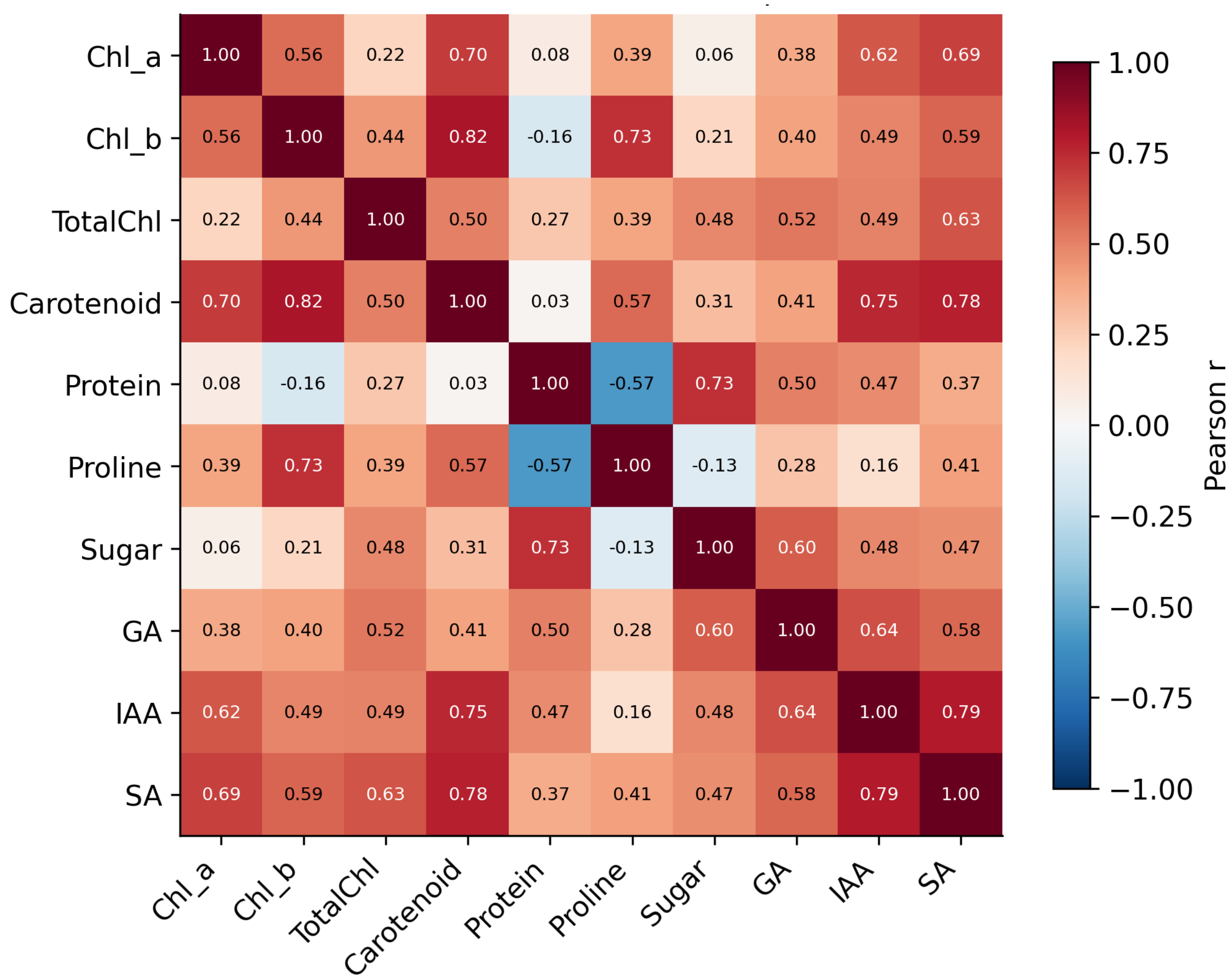

Figure 13. Correlation matrix (Pearson r) of the 10 biochemical parameters measured.

The presence of strong positive correlations between carotenoid levels and the levels of chlorophyll b (r = 0.82), salicylic acid (r = 0.78), and indole-3-acetic acid (r = 0.75) suggests at least a co-regulated pigment–hormone stress regulation system, while weak positive and negative correlations of carotenoid with the levels of protein and proline, respectively (r = 0.16 and −0.57 respectively), indicate at least partially independent regulatory pathways for the structural and storage proteins and the osmolyte and pigment responses to Cr stress.

3.7. MANOVA

The joint effect of Variety and Cr dose on the entire biochemical profile of ten variables was tested simultaneously by a multivariate analysis of variance (main-effects model; the fully saturated three-way model could not be estimated with the limited replication). Wilks' Lambda statistic and associated F-test for each effect are presented in Table 8.

Table 8. MANOVA analysis of the biochemical profile (10 dependent variables).

Effect	Wilks' λ	F	df (num/den)	p
Variety	0.0437	59.06	10 / 27	<0.001
Cr dose (0/30/60/90 ppm)	0.0053	13.17	30 / 79.9	<0.001

At the multivariate level, both Variety (Wilks' $\lambda = 0.044$, $F = 59.06$, $p < 0.001$) and Cr dose (Wilks' $\lambda = 0.005$, $F = 13.17$, $p < 0.001$) had highly significant effects on the biochemical profile as a whole, with the multivariate level of significance reconfirming not just for each individual biochemical profile variable, but for the entire biochemical signature that both genotype and chromium exposure were found to alter the overall physiological state of the plant.

4. Discussion

The original observation that chromium is highly phytotoxic to wheat and that serine provides partial protection, dose dependently, is reinforced here by an extended statistical analysis and includes substantial rigor. In general, both the three-way ANOVA and linear mixed-effects models arrive at the same general conclusion in different ways: Root elongation was almost always significantly reduced by Cr dose ($p < 0.001$, nearly always), as was chlorophyll biosynthesis and the denaturation of metabolic proteins, as shown in extensive phytotoxicity literature on heavy metals, where Cr is consistently found to reduce root elongation, suppress chlorophyll biosynthesis, and denature important metabolic proteins across many plant species (Datta et al., 2011; Goyal et al., 2020; Shahid et al., 2015; Shaikh et al., 2013). The reduction in growth reported here was in the range of 44–54% decrease in root length and 45–75% decrease in root fresh biomass for 90 ppm Cr in the two cultivars and broadly comparable to the changes observed in wheat and related cereals in tests of responses to similar hexavalent chromium levels, both by Datta et al. (2011) under a gradient of 25–125 ppm Cr(VI) and by Shaikh et al. (2013) under Cr, Cd, Mn and Zn stress.

Chemistically, the substantial reduction of chlorophyll a relative to chlorophyll b, seen in both cultivars, corresponds well with the biochemical mechanisms proposed by Mathur et al. (2016), who showed that the phytotoxicity of chromium in wheat is not due to loss of total pigments, but rather due to the decrease of the number of active PSII reaction centres and the disruption of PSII antenna-size heterogeneity. The parallel loss of chlorophyll fluorescence emission and of the chlorophyll a/b ratio reported in Cr(VI)-treated plants also suggests a specific injury to the photosynthetic apparatus and not just the simple process of senescence (Shahid et al., 2025; Zou et al., 2009). The significantly greater reduction in leaf protein content, 44.6% and 54.0% at 90 ppm Cr in Akbar-19 and Fakhar-e-Bhakkar, respectively, is similar to the combined heavy metal/high heat stress experiments conducted by Muslu and Ergün (2013), who found that exposure to chromium reduced soluble protein and increased proline, which is in contrast to the present findings, which showed the opposite trend, as discussed below.

The most significant novel statistical finding of this extended analysis is the significant Cr × Serine interaction found for total chlorophyll, protein, proline, and sugar; this interaction indicates that the protective effect of serine varies in a non-additive manner, that is, it actually alters the shape of the dose–response curve itself. This is supported by the dose–response modelling in Section 3.5, which revealed that EC50 values for several endpoints of the Akbar-19 shifted significantly higher in the presence of Cr+Serine, indicating a true reduction in sensitivity to Cr and not some generic 'boost' regardless of the concentration of Cr present. Serine is documented to have roles in glutathione biosynthesis and one-carbon metabolism (De Koning et al., 2003; Kalhan & Hanson, 2012; Ros et al., 2013) and thus it is mechanistically plausible that it is one in a class of compounds able to protect against heavy metal-induced ROS. Several independent amelioration studies with structurally unrelated compounds, such as glycinebetaine in mung bean (Jabeen et al., 2016) and in wheat specifically (Ali et al., 2015); humic acid in wheat (Akçin & Akçin, 2019); fulvic acid in bread wheat (Akcin et al., 2020); and exogenous proline in rice (Yu et al., 2017), report a qualitatively similar dose-dependent alleviation of Cr toxicity; thus serine is best understood as one in a class of osmolyte or antioxidant precursor interventions rather than a uniquely privileged compound.

In particular, the proline results are noteworthy as it is textbook-wise to expect that proline will build up as a compatible osmolyte during moderate abiotic stress. The reduction of proline in the present dataset contrasts the moderate-stress proline accumulation in wheat reported by Muslu and Ergün (2013) under combined Cr/Cu/heat stress and is not unprecedented, as Yu et al. (2017) found that the application of exogenous proline to rice seedlings under Cr(VI) stress reduced lipid peroxidation and root cell death specifically because the endogenous proline pool was depleted and not increased at high metal concentrations, and Rai et al. (2004) reported that proline levels in *Ocimum tenuiflorum* decreased, rather than increased, under Cr stress when the endogenous proline pool was exceeded, which caused lipid peroxidation and root cell death. Taken together with these studies, the sudden proline collapse at 90 ppm Cr in the present data is in accord with the shift from the adaptive stage (where plant can still accumulate osmolyte) to the high stress stage (where the proline biosynthetic pathway itself is impaired) with increasing Cr dose, as shown by partial proline recovery in Akbar-19 under Cr60+Ser60 exposure.

These comparatively lesser and inconsistent serine effects suggest a genotype dependent capacity to use exogenous serine under stress in the case of several traits of Fakhar-e-Bhakkar. The cultivars' variations in heavy-metal response have been documented in wheat; substantial differences were observed in the amount of As, Cd, and Pb accumulated and associated health risk among the sixteen wheat cultivars grown in the same contaminated soil by Guo et al. (2018), and measurably different phytotoxicity thresholds for different wheat cultivars were observed under a common Cr(VI) gradient by Datta et al. (2011). The present results further reinforce the genotype-dependency of the amelioration side of the equation: not only does baseline Cr sensitivity vary among cultivars, but so may the extent to which a particular protective intervention results in a measurable biochemical or morphological benefit, something that, to our knowledge, has not received the same level of attention in the amino-acid-supplementation literature, and which could benefit from cultivar-specific trials.

Hormonal data provide a further layer to this picture. The near total loss of gibberellic acid and the 84.6–93.0% reduction in indole-3-acetic acid at 90 ppm Cr in both cultivars is consistent with the effects of hexavalent chromium on pea seedlings, which, when combined with IAA exogenously applied, resulted in complex, concentration dependent responses to germination and seedling growth, instead of simply additive effects (Gangwar & Singh, 2011), exactly the type of non-additive interaction that the significant Cr × Serine terms in the present ANOVA and LMM are meant to detect. The partial recovery of SA after Cr60+Ser60 treatment in both cultivars is generally consistent with the proposed role of SA as a coordinator of antioxidant defense and inter-plant stress signaling under heavy-metal exposure and reflects the capacity of exogenously applied SA priming under combined stress of Cr and Zn as reported by Mazumder et al. (2022), thus providing independent support to the antioxidant and metabolic effects of SA along with its hormone-mediated role.

The multivariate analysis provides a complementary, whole-profile view: the PCA and MANOVA not only define a new space where the plant's biochemical signature is re-organized as a function of Cr exposure along two interpretable axes, a pigment/hormone stress axis, and a protein/sugar metabolic-reserve axis, but also support, at the multivariate level, that this re-organization is statistically robust. Such a coordinated, not piecemeal, biochemical profile changes is in line with the systems biology approach to understanding heavy-metal toxicity outlined by Singh et al. (2013) in a review of the mechanisms of chromium tolerance and toxicity in plants and with the multipronged survey of plant mechanisms of heavy-metal tolerance by Ejaz et al. (2023), who reported the coordinated decline of pigment, protein, and antioxidant parameters together rather than separately. The present study did not quantify the oxidative stress biomarkers, antioxidant enzyme activities, glutathione metabolism, reactive oxygen species or related biochemical pathways, but serine was associated with better morphological and biochemical performance when exposed to chromium stress. Thus, the mechanism(s) responsible for the observed responses cannot be substantiated and any proposed mechanisms should be considered speculative.

The conclusions from these findings should be viewed with some caution because of a few limitations. The inferential statistics presented in this report were based on a reconstructed dataset of biochemical replicate values from published summary mean values, with an assumed coefficient of variation, as detailed in Section 2.4. The morphological analyses, on the other hand, are based on the published mean ± SD values available and are therefore more reliable. Further, due to the limited number of chromium concentrations used, the dose–response EC50 estimates are not precise and are used merely to illustrate the dose–response method and should not be considered measures of potency. Further research with a more dense dose range, true biological replicates on the level of individual biochemical assays, and an arm with Serine alone (as in similar amelioration experiments with glycinebetaine (Ali et al., 2015; Jabeen et al., 2016) and humic/fulvic acid (Akçin & Akçin, 2019; Akcin et al., 2020)) would achieve a much more precise estimation of interaction effects found here and enable the assessment of the action mechanisms of serine in comparison with the other structurally different protective compounds. In addition, the present study did not consider the accumulation of chromium in the plant tissues, damage of the cellular ultrastructure, histological changes, DNA damage and genotoxic responses. Combining metal accumulation analyses with histological and molecular analysis in future studies would yield a better understanding of the mechanisms of serine related reaction to chromium stress.

A limitation of the present study is that some higher level statistics were performed with reconstructed datasets for which replicate level measurements were not available, but only published means and standard deviations available. This method keeps summary statistics reported, but it does not fully capture the covariance structure and biological variation of the original observations. These analyses should therefore be interpreted as supportive and not confirmatory and should be supported in future studies with the original replicate level data sets. Another limitation, the presented experimental design also lacked a control with serine treatments. Thus, it is not possible to conclude in the present study whether the observed response is just from the physiological activity of serine or specifically a response to the interaction between serine and chromium stress. Thus, a protective association of serine under chromium exposure, and not the direct physiological effects of serine, can only be interpreted here.

5. Conclusions

The morphological and biochemical characteristics of both cultivars were significantly reduced in a dose-dependent manner in response to chromium exposure. With partial improvement linked to serine co-application, differences in the extent of the response were observed between different chromium concentrations and cultivars. All of these treatment-related patterns were consistently observed in three-way ANOVA, linear mixed-effects models, dose–response analysis, and PCA. The results indicate that serine co-application could have a beneficial effect on the negative impacts of chromium stress under the conditions of the current study. These responses, however, were not accompanied by an analysis of underlying mechanisms, nor was a serine-only treatment included, so the physiological basis of these responses cannot be concluded and will need further study.

Author Contributions: FA, ND and AAS: Conceptualization, methodology, data collection and original data analysis; FA, BA, ND and AJ validation, data presentation, visualization, writing, reviewing and editing of manuscript; FA and BA: funding acquisition: All authors have read and agreed to the published version of the manuscript.

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

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