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Complementary Antioxidant and Photosynthetic Protection by Melatonin and Chitosan Biostimulants Alleviates Lead Toxicity in Quinoa

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Abstract

Melatonin and chitosan have emerged as potential biostimulants for mitigating heavy metal toxicity in plants. This study investigated the ameliorative effects of melatonin (MEL) and chitosan (CHI) foliar application on quinoa grown under lead (Pb) stress using a 3×3×3 factorial experiment with three Pb levels (0, 50, and 100 mg kg^{-1} soil) and three concentrations of each biostimulant (MEL: 0, 50, 100 μM; CHI: 0, 1, 3 g L^{-1}), measuring 27 physiological, biochemical, and yield traits in root and shoot. Lead significantly affected 26 of 27 traits ($p < 0.05$), reducing growth, photosynthetic efficiency, pigment content, and yield while increasing oxidative stress markers. Both MEL and CHI significantly affected 20 and 21 traits, respectively ($p < 0.05$). Notably, CHI uniquely improved vegetative growth parameters including leaf number ($F = 18.75$), leaf area ($F = 18.88$), plant height ($F = 10.00$), and stem diameter ($F = 21.30$) (all $p < 0.001$). Significant Pb×MEL×CHI three-way interactions were detected for six traits, including root volume, stem diameter, malondialdehyde content, activity of catalase and peroxidase, and chlorophyll b and carotenoids (all $p < 0.001$). Principal component analysis revealed that PC1 explained 75.5% of total variance, separating treatments primarily by Pb stress level. Combined MEL and CHI treatments produced additive protective effects, shifting plant physiological status toward unstressed controls. These findings demonstrate that melatonin and chitosan provide complementary amelioration of Pb toxicity in quinoa through coordinated enhancement of antioxidant defense and protection of photosynthetic pigments.

1. INTRODUCTION

Soil contamination with heavy metals represents one of the most significant environmental challenges facing global agriculture [1]. Among heavy metal pollutants, lead (Pb) is particularly concerning due to its persistence in soil, non-biodegradability, and severe phytotoxic effects [2]. Inside plant tissues, Pb interferes with several key physiological functions such as photosynthesis, mineral uptake, water regulation, and redox balance [3]. At the cellular scale, Pb exposure triggers an overproduction of reactive oxygen species (ROS), which in turn causes oxidative harm to membrane lipids, proteins, and nucleic acids [2, 4].

Quinoa (*Chenopodium quinoa* Willd.) has gained international recognition as a climate-resilient crop with exceptional nutritional properties [5]. Its ability to grow under marginal conditions, including saline and drought-prone environments, makes it an attractive candidate for cultivation in contaminated soils. However, like most crop species, quinoa is susceptible to heavy metal toxicity, which can significantly reduce its productivity and nutritional quality [5, 6].

Plants have evolved sophisticated defense mechanisms to cope with heavy metal stress, including enzymatic and non-enzymatic antioxidant systems [7]. Among the enzymatic defenses, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) are especially important because they detoxify ROS and help preserve redox equilibrium in cells [7, 8]. Through the sequential action of SOD, CAT, and POD, superoxide radicals are first converted to hydrogen peroxide and then detoxified to water and

oxygen, preventing oxidative damage to cellular constituents. Additionally, osmoprotectants like proline accumulate under stress conditions and contribute to osmotic adjustment, membrane stabilization, and direct ROS scavenging [9]. Beyond these enzymatic defenses, plants also deploy non-enzymatic antioxidants such as ascorbate, glutathione, and phenolic substances that provide additional layers of protection against heavy metal-induced oxidative injury [10]. The ascorbate-glutathione cycle represents a central hub for ROS detoxification in chloroplasts, while carotenoids protect the photosynthetic apparatus by dissipating surplus excitation energy and scavenging singlet oxygen [10, 11]. Together, these coordinated defense networks enable plants to maintain cellular homeostasis under heavy metal stress, although their capacity can be overwhelmed under severe contamination conditions [10].

Biostimulants have emerged as promising tools for enhancing plant stress tolerance. Melatonin (N-acetyl-5-methoxytryptamine), a ubiquitous molecule found in plants, has attracted considerable attention for its multifunctional roles in stress mitigation [12]. Melatonin functions as a strong antioxidant by directly neutralizing ROS and reactive nitrogen species, while also stimulating the activity of antioxidant systems and safeguarding photosynthetic pigments [13]. Furthermore, melatonin participates in the regulation of plant growth, root architecture, and stress-responsive gene expression [14]. Chitosan, a natural biopolymer derived from chitin, has demonstrated significant potential as a plant biostimulant [15]. It has been reported to enhance growth parameters, activate defense responses, and improve tolerance to a range of abiotic stresses, as well as heavy metal toxicity [16]. Chitosan application can stimulate root development, increase nutrient uptake, and enhance photosynthetic efficiency under stress conditions [17]. The polycationic nature of chitosan allows it to interact with cellular membranes and activate signaling cascades involved in stress responses [18].

Despite growing interest in these biostimulants, their individual and combined effects on heavy metal-stressed quinoa remain unexplored. Understanding how melatonin and chitosan influence physiological and biochemical responses under different Pb stress intensities could provide valuable insights for developing targeted amelioration strategies. Furthermore, investigating potential synergistic effects between these two biostimulants may reveal opportunities for integrated stress management approaches. Therefore, this study aimed to: (1) evaluate the individual effects of Pb contamination, melatonin, and chitosan on physiological, biochemical, and yield parameters of quinoa; (2) determine the combined ameliorative potential of melatonin and chitosan under Pb stress; and (3) identify potential synergistic interactions between these biostimulants across different Pb stress levels.

2. RESULTS

2.1. Effects of Lead Stress on Quinoa Performance

Lead contamination significantly affected 26 of the 27 measured traits ($p < 0.05$; Table 1). The only trait not significantly affected by Pb was F0 ($F = 2.69$, $p = 0.077$). Increasing Pb levels from low to high caused progressive and significant declines across all growth, physiological, and yield parameters.

Vegetative growth was severely impaired, with mean leaf number declining from 29.5 to 17.4 ($F = 480.04$, $p < 0.001$), mean leaf area from 14.9 to 7.9 cm² ($F = 296.05$, $p < 0.001$), and plant height from 77.6 cm to 38.9 cm ($F = 1,286.96$, $p < 0.001$). Root system development was similarly affected, with root weight decreasing significantly ($F = 1,025.20$, $p < 0.001$) and root volume declining from 780.2 to 464.9 cc ($F = 43,879.54$, $p < 0.001$).

Photosynthetic pigments decreased markedly with increasing Pb contamination (Fig. 1). Chlorophyll a decreased from 0.569 mg/g FW at control Pb to 0.202 mg/g FW at high Pb ($F = 1,007.31$, $p < 0.001$), chlorophyll b from 0.546 mg/g FW to 0.169 mg/g FW ($F = 132,185.92$, $p < 0.001$), total chlorophyll from 1.115 mg/g FW to 0.371 mg/g FW ($F = 3,947.22$, $p < 0.001$), and carotenoids from 0.499 mg/g FW to 0.123 mg/g FW ($F = 192,970.53$, $p < 0.001$).

Photosynthetic efficiency was severely compromised under Pb stress. SPAD values declined from 41.1 to 28.4 ($F = 514.47$, $p < 0.001$). Chlorophyll fluorescence parameters showed significant deterioration, with Fv/Fm decreasing from 0.786 to 0.740 ($F = 42.65$, $p < 0.001$), Fv from 600.0 to 479.2 ($F = 247.67$, $p < 0.001$), and Fm from 763.3 to 646.3 ($F = 290.40$, $p < 0.001$). Relative water content (RWC) decreased from 73.1% to 57.4% ($F = 113.49$, $p < 0.001$), while electrolyte conductivity (EC) increased from 21.7 to 27.0 ($F = 111.66$, $p < 0.001$) (Table 1 and Table S1).

Yield components showed significant reductions with increasing Pb contamination (Fig. 2). Grain yield decreased from 1,795 kg/ha at control Pb to 1,301 kg/ha at high Pb ($F = 194.09$, $p < 0.001$), representing a 27.5% reduction. Biological yield decreased from 500.6 g to 405.8 g ($F = 160.82$, $p < 0.001$), harvest index from 35.9 to 32.0 ($F = 109.60$, $p < 0.001$), and 1000-grain weight from 2.73 g to 1.60 g ($F = 227.73$, $p < 0.001$).

Oxidative stress markers increased dramatically with rising Pb stress (Fig. 3). The level of malondialdehyde (MDA) increased from 0.487 at control Pb to 0.863 at high Pb ($F = 43,556.60$, $p < 0.001$). Antioxidant enzyme activities showed parallel increases: catalase (CAT) activity from 0.360 to 0.737 ($F = 123,156.39$, $p < 0.001$), peroxidase (POD) activity from 0.244 to 0.622 ($F = 99,069.62$, $p < 0.001$), and proline content from 0.774 to 1.134 ($F = 49.08$, $p < 0.001$).

2.2. Individual Effects of Melatonin and Chitosan

Melatonin foliar application significantly affected 20 of the 27 measured traits ($p < 0.05$; Table 1). The most pronounced effects were observed on photosynthetic pigments and chlorophyll fluorescence parameters. Chlorophyll a increased with melatonin ($F = 108.16$, $p < 0.001$), chlorophyll b ($F = 15,636.82$, $p < 0.001$), total chlorophyll ($F = 445.20$, $p < 0.001$), and carotenoids ($F = 22,876.49$, $p < 0.001$). Fv/Fm increased significantly ($F = 18.48$, $p < 0.001$), along with Fv ($F = 30.85$, $p < 0.001$) and Fm ($F = 20.80$, $p < 0.001$). Yield parameters improved with melatonin: grain yield ($F = 14.53$, $p < 0.001$), biological yield ($F = 16.43$, $p < 0.001$), and 1000-grain weight ($F = 18.81$, $p < 0.001$). Melatonin significantly enhanced antioxidant machinery activities including MDA ($F = 5,288.60$, $p < 0.001$), CAT ($F = 14,536.06$, $p < 0.001$), and POD ($F = 11,601.73$, $p < 0.001$).

Chitosan foliar application significantly affected 21 of the 27 measured traits ($p < 0.05$; Table 1). Notably, chitosan uniquely improved several vegetative growth parameters that melatonin alone did not significantly affect. Chitosan significantly increased leaf number ($F = 18.75$, $p < 0.001$), leaf area ($F = 18.88$, $p < 0.001$), plant height ($F = 10.00$, $p < 0.001$), and stem diameter ($F = 21.30$, $p < 0.001$). Chitosan also enhanced root weight ($F = 24.45$, $p < 0.001$), root volume ($F = 106.19$, $p < 0.001$), and all photosynthetic pigments ($p < 0.001$). Post-spray physiological parameters improved with chitosan, including SPAD ($F = 38.42$, $p < 0.001$), Fm ($F = 33.22$, $p < 0.001$), Fv ($F = 36.97$, $p < 0.001$), and Fv/Fm ($F = 13.34$, $p < 0.001$). Yield components responded positively to chitosan, including grain yield ($F = 9.38$, $p < 0.001$), biological yield ($F = 5.04$, $p < 0.01$), harvest index ($F = 7.77$, $p < 0.01$), and 1000-grain weight ($F = 18.97$, $p < 0.001$). Chitosan significantly affected all oxidative stress markers ($p < 0.001$) except proline.

2.3. Interaction Effects

Twelve traits showed significant Pb×MEL interactions (Table 1), indicating that the ameliorative effect of melatonin depends on the intensity of Pb stress. Key interactions included plant height ($F = 5.39$, $p < 0.001$), SPAD ($F = 2.54$, $p < 0.05$), RWC ($F = 3.08$, $p < 0.05$), root volume ($F = 22.49$, $p < 0.001$), stem diameter ($F = 4.85$, $p < 0.01$), and all photosynthetic pigments and oxidative stress markers ($p < 0.001$).

Eight traits showed significant Pb×CHI interactions (Table 1). These included root volume ($F = 13.41$, $p < 0.001$), MDA ($F = 18.32$, $p < 0.001$), CAT ($F = 40.79$, $p < 0.001$), POD ($F = 30.75$, $p < 0.001$), chlorophyll a ($F = 2.64$, $p < 0.05$), chlorophyll b ($F = 49.12$, $p < 0.001$), total chlorophyll ($F = 4.78$, $p < 0.01$), and carotenoids ($F = 57.34$, $p < 0.001$).

Five traits showed significant MEL×CHI interactions (Table 1), including stem diameter ($F = 3.39$, $p < 0.05$), CAT ($F = 3.24$, $p < 0.05$), POD ($F = 5.30$, $p < 0.01$), chlorophyll b ($F = 7.75$, $p < 0.001$), and carotenoids ($F = 8.66$, $p < 0.001$).

Seven traits showed significant three-way interactions (Table 1), indicating complex interdependent effects among all three factors. Root volume showed a significant three-way interaction ($F = 4.81$, $p < 0.001$), with combined MEL and CHI producing the greatest increases. Stem diameter responses to MEL and CHI combinations were Pb-level dependent ($F = 2.74$, $p < 0.05$). Malondialdehyde exhibited the highest levels under combined high MEL and high CHI treatments at medium Pb stress ($F = 11.82$, $p < 0.001$). Catalase activity showed the strongest response to combined MEL + CHI treatment at medium Pb ($F = 37.56$, $p < 0.001$), with additive effects observed. Peroxidase activity demonstrated similar additive effects of MEL and CHI ($F = 23.80$, $p < 0.001$). Chlorophyll b content was highest under combined MEL and CHI treatments, with the strongest protective effect at medium Pb ($F = 33.19$, $p < 0.001$). Carotenoid content exhibited the largest increases under combined MEL + CHI application ($F = 54.35$, $p < 0.001$).

2.4. Simple Effects Analysis

Simple effects analysis of melatonin within each Pb×CHI combination revealed that melatonin remained effective across all Pb and CHI levels, though the magnitude of response varied (Table S1). Melatonin

was most effective at medium Pb across all CHI levels, consistent with a bell-shaped stress-response pattern. Similarly, chitosan was effective within each Pb×MEL combination, with the greatest growth-promoting effects observed at low to medium Pb stress (Table S1). Chitosan effects on vegetative growth parameters were most pronounced when combined with melatonin, suggesting complementary modes of action.

2.5. Correlation Patterns among Traits

Correlation analysis revealed strong and highly significant relationships among the measured traits (Fig. 4). Photosynthetic pigments formed a highly intercorrelated cluster. Chlorophyll b showed perfect correlation with carotenoids ($r = 1.00$, $p < 0.001$), and total chlorophyll was strongly correlated with chlorophyll a ($r = 0.996$, $p < 0.001$) and carotenoids ($r = 0.996$, $p < 0.001$). Oxidative stress markers were strongly interconnected. MDA was perfectly correlated with CAT ($r = 1.00$, $p < 0.001$) and nearly perfectly with POD ($r = 0.999$, $p < 0.001$). CAT and POD exhibited perfect correlation ($r = 1.00$, $p < 0.001$), suggesting highly coordinated antioxidant enzyme regulation.

Yield parameters showed strong positive correlations with photosynthetic traits. Grain yield was correlated with chlorophyll b ($r = 0.910$, $p < 0.001$), carotenoids ($r = 0.909$, $p < 0.001$), SPAD ($r = 0.890$, $p < 0.001$), and Fv/Fm ($r = 0.685$, $p < 0.001$). Conversely, yield exhibited strong negative correlations with stress markers: MDA ($r = -0.716$, $p < 0.001$), CAT ($r = -0.717$, $p < 0.001$), and POD ($r = -0.716$, $p < 0.001$).

2.6. Principal Component Analysis

PCA revealed clear separation of treatments according to Pb and biostimulant levels (Fig. 5). PC1 explained 75.5% of total variance and primarily separated treatments along the Pb stress gradient, with control Pb treatments positioned on the positive side and high Pb treatments on the negative side. PC2 explained 7.5% of the variance and further separated treatments according to biostimulant application within each Pb level. The top contributing traits to PC1 with strong positive coefficients were photosynthetic pigments (chlorophyll b, carotenoids, total chlorophyll) and growth parameters (plant height, grain weight per plant, SPAD). Traits with strong negative coefficients on PC1 were stress markers (CAT, POD, MDA) and electrolyte conductivity (EC).

Combined MEL and CHI treatments consistently shifted samples toward the positive side of PC1 within each Pb level, with the greatest shifts observed under medium Pb stress. The heatmap of treatment means further illustrated these additive effects (Supplementary Figure S1).

3. DISCUSSION

This study provides the first comprehensive evidence that combined melatonin and chitosan foliar application effectively alleviates Pb toxicity in quinoa through complementary mechanisms. The results demonstrate that while both biostimulants independently provide protection, their combination produces additive beneficial effects, particularly under moderate Pb stress conditions.

The decline in root volume and weight with increasing Pb levels suggests that roots, as the primary site of heavy metal contact, are particularly vulnerable to Pb toxicity, possibly resulting from Pb-induced disruption of cell division and elongation processes [28]. The significant reduction in photosynthetic pigments under Pb stress (Fig. 1) can be attributed to several mechanisms. Lead is known to block chlorophyll biosynthesis by suppressing δ-aminolevulinic acid dehydratase, an essential enzyme in that pathway [29, 30], while ROS generated by Pb exposure degrade pre-existing pigment molecules. The parallel decline in carotenoids is particularly concerning, as these compounds serve both as accessory pigments in photosynthesis and as antioxidants protecting the photosynthetic apparatus from photo-oxidative damage [31]. The decrease in Fv/Fm ratio under Pb stress indicates damage to photosystem II (PSII), specifically to the D1 protein of the reaction center [32]. The concurrent reduction in RWC and increase in EC suggest that Pb stress compromises membrane integrity and water relations, contributing to the overall decline in photosynthetic performance. The substantial increases in MDA, CAT activity, POD activity, and proline levels (Fig. 3) indicate that Pb exposure induces severe oxidative stress in quinoa. The correlation between these stress markers (Fig. 4) suggests a coordinated antioxidant response, where CAT and POD enzymes work synergistically to detoxify H_2O_2 generated during Pb stress, while proline accumulation contributes to osmotic adjustment and ROS scavenging [33]. As noted by Baxter et al. [34], the extent of oxidative injury depends on the equilibrium between ROS generation and their removal, and under severe Pb stress, this balance is disrupted, leading to lipid peroxidation and membrane damage.

One of the most significant findings of this study is the complementary nature of melatonin and chitosan effects. Melatonin primarily enhanced photosynthetic pigment content and antioxidant enzyme activities, consistent with its well-established role as a potent antioxidant and signaling molecule in plants [12]. As reviewed by Arnao and Hernández-Ruiz [35], melatonin acts as a multifunctional molecule that directly scavenges ROS, upregulates antioxidant enzyme activity, and protects chloroplasts from oxidative damage. Furthermore, melatonin has been reported to boost plant growth and abiotic stress tolerance through changes in root architecture, improved water use efficiency, and regulation of stress-related gene expression [14]. Chitosan, in contrast, improved vegetative growth parameters including leaf number, leaf area, plant height, and stem diameter (Table 1). These effects are consistent with the recognized biostimulant properties of chitosan in agricultural systems [15]. According to the report by Rojas-Pirela et al. [18], chitosan promotes plant growth under stress conditions through multiple mechanisms, including metal chelation, enhancement of nutrient uptake, stimulation of root development, and activation of defense signaling pathways. The metal-chelating capacity of chitosan, attributed to its reactive amino and hydroxyl functional groups, is particularly relevant for reducing heavy metal bioavailability in contaminated soils [17]. Dradrach et al. [16] demonstrated that soil application of chitosan combined with foliar melatonin spray significantly reduced heavy metal concentrations in wheat shoots, roots, and grain, while improving photosynthetic efficiency and grain nutritional quality. Similarly, in the present study, chitosan application reduced Pb toxicity symptoms and enhanced growth parameters that melatonin alone did not significantly affect. The improvement in chlorophyll fluorescence parameters (Fv/Fm, Fv, Fm) with both biostimulants indicates protection of PSII function.

This protection may result from enhanced antioxidant capacity that prevents oxidative damage to the photosynthetic electron transport chain. Masoumi et al. [36] reported that melatonin and nano-encapsulated melatonin improved chlorophyll fluorescence and photosynthetic pigments in tomato plants under salinity stress, while also upregulating aquaporin expression to enhance water relations. These findings parallel our observations of improved RWC and membrane stability in quinoa treated with melatonin and chitosan.

The significant three-way interactions for seven traits (Table 1) reveal complex interdependencies among Pb stress, melatonin, and chitosan. For antioxidant enzymes (CAT, POD) and stress markers (MDA), combined MEL + CHI treatments produced the greatest responses at medium Pb, suggesting additive activation of defense pathways. Such a bell-shaped trend suggests that the combined treatment most effectively boosts endogenous antioxidant capacity when stress is high enough to trigger defense responses but not so extreme that it overwhelms cellular repair systems [34]. For photosynthetic pigments (chlorophyll b, carotenoids), the three-way interaction reflects the combined protective effects of both biostimulants on pigment biosynthesis and stability. Iqbal et al. [37] reported that combining a soil amendment with foliar melatonin spray reduced Pb uptake in spinach by up to 95% in shoots and 84% in roots, while enhancing antioxidant enzymes activity and reducing oxidative stress markers. Our findings extend these observations to quinoa, demonstrating that chitosan can serve a similar protective function through its metal-chelating properties and growth-promoting effects. Carotenoids play dual roles as light-harvesting pigments and photoprotective agents that scavenge triplet chlorophyll and singlet oxygen [38], and their strong response to combined treatment ($F = 54.35$ for the three-way interaction) highlights their central role in stress amelioration. The synergistic effects observed between melatonin and chitosan in this study align with the findings of Al-Saadi et al. [39], who reported that combined melatonin and chitosan application significantly alleviated drought stress in saffron by maintaining RWC, reducing electrolyte leakage, enhancing catalase activity, and improving both vegetative and reproductive performance. These authors emphasized that the synergistic action of these two compounds helps maintain physiological homeostasis under stress conditions, a conclusion that is strongly supported by our PCA results showing that combined treatments produced the greatest shifts toward the physiological profile of unstressed controls.

The findings of this study support the potential use of combined melatonin and chitosan application as an integrated biostimulant strategy for crops grown in Pb-contaminated soils. The complementary effects—with chitosan promoting vegetative growth and melatonin enhancing stress tolerance mechanisms—suggest that combined application may provide more comprehensive protection than either biostimulant alone. Dradrach et al. [16] concluded that combining in-situ immobilization of heavy metals in soil with foliar melatonin application can act as a dual strategy in enhancing crop quality and remediating heavy metal-polluted soils simultaneously. Our results extend this concept to foliar application of both biostimulants, which may be more practical for field implementation. The PCA findings, which show that combined treatments produce the largest shifts toward the physiological profile of unstressed controls, offer multivariate statistical support for this integrated approach. PC1

explained 75.5% of total variance, demonstrating the dominant effect of Pb stress, while the separation along PC2 reflects the ameliorative effects of the biostimulants.

4. MATERIALS AND METHODS

4.1. Plant Materials, Experimental Design, and Treatments

This study was conducted as a factorial experiment based on a completely randomized design (CRD) with three replications under greenhouse conditions. Experimental treatments consisted of lead (Pb) stress at three levels [0 (control), 50, and 100 mg Pb per kg soil], foliar application of melatonin at three concentrations [0 (control), 50, and 100 μM], and chitosan foliar application at three concentrations [0 (control), 1, and 3 g L^{-1}]. The quinoa (*Chenopodium quinoa* Willd.) seeds used in this study were of the Titi Kaka variety (seed purity 99%, germination rate 95%, 1000-grain weight 2.6 g), obtained from Pakan Bazr Company, Isfahan, Iran. Prior to sowing, quinoa seeds were disinfected in 2% sodium hypochlorite for 1 minute, rinsed three times with distilled water, and then dried on filter paper [19].

The soil required for this experiment was collected from a depth of 0–30 cm from agricultural fields. Following air-drying, the soil (sandy clay loam with pH 7.81 and EC 1.21 dS m^{-1}) was mechanically ground and filtered through a 4-mm sieve. Soil spiking was performed by thoroughly mixing the soil with $PbCl_2$ solution to achieve concentrations of 0, 50, and 100 mg Pb per kg soil. The contaminated soils were then incubated under stable conditions (darkness, 25 ± 3°C, for 4 weeks at 60% water-holding capacity) to allow Pb equilibration with the soil matrix.

Plastic pots (40 cm height × 30 cm diameter) were filled with 7 kg of the prepared soil. Quinoa seeds were sown at a depth of 1 cm. After germination, healthy and vigorous seedlings were retained, and the remaining plants were thinned. Irrigation was performed according to soil moisture requirements throughout the growth period, and manual weeding was conducted as needed. Foliar application of the combined melatonin and chitosan treatments was performed at the vegetative growth stage and the flowering stage. One week after the second foliar application (at flowering), sampling was conducted for physiological and biochemical trait measurements.

The treatment codes used throughout this study are as follows: Pb1, Pb2, and Pb3 correspond to 0, 50, and 100 mg Pb per kg soil, respectively; Me0, Me1, and Me2 correspond to 0, 50, and 100 μM melatonin, respectively; and Chi0, Chi1, and Chi2 correspond to 0, 1, and 3 g L^{-1} chitosan, respectively.

4.2. Physiological, Biochemical, and Morphological Trait Measurements

Physiological traits including chlorophyll index (SPAD), electrolyte conductivity (EC) of leaves, chlorophyll fluorescence parameters, photosynthetic pigments, and relative water content (RWC) were measured for all treatments. Chlorophyll fluorescence traits—minimum fluorescence (F0), maximum fluorescence (Fm), variable fluorescence (Fv), and maximum quantum efficiency of photosystem II (Fv/Fm)—were

recorded with a fluorometer (OS-30p, OPTI-SCIENCES, USA). Before measurement, leaves were dark-adapted for 15 minutes using specialized clips. A SPAD-502 meter (Konica Minolta, Japan) was used to measure the chlorophyll index.

Photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids) were determined according to the method of Lichtenthaler [20]. Briefly, fresh leaf tissue (0.1 g) was homogenized in 80% acetone, and the volume was brought up to 10 mL. After centrifugation at 4000 rpm for 10 minutes, absorbance was measured at 663, 646, and 470 nm using a spectrophotometer. Pigment concentrations were calculated using standard equations and expressed as mg g^{-1} fresh weight (FW). Relative water content (RWC) was measured according to the method of Turner [21]. Ten fully developed reference leaves were collected from each treatment and immediately transported to the laboratory on ice. RWC was calculated as RWC (%) = [(FW − DW) / (TW − DW)] × 100, where FW is fresh weight, TW is turgid weight after 24 h in distilled water, and DW is dry weight after oven-drying at 80°C for 24 h. Electrolyte conductivity (EC) was measured according to the ISTA (2009) protocol [22].

Malondialdehyde (MDA) content was measured to assess the level of lipid peroxidation according to the method of Heath and Packer [23]. Absorbance was recorded at 532 and 600 nm, and MDA content was expressed as nanomoles per gram of fresh weight. Following the method of Bates et al. [24], proline content was measured by a ninhydrin-based colorimetric assay. Absorbance of the toluene layer was read at 520 nm.

For enzymatic extract preparation, 0.1 g of leaf tissue was pulverized and homogenized in 1.5 mL of phosphate-buffered saline (PBS, pH 7.8). The homogenate was centrifuged for 20 minutes at 4°C, and the resulting supernatant was used for enzyme activity measurements. Catalase (CAT) activity was measured according to Aebi [25] by recording the decrease in absorbance at 240 nm. CAT activity was expressed as mmol H_2O_2 decomposed per mg protein per minute. Peroxidase (POD) activity was determined according to Maehly and Chance [26] by measuring the rise in absorbance at 470 nm. POD activity was calculated with the extinction coefficient of tetraguaiacol (26.6 mM^{-1} cm^{-1}) and given as units per milligram of protein.

At the end of the growth period, six plants were randomly selected from each experimental unit. Plant height was measured from the soil surface to the tip of the panicle using a graduated ruler (cm). Stem diameter was measured at the middle of the first internode above the soil surface using a digital caliper (mm). Leaf number per plant was counted, and leaf area was measured using a leaf area meter (CI-202, CID Bio-Science, USA). Root volume was measured by the water displacement method using a graduated cylinder and expressed as cubic centimeters (cc). Thousand-grain weight was determined by counting eight replicates of 1000 healthy seeds and weighing with a digital balance (precision 0.001 g). Grain yield per plant was determined by harvesting, threshing, and weighing the grains of each plant. Biological yield was calculated as the total dry weight of aerial parts per plant. Harvest index was calculated as: Harvest index (%) = (Grain yield / Biological yield) × 100.

4.3. Statistical Analysis

All data were analyzed using Python (version 3.8) with the statsmodels (version 0.12.0), scipy (version 1.5.0), and scikit-learn (version 0.23.0) libraries. Data are presented as means ± standard error of the mean (SEM) for three replications (n = 3). Prior to analysis, normality was assessed using the Shapiro-Wilk test.

A three-way analysis of variance (ANOVA) was performed to evaluate the main effects of lead contamination (Pb; three levels: 1 = control, 2 = medium, 3 = high), melatonin foliar application (MEL; three levels: 0 = control, 1 = low, 2 = high), chitosan foliar application (CHI; three levels: 0 = control, 1 = low, 2 = high), and all two-way and three-way interactions on all measured traits. When ANOVA indicated significant effects ($p < 0.05$), Tukey's honestly significant difference (HSD) post-hoc test [27] was employed for mean separation. Compact letter display was used to indicate significant differences among treatment means, where means that share a common letter do not differ significantly at $p < 0.05$.

Simple effects analysis was conducted to examine: (1) the effect of melatonin within each Pb×CHI combination, and (2) the effect of chitosan within each Pb×MEL combination.

Pearson correlation coefficients were used to evaluate relationships among all 27 traits. Only correlations with $p < 0.05$ were treated as statistically significant. To visualize treatment effects and trait associations, PCA was carried out on data standardized to z-scores. Data were centered and scaled to unit variance prior to PCA. The first two principal components were retained for visualization, and trait coefficients were examined to identify variables contributing most to treatment separation.

All statistical tests were two-tailed, and significance was declared at $p < 0.05$. Significance levels in tables and figures are denoted as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ns (not significant).

5. CONCLUSIONS

This study demonstrates that melatonin and chitosan foliar application provides complementary amelioration of lead toxicity in quinoa through distinct but interconnected mechanisms. Melatonin primarily enhances antioxidant defense systems and protects photosynthetic pigments through its dual role as a direct ROS scavenger and signaling molecule. Chitosan additionally promotes vegetative growth parameters and may reduce heavy metal bioavailability through its metal-chelating properties. The significant Pb×MEL×CHI three-way interactions for seven key traits reveal complex interdependent effects, with combined treatments producing additive benefits particularly under moderate Pb stress. PCA confirmed that combined biostimulant treatments shift plant physiological status toward that of unstressed controls. These findings support the potential of integrated melatonin-chitosan foliar application as an effective strategy for improving quinoa productivity in Pb-contaminated soils, which may be more practical for field implementation.

Declarations

Competing interests

The authors declare no conflicts of interest.

Declaration
of Generative AI and AI-assisted Technologies in the Writing Process

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Declaration

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Author Contribution

Hadi Behzad: Investigation, Writing – original draft. Parisa Sheikhzadeh: Conceptualization, Supervision, Writing – review & editing, Funding acquisition. Nasser Zare: Project administration, Formal analysis, Writing – review & editing. Mitra Rostami: Investigation, Formal analysis, Writing – original draft.

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Data Availability

All datasets generated and analyzed during this study are provided within the published article and its Supplementary Information files.

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Figures

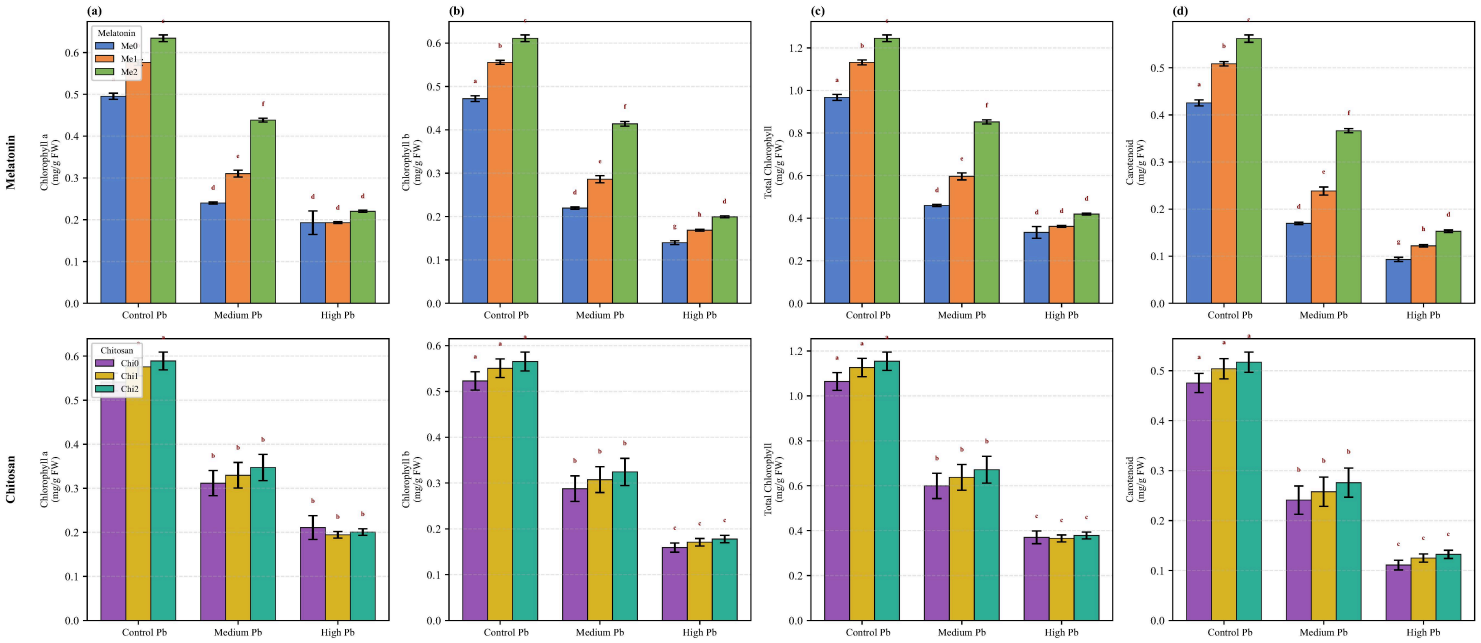


Figure 1

Photosynthetic pigment concentrations in quinoa plants under lead (Pb) contamination, melatonin (MEL), and chitosan (CHI) foliar application: (a) chlorophyll a, (b) chlorophyll b, (c) total chlorophyll, and (d) carotenoids. Bars represent mean ± SEM (n = 3). Different lowercase letters indicate significant differences among treatment combinations (Tukey HSD test, $p < 0.05$). Significant Pb×MEL×CHI three-way interactions were detected for chlorophyll b ($F = 33.19$, $p < 0.001$) and carotenoids ($F = 54.35$, $p < 0.001$).

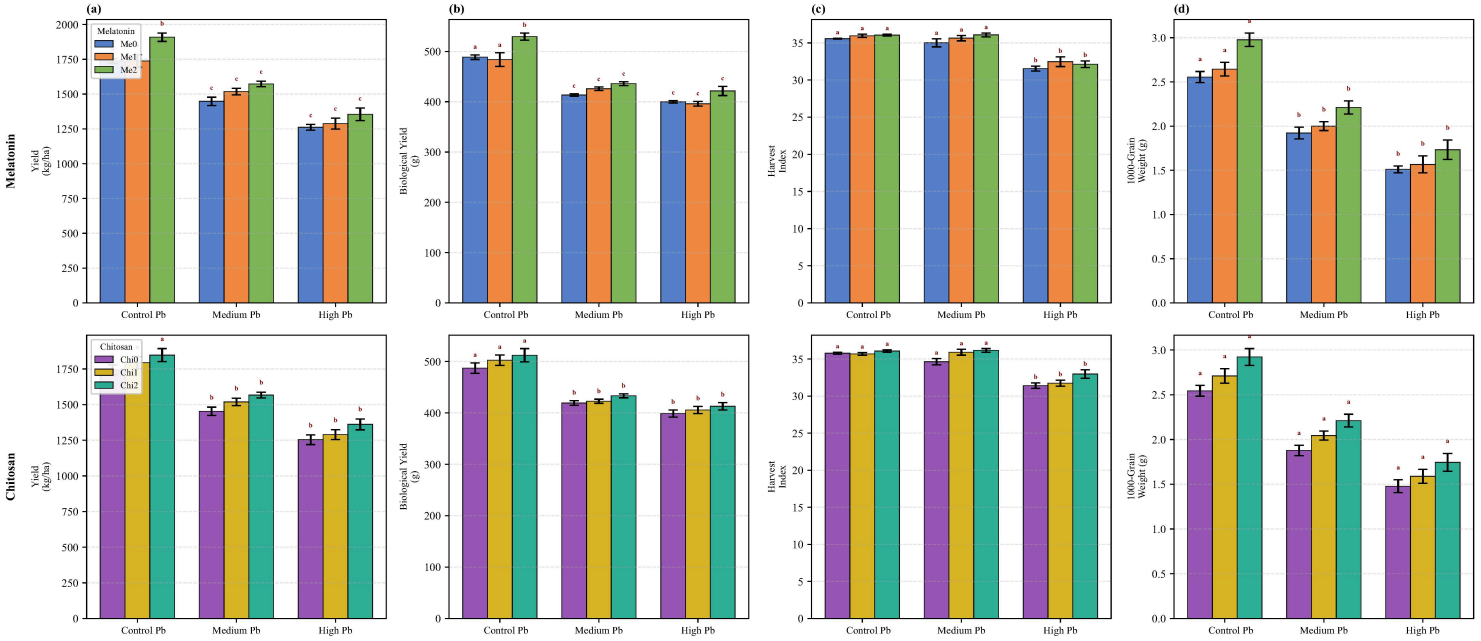


Figure 2

Yield components of quinoa under lead (Pb) contamination, melatonin (MEL), and chitosan (CHI) foliar application: (a) grain yield, (b) biological yield, (c) harvest index, and (d) 1000-grain weight. Bars represent mean ± SEM (n = 3). Different letters indicate significant differences among treatment combinations (Tukey HSD test, p < 0.05).

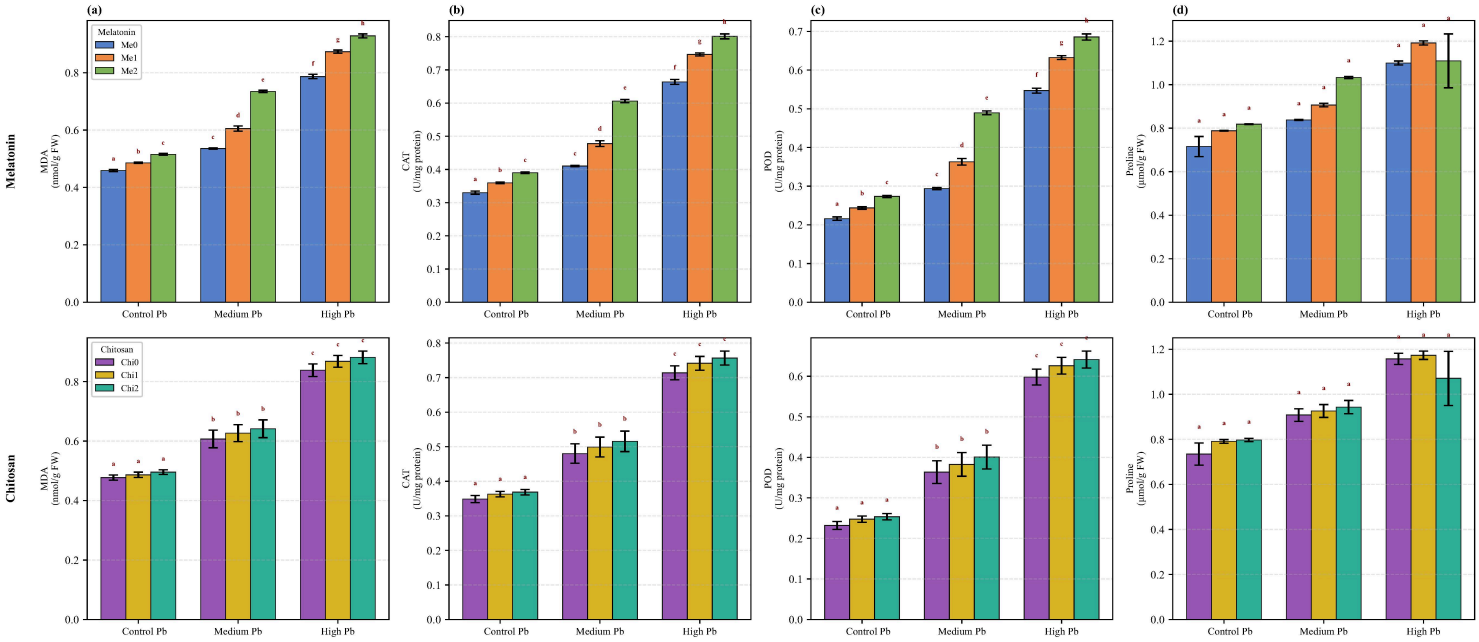


Figure 3

Biochemical stress markers in quinoa plants under lead (Pb) contamination, melatonin (MEL), and chitosan (CHI) foliar application: (a) malondialdehyde (MDA), (b) catalase (CAT), (c) peroxidase (POD), and (d) proline. Bars represent mean ± SEM (n = 3). Different lowercase letters indicate significant differences among treatment combinations (Tukey HSD test, p < 0.05). Significant Pb×MEL×CHI three-way interactions were detected for MDA ($F = 11.82$, $p < 0.001$), CAT ($F = 37.56$, $p < 0.001$), and POD ($F = 23.80$, $p < 0.001$).

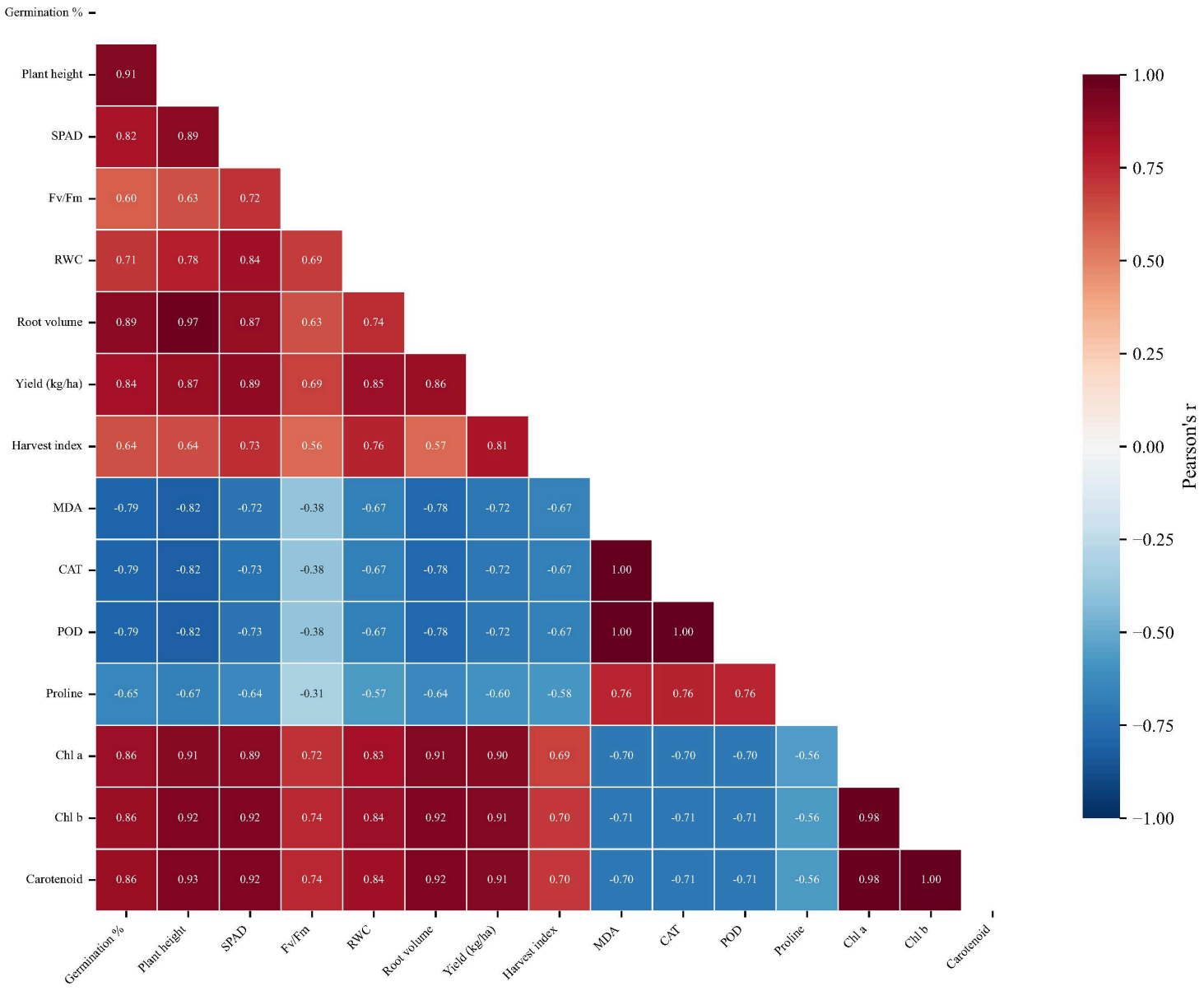


Figure 4

Pearson correlation matrix of key traits in quinoa under combined Pb, melatonin, and chitosan treatments. Color intensity represents correlation strength (red: positive; blue: negative). Only the lower triangle is displayed. All correlations shown are significant at $p < 0.05$.

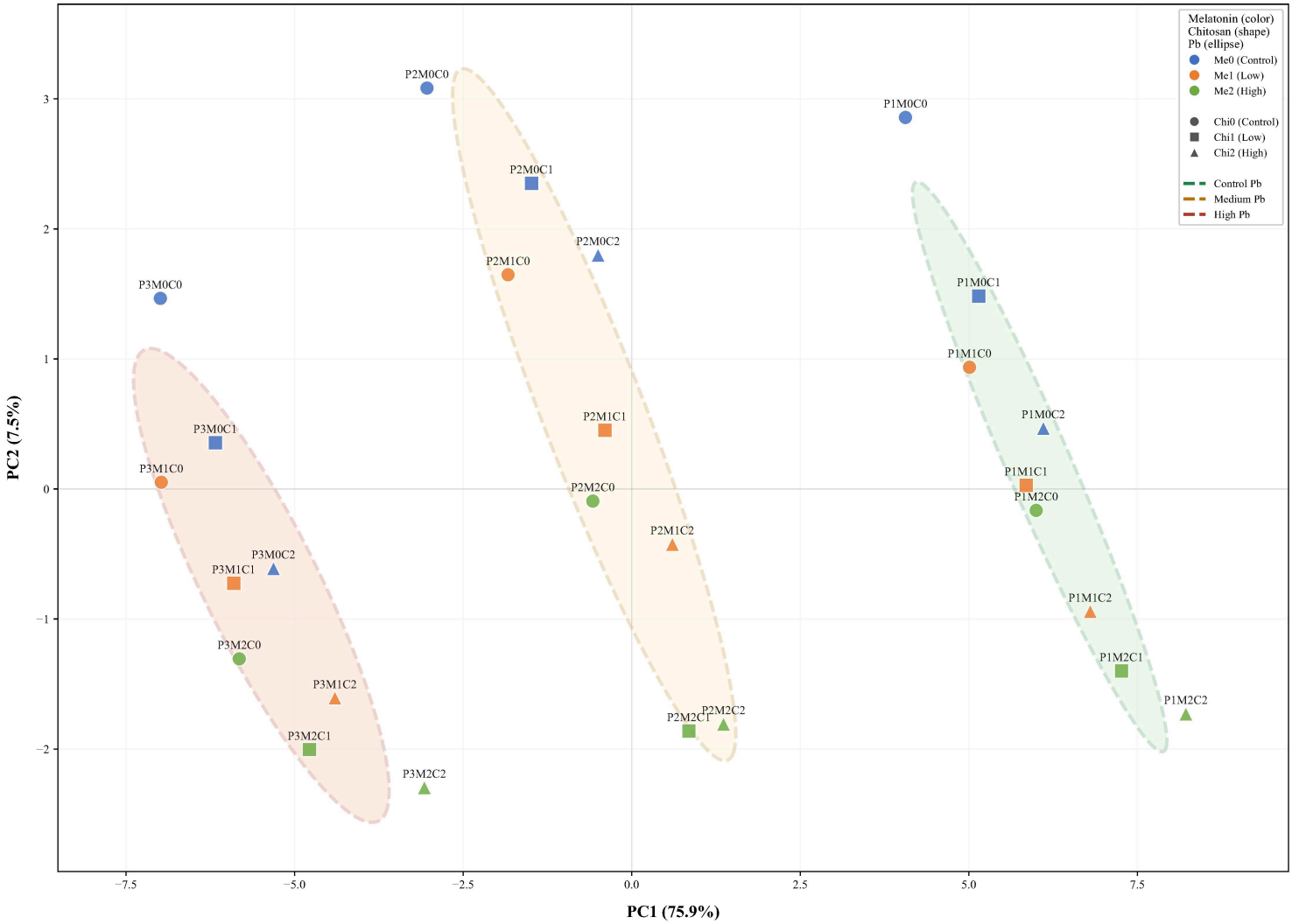


Figure 5

Principal component analysis (PCA) biplot of quinoa traits under lead (Pb) contamination, melatonin (MEL), and chitosan (CHI) foliar application. Dashed ellipses represent 68% confidence regions for Pb levels. Vectors represent the top contributing traits. PC1 explains 75.5%, and PC2 explains 7.5% of total variance (n = 3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1ANOVA.xlsx
- TableS1simple.xlsx
- FigS1Heatmap.pdf