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Protective effect of C-phycocyanin against lead nanoparticle – induced oxidative and genotoxic stress in alfalfa (*Medicago sativa* L.)

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ABSTRACT

Lead (Pb) is a serious threat for the plant growth and metabolism. Utilization of natural products, such as C-Phycocyanin, to ameliorate heavy metal stress is a successful and ecofriendly strategy for protection and enhancement of tolerance in plants growing under contaminated conditions. The nanotoxicological effects of lead nanoparticles (Pb NPs) and Pb NPs bound to C-phycocyanin (C-PC) on vegetative growth, chemical composition, oxidative burst, and DNA integrity in alfalfa plants (*Medicago sativa* L.) were studied using the plant as a model organism. Pb NPs at different concentrations (100–1000 mg L^{-1}) with and without C-phycocyanin were examined. Vegetative characteristic results indicated that the Pb NP exposure remarkably reduced plant height, branch number, as well as dry biomass and leaf area in a dose-response manner. Chemical composition analysis showed that the contents of chlorophyll, nitrogen, phosphorus, potassium and zinc decreased, while calcium increased under Pb stress. The exposure to Pb NPs also induced oxidative stress, increasing the levels of catalase, superoxide dismutase, ROS, lipid peroxidation and DNA damage. However, the addition of C-phycocyanin largely ameliorated such alterations, thereby stimulating growth and maintaining a good biochemical homeostasis, which protected cellular/genetic integrity from the damage caused by Pb. It was demonstrated that C-phycocyanin could be an efficient natural antioxidant and anti-lead nanoparticle protective agent, as well as plant defensive compound to combat the heavy metal stress.

Keywords: alfalfa, Pb NPs, C-phycocyanin, oxidative stress, DNA damage.

INTRODUCTION

Environmental pollution has become a global problem, as hazardous substances accumulate excessively in soil, water, and air ecosystems. Among these pollutants, heavy metals (Pb, Cd, Hg, and As) are some of the most persistent and toxic additives, since most of their forms do not degrade easily, and they tend to bioaccumulate in food chains. Long-term industrial operations, mining industries, and the application of polluted fertilizers have led to a significant increase in the levels of these elements in agricultural soils, which may harm not only food safety, but also sustainability (Mustapha *et al.*, 2025; Bao., 2025). The toxicity of heavy metals causes inhibition of growth, reduced photosynthetic efficiency, oxidative stress, as well as affects nutrient uptake and metabolism in plants (Mohamed, 2025).

In recent decades, due to the exponential growth of nanotechnology, a new kind of environmental pollution has arisen: accidental release of engineered NPs. They have been used in medicine, electronics and agriculture based on their unique physicochemical properties, but their deleterious release in the environment has raised serious ecological concerns (Rocco *et al.*, 2025). Due to their high surface-to-volume ratio, these particles particularly metal and metal oxide nanoparticles can efficiently interact with the biological system of plant or microbe, causing possible nanotoxicity to be induced (Jócsák *et al.*, 2022).

Pb NPs are one of the most hazardous pollutants due to nanometer scale and heavy metal characteristic. After uptake, NPs are able to penetrate plant tissues and accumulate within root as well as leaf cells to interfere with important metabolic process (Chen *et al.*, 2024). Different plant species exposed to Pb NPs show detrimental effects; notably, inhibited seed germination and stunted root length have been observed while chlorosis and necrosis were noticed in different plants (El-Shora *et al.*, 2021). Furthermore, the conversion of Pb NPs induces the generation of reactive oxygen species (ROS), resulting in overproduction rates of hydrogen peroxide and superoxide radicals, which raises oxidative stress, lipid peroxidation as well as membrane instability (Xu *et al.*, 2017; Nowicka, 2022). Such imbalance affects the photochemical apparatus, inducing degradation of proteins and nucleic acids engine, resulting in biomass reduction.

Alfalfa (*Medicago sativa* L.), an important forage legume, is highly sensitive to abiotic stress and is a good model for studying metal-induced phytotoxicity. The exposure of alfalfa to Pb NPs has been shown to alter morphometric characteristics, including root/shoot length, number of branches and leaf area, as well as physiological disruptions in terms of chlorophyll degradation and nutritional imbalances (Rahman *et al.*, 2024). These alterations are directly associated with oxidative stress, which alters the activities of antioxidative enzymes (superoxide dismutase SOD and catalase CAT) as well as those that protect against lipid peroxidation process, such as catalase CAT), and glutathione peroxidase GPx).

To mitigate these deleterious effects, the interest toward natural antioxidants and bioactive compounds produced by photosynthetic microorganisms has been growing. Among them, a blue pigment-protein complex named as C-phycocyanin (C-PC) purified from Arthrospira platensis or Spirulina has been extensively analyzed for its antioxidant and anti-inflammatory properties along with metal chelating nature (Lin *et al.*, 2022; Husain *et al.*, 2024). C-phycocyanin is a strong free radical scavenger that reduces oxidative stress by electron donation to counteract the effects of reactive oxygen species (Soror *et al.*, 2023). It also enhances the activities of the cellular antioxidant system as well as provides membrane stability against damaging stress by plants. In addition, heavy metals can be chelated by C-phycocyanin to decrease their bioavailability and plant translocation (Al-Yasiri and Alchalabi, 2021). Examining the synergistic effect of Pb NPs and C-phycocyanin is critical to elucidate the mechanism by which toxicity reduction, enhanced plant defense system work together. It has also been demonstrated that the exogenous application of C-phycocyanin brought about the recovery of photosynthetic pigments and growth parameters, as well as a decrease in heavy metal and nanoparticle-induced DNA damage in stressed plants (Mahanandia *et al.*, 2025). Therefore, investigating the alleviation of the Pb NP-induced toxicity in alfalfa by C-phycocyanin is important not only from a physiological stress perspective, but also for sustainable agriculture to reduce the potential hazards caused by nanoparticles. Henceforth, the aim of this study was to study the morphological and physiological adjustments in the alfalfa (*Medicago sativa* L.) plants irradiated with lead nanoparticles via a new route induced by Pb NPs as well as with applied prodefensive capping agent C-PC in comparison to its toxicity impact. The present experimentation will give an insight of C-phycocyanin into the amelioration of the Pb NP-mediated changes in growth, biochemical composition, oxidative equilibrium, and genetic stability to understand complete volatility phenomena for NP phytotoxicity to green solution.

MATERIALS AND METHODS

Experimental design

An experiment was conducted to investigate the impact of lead nano- particles (Pb NPs) alone and in combination with C-PC on the growth and physiological attributes of *Medicago sativa* L. (alfalfa). Four treatment groups were established:

- Control: untreated plants (distilled water was used for irrigation).
- Pb NPs: without any external agent (EA) at five concentrations (100, 300, 500, and 1000 mg L^{-1}).
- Pb NPs + C-Phycocyanin: combinations at equivalent Pb NP concentrations.
- C-phycocyanin alone: at matched concentrations (100–1000 mg L^{-1}).

The experiments were conducted in a CRD (complete randomized design) and replicated three times per treatment. The soil drencher was implemented every week for 4 weeks. The C-phycoyanin efficacy to alleviate the Pb nanoparticle-induced toxicity was investigated through morphological, biochemical and molecular analysis at the end of the experimental period of the plants.

Plant and growth conditions

Alfalfa (*Medicago sativa* L.) seeds were obtained from an authorized agricultural research center. The seeds were sterilized in 1% sodium hypochlorite for 3 min to remove contaminants and rinsed thoroughly with sterile distilled water. The seeds were germinated in plastic pots (20 cm diameter) filled with a soil–sand mixture (2:1, v/v). Plants were grown in a greenhouse at a constant temperature of 25 ± 2 °C, relative humidity of 65–70%, and a photoperiod of 16/8 h light/dark. The plants were irrigated at close intervals with distilled water to maintain the soil at about 70% of field capacity until treatments were applied.

Nanoparticle characterization and preparation

Lead nanoparticles (Pb NPs; >99.5% purity) were purchased from SkySpring Nanomaterials, USA (Product No.: 4835DL). The product obtained was grayish-black in color, as described by the manufacturer, and had an average particle diameter of 20–40 nm, a specific surface area of about 45–55 $m^2 g^{-1}$. The spherical nature of the particles was confirmed via SEM (scanning electron microscope) (Figure 1). Stock suspensions were prepared by dispersing Pb NPs in deionized water (1 g L^{-1}), and sonication was performed for 30 min at 40 kHz using a digital ultrasonic bath to facilitate uniform dispersion and avoid agglomeration. Suspensions were freshly drawn up before each application. All Pb NP manipulations were carried out under a chemical fume hood using appropriate PPEs (such as gloves, lab coats and respirators) for both inhalation and contact to avoid the potential toxicity of lead compounds.

Vegetative growth traits

The vegetative data of all treatments were taken on December 8, 2024. The measured growth variables were plant height (cm), number of branches per plant, leaf area (cm^2), fresh weight and dry weight (g). Height of the plants was recorded from soil > line to the apical tip by scale. The number of branches per plant was counted manually. Leaf area was estimated with a Laser Portable Leaf Area Meter (CI-202, Bio-Science, USA) following Johnson (1973). Fresh weights were measured immediately after harvest using an analytical balance (Model KERN AL J220-4NM). To calculate dry biomass, samples were oven-dried at 48 °C to constant weight.

Biochemical traits

Total chlorophyll was extracted with 80% acetone according to Mackinney (1941). Absorbance was measured at 645 and 663 nm using a UV-VIS spectrophotometer (Shimadzu UVmini-1240, Japan). The nutrient contents (N, P, K, Ca, and Mg) of the leaves were determined following wet digestion of samples using the method of Cresser

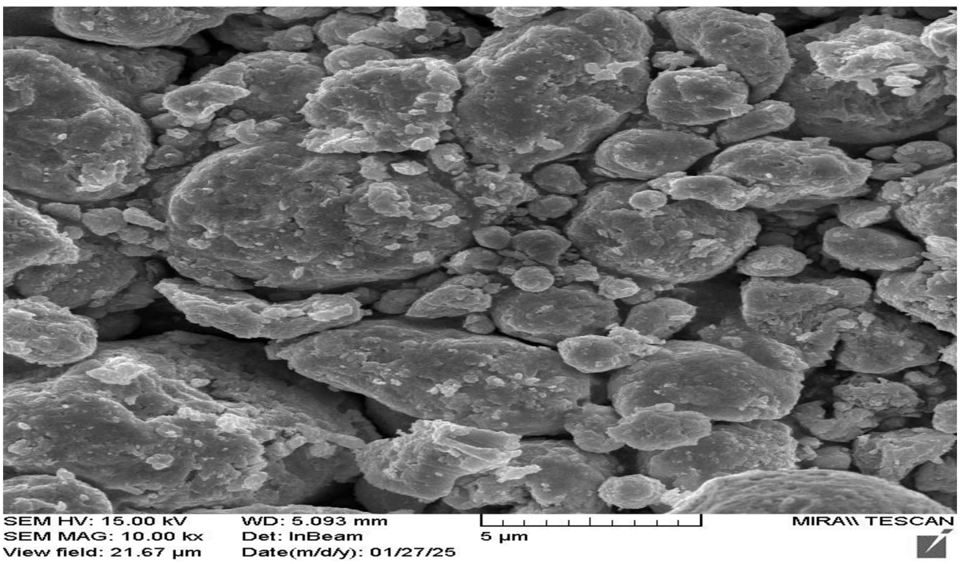

Figure 1. SEM image of Pb NPs powder (≥99.5% pure) indicating the spherical shape of particles and average particle size of 20 to 40 nm

and Parsons (1979). Nitrogen content was estimated using the Kjeldahl's method (Chapman and Pratt, 1962), phosphorus according to the vanadomolybdate spectrophotometric method (400 nm), potassium using a flame photometer (Jenway PFP7, Germany), and calcium, magnesium, and zinc by atomic absorption spectrophotometry (Shimadzu AA-7000). Total carbohydrates were determined using the anthrone method (Herbert *et al.*, 1971) after hydrolysis in diluted HCl followed by reaction with the anthrone reagent. Absorbance was read at 630 nm.

Measurement of oxidative stress and activities of antioxidant enzymes

Antioxidant enzymes were extracted from 1 g of fresh leaf tissue ground in a pre-chilled mortar and pestle with 2 mL phosphate-buffered saline (PBS, pH 7.0). The homogenate was centrifuged at 10.000 rpm for 30 min at 4 °C, and the supernatant was retrieved and diluted to a volume of 5 mL with PBS for enzymatic reactions (Bradford, 1976).

Superoxide dismutase (SOD)

SOD activity was measured according to Marklund and Marklund (1974) by the inhibition of pyrogallol auto-oxidation. Absorbance was read at 420 nm, and enzyme activity was expressed as μg mL^{-1}.

Catalase

To estimate catalase activity, the method of Johansson and Borg (1988), developed by Method A16, was used, and results were expressed as enzyme units per liter (U L^{-1}).

Glutathione peroxidase (GPx)

GPx activity was determined according to Rotruck *et al.* (1973). The oxidation of reduced glutathione (GSH) in the presence of H_2O_2 was followed at 412 nm using DTNB reagent. Activity was calculated as μmol GSH oxidized per mL enzyme extract.

Lipid peroxidation (MDA)

Lipid peroxidation was measured as described by Buege and Aust (1978) and Burtis and Ashwood (1999). The pink-colored MDA-TBA complex was spectrophotometrically determined at 532 nm and calculated as mmol MDA mg^{-1} of protein.

Reactive oxygen species (ROS)

The amount of ROS production was determined according to Venkidasamy *et al.* (2019). Absorbance was read at 560 nm, and ROS content was expressed as relative absorbance per mg fresh weight (oxidative damage intensity).

Vitamin C (ascorbic acid)

The ascorbic acid concentration was measured by DCPIP colorimetry, in which the reduction of the blue dye is proportional to the vitamin C content. Absorbance was determined, and the values were expressed as mg ascorbic acid per 100 g fresh weight.

Assessment of DNA damage (comet assay)

The alkaline Comet assay was performed, as described by Singh *et al.* (1988). Leaf nuclei were embedded on microscope slides in low-melting-point agarose and then lysed in lysis buffer at 37 °C for 2 min, followed by electrophoresis in the dark (70 V) for 60 min. The slides were neutralized, stained with ethidium bromide and examined under a fluorescence microscope. Nuclei were classified into low, medium and high-damage based on tail moment and length.

Statistical analysis

The data were analyzed using the SAS (9.3) package. The analyzed parameters were the influence of treatment, concentration and the interaction between both factors by using two-way ANOVA. Where differences were statistically significant, means were compared by LSD (least significant difference) at $p \leq 0.05$. Values are given as mean ± SE.

RESULTS

Morphological characteristics of alfalfa growth in the vegetative stage

The vegetative growth responses of alfalfa plants under various levels of Pb NPs and the joint effect with C-PC are displayed in Table 1.

The parameters measured were plant height, number of stems, green weight, dry weight and leaf area. The highest mean values of all vegetative traits were recorded in untreated plants, typical for unrestricted growth with no nanoparticle impact. There was a significant decline in all vegetative traits, not only solely with the Pb NP treatments, but with concentrations from 100 to 1000 mg L^{-1}. The highest reduction was found at 300 and 500 mg L^{-1}, with fresh and dry biomass of infected roots significantly decreased compared to controls.

The positive effect of C-phycocyanin on growth performance was partly observed when applied together with Pb NPs, mainly at low concentrations (100–300 mg L^{-1}) of Pb NPs. Co-application partially alleviated the adverse effects of Pb NPs on plant height, leaf area, and biomass but remained significantly lower than the untreated control. The ameliorative effect of C-phycocyanin decreased at higher concentrations (500–1000 mg L^{-1}), where the growth variables declined again.

C-Phycocyanin-only treatments resulted in a significant increase in vegetative growth at both test concentration levels. The height, branch number, leaf area, and fresh/dry weights of plants treated with 300–500 mg L^{-1} C-PC were significantly higher than or equal to those of the control. This proves that C-phycocyanin has a growth-promoting and protective effect on alfalfa plants under normal and stress conditions.

Collectively, these data suggest that the inhibitory effects on vegetative growth were dependent on the Pb NP exposure concentration, but were also partially counteracted by the protective and stimulatory actions of C-Phycocyanin, specifically at lower and moderate concentrations.

Table 1. Influence of lead nanoparticles (Pb NPs) and C-phycocyanin interaction on the vegetative growth traits of alfalfa (*Medicago sativa* L.) (Mean ± SE)

Treatments (mg L^{-1})		Vegetative growth traits				
		Plant height (cm)	Number of branches	Fresh weight (g)	Dry weight (g)	Leaf area (cm²)
Pb NPS	Control	32.3333±6.17342 A a	17±1.7321 A a	3.01±0.07095 A a	0.7527±0.0169 A a	2.55±0.4857 A a
	100	28±4.04145 A a	9±1 D b	1.11±0.04163 C c	0.2773±0.01065 D b	1.71±0.1701 E b
	300	20.3333±2.02759 C b	8±0.5774 C b	1.17±0.08327 D b	0.2927±0.02058 D b	1.77±0.07767 D b
	500	25±2.88675 A b	8±0.5774 D b	1.13±0.04041 E c	0.2827±0.00984 B b	1.71±0.11372 D b
	1000	23.3333±5.23874 C b	7±1.1547 B c	1.04±0.02517 E c	0.262±0.00656 D b	1.29±0.1601 E c
Pb NPS + C-Phycocyanin	Control	32.3333±6.17342 A a	17±1.7321 A a	3.01±0.07095 A a	0.7527±0.0169 A a	2.55±0.4857 A a
	100	24.3333±3.52767 A b	14±2.0817 B b	1.15±0.05686 C e	0.2877±0.0141 B c	1.99±0.18717 B c
	300	34.6667±6.48931 A a	16±1.7321 B a	1.71±0.14978 B b	0.4293±0.03943 A b	2.21±0.13317 C a
	500	27.6667±4.09607 A b	17±1 B a	1.52±0.1893 B d	0.378±0.04737 B b	2.13±0.34771 C b
	1000	23.6667±4.33333 C c	16±1.5275 A a	1.76±0.07371 C b	0.4393±0.0181 B b	2.42±0.09074 B a
C-phycocyanin	Control	32.3333±6.17342 A a	17±1.7321 A c	3.01±0.07095 A a	0.7527±0.0169 A a	2.55±0.4857 A b
	100	27±4.04145 A a	19±2.3094 A b	1.84±0.1159 A e	0.462±0.02829 A b	2.81±0.20881 A b
	300	25.3333±4.05518 B b	25±2.6458 A a	2.05±0.13204 A d	0.511±0.03296 A b	3.24±0.39879 A a
	500	26.6667±3.52767 A b	20±2.5166 A b	2.19±0.21362 A c	0.551±0.05372 A b	3.29±0.50659 A a
	1000	30.3333±4.40959 A a	15±2.6458 A d	2.32±0.17673 A b	0.581±0.04813 A b	3.19±0.37634 A a
LSD		5.52	1.87	0.13	0.12	0.36

Capital letters indicate significant differences between similar concentrations across different treatments.
Lowercase letters indicate significant differences among concentrations within each treatment.

Chemical composition of alfalfa biomass

Table 2 shows the chemical composition of alfalfa plants as influenced by Pb nanoparticles and C-phycocyanin treatments. The tested parameters included chlorophyll, N, P, K, Mg, Ca, and Zn contents, in addition to carbohydrate and protein quantities.

The treatment with Pb NPs alone significantly decreased almost all biochemical constituents compared to the control. The chlorophyll content declined dramatically, and the primary nutrient elements (N, P, K, and Zn) declined with increasing Pb concentration which indicated that the uptake of nutrients and metabolism were suppressed. The application of Pb NPs at 500, and 1000 mg L^{-1} resulted in the lowest average values of vegetative growth and showed the highest inhibition.

However, the concentration of Ca exhibited just the opposite effect under Pb treatment. These observations may imply that Ca has a function of preventing the charges and imbalance of heavy metal ions. The chemical composition values were substantially increased when C-phycocyanin was mixed with Pb NPs. The contents of chlorophyll, N, P and K were higher than the plants treated with only Pb that reveal a certain level restored metabolism (Table 2). The C-phycocyanin-only treatments exhibited significantly highest means

Table 2. Lead nanoparticle (Pb NPs)-induced changes in the chemical composition of alfalfa plants and the ameliorative role of C-phycocyanin (Mean ± SE)

Treatments mg L^{-1}		Chemical composition								
		Chlorophyll (%)	Magnesium (%)	Nitrogen (%)	Phosphorus (%)	Potassium (%)	Calcium (%)	Zinc (%)	Carbohydrates (%)	Protein (%)
Pb NPS	Control	78.957± 1.02599 A a	0.039± 0.00208 A b	2.45± 0.01732 A b	0.245± 0.00208 A c	2.003± 0.00208 A a	0.719± 0.00346 A d	18.411± 0.04852 A a	11.339± 0.10351 A e	15.3127± 0.10825 A a
	100	76.488± 1.19683 A b	0.002± 0.00058 E e	1.89±0.02082 E e	0.311± 0.00265 C a	1.672± 0.00208 D d	1.035± 0.00265 C a	12.431± 0.00416 D c	15.321± 0.00173 C b	11.8127± 0.13026 C b
	300	74.819± 0.2874 A b	0.057±0.001 D a	2.17±0.01528 C d	0.228± 0.00153 E d	1.071± 0.00153 E e	1.011±0.001 C b	12.932± 0.00153 C b	19.467± 0.00153 B a	13.5627± 0.09558 C a
	500	75.018± 0.6078 B b	0.018± 0.00153 D c	2.59±0.04041 C a	0.264± 0.00153 D b	1.943± 0.00153 C b	0.901± 0.00153 D c	11.22± 0.00265 E d	14.067± 0.00208 C c	16.1877± 0.25243 C a
	1000	74.311± 1.52137 D b	0.015±0.001 E d	2.31±0.01528 C c	0.208± 0.00153 D e	1.91±0.00252 C c	0.585± 0.00153 E e	8.164± 0.00306 E e	12.924± 0.00208 E d	14.4377± 0.09558 C a
Pb NPS + C-Phycocyanin	Control	78.957± 1.02599 A a	0.039± 0.00208 A e	2.45±0.01732 A c	0.245± 0.00208 A e	2.003± 0.00208 A c	0.719± 0.00346 A e	18.411± 0.04852 A a	11.339± 0.10351 A c	15.3127± 0.10825 A b
	100	76.798± 0.55608 A b	0.076± 0.00115 B c	2.03±0.03512 D E	0.355± 0.00058 B b	1.689± 0.00058 C e	1.048± 0.00058 B b	14.011± 0.00608 B C	19.927± 0.00208 A b	12.6877± 0.2194 B b
	300	76.072± 1.53883 A b	0.067± 0.00153 B d	2.34±0.02082 B d	0.332± 0.00173 C d	1.893± 0.00231 C d	1.024± 0.00346 B d	15.302± 0.00416 B b	20.487± 0.00208 A a	14.625± 0.13002 B b
	500	77.024± 0.9158 A b	0.085± 0.00115 B b	2.8267± 0.00882 B b	0.364± 0.00173 B a	2.504± 0.00265 B a	1.042± 0.00231 B c	15.513± 0.00473 C b	14.609± 0.00153 C b	17.5627± 0.10825 B a
	1000	77.911± 0.17951 B a	0.091±0.001 B a	2.87±0.02 B a	0.35±0.005 B c	2.173± 0.00503 B b	1.126±0.002 B a	13.195± 0.00252 E d	20.053±0.002 A a	17.9377± 0.12467 C a
C-phycocyanin	Control	78.957± 1.02599 A a	0.039± 0.00208 A a	2.45±0.01732 A e	0.245± 0.00208 A d	2.003± 0.00208 A e	0.719± 0.00346 A e	18.411± 0.04852 A a	11.339± 0.10351 A c	15.3127± 0.10825 A c
	100	75.753± 1.27301 A d	0.102± 0.00115 A c	3.43±0.02646 A a	0.414± 0.00208 A c	2.918± 0.00265 A a	1.088± 0.00252 A c	13.834± 0.00416 C e	17.519± 0.00115 B b	21.4377± 0.16523 A a
	300	76.718± 1.93144 A c	0.147± 0.00252 A b	3.29±0.01528 A c	0.454± 0.00153 A b	2.876± 0.00265 B b	1.231± 0.00153 A b	15.539± 0.00153 B c	19.923± 0.00208 B a	20.5627± 0.09558 A a
	500	77.261± 0.91712 A b	0.148±0.001 A b	3.14±0.01 A d	0.473±0.001 A a	2.833± 0.00153 A c	1.054± 0.00153 A d	16.541± 0.00361 B b	20.389± 0.00557 A a	19.6253± 0.06233 A b
	1000	79.754± 1.1572 A a	0.155± 0.00306 A a	3.39±0.02082 A e	0.455± 0.00208 A b	2.822± 0.00208 A d	1.265± 0.00321 A a	14.138± 0.00306 B d	17.008± 0.00252 C b	21.1877± 0.12994 A a
LSD		1.43	0.002	0.024	0.0024	0.0027	0.0029	0.26	0.53	0.015

Capital letters indicate significant differences between similar concentrations across different treatments.
Lowercase letters indicate significant differences among concentrations within each treatment.

for all characters studied with more pronounced trends at 300–500 mg L^{-1} suggesting its potential to enhance photo-synthesis and nutrient assimilation in both stressed and normal conditions.

Overall, these results suggest that Pb NPs consumed the important biochemical substances in alfalfa and C-phycocyanin ameliorated this effect of Pb on alfalfa by maintaining metabolites balance and antioxidant capacity was increased by chelating action.

Oxidative stress markers in alfalfa plants

The alfalfa plants exposed to Pb NPs and treated with C-phycocyanin exhibited significantly reduced oxidative stress response (Table 3). The parameters were CAT, SOD, MDA, AA (vitamin C), ROS and GPx.

Lead nanoparticles caused a significant elevation in oxidative stress markers. The enzymatic activities of CAT, SOD and GPx were also enhanced (significantly) with increasing Pb concentration, indicating induction of the antioxidant defense system. There was also a parallel increase in MDA and ROS levels, which suggested increment of lipid peroxidation and oxidative membrane injury. C-phycocyanin supplementation decreased the Pb NP + C induced oxidative stress by reducing enzyme activities, and MDA and ROS levels. The reduction was greater at moderate concentrations (300–500 mg L^{-1}), which implied that C-PC successfully scavenged ROS.

The C-Phycocyanin-treated NP plants retained similar (or even lower) enzyme and oxidative marker levels with the control, suggesting an antioxidant nature for the molecule.

In summary, Pb NPs increased the oxidative damage in alfalfa plants, which was significantly alleviated by C-phycocyanin by regulating the antioxidant and protecting cellular membranes.

Table 3. Changes in selected indicators of oxidative stress of alfalfa plants under the exposure to lead nanoparticles (Pb NPs) and supplemented with C-phycocyanin (Mean ± SE)

Treatments mg L^{-1}		Oxidative Stress Indicators					
		CAT U/l Mean±SE	SOD µg/ml Mean±SE	MDA (µmol/L) Mean±SE	Vit C mg/100g Mean±SE	ROS % Mean±SE	GPx (µmol/ml) Mean±SE
Pb NPS	Control	4±0.23094 A c	25±1.44338 A c	2.92±0.16859 A a	8.806±0.50841 A b	0.0082±0.00046 A d	6±0.34641 A d
	100	4±0.23094 B c	50±2.88675 A b	3.779±0.21818 A a	17.612±1.01683 A a	0.0183±0.00104 A b	12±0.69282 A c
	300	4±0.23094 B c	50±2.88675 A b	3.693±0.21322 A a	17.632±1.01798 A a	0.0146±0.00087 A c	6±0.34641 B d
	500	87±0.46188 B b	51±2.94449 A b	3.564±0.20577 A a	17.655±1.01931 A a	0.0148±0.00087 A c	18±1.03923 B b
	1000	16±0.92376 B a	53±3.05996y A a	3.521±0.20329 B a	18.131±1.04679 Aa	0.029±0.00167 A a	24±1.38564 B a
Pb NPS + C-phycocyanin	Control	4±0.23094 A b	25±1.44338 A a	2.92±0.16859 A a	8.806±0.50841 A b	0.0082±0.00046 A d	6±0.34641 B c
	100	4±0.23094 B b	24±1.38564 b A	2.092±0.12078 C a	8.806±0.50841 b B	0.012±0.00069 C c	7±0.40415 C b
	300	4±0.23094 B b	25±1.44338 C a	2.478±0.14307 C a	8.836±0.51015 B b	0.0131±0.00075 B b	6±0.34641 B a
	500	4±0.23094 C b	20.4±1.17779 C b	2.049±0.1183 C a	8.846±0.51072 B b	0.013±0.00075 B b	13±0.75056 B a
	1000	12±0.69282 C a	20±1.1547 D b	3.146±0.18163 C a	17.661±1.01966 A a	0.016±0.00092 B a	6±0.34641 A c
C-phycocyanin	Control	4±0.23094 A b	25±1.44338 A a	2.92±0.16859 A a	8.806±0.50841 A a	0.0082±0.00046 A d	6±0.34641 B b
	100	4±0.23094 B b	16.5±0.95263 C c	2.576±0.14873 B a	8.808±0.50853 B a	0.0127±0.00075 C c	18±1.03923 A a
	300	4±0.23094 B b	20±1.1547 D b	2.221±0.12823 C a	8.862±0.51165 B a	0.0129±0.00075 C c	7±0.40415 B b
	500	4±0.23094 C b	16.7±0.96417 D c	2.147±0.12396 C a	8.881±0.51274 B a	0.024±0.00139 C a	6±0.34641 C b
	1000	6±0.34641 D a	25±1.44338 C a	2.92±0.16859 C a	8.915±0.51471 B a	0.015±0.00087 C b	7±0.40415 C b
LSD		0.7	2.05	0.189	0.832	0.00094	1.09

Capital letters indicate significant differences between similar concentrations across different treatments.
Lowercase letters indicate significant differences among concentrations within each treatment.

Determination of DNA damage (comet assay)

Potential effects of Pb NPs and C-phycocyanin on DNA integrity in alfalfa plants the results of the comet assay (Table 4) were obtained to show the likely effects of Pb NPs and C-phycocyanin on DNA fragmentation in alfalfa. The nuclei with low damage were 100% of all in the mock plants indicating a preserved DNA without damages. The extent of DNA fragmentation was dose-dependent, with the amount of low-damage nuclei decreasing and higher damage medium- to high-damage (as well as main and tailing) nuclei increasing. DNA damage was particularly the highest at 500 mg L^{-1} (9–10% average DNA damage and a few high-damage nuclei), indicating the genotoxic potential of Pb NPs.

DNA integrity was remarkably improved, when the C-phycocyanin treatment took place in the presence of Pb NPs. The rate of mid-to-high damage dropped, while the percentage of intact nuclei rose. Virtually all of the high damage cells were recovered in the treatment with C-phycocyanin alone. These findings suggest the DNA-protective effects of C-PC that result in the attenuation of the Pb-induced genotoxic injury as a consequence of its extreme antioxidant and radical-scavenging behavior.

DISCUSSION

Alfalfa vegetative growth characteristics in the vegetative phase

Pb NPs caused a significant decrease in the vegetative growth of alfalfa including plant height, branch number, and fresh and dry weights of the plant as well as leaf area. These observations are also in line with previous reports, which have revealed that the Pb nanoparticles disturb these normal processes of the cell division, elongation and photosynthesis, which are responsible for the retardation of growth and decrease in biomass (Liu *et al.*, 2022; Rahman *et al.*, 2024). Reduction in growth parameters with elevated Pb levels might have been due to inhibited chlorophyll synthesis, unbalanced nutrient and oxidative membrane damage that disrupts photosynthate partitioning (Tan *et al.*, 2022; Kaur *et al.*, 2024).

The comparatively partial recovery in the C-PC + Pb NP treated groups also indicates that C-PC may be a bioprotectant against metal-induced damage. This pigment–protein complex also increases chloroplast stability and photosynthetic performance due to their powerful antioxidant and metal binding properties (Fernandes *et al.*, 2023; Castro-Gerónimo *et al.*, 2024). Furthermore, the Spirulina C-phycocyanin derived

Table 4. Genotoxic evaluation of lead nanoparticles on alfalfa (*Medicago sativa* L.) and the role of C-phycocyanin (comet assay)

Treatment		Low comet assay mean±SE	Medium comet assay mean±SE	High comet assay mean±SE
Pb NPS	Control	100±0 Aa	0±0 Ae	0±0 Ac
	100	98.3±0.579 Aa	0±0 Cd	1.7±0.580 Ab
	300	96.4±0.876 Bb	1.9±0.581 Ac	1.7±0.575 Ab
	500	88.7±0.669 Dd	9.5±0.574 Aa	1.8±0.580 Ab
	1000	92.3±0.549 Bc	5.1±0.579 Cb	2.6±0.578 Aa
Pb NPS + C-phycocyanin	Control	100±0 Aa	0±0 Ad	0±0 Da
	100	96.1±2.837 Bb	3.9±0.579 Bc	0±0 Da
	300	94.6±2.804 Cc	5.4±0.576 Bb	0±0 Da
	500	94.5±2.771 Bc	5.5±0.575 Cb	0±0 Da
	1000	92.2±2.902 Bd	7.8±0.575 Aa	0±0 Da
C-phycocyanin	Control	100±0 Aa	0±0 Ab	0±0 Ea
	100	98.8±2.122 Aa	1.2±0.580 Da	0±0 Ea
	300	98.6±0.576 Aa	1.4±0.578 Da	0±0 Ea
	500	98.5±0.873 Aa	1.5±0.574 Da	0±0 Ea
	1000	98.4±2.739 Aa	1.6±0 Da	0±0 Ea
LSD		1.76	0.534	0.300
Capital letters indicate significant differences between similar concentrations across different treatments. Lowercase letters indicate significant differences among concentrations within each treatment.				

protein has been shown to enhance plant vigor under a different abiotic stress by maintaining the membrane stability and nutrient uptake (Mahanandia *et al.*, 2025; Touzout *et al.*, 2025). Therefore, the promotion of vegetative qualities in alfalfa stands for the joint effect of C-PC on alleviating the Pb-induced cytotoxicity and reestablishing metabolic homeostasis.

Phytochemicals in alfalfa plants

The depression in chlorophyll, nitrogen, phosphorus, potassium, magnesium and zinc contents under the stress of Pb NP are in agreement with earlier findings (Al-Khayri *et al.*, 2023; Rahman *et al.*, 2024). Pb disrupts ion pumps, channels and enzymes systems which inhibit the process of photosynthesis and protein synthesis (Tan *et al.*, 2022; Kumari *et al.*, 2024). The enhanced concentration of calcium exposed to Pb could be a compensatory response, given that Ca is involved in membrane stabilization and alleviation of the toxic effects caused by ions (Panahirad *et al.*, 2025).

On the other hand, the marked improvement in chemical composition when using C-PC might be due to their ability to improve nutrient absorption and metabolism. C-PC can recover the chlorophyll content and stimulate nitrogen assimilation through its antioxidants and chelation actions (Grover *et al.*, 2021; Jach *et al.*, 2022). Also, Spirulina extracts studies have revealed that the products rich in phycocyanin enhance both the photosynthetic pigment content and the accumulation of macronutrients against heavy metals (Fernandes *et al.*, 2023; Mahanandia *et al.*, 2025). Increased levels of proteins and carbohydrates in the C-PC-fed plants suggest enhanced physiological functions and the recovery of photosynthetic efficiency.

Oxidative stress markers of alfalfa seedlings

Alfalfa showed significant oxidative stress upon the exposure to Pb NPs, as evidenced by increased activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), and augmented levels of malondialdehyde (MDA) and reactive oxygen species (ROS). This fits well with previous results indicating that the Pb-induced oxidative stress induces antioxidant enzymes as a defense against excessive ROS accumulation (Tan *et al.*, 2022; Hussan *et al.*, 2024). However, when the Pb concentrations were high and activities of lipid peroxidation and enzyme increased beyond normal levels, some cells would be destroyed (Yang *et al.*, 2020; Niu and Xiang, 2018), leading to a decrease in antioxidant substance. C-phycocyanin could significantly ameliorate oxidative stress by rebalancing the enzyme activities and decreasing MDA level as well as ROS content. This protective role is due to its capacity of directly countering free radicals and activating endogenous antioxidant systems, including the induction of SOD; CAT and glutathione systems (Grover *et al.*, 2021; Castro-Gerónimo *et al.*, 2024; Jaiswal *et al.*, 2025). The phycocyanobilin chromophore allows C-PC to scavenge hydroxyl and superoxide radicals, maintaining redox homeostasis (Wu *et al.*, 2016; Bellamy-Carter *et al.*, 2022). These data clearly demonstrate that C-PC can act not only as a ROS scavenger, but also as a biochemical modulator protecting the membrane from heavy metal-induced oxidative injury.

DNA damage evaluation (comet assay)

Lead at the nano-scale caused concentration-dependent DNA fragmentation in alfalfa, as indicated by a greater proportion of medium- and high-damage nuclei. Genotoxic responses, such as those described above have also been reported in other plants subjected to the Pb stress, where strand breaks and chromosomal instability were attributed to oxidative radicals and interference with metal ion (Rahman *et al.*, 2024; Panahirad *et al.*, 2025). The maximum DNA damage at 500 mg L^{-1} suggests a severe state of oxidative imbalance, probably due to a significant ROS generation and inhibition of repair mechanisms (Ceramella *et al.*, 2024).

In contrast, C-phycocyanin co-application mitigated the DNA damage and protected nuclear architecture. This is in agreement with the C-PC suppression of genotoxicity by its radical-scavenging, metal-chelating and membrane-stabilization abilities (Grover *et al.*, 2021; Al-Yasiri and Alchalabi, 2021; El-Sayed *et al.*, 2022). C-PC antioxidant activity prevents nucleic acid from Pb–induced peroxidation and maintains genomic stability for normal cellular function. Thus, incorporation of photoautotrophic plant organisms can serve as an efficient method of promoting genoprotective response against the nanoparticle-induced oxidative stress.

CONCLUSIONS

The exposure to Pb NPs significantly inhibited alfalfa (*Medicago sativa* L.) growth by reducing plant height, leaf area, and biomass, along with a clear decline in chlorophyll and essential nutrients (N, P, K, and Zn). These effects were accompanied by elevated oxidative stress, evidenced by increased ROS, MDA, and antioxidant enzyme activities, as well as DNA fragmentation. Conversely, the treatment with C-PC effectively mitigated Pb toxicity. C-PC improved vegetative growth and nutrient balance, restored chlorophyll content, and reduced oxidative and genotoxic stress. Overall, C-PC acts as a strong natural antioxidant and metal-chelating compound that enhances physiological resilience and promotes plant tolerance under heavy metal stress.

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