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The Impact of Titanium Dioxide Nanoparticles (TiO_2 NPs) on the Vegetative Characteristics of Alfalfa (*Medicago sativa L.*) and the Ameliorative Role of C-Phycocyanin

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ABSTRACT

This work investigated the impacts of C-Phycocyanin on Titanium Dioxide Nanoparticles (TiO_2 NPs)-induced stress in alfalfa (Medicago sativa L.) plants. The study focused on evaluating the effects of TiO_2 NPs on the vegetative growth, biochemical composition, oxidative stress markers, and DNA integrity of alfalfa plants. TiO_2 NPs stressed the alfalfa plants in a concentration-dependent manner through decrease in plant height, branch number, leaf area, and biomass production. Biochemical parameters like chlorophyll, nitrogen, and potassium contents were reduced, revealing diminished physiological and photosynthetic activities. Contrarily, the co-application of TIO_2 NPs and C-Phycocyanin mitigated the detrimental effects, as they promoted the growth and biochemical parameters recovery at low and moderate nanoparticle concentrations. TIO_2 NPs treatment increased the level of Catalase, Superoxide Dismutase, Malondialdehyde, Reactive Oxygen Species (CAT, SOD, MDA, and ROS) oxidative stress biomarkers, while C-Phycocyanin application decreased these indicators values, masking their scavenging potential. DNA integrity analysis through Comet Assay suggested that C-Phycocyanin effectively resist cells from TIO_2 NPs -induced DNA damage and retain genomic stability. Therefore, co-application of C-Phycocyanin mitigated these adverse effects, demonstrating its potential as a natural biostimulant that enhances plant tolerance to nanoparticle-induced phytotoxicity.

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INTRODUCTION

Nanomaterial pollution is one of the most urgent contemporary environmental problems due to the rapid development of nanotechnology and the wide industrial use of engineered nanoparticles. Nanoscale materials are characterized by several unique physicochemical properties, such as small particle size, high surface area, and reactivity, making them particularly attractive for application in the industry, cosmetics, paints, medicine, and agriculture. Nevertheless, these same properties ensure the materials' long-term persistence and potential for toxicity upon release into the environment (El-Saadony *et al.*, 2022; Alabdallah *et al.*, 2024). Titanium dioxide nanoparticles are among the most commonly used nanomaterials, primarily because of their high production volume and extensive implementation in numerous consumer goods. The accumulation of TIO_2 NPs in soil and water ecosystems has already resulted in significant concerns related to the ecological footprint of such nano pollution. Once in the environment, TiO2 NPs interact with soil matrices, microorganisms, and plants, potentially disrupting the

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biological and biochemical balance (Agarwal and Chibber, 2017; Kong *et al.*, 2022).

Plants, as the primary recipients of soil-based pollutants, are highly sensitive to nanoparticle activity. The interaction between TiO2 NPs and plant tissue is concentration-, exposure time-, and nanoparticle-size dependent. At low concentrations, TiO2 NPs facilitate seed germination, chlorophyll synthesis, and photosynthesis by modulating reactive oxygen species levels and promoting antioxidant enzymes (Gohari *et al.*, 2020; Carbajal-Vázquez *et al.,* 2025). High concentrations, however, derail these processes by inducing oxidative stress through ROS overproduction, lipid peroxidation, and cell membrane and organelle damage. The stress-induced chlorosis, growth retardation, and metabolic disruption are indicators of TiO2 NP efficiency and toxicity (Babaei *et al.*, 2025; Mohajjel Shoja *et al.*, 2021).

The possible toxicological mechanism, with regards to TiO_2 NP toxicity exposure to plants, is related in part to an imbalance produced between Reactive oxygen species (ROS) generation and the counter plant antioxidant defense. If ROS are present in excessive amounts, they can damage macromolecules including proteins, lipids and nucleic acids and also cause DNA fragmentation and cell death. This oxidative stress may result in priming defense enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) to respond against free radicals simultaneously. Nevertheless, at high doses of nanoparticles that overcome the plant's defense capabilities, cytotoxic and genotoxic effects are observed (Kumari *et al.,* 2024; Carbajal-Vázquez *et al.,* 2025).

Medicago sativa L. (alfalfa) is a widely used forage crop that has high protein content, and wide adaptability to environmental changes in the agroecosystems. Because of its response to stress and importance in maintaining soil fertility during nitrogen fixation, alfalfa is recognized as a promising bioindicator for evaluation of environmental pollutants including , TiO2 NPs has been shown to modify the vegetative characteristics as well as pigment content and antioxidant enzyme activity of alfalfa, indicating that alfalfa possesses a flexible strategy in response to nanoparticle stress according to dose and time exposure (Páramo *et al.*, 2023; Acosta-Slane *et al.*, 2024).

The attenuation of the adverse effects induced by NPs on plants has received extensive attention. One such promising strategy is to use naturally available antioxidants, which can act as ROS scavengers and reinforce cellular protection mechanisms. Among them, phycocyanin (C-PC), derived from Spirulina platensis a phycobiliprotein, has culture demonstrated impressive antioxidative, anti-inflammatory and cytoprotective properties (Romay *et al.*, 2003; Fernandes *et al.*, 2023). C-PC protects against oxidative stress by scavenging free radicals directly and promoting the expression of CAT, SOD, and GPx indirectly. This response to stress reduces lipid peroxidation, and enables the maintenance of a redox balance (Farooq *et al.*, 2014; Dong *et al.*, 2022). It is also connected with the activation of Nrf2 signaling that is crucial to control gene expression associated with oxidative stress responses (Puengpan *et al.*, 2024; Nguyen *et al.*, 2025).

It has been previously reported that C-PC ameliorates TiO_2 NP-mediated toxicity in animals by regenerating biochemical components and shielding the integrity of cellular structure (Sayed *et al.*, 2022). To the best of our knowledge, these results also show a protective role against oxidative damage and for maintaining plant homeostasis of C-PC treatment after nanoparticle exposure. Furthermore, given both its nature of natural product, biodegradability and biocompatibility, C-PC can be considered also as a sustainable and environmental-safe bio-stimulant meeting the trends of green agricultural practices (Rudi *et al.*, 2024).

We hypothesized that the application of C-PC would alleviate TiO_2 NP-induced phytotoxicity in alfalfa by enhancing the antioxidant defense system, protecting photosynthetic machinery, and maintaining genomic stability. Therefore, the objectives of this study were to evaluate the effects of TiO_2 NPs on alfalfa's growth, biochemistry, oxidative stress, and DNA integrity, and to determine the protective efficacy of C-PC co-application.

MATERIALS AND METHODS

Plant Material And Growth Conditions

Seeds of *Medicago sativa* L. (alfalfa) were obtained from a certified agricultural research center and surface-sterilized with 1% sodium hypochlorite for 3 minutes, then thoroughly rinsed with distilled water. The seeds were germinated in plastic pots (20 cm diameter) containing a soil–sand mixture (2:1, v/v). Plants were maintained in a greenhouse at 25 ± 2°C, relative humidity 65–70%, and a 16/8 h light/dark cycle. Irrigation was performed with distilled water to sustain 70% of field capacity until treatments were initiated.

C-Phycocyanin Source and Application

C-Phycocyanin (C-PC) was obtained from Arthrospira platensis (organic Blue Spirulina) powder supplied by Micro Ingredients Inc. (Montclair, CA, USA). The product was USDA Organic certified, GMO-free, and contained no fillers or preservatives. A stock solution (purity ≥95%) was freshly prepared in distilled water before use. C-PC was applied as a foliar spray at concentrations identical to those of TiO_2 nanoparticles (100, 300, 500, and 1000 mg L^{-1}). Treatments were applied at regular intervals throughout the experimental period.

Nanoparticle Characterization And Preparation

Titanium dioxide nanoparticles (TiO_2 NPs, rutile phase, 99.5% purity) were purchased from SkySpring Nanomaterials, USA (Product No. 7920DL). According to the manufacturer's datasheet, the particles were white in appearance with an average size range of 10–30 nm, a specific surface area of ~50 m^2 g^{-1}, and minimal impurities (Nb <150 ppm, Al <100 ppm, Ca <100 ppm, Hg <0.5 ppm, Mg <100 ppm). SEM measurement evidenced the shape and approximate size of the nanoparticles. Stock suspensions were in deionized water and sonicated for 30 min at 40 kHz before use to achieve even dispersion (Figure 1).

Experimental Design

The experiment was conducted in a completely random with CRD (Complete Randomized

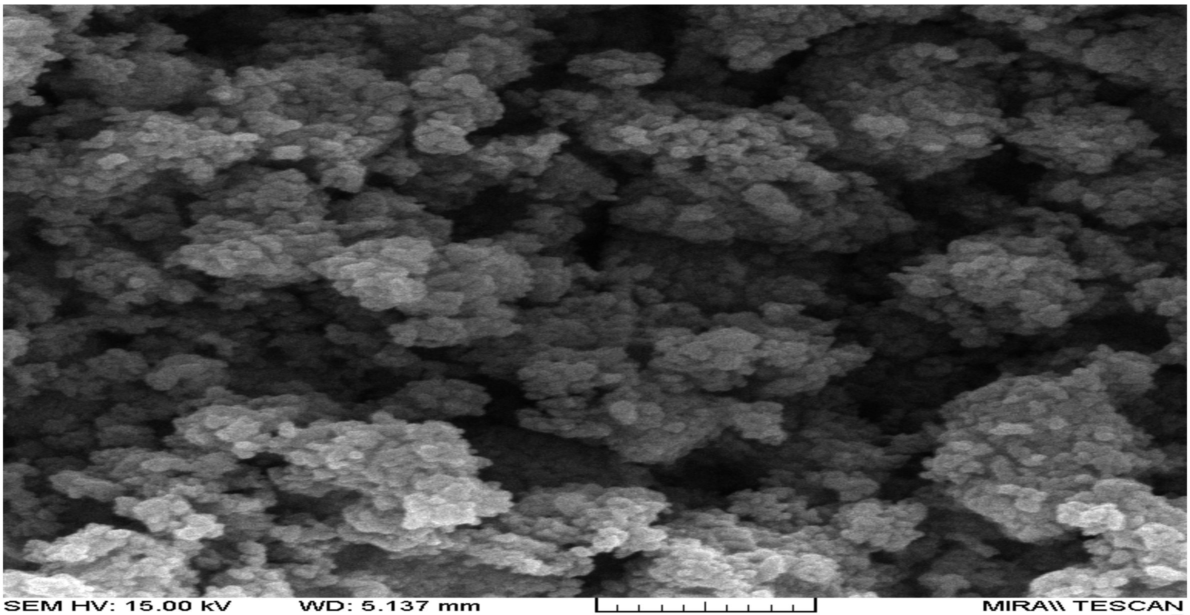

Fig. 1. Scanning Electron Microscopy (SEM) image of titanium dioxide nanoparticles (TiO_2, rutile phase, 99.5% purity) with spherical shape and particle size distribution ranging from 10 to 30 nm.

Design) using three replications per treatment. There were 5 plants in each replicate, all growing under the same environmental conditions.

Treatment Details

Plants were grouped as the following's treatments;

Control: untreated plants.

TiO_2 NPs solely: 100, 300, 500 and 1000 mg L^{-1}.

C-PC alone: 100, 300, 500 and 1000 mg L^{-1}.

Combined treatments: TiO_2 NPs (100, 300, 500, and 1000 mg L^{-1}) + C-PC (100, 300, 500, and 1000 mg L^{-1}).

All treatments were derived from same growth conditions and all following morphogenetic, biochemical and genotoxic parameters were recorded after 21 days of treatment.

Vegetative Growth Traits

Vegetative aspects the vegetative parameters were measured on December 8, 2024 for all treatments. Plant height (cm), number of branches per plant, leaf area (cm^2) and fresh and dry biomass were recorded. Height from the soil surface to the apical shoot and branches was estimated by using a metric tape, and number of branches were counted manually. Leaf area was calculated using a Laser Portable Leaf Area Meter (CI-202, Bio-Science, USA) as described in Johnson (1973). Fresh and dry weights of vegetative parts were taken using an analytical balance (Model KERN AL J220-4NM); samples were dried in oven at 48°C to constant weight, to obtain their dry mass.

Biochemical Traits

Total chlorophyll was estimated with 80% acetone extraction, following Mackinney (1941) and spectrophotometric readings at 645 and 663 nm in a UV–VIS range Shimadzu UVmini-1240.

The leaf contents of Nitrogen, Phosphorus, Potassium, Calcium, Magnesium, Zinc (N, P, K, Ca, Mg and Zn) were determined by the wet digestion procedure (Cresser and Parsons, 1979).

Nitrogen was determined by the Kjeldahl method (Chapman and Pratt, 1962), phosphorus via the vanadomolybdate spectrophotometric method at 410 nm, and potassium determined using a Flame Photometer (PFP7, Jenway, Germany). Calcium, magnesium, and zinc were determined using Atomic Absorption Spectrophotometry (Shimadzu AA-7000, Japan).

Total carbohydrate was hydrolyzed with diluted hydrochloric acid (HCl) according to the anthrone method developed by Herbert *et al.* (1971); the absorbance was recorded at 630 nm.

Measurement Of Oxidative Stress And Antioxidant Enzyme Activities

Antioxidant enzymes were extracted according to the standard method using phosphate buffered saline (PBS) as described by Giannopolitis and Ries (1977) and Aebi (1984), with minor modifications.

Superoxide Dismutase (SOD)

SOD activity was measured as described by the method of Marklund and Marklund (1974). based on the inhibition of pyrogallol autoxidation. The absorbance was read at 420 nm and the enzyme activity was reported as µg m/L.

Catalase (CAT)

The catalase activity was measured according to Johansson and Borg (1988). was determined using Method A16, Enzyme was reported in units per liter (U/L).

Glutathione Peroxidase (GPX)

GPx activity was determined according to Rotruck *et al.* (1973). The protocol assesses the oxidation of reduced glutathione (GSH) in the presence of hydrogen peroxide by DTNB reagent and the absorbance is read at 412 nm. The activity was presented as the amount (in µmol) of GSH oxidized per mL of enzyme extract.

Lipid Peroxidation (MDA)

Lipid peroxidation Lipid peroxidation was estimated using thiobarbituric acid (TBA) as described by Buege and Aust (1978) and Burtis and Ashwood, (1999). This is a pink-coloured complex of MDA and TBA, which is determined at 532 nm. Results were reported as mmol MDA/mg protein.

Reactive Oxygen Species (ROS)

The ROS content was determined as described by Venkidasamy *et al.* (2019). The amount of inhibition of ROS is determined by absorption at 560 nm, (in the leaves) and indicates the severity of oxidative stress in the leaf.

Vitamin C (ASCORBIC ACID)

The ascorbic acid content was determined by 2,6-Dichlorophenol Indophenol (DCPIP) colorimetric assay: 29 the reduction of the blue dye reflects an end point that is indicative of vitamin C content, and absorbance were determined; values were expressed in mg/100 g fresh weight.

Measurement Of Dna Damage (COMET ASSAY)

DNA damage was measured by alkaline Comet assay (Singh *et al.*, 1988). Leaf nuclei were embedded in low melting point agarose, attached to a gel electrophoresis apparatus and lysed for 2 min at 37 °C in lysis buffer., Electrophoresis was then carried out using a voltage of 70 V for 60 min. Slides were neutralized and stained with ethidium bromide, and observed under a fluorescence microscope. Cells were defined as low-, moderate-, or high-damage cells according to tail intensity and length.

Statistical Analysis

Statistical analyses were performed with the SPSS (version 25.0, IBM Corp., Armonk, NY, USA). Two-way ANOVA was applied to the study factors (treatment and concentration) and their interaction on the responses. When differences between means were significant (according to the t test), mean values were compared by LSD test at $p \leq 0.05$. The results are presented as the mean ± SE.

RESULTS AND DISCUSSION

Characteristics Of Alfalfa Plant Growth During The Vegetative Stage

The vegetative growth characters of alfalfa plants in response to TiO2 NPS as well as their interaction with C-Phycocyanin are shown at Table (1). The parameters determined were plant height, number of branches, fresh weight, dry weight and leaf area.

The highest mean values for all vegetative traits were recorded at the untreated control plants, suggesting normal alfalfa growth without any interference of nanoparticle. In treatment only with TiO 2 NPs, a decrease of all vegetative traits in the range between 100 and 1000 mg L^{-1} was evident. The greatest reduction was observed at 300 and 500 mg L^{-1} treatments, where fresh and dry biomass were significantly lower than the control.

Based on Table (1), the minimum average leaf area was observed for the plants subjected to TiO_2 NPs at 500 mg L^{-1} which was equal to 1.51 cm^2, representing the most inhibitory effect of nanoparticles on vegetative growth. However, the maximum mean leaf area was registered in

Table 1. Vegetative traits of alfalfa plants grown with TiO_2 nanoparticles and C-phycocyanin

Treatments |		Plant height (cm) Mean±SE	Number of branches Mean±SE	Fresh weight (g) Mean±SE	Dry weight (g) Mean±SE	Leaf area (cm^2) Mean±SE
TiO2 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	26±4.3589 A b	9±1.5275 C b	1.12±0.03786 C c	0.2807±0.00837 C b	1.74±0.19035 D b
	300	24±3.05505 B b	7±1 E c	1.16±0.02646 E c	0.289±0.007 C b	1.56±0.17616 E b
	500	25±4.50925 A b	7±0.5774 D c	1.45±0.19553 C b	0.3627±0.04859 B b	1.51±0.02646 E b
	1000	29±3.78594 A a	8±1 B b	1.09±0.02646 C c	0.271±0.00608 C b	1.59±0.2512 D b
TiO2 NPS +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 a
	100	25.3333±3.52767 A b	10±1 C b	1.35±0.12342 B c	0.3377±0.03069 A b	1.75±0.10214 C c
	300	30.6667±6.35959 A a	8±0.5774 C a	1.21±0.10504 C d	0.3027±0.02717 B c	2.23±0.15716 B a
	500	25.6667±3.4801 A b	11±0.5774 C b	1.44±0.09074 D c	0.362±0.02265 B b	2.51±0.20306 B a
	1000	24±2.08167 B b	9±1 B d	1.77±0.11719 B b	0.4427±0.02961 B b	2.19±0.2318 C b
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.

plants treated with C-Phycocyanin at 500 mg L^{-1} (3.29 cm^2) indicating a significant stimulatory effect of C- Phycocyanin on the expansion and photosynthesis performance of leaves.

However, with the combined treatments, plants treated with TiO_2 NPs + C-Phycocyanin (500 mg L^{-1}) showed a positive leaf area of 2.51 cm^2 indicating an apparent reduction from the inhibition caused by TiO_2. Thus, the results presented here indicate that C-Phycocyanin treatment significantly prevents the growth-suppressive effects of TiO2 NPs and partially restores leaf development, suggesting a protective or synergistic effect when both treatments are used simultaneously.

The growth parameters improved partially when C-Phycocyanin was supplemented in combination with TiO 2 NPs. At lower concentrations (100–300 mg L^{-1}), application of C-Phycocyanin reduced the decrease in plant height and leaf area from TiO_2 treatments alone although remained insignificantly higher than those reported when only TiO_2 NPs applied. However, in higher concentrations (500 and 1000 mg L^{-1}), the ameliorative effect of C-Phycocyanin decreased, and growth parameters fell again in comparison to the control.

Vegetative performance was significantly enhanced from C-Phycocyanin alone, over its concentration range. Plants treated with 300 and 500 mg L^{-1} of C-PC presented significant improvements in plant height, number of branches, fresh/dry weight and leaf area compared to the other treatments, which reached or exceeded control values. It indicates that C-Phycocyanin has a positive physiological effect on growth and photosynthetic ability in natural condition.

Collectively, the results implicated that TiO_2 NPs have a significant inhibitory effect on vegetative growth in a concentration-dependent manner and C-Phycocyanin has protective or stimulatory effects, especially when applied alone or mixed with TiO_2 NPs at lower concentrations.

These results were statistically supported at $p \leq 0.05$, confirming the significant influence of both TIO_2 NPS and c-phycocyanin treatments on vegetative characterizations in alfalfa plants.

The suppressive effect of TiO_2 NPs on plant vegetative parameters could probably be due to the ability of NPs to occlude stomatal pores, change water balance and induce restriction on gas exchange and transpiration efficiency. The lower leaf area and height that we observed agree with the results of Iqbal *et al.* (2023), who showed that the formation of nanoparticles hinders CO_2 diffusion, decreases turgor pressure, and ultimately leads to a decrease in photosynthetic efficiency. The increased oxidative stress induced by exposure to TiO_2 results in a premature senescence of photosynthetic tissue, and reduces the content of chlorophylls and interferes with light-harvesting processes.

In contrast, the application of C-Phycocyanin (C-PC) resulted in improved recovery of all morphological characters in plants. The bioactive property of pigment, particularly on phycobiliprotein like structure, engenders the antioxidant and metal chelator capabilities to its physiological function that modulate the uptake or internal toxicity of NPs (Husain *et al.*, 2024). C-PC scavenged Reactive Oxygen Species (ROS) and preserved membrane fluidity, which resulted in recovery of cell expansion and tissue hydration. The increase in leaf area and biomass of C-PC–treated plants comply with its reported function toward promoting chloroplast integrities, and enhancing osmotic adjustment during nanoparticle stress (Sayed *et al.*, 2022; Wang *et al.*, 2022).

Composition of Chemicals in Alfalfa Plants

The data presented in Table (2, 3) indicated the effect of titanium dioxide nanoparticles (TiO_2 NPs) and its interaction with C-Phycocyanin on some biochemical characteristics for alfalfa plants: chlorophyll-a, magnesium, nitrogen, phosphorous, potassium, calcium, zinc contents as well as carbohydrates and protein contents.

The untreated control plants showed normal chlorophyll and nutrient contents, indicating that the physiological status was optimal in the absence of any addition. The concentrations of most chemical constituents, however, exhibited a significant decrease when exposed to TiO 2 NPs alone. With increased TiO_2 NP supply from 100 to 1,000 mg L^{-1}, chlorophyll content, macronutrients (N, P and K), and micronutrients including Zn and Ca decreased gradually. The lowest nutrient accumulation and chlorophyll contents were observed at 300 mg L^{-1}, indicating that a higher concentration of nanoparticles limits mature stages of photosynthetic pigment synthesis and nutrients absorption.

The supplementation of TiO_2 NPs with C-Phycocyanin, however, ameliorated these negative effects. The addition of C-Phycocyanin enhanced chlorophyll synthesis and nutrient balance in leaves when compared with TiO2 NPs treatments only. This increase was more pronounced at 100 and 300 mg L^{-1}, where the substances in nitrogen, potassium and carbohydrates increased showing a partial recovery of plant metabolic activity.

When C-Phycocyanin was applied alone, it significantly increased all measured biochemical parameters compared to control as well nano particle treatments. The maximum values of chlorophyll, nitrogen, potassium and protein content were recorded at 300–500 mg L^{-1} of C-Phycocyanin suggesting its stimulatory role in photosynthesis, nutrient assimilation and protein formation. These findings indicate the feasibility of C-Phycocyanin in promoting metabolic efficacy and alleviating oxidative or ionic stress induced by nanoparticles. The potential mediating action of C-Phycocyanin (C-PC) to improve metabolic efficiency and alleviate oxidative stress is mainly attributed to it strong antioxidants effects and protection.

Table 2. Biochemical measurements in alfalfa plants treated with TiO_2 nanoparticles and C-phycocyanin

Treatments		**Chlorophyll (%)**	**Magnesium (%)**	**Nitrogen (%)**	**Phosphorus (%)**	**Potassium (%)**
TiO2 NPS	Control	78.957±1.02599 A a	0.039e±0.00208 A b	2.45±0.01732 A a	0.245±0.00208 A b	2.003±0.00208 A a
	100	75.132±1.21409 A b	0.023±0.00252 C d	2.17±0.01528 C b	0.146±0.00153 E c	1.561±0.00153 E e
	300	74.944±2.09978 A c	0.051±0.00173 E a	1.47±0.01732 D e	0.306±0.00208 D a	1.655±0.00208 D c
	500	78.017±1.28204 A a	0.019±0.00153 D e	1.88±0.01528 E d	0.226±0.00153 E c	1.859±0.00153 E b
	1000	76.469±1.79115 C b	0.036±0.001 D d	2.12±0.01 E c	0.177±0.001 E d	1.604±0.00153 E d
TiO2 NPS +C-Phycocyanin	Control	78.957±1.02599 A a	0.039±0.00208A c	2.45±0.01732 A a	0.245±0.00208 A e	2.003±0.00208 A b
	100	76.809±0.89603 A b	0.028±0.00173 D d	2.24±0.01528 B b	0.2843±0.00167 D d	1.757±0.00153 B b
	300	75.612±1.21839 A c	0.084±0.00153 C b	1.47±0.02 D e	0.382±0.002 B a	2.954±0.00351 A a
	500	78.186±1.01873 A a	0.029±0.00153 C d	1.96±0.03055 D d	0.345±0.00208 C b	1.926±0.00231 D c
	1000	77.469±1.2021 B b	0.087±0.00153 C a	2.17±0.01528 D c	0.321±0.00153 C c	1.748±0.001 D e
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
	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
	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
	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
	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
LSD		1.43	0.002	0.024	0.0024	0.0027

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

C-PC is also associated to the chromophore phycocyanobilin and presents an extrinsic ROS-scavenging activity, which occurs by a direct scavenging of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2). This scavenging activity is thought to limit lipid peroxidation and membrane leakage, hence preserving cellular structure under stress conditions. Additionally, C-PC activates enzymatic antioxidants including superoxide dismutase (SOD) and catalase (CAT), that also play an important role in scavenging ROS as well as the restoration of redox homeostasis (Romay *et al.*, 1998; Sayed *et al.*, 2022; Husain *et al.*, 2024).

This C-PC protects the chloroplast structural organization and photosynthetic apparatus against oxidative damage, maintaining a higher efficiency of photosynthesis and the assimilation of nutrients resulting in an increase in chlorophyll, nitrogen, potassium, and protein contents. This implies that C-PC is not only involved in reducing the oxidative and ionic stress of nanoparticles, but also increases the efficiency to metabolize energy and synthesize protein (Nurjannah *et al.*, 2025).

The present results agree with previous studies suggesting that C-Phycocyanin acts against oxidative stress-mediated damage favouring antioxidant protection of biological systems under different stress conditions. For instance, Sayed *et al.* (2022) indicated that C-Phycocyanin reduced TiO_2 nanoparticle-induced oxidative dysfunctions through the modulation of antioxidant enzyme activities and lipid peroxidation in animal tissues. Similarly, Husain *et*

Table 3. Biochemical measurements in alfalfa plants treated with TiO_2 nanoparticles and C-phycocyanin

Treatments		Calcium (%)	Zinc (%)	Carbohydrates (%)	Protein (%)
TiO2 NPS	Control	0.719±0.00346 A e	18.411±0.04852 A a	11.339±0.10351 A e	15.3127±0.10825 A a
	100	1.011±0.00153 D a	12.194±0.00379 E b	17.608±0.00208 B a	13.5627±0.09558 B b
	300	0.789±0.00208 E c	10.904±0.00361 D c	12.412±0.00208 D d	9.1877±0.10825 D e
	500	0.777±0.00153 E d	12.168±0.00473 D b	13.492±0.00153 D c	11.75±0.09525 D c
	1000	0.992±0.00153 D b	10.983±0.00557 C c	15.825±0.00208 D b	13.2503±0.06267 D d
TiO2 NPS +C-Phycocyanin	Control	0.719±0.00346 A e	18.411±0.04852 A b	11.339±0.10351 A d	15.3127±0.10825 A a
	100	0.991±0.00153 E b	15.328±0.00208 A d	19.604±0.00208 A a	14±0.09525 B b
	300	0.933±0.00351 D c	20.281±0.00451 A a	17.314±0.003 C b	9.188±0.125 D b
	500	0.916±0.00265 C d	17.936±0.00361 A c	15.901±0.00208 B c	12.25±0.19094 D b
	1000	1.108±0.001 C a	14.644±0.00252 A e	19.192±0.00208 B a	13.5627±0.09536 C b
C-Phycocyanin	Control	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	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	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	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	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		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.

al. (2024) reported that the C-PC supplementation would be beneficial in redox stability and metabolic performance through protecting mitochondrial function while reduce ROS increase. These findings corroborate the present results that established C-Phycocyanin as an effective bio-natural agent to be used in order to safeguard metabolism and overcome nanoparticle-induced stress.

Overall, TiO_2 NPs negatively influenced the biochemical status of alfalfa depending on the concentration of exposed test and in contrast C-Phycocyanin alone or in combination was a bio-enhancer due to the increase in the chlorophyll content as well as overall nutrient content. These results were statistically supported at $p \leq 0.05$, confirming the significant influence of both TIO_2 NPS and c-phycocyanin treatments on chemicals composition in alfalfa plants.

In biochemical parameters, the more substantial decreasing values of chlorophyll, nitrogen, phosphorus, potassium and calcium indicated metabolic disarray as well as disorder in nutrient uptake due to TiO_2 NPs in MLE. Root membrane disruption and inhibition of ion transport have been suggested to contribute to the similar nutrient depletion under nanotoxic stress (Emamverdian *et al.*, 2022). Degradation of chlorophyll at high TiO_2 loadings could be a consequence of magnesium removal from the chlorophyll molecule or due to inhibition of enzymes involved in the biosynthesis of chlorophyll (Blas-Valdivia and Moran-Dorantes, 2022).

C-Phycocyanin supplementation, however, significantly ameliorated these biochemical effects. Based on its antioxidative and regulatory ability, NPK significantly increased the

Table 4. Oxidative stress indicators in alfalfa under TiO_2 nanoparticles and C-phycocyanin treatments

Treatments		CAT U/I 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
TiO2 NPS	Control	4±0.23094 A e	25±1.44338 A c	2.92±0.16859 A b	8.806±0.50841 A c	0.0082±0.00046 A d	6±0.34641 A e
	100	8±0.46188 A d	25±1.44338 B c	3.163±0.18262 B b	17.612±1.01683 A b	0.0144±0.00081 B b	13±0.75056 A d
	300	12±0.69282 A c	37.5±2.16506 B b	3.178±0.18348 B b	17.704±1.02214 A b	0.0124±0.00069 B c	18±1.03923 A c
	500	32±1.84752 A b	50±2.88675 A a	3.693±0.21322 A b	18.448±1.0651 A a	0.0143±0.00081 A b	48±2.77128 A b
	1000	36±2.07846 A a	52±3.00222 A a	5.11±0.29503 A a	18.902±1.09131 A a	0.0298±0.00173 A a	54±3.11769 A a
TiO2 NPS +C-Phycocyanin	Control	4±0.23094 A b	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 b
	100	4±0.23094 B b	16.7±0.96417 C d	3.135±0.181 B a	8.806±0.50841 B b	0.0128±0.00075 C c	6±0.34641 B b
	300	8±0.46188 A a	16.2±0.93531 E d	3.155±0.18215 B a	8.856±0.5113 B b	0.0129±0.00075 B c	6±0.34641 B b
	500	4±0.23094 C b	35.7±2.06114 b B	3.006±0.17355 B a	17.612±1.01683 a A	0.0132±0.00075 B a	12±0.69282 B a
	1000	4±0.23094 E b	42.9±2.47683 B a	2.147±0.12396 D a	17.904±1.03369 A a	0.0123±0.00069 C b	6±0.34641 A b
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.

nitrogen metabolism which led to better retention of minerals in the leaves. It appears that rise in chlorophyll and total carotenoid content of C-PC–treated plants is associated with the stabilization of photosynthetic pigments which can protect them from photo-oxidative damages. Abdel-Wahhab *et al.* (2021) and Yu *et al.* (2022) concluded C-PC increases the expression of ROS-related enzymes (peroxidases and MDHAR), $NADP^+$/NADPH ratio, and protects chloroplast membranes.

Additionally, the increases in carbohydrate and protein contents of C-PC treated plants indicate enhanced metabolic efficiency. These results are in line with those reported by Soror *et al.* (2023), who showed that C-Phycocyanin improves both carbon fixation and nitrogen assimilation during abiotic stress by stabilizing RuBisCO activity and amino acid biosynthesis. Thus C-PC not only recuses cells from oxidative stress but also completes pivotal biochemistry of cell machinery leading to plant growth and productivity.

Stress Marker Analysis in Alfalfa Plants

The data in Table (4) showed the influence of titanium dioxide nanoparticles (TiO_2 NPs) and their intersection with C-Phycocyanin on several indicators of oxidative stress [catalase, CAT; superoxide dismutase, SOD; malondialdehyde, MDA; Vitamin C, Vit C; reactive oxygen species, ROS and glutathione peroxidase GPx] in alfalfa plant.

All enzymatic and non-enzymatic antioxidants indices in control plants remained within their physiological range, with respect to oxidative status (Table 3). Alternatively, exposure to TiO_2 NPs alone resulted in a rapid and significant concentration-dependent increase of oxidative stress markers. CAT, SOD and GPx enzymes were markedly enhanced in the two higher concentrations of TiO_2 NPs (500 and 1000 mg L^{-1}), concomitant with high levels of MDA and ROS. These findings support the induced oxidative stress by the increase of reactive oxygen species and activation of antioxidant response as a protective mechanism.

The beneficial metabolic efficiency increases and oxidative stress reduction by C-Phycocyanin (C-PC) can be considered as a result of nutraceutical and redox-modulating action. As reported by Citi *et al.* (2024), the C-PC of *Arthrospira platensis* functions as a potent antioxidant and regulator of redox signaling, stabilizing chloroplast and mitochondrial activities. This protection sustains energy metabolism, photosynthetic activity and nutrient uptake under stress. This would in turn account for the elevated content of chlorophyll, nitrogen, potassium and protein observed in plants treated with C-PC compared to the values found for control and TiO_2 NP treatments. Therefore, the findings of this study further support the recent evidence on C-Phycocyanin promoting cellular protection as well as metabolic efficacy upon oxidative stress.

Loading C- Phycocyanin with TiO_2 NPs significantly lower the level of oxidative stress. At low and moderate concentrations (100–500 mg L^{-1}), the combined treatment significantly reduced ROS and MDA contents compared to TiO_2 NPs alone, but CAT activity and SOD was kept at a moderate level. This indicated that C-Phycocyanin acted as a natural antioxidant, which scavenged free radicals and reduced the cellular damage caused by TiO_2 nanoparticles.

Plants treated with C-Phycocyanin alone also showed the lowest oxidative stress compared to all treatments. The MDA and ROS levels were near that of the control, enzyme antioxidants--CAT, SOD kept stable activity and balance. The relative increase in levels of vitamin C and GPx induced in these plants reflects the belonging of C-Phycocyanin to an antioxidative/protective modulation condition, thus maintaining redox homeostasis.

In general, the results demonstrated that TiO_2 NPs caused oxidative stress in alfalfa seedlings, and C-Phycocyanin (especially when used alone or coapplied at lower levels) remarkably reduced this stress and improved antioxidant defense responses.

These results were statistically supported at $p \leq 0.05$, confirming the significant influence of both TIO_2 NPS and c-phycocyanin treatments on antioxidant parameters in alfalfa plants.

The values we found in the indicators of oxidative stress evaluated CAT, SOD, MDA, ROS and GPx show quite well the double effects caused on nanoparticles as a stressor through inducer of defense by enzymatic action. In the current study, exposure of TiO_2 NPs elicited dramatically increased SOD and CAT activities at the highest concentration which is a common characteristic for causing high levels of ROS generation. Accumulation of MDA reflected the peroxidation of membrane lipids confirming oxidative damage (Ramadan *et al.*, 2022). This is in line with Tuncsoy and Mese's (2021) report that TiO2 NPs lead to the generation of free radicals thereby causing electrolyte leakage and lowering enzymatic activity.

Intraperitoneal administration of C-Phycocyanin substantially reduced these oxidative parameters. Direct ROS scavenging ability of this molecule, which serves in electron donation and neutralization of hydroxyl, super oxide radicals also decrease ROS accumulation. MDA and ROS levels were reduced, and activities of antioxidant enzymes reached the physiological range. Husain *et al.* (2024) and Sayed *et al.* (2022) have reported, C-PC stimulates the ascorbate–glutathione cycle and thus reduces exhaustion of non-enzymatic antioxidants as vitamin C and reduced glutathione, ensuring redox homeostasis in cells. The reduced lipid peroxidation and enhanced vitamin C levels found in the present study strengthen the antioxidant synergy of C-Phycocyanin under TiO_2 stress.

Comet Assay to Determine the Dna Damage

The data in Table (5) showed the effects of TiO2NPs and their interaction C-Pc on DNA

Table 5. DNA integrity (Comet test) in alfalfa plants exposed to TiO_2 nanoparticles and C-phycocyanin (Comet test)

المعاملات		Low Comet Assay Mean±SE	Medium Comet Assay Mean±SE	High Comet Assay Mean±SE
TiO2 NPS	Control	100±0 Aa	0±0 Ac	0±0 Ad
	100	90.2±1.833 Cb	9.8±0.578 Ab	0±0 Bc
	300	88.2±1.613 Dc	11.8±0.577 Aa	0±0 Bb
	500	92.3±1.126 Cb	6.2±0.573 Bc	1.5±0.575 Ba
	1000	91.4±2.348 Cb	6.9±0.574 Bc	1.7±0.576 Ba
TiO2 NPS +C-Phycocyanin	Control	100±0 Aa	0±0 Ae	0±0 Aa
	100	97.1±1.550 Ab	2.9±0.580 Cd	0±0 Ca
	300	95.5±1.519 Bb	4.5±0.575 Cc	0±0 Ca
	500	94.8±0.850 Bb	5.2±0.580 Cb	0±0 Ca
	1000	93.2±2.026 Bb	6.8±0.580 Ba	0±0 Ca
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.

integrity in alfalfa plants using the Comet Assay test. Percentage of low, medium and high DNA fragmented cells were determined in the assay.

Control plant cells, which were not treated with any substance, had 100% of cells at the frequency of low DNA damages; none showed medium or high fragmentation frequencies, implying active genomic stability in normal growing conditions.

The DNA damage was increased in a TiO_2 NPs concentration-dependent manner when the cells were treated with TiO_2 NPs alone. By increasing the TiO_2 NP concentration from 100 to 1000 mg L^{-1}, the percentage of cells with medium size comet tails was increased and that of low-damage category decreased. This trend reveals that exposure to TiO_2NPs induced a genotoxicity effect, which can be associated with reactive oxygen species accumulation that interacted with the cellular DNA.

When C-Phycocyanin was administered together with TiO_2 NPs, a significant decrease of DNA damage compared to the effect caused by exposure to TiO_2 NPs alone was observed. Most of the cells (>93%) were still scored as low damage, even at increased nanoparticle concentrations and only a small percentage of the cells showed medium comets with nearly no representation of high DNA fragmentation. It suggested the protective effect of C-Phycocyanin against nanoparticle mediated genotoxicity, possibly due to its potent antioxidant and radical-scavenging nature.

C-Phycocyanin alone was not effective protection, as DNA remained highly stable, and the comets were almost indistinguishable from the comet profiles of control plants (brokenly 98–100% with a relatively low percentage in comet) (and it did not show signals of medium or high-level damage). These findings document that C-Phycocyanin does not only offset nanoparticle mediated DNA damage, but also sustains the genetic integrity of the genome.

In conclusion, TiO_2 NPs induced a concentration-dependent genotoxicity in alfalfa plants and C-PC (single or combined) conferred significant protection to the cellular DNA by decreasing fragmentation and maintaining chromosomal stability.

These results were statistically supported at $p \leq 0.05$, confirming the significant influence of both TIO_2 NPS and c-phycocyanin treatments DNA Damage in alfalfa plants.

The Comets Assay results strongly support the genotoxic power of TiO2 NPs. The raise of medium/high comet proportions after plants treatment indicate DNA strand breaks and chromosomal instability as a result of oxidative stress damage (Zamora-Ledezma *et al.*, 2025). ROS can damage purine and pyrimidine bases, inducing the possible mutations or replication fidelity overload. These genotoxicity observations have been detected in different plant species, indicating that exposure to NPs may impair genome stability (Sun *et al.*, 2024).

C-Phycocyanin, on the other hand, significantly decreased DNA fragmentation and kept the nuclear integrity. The ability to quench singlet oxygen and chelate metal ions by the pigment also contributes to ROS-mediated nucleic acid oxidation (Cao *et al.*, 2024). The large proportion of low-damage comets in C-PC–treated plants reflect efficient DNA repair and protection of chromatin. Abdelhameed *et al.* (2024) to the point that C-PC stimulates repair enzyme transcription (e.g., DNA glycosylases) and maintains histone acetylation for efficient protection of the genome. These could critically account for the near-normal DNA profiles in plants exposed to C-PC alone or in combination with TiO_2 NPs.

Taken together, the physiological, biochemical and genotoxic investigations show that TiO_2 NPs display a multivectorial toxic action against alfalfa plants by way of oxidative/ionic/genetic routes. c-Phycocyanin neutralizes the toxicity by favouring recovery of redox equilibrium, enhancing nutrient metabolism, and safeguarding genetic components. Owing to the pigment's antioxidant power, as well as its metal-chelating properties, it functions as an efficient bioprotectant against nano-stress by not interfering with essential plant processes.

Hence, applying C-Phycocyanin in agriculture management programs under nanoparticles pollution, might be a safe method to ensure the crop health and productivity maintenance over time from an environmental and biological sustainable perspective (Kanta *et al.*, 2024).

CONCLUSION

This research demonstrated that titanium dioxide nanoparticles (TiO_2 NPs) negatively affect alfalfa growth and physiology in a dose-dependent manner, leading to reduced biomass, impaired pigment content, and oxidative stress–related damage. However, the inclusion of C-phycocyanin (C-PC) significantly alleviated these negative impacts by improving plant growth, restoring chlorophyll levels, and reducing oxidative markers. Beyond these direct effects, the findings point to a broader implication: natural biomolecules such as C-phycocyanin could play a vital role in protecting crops against nanoparticle-induced toxicity. Using such compounds offers a sustainable and eco-friendly approach to maintaining plant productivity in environments increasingly exposed to engineered nanomaterials. Overall, C-phycocyanin shows strong potential as a biological mitigator against nanoparticle stress, and its integration into agricultural management practices may contribute to safer and more sustainable crop production systems.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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