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Nano-TiO_2-Mediated Water Stress Tolerance in *Lupinus albus* L.

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ABSTRACT

The effect of foliar spraying by micrometer-sized TiO_2 particles (TiO_2-Bulk) and TiO_2-nanoparticles (TiO_2-NPs) at different concentrations (50, 150, 300, and 600 ppm) on *Lupinus albus* subjected to different levels of water availability (25%, 50%, 75%, and 100% of field capacity) were examined. Under drought stress circumstances, all potential associations between plant enzymatic activities (CAT, APX and GPX), proline and membrane damage monitors (H_2O_2 concentration, lipid peroxidation and membrane injury index) were evaluated. Significant relationships between both types of TiO_2 and enzymatic activities were found, especially in the roots of *Lupinus*. All enzyme activities were elevated by decreasing water availability, indicating to increasing the efficiency of antioxidant defense mechanisms. These activities were diminished in some enzymes by foliar spraying by bulk or nano-TiO_2, specially at low concentrations, suggesting possible roles of TiO_2 in increasing the capability of plants in attenuating ROS-induced oxidative damage. Lipid peroxidation was positively correlated with H_2O_2 concentration that increased by water stress in the plant shoots and roots. The unique grouping of treatments based on the type and concentration of TiO_2 and on the water stress level was further highlighted by Cluster Analysis to reflect the interdependent impact of both factors on plant responses. Treatments with bulk or nano-TiO_2 particles showed a propensity to strengthen antioxidant defense mechanisms in *Lupinus albus* under moderate water stress. The protective properties of these particles seemed to be lessened under extreme water stress (25% FC), indicating that both kinds of TiO_2 particles may worsen oxidative stress in the plant under severe water deficit especially under higher concentration.

INTRODUCTION

Under water scarcity stress, plants respond intricately, altering every physiological function as well as their macro and micro morphology. Reactive oxygen species (ROS) accumulate in water deprivation stress, mainly in mitochondria and chloroplasts, and produce oxidative stress, just like in other abiotic stressors. One of the ways that plants protect themselves from abiotic stresses is by scavenging reactive oxygen species (ROS). Consequently, a range of antioxidants that reduce oxidative damage and promote stress tolerance are naturally produced by plants. Among these are antioxidant enzymes including superoxide dismutase, peroxidase, and catalase. Oxidative damage occurs in plants under

water deficiency stress, disrupting the photosynthetic machinery and reducing chlorophyll levels [1,2].

Nanotechnology refers to the manipulation and study of materials at the nanoscale, specifically between 1 and 100 nanometers, where unique physical and chemical properties arise, offering new applications across industries and research domains [3]. Titanium (Ti) is the ninth most abundant element in the Earth's crust and occurs in oxidation states of Ti^{2+}, Ti^{3+}(unstable), and Ti^{4+}(stable). With an annual production of up to 38,000 metric tons in the US, TiO_2 is one of the most produced nanoparticles in the world. By 2025, production is predicted to reach 2.5 million metric tons [4]. Recently, a number of disciplines, including botany, have shown a considerable deal of interest in nanotechnology. One of the most produced and extensively utilized nanoparticles worldwide is nano-titanium dioxide (Nano-TiO_2) [5]. These nanoparticles have already infiltrated agroecosystems due to their widespread use and applications, exposing plants to Nano-TiO_2. Human exposure to these nanoparticles will rise as titanium makes its way into food chains. Thus, some authors reviewed the assessment of the harmful consequences caused by Nano-TiO_2 and its effects on human health [6,7].

Different climatic conditions, plant species variety, and titanium application concentrations will all affect how titanium affects plants differently [8,9]. Utilizing nano-TiO_2 in agriculture has been shown to increase crop productivity and protection. However, a number of options must be investigated to guarantee its safe and sustainable use. Thorough ecotoxicological research is essential, with an emphasis on the long-term impacts on the soil microbiota and possible effects on organisms that are not the intended target, such as beneficial insects and earthworms.

Furthermore, according to recent research, rutile, anatase, and TiO_2 crystalline phases have a different impact on physiological, biochemical, and genetic plant factors [10,11]. Plant anatase is more poisonous than rutile, according to available literature. Compared to a mix of anatase and rutile, Silva et al. [12] found that anatase toxicity was higher for wheat seed germination and membrane permeability. According to Giorgetti et al [13], anatase, either alone or in combination with rutile, caused more oxidative stress and ultrastructural damage to pea plant roots than pure rutile. It is unacceptable to neglect the consequences for human health, necessitating evaluations of crop hazards and possible exposure to farmworkers. The cost-effectiveness, effect on small-scale farmers, and cultural, regional, and ethical considerations should all be taken into account when assessing the socioeconomic and ethical implications of nano-TiO_2 in agriculture. Because nano-TiO_2 is widely used, a great deal of research has been made on how these nanoparticles affect plant growth and development. Plants are significantly impacted physiologically by nano-TiO_2, which has stimulatory effects at low concentrations but becomes poisonous at greater ones [9,14,15]. The effect of nano-TiO_2 on plants has revealed that they behave in a hormetic-like manner. Low-dose stimulation and high-dose inhibition are the hallmarks of hormesis, a dose-response phenomena [16]. When it comes to using nano-TiO_2, the creation of control-release formulations may reduce exposure to the environment, and novel delivery techniques like foliar sprays or seed coatings may maximize advantages. It is essential to comprehend the dose-dependent environmental fate of nano-TiO_2, particularly its mobility, persistence, and degradation in different soil types as well as its potential to leak into groundwater. A high nano-TiO_2 concentration (1000 μg L^{-1}) has been demonstrated by Szymánska et al. [17] to cause lipid peroxidation and other adverse consequences, such as a decrease in biomass and chlorophyll content. Additionally, the level of H_2O_2 was markedly increased, as were the activities of antioxidant enzymes SOD, APX, and CAT. Lipid peroxidation produces malone dialdehyde (MDA), a

secondary product that is frequently utilized as an indicator of the oxidative stress response. It has frequently been demonstrated that its hormetic response to an increase in nano-TiO_2 concentration signals membrane lipid damage and additional premature senescence.

According to Sompornpailin and Chayaprasert [18], using titanium nanoparticles to *Nicotiana tabacum* improved the antioxidant activity, photosynthetic pigments, and cell membrane stability. Additionally, Khan et al. [19] shown that when Vicia faba was exposed to a shortage in water supply, Nano-TiO_2 enhanced the production of enzyme and non-enzyme antioxidants. Hong et al [20] and Liu et al [21] reported an increase in the activities of SOD, CAT, POD and a decrease in accumulation of ROS when plants were exposed to TiO_2 nanoparticles.

Some nanomaterials can disrupt the plasma membrane, inducing the formation of pores for crossing into the cell [22] and reaching directly the cytosol without being encapsulated in any organelle [23]. Faraji and Sepehri [24] reported that Nano-TiO_2 reduced the H_2O_2 and MDA contents in *Triticum aestivum***.** The application of TiO_2 NPs enhanced the activities of antioxidants also decreased the rate of production of MDA and H_2O_2 in the plants [25].

Four questions were addressed in this study. First, do lupin plants respond to foliar application of Nano-TiO_2 in the same way as Bulk-TiO_2? Second, what effects do rise TiO_2 concentrations have on cell membranes and certain enzyme activity? Third, is it possible for Nano-TiO_2 to somewhat mitigate the effects of drought? Fourth, does drought act as an abiotic stressor on plants in conjunction with nano-TiO_2? Since foliar spraying is used to deliver the TiO_2, an additional goal of this study was to ascertain whether the root will be affected similarly to the shoot under different levels of water availability.

MATERIALS AND METHODS

1. Experimental design and treatments

The purpose of this experiment was to determine how different concentrations of nanoparticles or bulk titanium dioxide (TiO_2-NPs or Bulk-TiO_2) affected the integrity of the cell membranes and certain antioxidant enzymes of *Lupinus albus* L. (Lupin) plants under varying water availability conditions. The experiment began on December 1, 2020, in the greenhouse of the Botany Department, Assiut University, in a field setting. During December and January, the average temperature was 23±2, the lowest temperature was 9±1.7, and the relative humidity was 38% ±1.

In pots with 3 kg of soil (2:1 clay: sand by weight), about 15 seeds were sown. The field capacity, which was determined to be roughly 24% of dry soil (the welting point of lupin was 8%), was reached by irrigating the 120 pots with tap water. After approximately a month of growing, the number of individuals was thinned to 7 homogeneous plants per pot. Each pot was given 50 ml of Hoagland's solution at a tenth strength, along with 10 ml of irrigation water day after day to prevent nutrient deprivation.

Three replications of a factorial experiment using randomized complete block design (RCBD) were conducted. The 120 pots were separated into 10 groups, with three pots serving as replicates of the four water levels (100%, 75%, 50%, and 25% of field capacity). Eight groups were sprayed with 50, 150, 300, and 600 ppm Nano-TiO_2 or Bulk-TiO_2, while the first group served as the control. The second group was foliary sprayed with distilled water. The foliar spraying was done after ten days of establishment at varying water levels, when the plants had roughly three pairs of leaves. A total of 15 ml of spraying solution was used to spray each pot twice at 5-day intervals. After 25 days from the first spraying (the plants were with 6 pairs of leaves) the plants were harvested for different measurements.

The Nano-TiO_2 with particle size less than 25 nm, purity 99.7% and surface area of 45–55 $m^2.g^{-1}$ was purchased from Sigma-Aldrich Company. Nano-TiO_2 and Bulk-TiO_2 particles, separately, were dissolved in distilled water just before using and scattered by ultrasonic vibration "BANDELIN SONOPULS HD 2070" homogenizer at 100 W and 40 kHz for 10 min.

2. Membrane stability index and electrolyte leakage

The test for cell membrane stability was conducted according to Premachandra et al [26]. Ten fresh leaf discs were immersed in 25 milliliters of bi-distilled water for 24 h at room temperature after being rinsed three times with the same water. The bathing solution's electric conductivity was assessed using a conductometer (YSI Model 35, Yellow Springs, OH, USA). Samples were then autoclaved and allowed to cool to ambient temperature before the EC was measured again. Cell membrane stability index, or membrane injury, was evaluated as percentage injury according to the following equation:

$$\text{Membrane injury index} = \left[1 - \frac{(1 - {T_1}/{T_2})}{(1 - {C_1}/{C_2})}\right] \times 100$$

In addition, the electrolyte leakage was calculated relative to that of control plants as following:

$$\text{Electrolyte leakage} = \frac{T_1}{T_2} \Big/ \frac{C_1}{C_2}$$

Where T_1 and T_2 are EC values of the treated plants, while C_1 and C_2 represent the EC values of control plants before and after autoclaving, respectively.

3. Determination of lipid peroxidation (malondialdehyde content)

With a few minor adjustments, the Hodges et al [27] method was used to calculate the amount of malondialdehyde (MDA) in lupin leaves, which indicates the degree of lipid peroxidation. The thiobarbituric acid reaction was used to measure the amount of MDA, a byproduct of lipid peroxidation. 4 ml of ethanol 80% with 2% dimethyl sulfoxide was used to homogenize 0.2 g of fresh leaves, and the resulting homogenate was centrifuged for 10 minutes at 10,000 rpm. Three milliliters of 20% TCA containing 0.65% thiobarbituric acid (TBA) were added to each one milliliter aliquot. After 30 minutes of heating at 95 ^oC, the mixture was rapidly cooled on an ice bath. After that, the mixture was centrifuged at 6000 rpm for 15 minutes and the absorbance of the supernatant was measured at 532 nm using UV2000/2200 Spectrophotometer (Ray Wild Limited Compony, Germany). Blanks with the same weight from the same leaf or opposite one were proceeded as the samples but without TBA. After subtracting blank reading, the level of lipid peroxidation was expressed as nmol g^{-1} FW of MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1}.

4. Determination of H_2O_2

With a few modest adjustments, the hydrogen peroxide level of lupin leaves or roots was determined spectrophotometrically as described by Sellers [28]. Two millilitres of cold acetone and three millilitres of double-distilled water were used to extract a specified weight (0.1 g) of tissue. Three millilitres of the supernatant were combined with one millilitre of 0.1 M potassium titanium (IV) oxalate dihydrate [$K_2TiO(C_2O_4)_2.2H_2O$] following ten minutes of centrifugation at 6000 rpm in 5 M sulphoric acid. At 400 nm, the yellow-orange colour intensity was measured. The same weight of leaves or roots was

boiled for one minute to create a blank, which was then processed as the original sample. H_2O_2 was calculated using the molar absorptivity of 935 L mol^{-1} cm^{-1} as µmole g^{-1} FW.

5. Determination of proline

The acid–ninhydrin method, as described by Bates et al [29], was used to measure the concentration of proline. Specifically, 3 ml of 3% sulfosalicylic acid were used to homogenize 0.2 g of fresh leaves, and the resulting homogenate was centrifuged for 10 minutes at 10,000 rpm. In a test tube, 400 µl of acid-ninhydrin, 400 µl of glacial acetic acid, and 200 µl of 3% sulfosalicylic acid were combined with 1 ml of the mixture. The reaction was allowed to run for an hour at 96°C before being stopped in an ice bath. Four milliliters of toluene were used to extract the reaction mixture, which was then well mixed. Toluene was used as a blank for measuring the absorbance at 520 nm after the chromophore containing it was aspirated from the aqueous phase and warmed to room temperature. The proline concentration was determined from a standard curve and calculated as µg g^{-1} FW.

6. Assay of antioxidant enzymes

Preparation of enzyme extract: After 0.5 g of roots or leaf tissues were finely powdered in liquid N_2, they were homogenized in 5 ml of 100 mM potassium phosphate buffer (pH 7.8) that contained 0.1 g of polyvinylpyrrolidone (PVP) and 0.1 mM ethylenediamine tetraacetic acid-disodium salt (Na_2-EDTA). The homogenate was centrifuged under cooling (4 ^oC) for 10 minutes at 18,000 rpm, and the supernatants were gathered and used for catalase, guaiacol peroxidase and ascorbate peroxidase assay. Using a spectrophotometer, all colorimetric measurements were performed at 20^oC. In this extract, the content of soluble proteins was determined according to Lowry et al [30] method and the specific activity of enzymes was expressed as "units mg^{-1} protein min^{-1}".

The modified Aebi [31] approach was used to measure the rate of H_2O_2 dissociation to O2 and water for one minute in order to evaluate catalase (CAT; EC 1.11.1.6). 2.8 ml of 50 mM potassium phosphate buffer (pH 7) and 100 µl of enzyme extract made up the assay media (3 ml). The reaction started by adding 100 µl of 10 mM H_2O_2. For one minute, the absorbance drop at 240 nm was noted.

The method of Zaharieva et al [32] was modified to measure guaiacol peroxidase (GPX; EC 1.11.1.7). The substrate used was guaiacol. POD was determined in a 1.3 ml reaction mixture using 1 ml 30 mM phosphate buffer (pH = 7), 100 µl enzyme extract, 100 µl 6.5 mM H_2O_2, and 100 µl 1.5 mM guaiacol. The formation of tetra guaiacol was measured at 470 nm.

Ascorbate peroxidase (APX; EC 1.11.1.11) was measured according to Nakano and Asada [33] by monitoring the rate of ascorbate oxidation at 290 nm. The reaction mixture contained 1.6 ml of 50 mM potassium phosphate buffer (pH = 7), 0.1 mM Na_2-EDTA, 5 mM H_2O_2, 0.5 mM ascorbic acid and 50 µl enzyme extract. The decrease in absorbance at 290 nm was monitored to calculate the activity of APX.

7. Statistical analyses

Data were subjected to statistical analysis using SPSS (version 21). One-way ANOVA was performed followed, when the effect was significant at $P \leq 0.05$, by the post hoc Duncan's multiple-range test (at $P \leq 0.05$) for comparison between means of parameters at each level of water availability or at each concentration of the spraying solution. Two-way ANOVA was carried to achieve the effect of water availability, Nano- or Bulk-TiO_2 and their interaction on different parameters estimated in leaves or roots and eta square "η^2" was calculated as: $\eta^2 = SS_{Effect} / SS_{Total}$ to achieve the size effects of each factor or the interaction between factors. Heatmap and Cluster Analysis were used to

illustrate the expression levels of various phytochemicals and their associated activities across different Ti_2O × drought levels. These two statistical techniques showed all possible correlations (and/or) regressions among all assessed chemical profile.

RESULTS

1. Oxidative stress indicators

1.1. H_2O_2 generation

The hydrogen peroxide (H_2O_2) concentration in lupin leaves exhibited a continuous and significant increase under conditions of reduced water availability across all treatments of TiO_2 (Table 1). Notably, the application of water spray markedly diminished H_2O_2 levels compared to the control group. H_2O_2 concentrations in lupin leaves escalated with rising concentrations of both Nano- and bulk-TiO_2; however, nano-TiO_2 treatments consistently resulted in higher H_2O_2 levels than bulk-TiO_2.

A similar trend was observed in lupin roots, where H_2O_2 levels increased with higher concentrations of Nano-TiO_2 (Table 1). Conversely, increasing bulk-TiO_2 concentrations did not produce significant variations in H_2O_2 levels in the roots. The lowest H_2O_2 levels were recorded in roots treated with 150 ppm bulk-TiO_2, while the highest were found in roots exposed to 600 ppm nano-TiO_2. As in leaves, H_2O_2 content in lupin roots demonstrated a proportional and significant increase with diminishing soil moisture availability. Throughout all levels of water availability, H_2O_2 generation were significantly lower in the roots of plants treated with bulk-TiO_2 compared to both control and water-sprayed plants. Additionally, roots of plants treated with 50 and 150 ppm Nano-TiO_2 exhibited significant reduction in H_2O_2 content relative to the control plants. Across all water availability status, the H_2O_2 content in roots was consistently lower in bulk-TiO_2 treated plants than in those treated with nano-TiO_2. Overall, the application of water spray significantly mitigated H_2O_2 levels in lupin roots.

1.2. Lipid peroxidation

The results pertaining to malondialdehyde (MDA) content, a widely recognized biomarker for lipid peroxidation, were assessed in the leaves and roots of *Lupinus albus*. Table 1 shows the impact of water deficit stress and the application of two types of TiO_2 on lipid peroxidation in *L. albus* tissues. Notably, the application of water spray resulted in a non-significant reduction in MDA levels compared to the control group. Lipid peroxidation in leaf cells exhibited a proportional and significant increase with diminishing soil moisture availability, transitioning from full field capacity (FC) to 25% FC.

At all moisture levels, MDA content in the leaves those treated with either nano- or bulk-TiO_2 significantly surpassed that of control and water-sprayed plants, with the exception of the 600-ppm nano-TiO_2 treatment, which induced a non-significant decrease. The most pronounced lipid peroxidation was observed in leaves of lupin plants subjected to 50 and 150 ppm nano-TiO_2, whereas minimal peroxidation was noted in those treated with 600 ppm Nano-TiO_2. Across all FC levels, foliar application of 50 and 150 ppm nano-TiO_2 resulted in higher MDA content compared to bulk-TiO_2; conversely, treatments with 300 and 600 ppm bulk-TiO_2 yielded greater MDA levels than their nano-TiO_2 counterparts.

In lupin roots, lipid peroxidation also demonstrated a significant and proportional increase in response to decreasing soil water availability. The application of water significantly mitigated lipid peroxidation in roots compared to control and TiO_2 -treated plants. At all moisture levels, MDA content was significantly lower in roots treated with

either Nano- or Bulk-TiO_2 compared to control. Table 1 indicates that lipid peroxidation in lupin roots was effectively reduced through the application of 600 ppm nano-TiO_2 and 50 ppm bulk-TiO_2 .

1.3. Cell membranes injury and electrolyte leakage

According to data obtained from Table 1, electrolyte leakage and cell membrane damage in lupin leaves matched and followed the same pattern. Both were reduced by watering the plants, and they were subsequently and primarily greatly increased by nano- or bulk-TiO_2 or by lowering the water availability level. However, plants treated with 300 and 600 ppm bulk-TiO_2 had the largest estimated electrolyte leakage. Compared to plants treated with nano-TiO_2, those treated with bulk-TiO_2 showed greater increases in electrolyte leakage and cell membrane damage. When plants were sprayed with high concentrations of nano-TiO_2 (300 and 600 ppm), electrolyte leakage rose dramatically; however, when plants were treated with 50 and 150 ppm, it did not change significantly.

Table 1. The effect of concentrations of nano- or bulk-TiO_2 on generation of H_2O_2 (µmole g^{-1} FW), lipid peroxidation (nmol MDA g^{-1} FW) and membrane injury index in leaves and roots of *Lupinus albus* grown on different levels of water availability. The data are averages across all concentrations of TiO_2 in each level of water availability (n= 18), and across all levels of water availability in each concentration of TiO_2 (n= 12). Comparison between nano- and bulk-TiO_2 at each level of FC or concentration of TiO_2 was achieved from one-way ANOVA, while the comparison between different levels of each factor (water availability or TiO_2) was achieved from two-way ANOVA and Duncan's test at $P \leq 0.05$.

Organ		H_2O_2			MDA			Mem. injury		
	TiO_2 / Treatment	NPs	Bulk	Sign.	NPs	Bulk	Sign.	NPs	Bulk	Sign.
Leaves	Full FC	$17.1^{a}\pm1.4$	$12.3^{a}\pm0.9$	**	$99.6^{a}\pm3.8$	$95.6^{a}\pm2.1$	ns	$2.8^{a}\pm0.8$	$5.0^{a}\pm1.3$	ns
	75% FC	$21.7^{b}\pm1.2$	$17.4^{b}\pm1.1$	*	$109.0^{b}\pm4.0$	$107.4^{b}\pm2.5$	ns	$4.1^{b}\pm0.6$	$6.9^{b}\pm1.1$	*
	50% FC	$29.3^{c}\pm2.3$	$23.2^{c}\pm1.3$	*	116.7c±4.7	$115.1^{c}\pm3.2$	ns	5.3c±0.7	$7.8^{c}\pm0.9$	*
	25% FC	$40.6^{d}\pm1.7$	$37.0^{d}\pm0.7$	ns	$124.3^{d}\pm4.5$	$127.0^{c}\pm3.6$	ns	$6.3^{d}\pm0.8$	$9.7^{d}\pm1.1$	*
	Control	$23.6^{b}\pm1.55$	$23.6^{c}\pm1.55$		$101.2^{b}\pm2.04$	$101.2^{b}\pm2.04$		$2.7^{b}\pm0.34$	$2.7^{b}\pm0.34$	
	H_2O-sprayed	$17.8^{a}\pm2.36$	$17.8^{a}\pm2.36$		$95.2^{a}\pm1.72$	$95.2^{a}\pm1.72$		$1.6^{a}\pm0.39$	$1.6^{a}\pm0.39$	
	50 ppm	$25.2^{b}\pm2.3$	$20.3^{b}\pm2.9$	ns	$133.4^{d}\pm3.8$	$112.4^{c}\pm5.3$	**	$3.0^{bc}\pm0.4$	$6.2^{c}\pm1.1$	*
	150 ppm	$29.1^{c}\pm2.9$	$20.4^{b}\pm2.8$	*	$134.0^{d}\pm3.2$	$124.2^{d}\pm4.6$	ns	$3.5^{c}\pm0.3$	$9.4^{d}\pm0.5$	**
	300 ppm	$30.8^{c}\pm2.5$	$24.6^{c}\pm3.2$	ns	$117.6^{c}\pm3.2$	$123.1^{d}\pm4.1$	ns	$7.5^{d}\pm0.5$	$10.9^{e}\pm0.5$	**
	600 ppm	$36.4^{d}\pm3.8$	$28.1^{d}\pm2.7$	ns	$92.7^{a}\pm2.7$	$111.5^{c}\pm2.9$	**	$9.5^{e}\pm0.6$	$13.2^{f}\pm0.6$	**
Roots	Full FC	$11.8^{a}\pm1.1$	$7.7^{a}\pm1.2$	*	$76.4^{a}\pm2.8$	$72.8^{a}\pm2.6$	ns			
	75% FC	$14.3^{b}\pm0.8$	$9.7^{b}\pm1.2$	**	$89.9^{b}\pm4.4$	$83.6^{b}\pm4.2$	ns			
	50% FC	$18.4^{c}\pm0.9$	$12.0^{c}\pm1.5$	**	$105.0^{c}\pm5.1$	$98.0^{c}\pm4.5$	ns			
	25% FC	$20.3^{d}\pm0.9$	$13.9^{d}\pm1.4$	**	$116.3^{d}\pm5.5$	$111.0^{d}\pm4.6$	ns			
	Control	$21.1^{e}\pm0.50$	$21.1^{d}\pm0.50$		$118.8^{e}\pm5.06$	$118.8^{d}\pm5.06$				
	H_2O- sprayed	$14.5^{b}\pm0.96$	$14.5^{c}\pm0.96$		$73.7^{a}\pm2.89$	$73.7^{a}\pm2.89$				
	50 ppm	$11.81^{a}\pm1.04$	$7.63^{b}\pm0.4$	**	$110.1^{d}\pm8.83$	$76.9^{a}\pm3.4$	**			
	150 ppm	$12.7^{a}\pm0.8$	$6.67^{a}\pm0.7$	**	$100.0^{c}\pm3.0$	$82.9^{b}\pm4.0$	**			
	300 ppm	$17.3^{c}\pm1.4$	$7.31^{ab}\pm0.7$	**	$100.8^{c}\pm2.2$	$98.8^{c}\pm4.1$	ns			
	600 ppm	$19.8^{d}\pm1.1$	$7.56^{b}\pm0.7$	**	$78.0^{a}\pm3.3$	$96.7^{c}\pm4.7$	**			

2. Defensive strategies

2.1. Proline accumulation

As illustrated in Table 2, proline concentrations in the leaves of *Lupinus albus* exhibited a significant and proportional increase in response to decreasing soil water availability. In particular, proline levels in leaves of plants subjected to water scarcity (50% and 25% field capacity, FC), regardless of TiO_2 application, were significantly elevated compared to those grown under full FC. Notably, proline content surged in plants experiencing 25% available water, reaching approximately 1.5 to 4.2 times the levels observed in plants maintained at full FC. Similarly, proline concentrations in the roots of lupin plants under water deficit conditions, independent of TiO_2 treatment, were significantly higher than those in plants grown under full FC (Table 2). Consistent with the leaf data, proline levels in roots of plants at 25% FC increased dramatically, ranging from 2.5 to 4.1 times the levels found in fully irrigated plants.

2.2. Enzymatic antioxidant activities

To evaluate if nono- or bulk-TiO_2 have a role in the of antioxidant enzymes, catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) activities were assayed in leaves and roots of *L. albus*.

2.2.1. Catalase (CAT)

The results presented in Table 2 demonstrate significant variations in CAT activity in the leaves and roots of *Lupinus albus* in response to differing levels of water availability and TiO_2 treatments. Across all moisture conditions, CAT activity was markedly reduced in plants treated with bulk-TiO_2 compared to control, water-sprayed plants, or those treated with nano-TiO_2. Generally, CAT levels in lupin leaves increased more significantly with nano-TiO_2 treatment than with bulk-TiO_2.

At all water availability levels, CAT activity in leaves decreased significantly in response to nano-TiO_2 up to 300 ppm, but rebounded with the 600-ppm treatment, approaching levels observed in control or water-sprayed plants. The highest CAT activities were recorded in water-sprayed plants, whereas treatments with 300 and 600 ppm bulk-TiO_2 resulted in considerable inhibition of CAT activity.

In the roots, CAT activity similarly decreased in plants treated with either nano- or bulk-TiO_2 compared to control or water-sprayed plants. The lowest CAT activities were predominantly observed in roots exposed to 300 and/or 600 ppm bulk-TiO_2. Furthermore, CAT activity did not differ significantly between plants treated with bulk-TiO_2 and those treated with nano-TiO_2. Notably, leaves and roots of lupins exhibited differential responses to water deficit stress; CAT activity in leaves increased with escalating water deficit, regardless of TiO_2 concentration or type, while the opposite trend was observed in the roots (Table 2).

2.2.2. Guaiacol peroxidase (GPX)

The data presented in Table 2 indicate that, irrespective of the type or concentration of TiO_2, GPX activity in the leaves and roots of *L. albus* exhibited a gradual and significant increase with decreasing water availability. Notably, GPX activity was generally higher in plants treated with bulk-TiO_2 compared to those subjected to nano-TiO_2 treatments. In leaves, GPX activity decreased significantly or non-significantly in response to various concentrations of nano-TiO_2 compared to control or water-sprayed plants. Water spraying resulted in a significant reduction in GPX activity. The highest GPX activities were observed in plants treated with 600 ppm bulk-TiO_2, while the lowest were recorded in those treated with 50 ppm nano-TiO_2.

In the roots, GPX activity showed a slight increase with 50 and 150 ppm bulk-TiO_2 treatments but was markedly inhibited by 300 and 600 ppm across all water availability

levels. Consequently, the lowest GPX activities were associated with these higher bulk-TiO_2 treatments. Additionally, GPX activity in plants treated with 50 and 150 ppm bulk-TiO_2 was greater than in those treated with equivalent concentrations of nano-TiO_2 ; however, this trend reversed at 300 and 600 ppm treatments.

2.2.3. Ascorpate peroxidase (APX)

The results indicate that as water deficit stress increased, ascorbate peroxidase (APX) activity in *Lupinus albus* was generally inhibited. Notably, APX activity in the leaves of lupins treated with nano-TiO_2 was significantly elevated across all levels of water availability compared to control plants. In roots of water-sprayed plants grown at 50% and 25% field capacity (FC), APX activity decreased significantly relative to control.

In contrast, APX activity in the roots of lupin plants treated with nano-TiO_2 was significantly lower than in control plants across all moisture conditions. The data also revealed a substantial reduction in APX activity with 600 ppm bulk-TiO_2 treatment at all levels of water availability. Furthermore, spraying lupin plants with 150 and 300 ppm bulk-TiO_2 resulted in higher APX activity compared to those treated with 50 and 600 ppm. Both concentrations of bulk-TiO_2 enhanced APX activity more effectively than their nano-TiO_2 counterparts.

Table 2: The effect of concentrations of nano- or bulk-TiO_2 on accumulation of proline (µg g^{-1} FW), and activities of CAT, GPX and APX (units mg^{-1} protein min^{-1}) in leaves and roots of *L. albus* grown on different levels of water availability. Statistics as in Table 1.

Organ		Proline			CAT activity			GPX activity			APX activity		
	TiO_2 / Treatment	NPs	Bulk	Sign	NPs	Bulk	Sign.	NPs	Bulk	Sign	NPs	Bulk	Sign
Leaves	Full FC	$184.3^{a}\pm11.3$	$143.2^{a}\pm6.3$	**	$7.41^{a}\pm0.4$	$6.3^{a}\pm0.5$	ns	$54.9^{a}\pm1.5$	$62.1^{a}\pm1.6$	**	$259.0^{b}\pm14.7$	$327.7^{d}\pm18.1$	**
	75% FC	$235.4^{b}\pm4.0$	$197.2^{b}\pm8.4$	**	$8.5^{b}\pm0.44$	$7.0^{b}\pm0.6$	*	$62.8^{b}\pm2.3$	$76.3^{b}\pm2.6$	**	$227.4^{a}\pm8.0$	$278.0^{c}\pm17$	*
	50% FC	$321.9^{c}\pm12.6$	$325.2^{c}\pm6.1$	ns	$9.6^{c}\pm0.3$	$7.6^{c}\pm0.5$	**	$71.3^{c}\pm2.3$	$85.4^{c}\pm2.3$	**	$233.3^{a}\pm12.0$	$222.8^{b}\pm11.4$	ns
	25% FC	$512.0^{d}\pm28.5$	$475.0^{d}\pm14.3$	ns	$10.4^{d}\pm0.3$	$8.3^{d}\pm0.6$	**	$86.5^{d}\pm2.4$	$101.3^{d}\pm1.7$	**	$224.2^{a}\pm16.0$	$183.4^{a}\pm8.3$	*
	Control	$297.7^{c}\pm28.3$	$297.7^{d}\pm28.3$		$10.1^{d}\pm0.19$	$10.1^{c}\pm0.19$		$79.6^{e}\pm2.37$	$79.6^{c}\pm2.37$		$215.5^{a}\pm6.00$	$215.5^{a}\pm6.00$	
	H_2O-sprayed	$281.7^{b}\pm28.4$	$281.7^{b}\pm28.4$		$10.6^{d}\pm0.20$	$10.6^{c}\pm0.20$		$71.5^{c}\pm2.70$	$71.5^{a}\pm2.70$		$226.9^{ab}\pm16.6$	$226.9^{a}\pm16.6$	
	50 ppm	$256.2^{a}\pm12.4$	$262.0^{a}\pm18.6$	ns	$9.3^{c}\pm0.3$	$6.6^{b}\pm0.3$	**	$56.3^{a}\pm2.1$	$74.7^{b}\pm6.3$	*	$235.9^{b}\pm16.0$	$232.7^{a}\pm12.0$	ns
	150 ppm	$297.8^{c}\pm28.9$	$288.2^{bc}\pm39.4$	ns	$8.6^{b}\pm0.3$	$6.2^{b}\pm0.3$	**	$61.8^{b}\pm4.2$	$83.0^{d}\pm4.3$	**	$237.8^{b}\pm11.1$	$231.8^{a}\pm16.8$	ns
	300 ppm	$362.4^{d}\pm49.9$	$291.2^{cd}\pm45.1$	ns	$6.4^{a}\pm0.5$	$5.3^{a}\pm0.3$	ns	$69.0^{c}\pm4.5$	$86.2^{e}\pm4.1$	**	$240.1^{b}\pm15.7$	$256.7^{b}\pm16.3$	ns
	600 ppm	$384.9^{e}\pm55.8$	$289.9^{bcd}\pm48.8$	ns	$8.9^{bc}\pm0.6$	$5.1^{a}\pm0.2$	**	$75.0^{d}\pm3.9$	$92.6^{f}\pm4.8$	**	$259.6^{c}\pm17.9$	$354.0^{c}\pm31.1$	*
Roots	Full FC	$341.4^{a}\pm8.6$	$396.5^{a}\pm23.4$	*	$11.5^{c}\pm0.49$	$10.6^{d}\pm0.6$	ns	$3.9^{a}\pm0.09$	$3.8^{a}\pm0.18$	ns	$118.2^{c}\pm4.87$	$118.0^{c}\pm4.5$	ns
	75% FC	$394.0^{b}\pm7.2$	$440.2^{b}\pm24$	ns	$9.33^{b}\pm0.32$	$9.1^{c}\pm0.32$	ns	$4.5^{b}\pm0.07$	$4.2^{b}\pm0.18$	ns	$108.1^{b}\pm3.7$	$120.4^{c}\pm2.64$	**
	50% FC	$640.2^{c}\pm40.2$	$567.2^{c}\pm29.2$	ns	$8.6^{b}\pm0.26$	$7.6^{b}\pm0.33$	*	$5.2^{c}\pm0.07$	$4.8^{c}\pm0.22$	ns	$103.9^{b}\pm4.5$	$111.7^{b}\pm5.3$	ns
	25% FC	$1273.0^{d}\pm19.2$	$1235.0^{d}\pm23.5$	ns	$7.3^{a}\pm0.3$	$6.9^{a}\pm0.32$	ns	5.9d±0.18	$5.5^{d}\pm0.28$	ns	$90.8^{a}\pm6.55$	$101.6^{a}\pm7.9$	ns
	Control	$584.4^{a}\pm70.8$	$584.4^{a}\pm70.8$		$10.4^{c}\pm0.49$	$10.4^{c}\pm0.49$		$5.2^{c}\pm0.26$	$5.2^{d}\pm0.26$		$114.7^{d}\pm4.93$	$114.7^{c}\pm4.93$	
	H_2O-sprayed	$597.6^{a}\pm74.6$	$597.6^{a}\pm74.6$		$10.4^{c}\pm0.38$	$10.4^{c}\pm0.38$		$4.7^{a}\pm0.12$	$4.7^{c}\pm0.12$		$104.5^{c}\pm5.90$	$104.5^{b}\pm5.90$	
	50 ppm	$639.7^{b}\pm109.2$	$599.1^{a}90.36$	ns	$9.4^{b}\pm0.5$	$7.9^{b}\pm0.5$	ns	$4.8^{a}\pm0.3$	$5.2^{d}\pm0.3$	ns	$89.0^{a}\pm5.7$	$112.0^{c}\pm3.2$	**
	150 ppm	$655.9^{b}\pm120.9$	$587.9^{a}111.1$	ns	$9.5^{b}\pm0.6$	$8.5^{b}\pm0.5$	ns	$4.9^{ab}\pm0.2$	$5.3^{d}\pm0.2$	ns	$97.6^{b}\pm3.8$	$131.3^{d}\pm3.6$	**
	300 ppm	$715.9^{c}\pm117.1$	$755.1^{b}103.5$	ns	$7.9^{a}\pm0.5$	$7.1^{a}\pm0.3$	ns	$4.8^{a}\pm0.2$	$4.0^{b}\pm0.1$	**	$125.1^{e}\pm3.1$	$128.0^{d}\pm3.6$	ns
	600 ppm	$779.4^{d}\pm121.6$	$814.3^{c}98.7$	ns	$7.5^{a}\pm0.4$	$7.0^{a}\pm0.4$	ns	$5.1^{bc}\pm0.3$	$3.1^{a}\pm0.2$	**	$100.6^{bc}\pm5.5$	$87.0^{a}\pm4.7$	ns

3. Effect size quantification

Data in Table 3 presents the eta squared (η^2) values, indicating the magnitude of effect for different factors affecting various physiological parameters in the leaves and roots of *L. albus.* To quantify the effect size of each factor, η^2 was calculated by dividing the sums of squares for the effecting factor (SS_{effect}) by the total sums of squares for all effects,

including errors and interactions (SS_{total}) in the ANOVA. As shown in Table 3, in lupin leaves treated with Nano- or bulk-TiO_2 , water availability exerted the most significant influence on the variations in H_2O_2 and proline content. Specifically, nano-TiO_2 represented the primary effect on MDA content and membrane injury, accounting for over 73% of the variance. In plants treated with bulk-TiO_2 , 78.6% of variance was attributed to bulk-TiO_2, which emerged as the main effect on membrane injury.

In the leaves of lupin, both nanoparticle (NP) treatments and bulk-TiO_2 (BPs) demonstrated significant effects on catalase (CAT) activity, with η^2 values of 0.492 and 0.850, respectively, indicating their substantial influence. Noteworthy effects were also observed for guaiacol peroxidase (GPX), with NPs showing an η^2 of 0.284 and BPs at 0.178, suggesting that TiO_2 treatments markedly enhance GPX activity. The role of ascorbate peroxidase (APX) was particularly pronounced with BPs ($\eta^2 = 0.341$) compared to NPs ($\eta^2 = 0.058$). Proline accumulation was significantly impacted by both factors, with water deficit (WD) exhibiting a notably high effect size of 0.767. Additionally, significant effects on H_2O_2 and malondialdehyde (MDA) levels were observed, particularly in relation to NPs and WD, emphasizing their roles in mediating oxidative stress responses. Notably, the highest η^2 values for membrane injury were recorded for both TiO_2 treatments, indicating a pronounced impact on membrane stability.

In the roots, both nano- and bulk-TiO_2 treatments indicated that water availability had the most substantial effect on proline content. Both water deficit (WD) and nanoparticles (NPs) significantly influenced H_2O_2 and MDA levels, with BPs being the most critical factor affecting H_2O_2 content. The interaction between levels of water deficit stress and NPs or BPs exhibited a weak effect on the parameters measured. According to the two-way ANOVA, the two primary factors—water deficit stress and the type of TiO_2 —and their interaction significantly impacted nearly all measured parameters in both leaves and roots of lupins.

In the roots, similar trends were observed, with both NPs ($\eta^2 = 0.278$) and BPs ($\eta^2 = 0.408$) exhibiting significant effects on CAT activity, suggesting a robust antioxidant response. GPX activity was significantly enhanced by both treatments, with NPs at $\eta^2 = 0.047$ and BPs at $\eta^2 = 0.533$. Furthermore, significant contributions to APX activity were noted for both NPs ($\eta^2 = 0.262$) and BPs ($\eta^2 = 0.395$). Interaction effects were particularly pronounced for proline accumulation, with WD showing a high η^2 value of 0.938, reflecting its critical role under stress conditions. Lastly, both treatments significantly influenced oxidative stress markers, with BPs exhibiting the highest η^2 values for H_2O_2 and MDA levels.

4. Multivariate analysis

Figure 1 provides a comprehensive analysis of how varying concentrations of TiO_2 compounds and drought stress levels affect the physiological responses of *L. albus*. The figure presents a dendrogram and heatmap analyzing the responses of *L. albus L.* to varying concentrations of nano-TiO_2 and bulk-TiO_2 under different drought stress levels.

The dendrogram provides additional insight, showing how different treatments cluster based on their physiological responses. Treatments with similar effects on antioxidant enzymes and oxidative stress indicators group closely, indicating similar physiological mechanisms at play. The dendrograms in panels A and D (hierarchical clustering, Figure 1) highlight distinct clustering patterns based on the treatments, suggesting that both nano-TiO_2 and bulk-TiO_2 compounds.

Table 3: The eta squared (η^2) for the magnitude of effect of Nano- (NPs) or bulk-TiO_2 (BPs), water deficit stress (WD) and the interaction between them on changes of each studied variables in Lupin leaves and roots. The significant effect of each factor on the parameters is achieved from the two-way ANOVA

Organ	spray	Factor	CAT	Sign.	GPX	Sign.	APX	Sign.	Proline	Sign.	H_2O_2	Sign.	MDA	Sign.	Mem. inj.	Sign.
Leaves	Nano-TiO_2	NPs	0.492	***	0.284	***	0.058	***	0.101	***	0.268	***	0.738	***	0.761	***
		WD	0.356	***	0.634	***	0.06	***	0.767	***	0.616	***	0.213	***	0.166	***
		WD*NPs	0.07	ns	0.05	***	0.791	***	0.128	***	0.068	***	0.013	ns	0.046	***
		Error	0.083		0.033		0.091		0.005		0.048		0.036		0.027	
	Bulk-TiO_2	BPs	0.85	***	0.178	***	0.341	***	0.007	***	0.11	***	0.405	***	0.786	***
		WD	0.097	***	0.732	***	0.464	***	0.916	***	0.828	***	0.479	***	0.126	***
		WD*BPs	0.006	ns	0.064	***	0.118	***	0.073	***	0.031	***	0.053	**	0.062	***
		Error	0.047		0.026		0.078		0.004		0.031		0.063		0.026	
Roots	Nano-TiO_2	NPs	0.278	***	0.047	***	0.262	***	0.031	***	0.476	***	0.452	***		
		WD	0.533	***	0.742	***	0.185	***	0.938	***	0.44	***	0.395	***		
		WD*NPs	0.057	**	0.125	***	0.489	***	0.026	***	0.044	***	0.134	***		
		Error	0.132		0.086		0.065		0.004		0.04		0.02			
	Bulk-TiO_2	BPs	0.408	***	0.533	***	0.395	***	0.074	***	0.795	***	0.486	***		
		WD	0.418	***	0.347	***	0.096	***	0.914	***	0.153	***	0.424	***		
		WD*BPs	0.08	**	0.073	***	0.434	***	0.008	***	0.04	***	0.063	***		
		Error	0.094		0.046		0.076		0.004		0.013		0.028			

The data presented in the figure clearly indicate that the physiological responses of *Lupinus albus* to drought stress were not significantly enhanced by treatments with either nanoparticle or bulk titanium dioxide, irrespective of the concentration applied. Specifically, there were no discernible differences in plant deterioration under severe drought condition (FC = 25%) among the various TiO_2 treatments. Most plants subjected to both TiO_2 application types + 25% moisture displayed similar responses to the control (only drought without TiO_2).

Furthermore, the application of low concentrations of titanium (50 and 150 ppm) – without drought stress – showed no significant improvements or detriments in key physiological indicators. These low-concentration treatments clustered within the same performance group as the control treatment (100%), suggesting a lack of efficacy in enhancing plant resilience under non-stress conditions.

Conversely, the application of high concentrations of titanium (300 and 600 ppm) resulted in notable deterioration of plant health. These plants exhibiting conditions comparable to those subjected to drought stress (50%), even when maintained under optimal moisture levels (100% and 75% field capacity). This finding underscores the

potential phytotoxicity of elevated titanium concentrations, warranting further investigation into the interactions between TiO_2 treatments and environmental stressors in *L. albus*.

The heatmap employs a color gradient to depict correlation coefficients, with blue indicating positive correlations and red indicating negative correlations. The size of the circles represents the strength of these correlations. The heatmaps (correlation analysis) in panels C and F (Figure 1) reveal critical relationships between physiological traits. The heatmap reveals distinct correlation patterns among various physiological parameters measured in *L. albus*. Variables such as GPX and APX show strong positive correlations with proline accumulation and oxidative stress indicators MDA, H_2O_2 and membrane leakage) suggesting a robust antioxidant response under stress. The observed significant positive correlations – under NP*drought treatments – revealed that shoot and root proline were the crux of the matter; they directly coupled with hydrogen peroxide and other membrane injury indicators. Also, this vital osmolyte (proline) was indirectly coupled with GPX and CAT (especially in shoots). Meanwhile, under bulk-TiO_2*drought treatments, shoot and root proline and GPX were also significantly coupled with membrane injury indicators, but surprisingly, all of them were negatively correlated with CAT and APX.

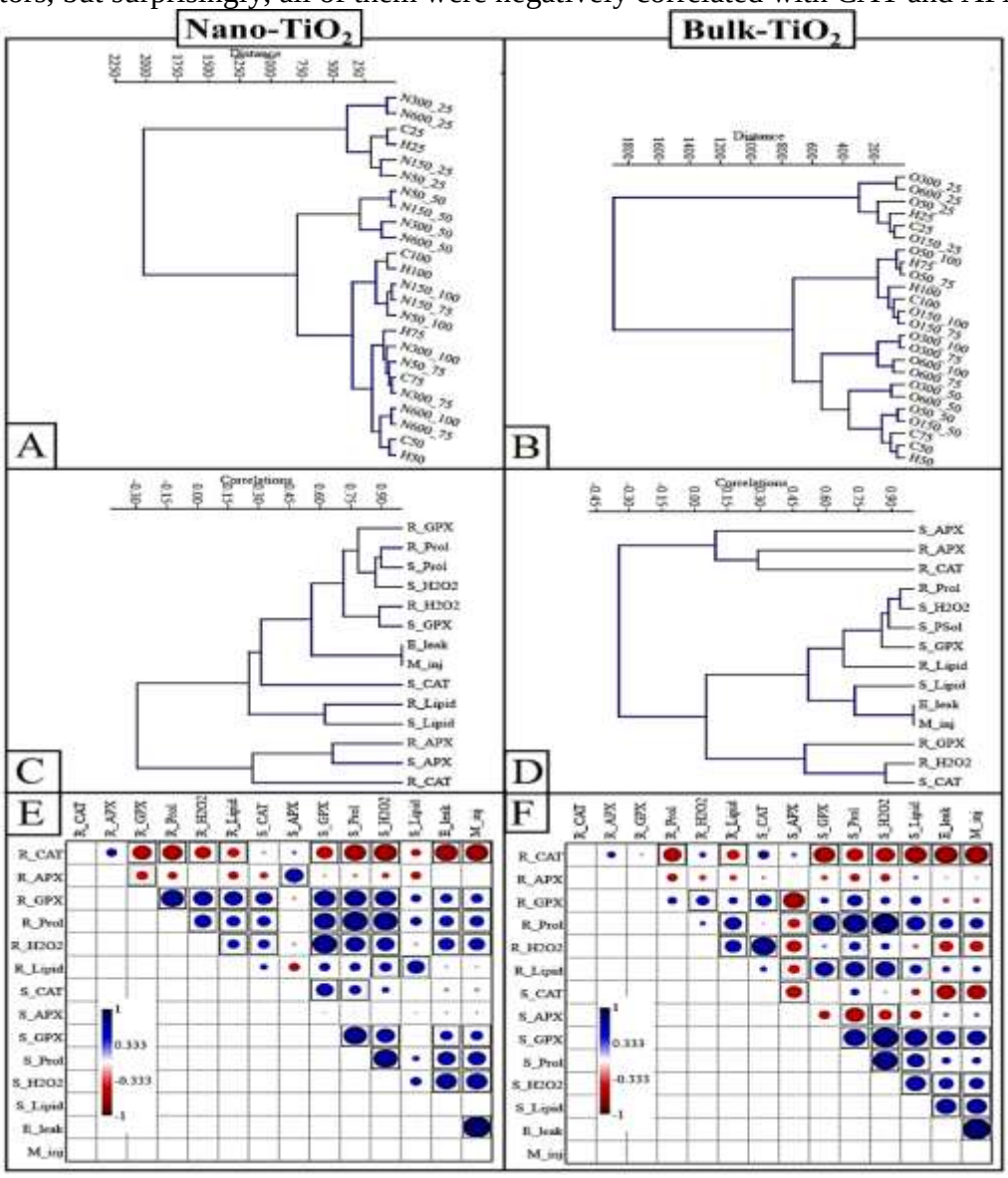

Figure 1: Dendrogram hierarchical clustering (**A** and **B**) and Heat-map (**C-F**) representing *L. albus* responses under the influence of both nano-TiO_2 (**A**, **C** and **E**) and bulk- TiO_2 (**B**, **D** and **F**) concentrations and drought stress levels. Heatmap color gradient indicates positive (blue) and negative (red) correlations, with circle sizes representing the strength of the correlation.

DISCUSSION

Earlier researchers have shown that nano-TiO_2 can act as a growth promoter in some plants, but different species exhibit varying responses [34]. Why such variability occurs and how it will impact the broader agricultural ecosystem still needs to be investigated. This study investigated the effects of foliar application of nano-TiO_2 and bulk TiO_2 on lupin plants under different water deficit conditions. The findings showed complex relationships between TiO_2 type, concentration, and water availability that affect several important physiological parameters associated with antioxidant defense and oxidative stress. Under abiotic stresses, pronounced ROS formation can cause cellular macromolecules such proteins, carbohydrates, membrane lipids, and nucleic acids to oxidize [35]. In this study, H_2O_2 and lipid peroxidation, along with the observed cell membrane damage, were the indicators of oxidative stress in *Lupinus albus*. When 10–30 mg L^{-1}nano-TiO_2 was present in onions, the activity of CAT and GPOX was increased; however, as concentrations increased, their activities reduced [36]. Hu et al. [37] discovered that while the lower dose ($\leq$50 mg L^{-1}) had no impact, nano-TiO_2 between 100 and 400 mg L^{-1} dramatically increased the antioxidant enzyme activity in both shoots and roots of coriander plants. Wheat plants had the same response, with low concentrations of nano-TiO_2 (<400 mg/kg) causing antioxidant enzyme activation and negative effects at higher concentrations (600 mg kg^{-1} soil) [38]. SOD activity increased in onion plants in a concentration-dependent manner, while CAT and POD activities increased when onion is treated with 10–30 mg L^{-1} , but decreased when it is treated with 40 and 50 mg L^{-1} nano-TiO^2 [36]. Wheat plants treated with 0-600 mg kg^{-1} soil of nano-TiO_2 exhibit a decreased level of electrolyte leakage, H_2O_2, and MDA in comparison to control plants, while under 600 mg kg^{-1} these parameters increased [38].

This study indicates that nano-TiO_2 generally led to higher H_2O_2 levels in both leaves and roots of lupin compared to bulk-TiO_2, particularly in roots. This leads to a suggestion that nano-TiO_2 may induce a greater oxidative burst. Although it may be harmful, elevated H_2O_2 can also function as a signaling molecule in stress reactions. In lupin roots, H_2O_2 levels decreased to less than 20 µmole g^{-1} FW, whereas in leaves, they ranged from 12 to 40 µmole g^{-1} FW. Hydrogen peroxide is produced in the active tissues of leaves, primarily in peroxisomes but also in other plant cell organelles like mitochondria and chloroplasts. In all cell sections, this ROS will typically be balanced between production and scavenging. According to Lei et al. [39], applying nano-TiO_2 improved photosynthetic rate, chlorophyll production, and antioxidant and rubisco enzyme activity, all of which led to an increase in the yield of crops. The observation that H_2O_2 increased with increasing water deficit, regardless of TiO_2 treatment, is consistent with the established link between drought stress and oxidative stress. Intracellular ROS signaling aspects have been highlighted, defining the intracellular production of H_2O_2 as "a necessary evil for cell signaling" [40]. A highly branched root system with several lateral roots and a shorter primary root was developed by plants subjected to varying amounts of H_2O_2 [41]. Expansion of the root system, delivered by H_2O_2 signaling, may be a required adaptive response toward water deficit stress. Also, MDA, which is a marker of lipid peroxidation, generally increased with both increasing water deficit and TiO_2 concentration in both leaves and roots. This confirms, on the other hand, that both drought and high levels of TiO_2

application, particularly in leaves, combined to induce oxidative damage to membranes. The non-significant increase of MDA in leaves treated with low concentrations of nano-TiO_2 reflects the mitigating effect of nano-TiO_2 in these specific tissue under stress conditions. However, the significant decrease of MDA in leaves by high concentrations of nano-TiO_2 may support the evidence that penetration of nano-TiO_2 increases with increasing the concentration of foliar spraying solution and hence improve the mitigation effect.

The use of nano-TiO_2 has been shown to have some benefits, including increased biomass and shoot and root growth [42–44]. Another strategy that plants take to cope with cell dehydration due to drought stress is accumulation of compatible organic solutes such as proline. Proline helps to stabilize subcellular structures in the cell cytosol in addition to serving as an osmolyte for osmotic adjustment [45]. Proline, a common osmoprotectant, accumulated in both leaves and roots of lupin in response to water deficit, as expected. The increased proline levels in TiO_2-treated plants, particularly with nano-TiO_2, led to a suggestion that TiO_2 application may further exacerbate the stress experienced by the plants, leading to enhance proline accumulation as a protective mechanism. There are some studies that have also highlighted the role of proline as a signaling molecule and a ROS scavenger [46,47]. The interplay between proline accumulation and antioxidant enzyme activity is complex and can vary depending on the plant species and stress conditions. While some studies have reported a synergistic effect between proline and antioxidant enzymes [48], others have shown that proline can also act independently of these enzymes in protecting against oxidative stress [49].

Proline content surged in lupin plants subjected to 25% available water, reaching approximately 1.5- to 4.2-fold of the levels observed in plants maintained at full FC. When comparing drought-stressed dragonhead plants to control plants receiving regular irrigation, Mohammadi et al. [50] discovered that the proline content of their leaves increased. In comparison to untreated plants under water-deficit stress, the dragonhead plants treated with 10 ppm nano-TiO_2 exhibited a considerably greater relative water content and significantly more leaf proline. This supports the idea that proline may be synthesized more readily by nano-TiO_2. Also, Shallan et al [51] reported the same result in drought-stressed cotton plants where nano-TiO_2 or nano-SiO_2 caused an increase of proline content. Further investigation into the underlying mechanisms of such responses will be essential for optimizing nano-TiO_2 applications in sustainable agricultural practices.

The activities of three key antioxidant enzymes: catalase (CAT), Guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) which examined in this study showed differential responses to nano- and bulk-TiO_2. Nano-TiO_2 generally increased CAT activity in leaves compared to bulk-TiO_2, and this will mitigate the oxidative stress in these tissues. However, this effect was not observed consistently across all concentrations or between leaves and roots. Bulk-TiO_2 generally increased GPX activity in leaves, while nano-TiO_2 increased it in roots. However, the two types of TiO_2 may differentially activate GPX in different tissues. The increase in GPX activity with increasing water deficit further highlights the importance of this enzyme in drought stress tolerance. APX activity in leaves was primarily increased by bulk-TiO_2 at the highest concentration. In roots, APX activity was also higher with bulk-TiO_2 at specific levels of FC and concentration. This suggests a more prominent role for APX in the bulk-TiO_2-mediated stress response, particularly in roots [8].

The interaction between TiO_2 treatments and water availability was significant for enzymatic activities. The strong interaction effect observed between water availability and nano-TiO_2 on APX activity may indicate to the combined effect of stressors in enhancing

the plant's antioxidant capacity, a mechanism that is crucial for maintaining cellular homeostasis under stress. In the roots, while water availability predominantly influenced proline and enzymatic activities, the modest effect sizes associated with TiO_2 treatments suggest that root responses might be more resilient or less susceptible to TiO_2 -induced changes compared to foliar responses. This differential response may highlight the complexity of root versus shoot interactions in stress adaptation [50,52].

This study demonstrates that both nano- and bulk-TiO_2 can differentially influence the oxidative status and antioxidant defense systems of lupin plants. Nano-TiO_2 appears to induce a greater oxidative burst, particularly in roots, while bulk-TiO_2 seems to elicit a stronger APX response. The changing trend in the activities of different antioxidant enzymes may be attributed to the foliar application method used in this study. Compared to CAT, APX has a greater affinity for H_2O_2 and requires a reductant (ascorbate) to scavenge it. Every cellular compartment that produces ROS contains APX [53]. This could help to explain why APX activity in lupin leaves and roots was affected differently by Nano- or Ord-TiO_2 than it was by CAT or GPX. The increase in APX activity in lupin roots by high concentrations of Nano-TiO_2 was accompanied by a decrease in H_2O_2 generation. This supports the concept that, while CAT may primarily act as a bulk scavenger for excess ROS formation under stress conditions, APX may act as a fine regulator of intracellular ROS steady-state levels, perhaps at signaling levels [53–55]. The activity of APX was reduced in lupin leaves by a combination of high concentrations of Nano-TiO_2 and high levels of water deficit. Certain plant antioxidative mechanisms may be harmed when high levels of two or more stressors are present. Antioxidant enzymes like SOD, CAT and APX are essential for protecting plant cells from oxidative stress, which is brought on by an imbalance of reactive oxygen species [56]. Superoxide radicals are specifically converted by SOD into hydrogen peroxide, which CAT subsequently breaks down into oxygen and water without using up any cellular energy. Because APX protects chloroplasts and other organelles from oxidative damage, these enzymes are essential for reducing the harmful effects of stressors. Antioxidant enzymes could therefore serve as early biomarkers to evaluate how nano-TiO_2 affects plant systems in the environment [57, 58].

The η^2 analysis elucidates the intricate dynamics between TiO_2 treatments and different levels of water availability in *Lupinus albus*, highlighting the significant effects which these factors have on physiological parameters such as H_2O_2, proline, and enzymatic activities. The η^2 values revealed that water availability exerted the most considerable effect on GPX activity in the leaves of lupin treated with either Nano- or bulk-TiO_2. Additionally, both TiO_2 types had a pronounced effect on CAT activity in the leaves. The interaction between varying water availability levels and Nano-TiO_2 exerted the greatest influence on APX activity in treated plants. In the roots of lupin treated with Nano-TiO_2, water availability primarily affected CAT and GPX activities. Each level of field capacity and bulk-TiO_2 also demonstrated significant effects on the enzymatic activities. APX activity in both leaves and roots was significantly impacted by the interaction between varying water availability levels and TiO_2 treatments, with bulk-TiO_2 exhibiting a comparatively large effect size. Overall, the two-way ANOVA confirmed that the interaction of the main factors, water deficit stress and TiO_2 type, significantly influenced nearly all enzymatic activities assessed in the leaves and roots of *Lupinus albus*.

The dendrogram and heatmap underscore the complex interactions between TiO_2 treatments and levels of water availability, providing valuable insights into strategies for improving the resilience of *Lupinus albus* challenged with water deficit. Further exploration of these relationships could lead to enhanced agricultural practices aimed at increasing crop

tolerance to environmental stressors. The positive correlations between antioxidant enzyme activities and proline accumulation underscore the plant's adaptive mechanisms for managing oxidative stress under drought conditions. Treatments with nano-TiO_2 appear to confer enhanced resilience, as evidenced by the synergistic effects suggested by the positive correlations among specific physiological parameters. Conversely, the application of bulk-TiO_2 exerted a shift from positive to negative correlations between proline and the antioxidant enzymes such as GPX and CAT. This shift indicates a potential alteration in the plant's antioxidant responses, raising important questions about the underlying mechanisms played by bulk-TiO_2 . The findings reveal that while proline accumulation plays a critical role in mitigating oxidative damage during drought stress, the type of TiO_2 treatment applied may significantly influence the plant's physiological pathways. Further investigations are warranted to elucidate these complex relationships and their implications for enhancing plant stress resilience.

CONCLUSION

The present study contributes to a deeper understanding of how TiO_2 , particularly in its nanoscale form, can influence the physiological responses of *L. albus* under water deficit. It is evident, from the multivariate analysis, that nano-TiO_2 is pivotal in modulating plant responses to environmental stressors. Additionally, it is crucial to investigate the long-term effects of nano-TiO_2 treatments on plant health and soil ecosystems, as well as the specific pathways involved in these interactions. Careful consideration is warranted regarding the use of nanoparticles in economically important plants, particularly concerning their integration into the food chain, necessitating comprehensive studies to ensure both efficacy and safety for agricultural sustainability.

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