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Different Physiological and Biochemical Responses of Bamboo to the Addition of TiO₂ NPs under Heavy Metal Toxicity

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Abstract: Bamboo forests cover a remarkable area of Chinese forestland. Recently, titanium dioxide nanoparticles (TiO₂ NPs) have been used for plant protection against abiotic stress. In this study, an in vitro tissue culture experiment was conducted to determine the impact of titanium on plant tolerance to two different heavy metals (Cu and Pb). Bamboo plants (*Arundinaria pygmaea* L.) were grown using five concentrations of TiO₂ NPS (0, 50, 80, 100, and 150 µM) without or with 100 µM Cu and 100 µM Pb for 30 days. The results showed that while Cu and Pb increased the generation of Reactive oxygen species (ROS) compounds in plants, TiO₂ NP treatments played a positive role in reducing oxidative stress, as indicated by the decrease in ROS compounds, the extent of lipoperoxidation, and soluble proteins. On the other hand, the use of TiO₂ NPs increased the total antioxidant capacity, chlorophyll content and general plant biomass. Moreover, the addition of TiO₂ NPs significantly reduced Cu, and Pb accumulation in roots, stems, and shoots. We concluded that TiO₂ NPs have the ability to reduce oxidative stress in plants by increasing the antioxidant capacity, improving the level of injury, and protecting cell membranes via reducing lipoperoxidation (reduction of Malondialdehyde (MDA) content). However, the results indicated that the efficiency of TiO₂ NPs was related to the type and concentration of heavy metal, as TiO₂ NPs were more effective for Cu than Pb. Additionally, a high concentration of TiO₂ NPs resulted in the greatest enhancement in plant growth and development under heavy metal stress.

Keywords: titanium dioxide nanoparticles; bamboo species; copper (Cu); lead (Pb); antioxidant activity; oxidative stress

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1. Introduction

Soil contamination with heavy metals caused by anthropogenic activities and natural processes is a major threat to human health as these metals can easily enter the human food chain [1]. Among heavy metals, Cu and Pb are considered the most polluting and most toxic elements, respectively, in the farmland soils of China [2]. Excessive concentrations of Cu, in the Cu²⁺ form, and lead excess in plants can trigger the production of reactive oxygen species (ROS) and the generation of oxidative stress in cell walls [3] resulting in a disturbance in the balance of crucial ions in plant cells [4]. Copper excess can lead to the disruption of enzymatic activities and cell membrane permeability, eventually reducing respiration and photosynthetic efficiency [5].

The destructive impact of Pb reduces photosynthetic capacity [6] and inhibits shoot and root growth in plants. [7].

TiO₂ NPs have the potential to ameliorate the deleterious effects of various stressful factors on the biochemical and physiological traits of plants [8]. In recent years, many

researchers have reported that TiO₂ NP applications can significantly improve photosynthetic properties, chlorophyll (Chl) synthesis, and dry weight in plants. This is attributed to the role of TiO₂ NPs in increasing antioxidant capacity and protecting plant cell membranes [9–11]. For example, TiO₂ NPs have been reported to increase the protein content, the total nitrogen content, oxygen and biomass levels [12], the transfer of electrons and light energy by chlorophyll [13], and seedling growth [14]. Additionally, it has been reported that the application of TiO₂ NPs can play an important role in decreasing cadmium toxicity in soybean [15] and green algae (*Chlamydomonas reinhardtii* P.A. Dang) [16]. In contrast, some evidence shows that TiO₂ NPs have a negative impact on plant metabolism [17,18]. However, the efficiency of nanoparticles in increasing plant growth, especially under stress, depends on the concentration, type, and size of the nanomaterial, as well as the plant species [19], which were reported in our previous studies where different types of nanoparticles (SiO₂NPs and TiO₂ NPs) were used to decrease the concentration levels of heavy metals in bamboo species [20,21]. However, there are not enough studies exploring the possibility of TiO₂ NPs to ameliorate heavy metal toxicity and to reduce oxidative stress. Given the hypothesis that titanium nanoparticles stimulate plant growth under stress, we investigated the possible physiological and biochemical responses of bamboo species to heavy metals and made attempts to identify the involved mechanisms.

Bamboo plants with rapid growth and high biomass production are known as one of the important forest resources [22]. Bamboo plants are classified into 500 species and 48 genera and cover a remarkable area of Chinese forestland [23,24]. Also, bamboo is estimated to cover 31.5 million ha of the world's forestland [25]. This tropical plant is a major source of nourishment and income for a large population of local people in the western and southern areas of China [26]. On the other hand, soil contamination has become a major dilemma in this part of China's farmland [2]. It has been reported that 16.1% of Chinese soils are tainted, of which, approximately 82% are contaminated by the accumulation of heavy metals such as lead, arsenic and cadmium [27]. Therefore, investigating ways to reduce contamination exposure for bamboo plants can have a direct impact on the health and economy of the human population in this region. This study aimed to address the environmental concerns related to heavy metals by investigating various responses of bamboo growth to the addition of TiO₂ NPs under two different types of heavy metal stress (Cu and Pb) and to understand how bamboo species can respond to TiO₂ NPs.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Bamboo samples were collected from the bamboo garden of Nanjing Forestry University where bamboo plants have been growing since 1982. Plant material was sampled from single clone 1-year-old branches of *Arundinaria pygmaea* obtaining ten-millimeter-long nodal explants under tissue culture conditions. For proliferation, axillary shoot production, and shoot expansion, tissue culture was conducted using MS medium [28] (Murashige and Skoog, 1962), which was supplemented with kinetin (KT) (0.5 mM), 6-benzyl amino purine (6-BA) (4 mM), sucrose (30 g/L), and agar (7–10 g/L). Young shoots were induced to proliferate roots. The young shoots were then transferred to glass Petri dishes with a diameter of 60 mm containing MS medium, which consisted of 4 µM nicotinic acid, 1.2 µM thiamine-HCl, pyridoxine (3 µM), myoinositol (0.6 mM), different concentrations of TiO₂ NPs and heavy metals, along with sucrose (30 g/L), and agar (7–10 g/L). An amount of 0.1 mM IAA was used as a hormone growth factor.

Treatments included 5 concentrations of TiO₂ NPs (0, 50, 80, 100 and 150 µM) with or without two types of heavy metals (100 µM Cu and 100 µM Pb) in 5 replicates. Bamboo planting was conducted in an inoculation hood (Air Tech, Huntington Beach, CA, USA) under ultraviolet light and fluorescent white lamps, which provided light at wavelengths of 350–750 nm. The temperature was maintained between 15 °C and 30 °C. Finally, the plants were moved to a special chamber for plant tissue culture and grown under controlled

conditions with a photoperiod of 16 h and temperatures of 30/25 °C and 17/22 °C during the light and dark periods, respectively, for 30 days.

TiO₂ NPs were purchased from Nanjing Jiancheng Company (Nanjing, China), which is located in Jiangsu Province, China. The TiO₂ NPs were a powder with 99.8% pure nano titanium with a diameter of 25 nm. The range of heavy metal levels and titanium concentrations was chosen based on the high and low tolerance range of our species according to the previous studies.

2.2. Biomass and Shoot Length Determination

Plant shoots and roots were thoroughly washed with deionized water. The leaves were dried in an oven (vacuum drying oven (DZF-6090)) under 110 °C for 20 min. The samples were dried to a constant dry weight at 80 °C, and used for the calculation of biomass. The calculations were performed with five replicates for each treatment. The shoot length was measured at the beginning and end of the study.

2.3. Antioxidant Activities

Samples of bamboo leaves from each treatment (0.5 g) were ground in a mortar in liquid nitrogen. Then, the samples were maintained at 2–8 °C, and the obtained powder was placed in a test tube containing 2 mg of phosphate buffer (pH 7.8). The samples were centrifuged at 2000–3000 × *g* for 20 min and then the supernatant was separated and used to measure antioxidant activities. SOD activity was assessed according to the photoreduction of nitro blue tetrazolium (NBT) using the method of Zhang (1992) [29]. Then, 0.1 mL of the sample was added to 0.2 mL of NBT, 0.2 mL of MET, 0.2 mL of EDTA, 0.2 mL of Rib, and 3.1 mL of buffer at pH 7.0 in a test tube. The color of the solution changed with exposure to light for 10–20 min. The enzyme activity of superoxide dismutase (SOD, EC 1.15.1.1) was quantified by OD measurement using a spectrophotometer (Beijing Purkinje TU-1810 UV-vis spectrometer).

The activity of peroxidase (POD, E.C. 1.11.1.7) was assessed based on the protocol of Zhang [29]. Therefore, 0.2 mL of H₂O₂, 4 mL of 2-methoxy phenol, and 0.8 mL of buffer solution (pH7) were added to 20 mL of the sample in a test tube. The supernatant was read at 470 nm.

Catalase activity (CAT, EC 1.11.1.6) was assessed by Aebi's method [30] according to two H₂O₂ reactions analyzed at 240 nm. In this method, 0.1 mL of the sample was added to 1 mL of Tris-HCl, 1.6 mL of water, and 0.2 mL of H₂O₂ in a test tube. To determine the CAT content, the resulting soluble sample was assessed several times (two or three times) at 230 nm (Beijing Purkinje TU-1810 UV-vis spectrometer).

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was quantified according to the method of Nakano and Asada [31]. For this purpose, 600 µL of 0.1 mM EDTA, 100 µL of an enzymatic extract, 400 µL of 0.5 mM ascorbic acid, and the optimum amount of Na-phosphate (pH 7.0) (0.05 M) were added to the sample (20 mL) in one test tube. Next, 400 µL of 3% H₂O₂ was added to the reaction and the reduction of the resulting supernatant was measured after 60 s at an absorbance of 290 nm. The activity of APX was recorded through an extinction coefficient ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$).

For the determination of glutathione reductase (GR) activity, one commercial chemical kit provided by the Nanjing Jiancheng Technology Co. was used. The chemical assay kit contained 0.1 mM EDTA, 2% PVP and 0.5% (*w/v*) Triton-100, which were added to our bamboo samples. In the next step, the resulting supernatant was centrifuged at an optimum speed of 10,000 × *g* at 4 °C for 10 min. To determine the activity of GR, the resulting supernatant was tested according to the manufacturer's instructions.

The activity of phenylalanine ammonia-lyase (PAL) was determined according to the method of [32]. Based on this method, 0.5 g of leaf samples were homogenized with a mortar and pestle. The samples were transferred to an ice bath consisting of 1 mM EDTA, 5.0 mM thioalcohol, and 5 mL of 50 mM borate buffer (pH = 8.8). The homogenate was centrifuged at 4 °C for 10 min 13,000 × *g*. Then, the reagents, 1.0 mL of 20 mM L-

phenylalanine and 2 mL of 50 mM borate buffer (pH 8.8), were added to 0.2 mL of the crude homogenate. For fixation, the reaction mixture was exposed to 0.25 mL of 5 M HCl and incubated for 30 min at 40 °C. In the final step, the activity of PAL was recorded by a spectrometer based on the enhancement in absorbance at 290 nm.

Hydrogen peroxide (H₂O₂), soluble protein (SP), and malondialdehyde (MDA) contents

The levels of hydrogen peroxide (H₂O₂) were assessed by an assay kit purchased from one local company in Nanjing city (Nanjing Jiancheng). To measure the H₂O₂ content, leaf samples were stored in liquid nitrogen (LN₂) at low temperatures (−80 °C or −20 °C) for 7 days. During this time, the samples lost as much as 60% of their H₂O₂. For analysis of the samples, in the next step, the samples were separated from liquid nitrogen and weighed before thawing. Then, the samples were ground by exposure to liquid nitrogen in one pre-chilled mortar and pestle. In the last step, a modified ferrous ammonium sulfate/xenol orange (FOX) method was applied to record the H₂O₂ content in the extract.

The content of soluble proteins was assessed based on the Bradford method [33], which measures the alteration in levels of proteins with Coomassie Brilliant Blue (G25). For this purpose, 0.1 mL of Coomassie Brilliant Blue G25 was mixed with 50 mL of 90% ethanol, 100 mL of H₃PO₄, and 1000 mL of water in a tube. Finally, the content of soluble protein was recorded using a spectrometer.

MDA levels were determined according to the method of Kai and Feng [34], which is based on the reaction of thiobarbituric acid (TBA). Based on this protocol, 0.5 g of our sample was homogenized with trichloroacetic acid (TCA) (10%) and then centrifuged, 4000 × g for 10 min. The obtained supernatant (2 mL) was homogenized with 2 mL of TBA (0.6%) and kept in an oven for 20 min at 100 °C. Then, the cooling process was conducted instantaneously in an ice bath. The final solution was centrifuged, 4000 × g for 15 min, and a spectrometer was used to record the absorbance at 600, 532, and 450 nm.

2.4. Determination of Chlorophyll and Carotenoid Contents

Chlorophyll, including chlorophyll *a* and chlorophyll *b*, and carotenoid contents were estimated by using the method of [35]. An amount of 0.5 g of a bamboo leaf sample was crushed in a mortar with liquid nitrogen and pulverized. The obtained powder was used to prepare the liquid sample extract, which was added to 20 mL of 80% acetone at 0 to 4 °C. Extract samples were centrifuged at 6000 × g for 10 min. Finally, chlorophyll *a*, chlorophyll *b*, and carotenoid contents were measured at 663, 645, and 470 nm. The final data were obtained by the following formulas and represented in units of mg/g fresh weight:

$$\text{Chlorophyll } a = 12.25A_{663} - 2.79A_{647}.$$

$$\text{Chlorophyll } b = 21.50A_{647} - 5.10A_{663}.$$

$$\text{Carotenoid} = 1000A_{470} - 1.82\text{Chl } a - 95.15\text{Chl } b/225.$$

2.5. Determination of TiO₂ NP and Heavy Metal Accumulation in Bamboo Roots, Stems, and Leaves

Root, stem, and leaf samples were carefully washed with water and then dried. Then, nitric acid (70%) was added for 20 min at 70 °C. The preparation of samples (0.5 g) was completed by centrifugation at 12,000 × g for 5 min to obtain the supernatants. The Ti and heavy metal (Cu and Pb) contents were recorded by atomic absorption spectrometry (AAnalyst 800, Perkin Elmer, Waltham, MA, USA). To obtain the element contents, the parameters of the instruments were optimized. Determination of metal standards was conducted based on 2.5% nitric acid (Spectrascan). The calibration of the verification standard (Perkin Elmer), a standard including all the elements in the inorganic target analyst list (TAL), was run at optimum intervals in an unattended automatic analysis run.

2.6. Statistical Analysis

The experimental data were analyzed with a 2-way factorial design with five replicates under completely randomized design conditions. The R statistical software package was

used for the analysis of variance (ANOVA). For comparison of the mean differences between treatments, Tukey's test was used at the $p < 0.05$ probability level.

3. Results

3.1. Impact of the Combination of Different Concentrations of TiO_2 NPs with 100 μM Cu and 100 μM Pb on Antioxidant Activities in Bamboo

The results on antioxidant activities (SOD, POD, CAT, APX, GR and PAL) showed significant differences between the various levels of TiO_2 NPs and 100 μM heavy metals (Cu and Pb) ($p < 0.001$). The 150 μM TiO_2 NPs $\times$ 100 μM Cu and Pb treatment had the greatest impact on the stimulation of antioxidant capacity, with 2.0-, 1.8-, 0.99-, 3.1-, 1.2-, and 0.79-fold enhancements in SOD, POD, CAT, APX, GR, and PAL capacities, respectively, in comparison with those of the control treatment (Table 1). This result revealed the positive impact of TiO_2 NPs on the antioxidant capacity in bamboo species under 100 μM Cu and Pb treatment. On the other hand, 100 μM Cu and 100 μM Pb alone did not increase the antioxidant activity, with 63% and 70% reductions in SOD activity, 45% and 65% reductions in POD activity, 44% and 51% reductions in CAT activity, 59% and 80% reductions in APX activity, 42% and 49% reductions in GR activity, and 37% and 42% reductions in PAL activity, respectively, compared with the those of the control treatment. According to Figure 1, antioxidant activities increased with increasing TiO_2 NP concentration in combination with 100 μM Cu and 100 μM Pb, the greatest increase in antioxidant activities by TiO_2 NPs was related to APX, SOD, POD, GR, CAT, and PAL, with 118%, 86%, 84%, 53%, 46%, and 36% enhancements, respectively, in comparison with those of the control treatment.

Table 1. The change in antioxidant enzymatic activities and H_2O_2 , MDA, and soluble protein contents at the various levels of TiO_2 NPs and heavy metals (Cu and Pb) compared to those of the control treatment. The arrows show the percentage reduction of indexes.

Concentration of TiO_2 NPs—100 μM (Cu and Pb)	SOD	POD	CAT	APX	GR	PAL	H_2O_2	MDA	SP
50 μM TiO_2 NPs	14.9%	22.1%	13.8%	18.5%	26.5%	12.5%	10.3% ↓	16.7% ↓	12.7% ↓
50 $\times$ 100 μM (Cu)	53.1%	38.4%	25.4%	46.1%	47.7%	17.5%	17.3% ↓	26.6% ↓	14.2% ↓
50 $\times$ 100 μM (Pb)	51.2%	67.6%	27.9%	125%	23.1%	10.8%	15.1% ↓	35.0% ↓	11.3% ↓
80 μM TiO_2 NPs	24.1%	36.9%	17.6%	25.9%	39.1%	28.1%	15.5% ↓	45.4% ↓	21.8% ↓
80 $\times$ 100 μM (Cu)	80.4%	47.8%	42%	64.1%	56.8%	25.7%	21.2% ↓	40.4% ↓	19.7% ↓
80 $\times$ 100 μM (Pb)	79.51%	95.2%	34.4%	153%	89.4%	18.9%	18.9% ↓	25.5% ↓	15.4% ↓
100 μM TiO_2 NPs	36.1%	53.2%	26.2%	40.7%	52.6%	34.3%	22.4% ↓	39.4% ↓	34.5% ↓
100 $\times$ 100 μM (Cu)	140%	100%	76.4%	129%	54.5%	55.5%	34.3% ↓	54.4% ↓	35.1% ↓
100 $\times$ 100 μM (Pb)	140%	168%	71.7%	281%	102%	48.6%	31.7% ↓	57.7% ↓	28.8% ↓
150 μM TiO_2 NPs	48.9%	65.6%	28.6%	51.2%	61.1%	40.6%	33.9% ↓	48.3% ↓	43.6% ↓
150 $\times$ 100 μM (Cu)	200%	137%	99.7%	169%	67.1%	79.2%	40.6% ↓	60.4% ↓	43.9% ↓
150 $\times$ 100 μM (Pb)	174%	187%	90.7%	315%	128%	56.7%	34.6% ↓	60.4% ↓	32.9% ↓

3.2. Impact of the Combination of Different Concentrations of TiO_2 NPs with 100 μM Cu and 100 μM Pb on Hydrogen Peroxide, Methylenedioxymphetamine, and Soluble Protein Contents in Bamboo

The results revealed a certain significant difference between the different contents of H_2O_2 , MDA, and soluble protein ($p < 0.001$). Accordingly, 100 μM heavy metals (Cu and Pb) increased ROS contents, cell lipoperoxidation extent, and soluble protein contents, while different concentrations of TiO_2 NPs alone and in combination with heavy metals decreased the deleterious effects of elevated H_2O_2 , MDA, and SP contents in cells, which could inhibit cell injury under metal stress (Figure 2). The greatest increases in H_2O_2 , MDA, and SP contents were related to treatments with 100 μM Cu and 100 μM Pb, with 58% and 73% increases in the H_2O_2 content, 132% and 210% increases in the MDA content, and 65% and 76% increases in the soluble protein content, respectively, in comparison with those of the control. On the other hand, the cell injury extent decreased with the increase in

TiO₂ NPs, such that the concentration of 150 µM TiO₂ NPs in combination with 100 µM Cu and 100 µM Pb reduced the H₂O₂, MDA and SP contents by 40.6%, 60.4%, and 43.9%, respectively, in comparison with those of the control treatment. However, this impact on 100 µM Cu was more effective than that on 100 µM Pb (Table 1).

3.3. Impact of the Combination of Different Concentrations of TiO₂ NPs with 100 µM Cu and 100 µM Pb on the Contents of Chlorophyll-a, Chlorophyll-b, Total Chlorophyll and Total Carotenoids

Chlorophyll-a, chlorophyll-b, total chlorophyll, and carotenoid contents were significantly different between the various concentrations of TiO₂ NPs and 100 µM Cu and 100 µM Pb ($p < 0.001$). TiO₂ NP concentrations showed an increasing impact on chlorophyll and carotenoid contents with the exposure to 100 µM Cu and 100 µM Pb. The results: the greatest enhancement in chlorophyll and carotenoid contents was attributed to 150 µM TiO₂ NPs in combination with 100 µM Cu and 100 µM Pb, with 52.5%, 67.5%, 87.9%, and 77.5% increases in chlorophyll-a, chlorophyll-b, and total chlorophyll and carotenoid contents, respectively, in comparison with those of the control treatment. On the other hand, the results indicated that the 100 µM Cu and 100 µM Pb treatments reduced pigment concentrations with 39.1% and 42.2% reductions in chlorophyll-a content, 81.2% and 93% reductions in chlorophyll-b content, 54.4% and 59% reductions in total chlorophyll content, and 61.7% and 67.8% reductions in carotenoid content, respectively, in comparison with those of the control treatment (Table 2).

Figure 1. Cont.

Figure 1. Impacts of TiO₂NP levels on the antioxidant enzyme activities (SOD (a), POD (b), CAT (c), APX (d), GR (e), and PAL (f)) of *A. pygmaea* L. with 100 μM Cu and 100 μM Pb. The treatments contained various concentrations of TiO₂ NPs alone or in combination with 100 μM Cu and 100 μM Pb. The capital letters show statistically significant differences across various levels of TiO₂ NPs alone or in combination with 100 μM Cu and 100 μM Pb (the bars with the same colors), while the lowercase letters show statistically significant differences within each concentration of TiO₂ NPs alone or in combination with 100 μM Cu and 100 μM Pb (the bars with different colors) according to Tukey's test ($p < 0.05$).

3.4. Determination of TiO₂ NP Accumulation and Heavy Metal Contents in Roots, Stems, and Leaves

The amount of TiO₂ NP accumulation and heavy metal contents (Cu, Pb) were determined in the roots, stems, and shoots of bamboo species (Table 3). The highest accumulation of TiO₂ NPs was found with 150 μM TiO₂ NPs alone, 150 μM TiO₂ NPs in combination with 100 μM Cu and 150 μM TiO₂ NPs in combination with Pb, with 21.5 μg/L, 11.4 μg/L, and 10.8 μg/L in leaves, 22.7 μg/L, 13.8 μg/L, and 11.12 μg/L in stems, and 30.5 μg/L, 17.6 μg/L, and 12.8 μg/L in roots, respectively. Additionally, Ti accumulation in roots was greater than that in shoots and stems. On the other hand, the results showed that, with increasing concentrations of TiO₂ NPs, the amount of heavy metal accumulation decreased, the lowest amount of heavy metals was found for the combination of 150 μM TiO₂ NPs with 100 μM Cu and 100 μM Pb, resulting in 47% and 42% reductions in shoots, 41% and 38% reductions in stems, and 34% and 33% reductions in roots, respectively, in comparison with those of the control treatment. The highest accumulation of heavy metals was attributed to the 100 μM Cu and 100 μM Pb treatments (14.8 μg/L, 18.5 μg/L in shoots, 17.02 μg/L, and 20.8 μg/L in stems and 20.5 μg/L and 24.2 μg/L in roots).

3.5. Impact of the Combination of Various Levels of TiO₂ NPs in Combination with 100 μM Cu and 100 μM Pb on the Root and Shoot Dry Weight and Shoot Length

Biomass indexes for root and shoot dry weight, as well as shoot length, showed an obvious significant difference between different levels of TiO₂ NPs and heavy metal concentrations ($p < 0.001$). According to the data, the highest increase in plant biomass was found for the 150 μM TiO₂ NP treatment in combination with 100 μM Cu and 100 μM Pb, with 95.1%, 58.5%, and 97.2% increases in shoot and root dry weight and shoot length, respectively, in comparison to those of the control treatment, which was similar to the results obtained for antioxidant activities and chlorophyll contents (Table 4). On the other hand, it was clear that 100 μM Cu and 100 μM Pb reduced the biomass indexes, with 0.35 g and 0.30 g reductions in shoot dry weight, 0.41 g and 0.45 g reductions in root dry weight, and 5.7 cm and 7.18 cm reductions in shoot length, respectively, in comparison with those of the control treatment (Figure 3).

Figure 2. The impacts of TiO₂ NP levels on H₂O₂ (a), MDA (b), and SP (c) contents in *A. pygmaea* with 100 μM Cu and 100 μM Pb. The treatments contained various levels of TiO₂ NPs alone or in combination with 100 μM Cu and 100 μM Pb. The capital letters show statistically significant differences across various levels of TiO₂ NPs alone or in combination with 100 μM Cu and 100 μM Pb (the bars with the same colors), while the lowercase letters show statistically significant differences within each level of TiO₂ NPs alone and in combination with 100 μM Cu and 100 μM Pb (the bars with different colors) according to Tukey's test ($p < 0.05$).

Table 2. The impact of the combination of TiO₂ NPs with 100 μM Cu and 100 μM Pb on the contents of chlorophyll-*a*, chlorophyll-*b*, total chlorophyll, and carotenoids. Each data point is the mean $\pm$ SE of five replicates. The treatments included four concentrations of TiO₂ NPs (50, 80, 100, 150 μM) alone and in combination with 100 μM Cu and 100 μM Pb. The capital letters show statistically significant differences across different concentrations of TiO₂ NPs alone or in combination with 100 μM Cu and 100 μM Pb, while the lowercase letters show statistically significant differences within each concentration of TiO₂ NPs alone and in combination with 100 μM Cu and 100 μM Pb, according to Tukey's test ($p < 0.05$). They are subscribed on top of the numbers.

TiO ₂ NP Levels	Heavy Metals	Chl- <i>a</i>	Chl- <i>b</i>	T. Chl	Carotenoids
μM	μM	(μg g ⁻¹ F.w.)	(μg g ⁻¹ F.w.)	(μg g ⁻¹ F.w.)	(μg g ⁻¹ F.w.)
0	-	8.89 $\pm$ 1.13 ^{Ab}	4.30 $\pm$ 0.85 ^{Ba}	13.10 $\pm$ 1.65 ^{Ca}	0.38 $\pm$ 0.081 ^{Ba}
0	100 μM Cu	5.38 $\pm$ 0.66 ^{Ab}	0.82 $\pm$ 0.08 ^{Bb}	6.15 $\pm$ 0.67 ^{Cb}	0.15 $\pm$ 0.088 ^{Cb}
0	100 μM Pb	5.13 $\pm$ 0.84 ^{Ab}	0.31 $\pm$ 0.08 ^{Cb}	5.46 $\pm$ 0.86 ^{Cb}	0.127 $\pm$ 0.066 ^{Bb}
50 μM	-	9.10 $\pm$ 0.52 ^{Aa}	5.63 $\pm$ 0.94 ^{Ba}	14.51 $\pm$ 1.40 ^{BCa}	0.46 $\pm$ 0.090 ^{Ba}
50 μM	100 μM Cu	7.46 $\pm$ 0.94 ^{Ab}	1.81 $\pm$ 0.83 ^{Bb}	9.26 $\pm$ 0.79 ^{Bb}	0.22 $\pm$ 0.079 ^{BCb}

Table 2. Cont.

TiO ₂ NP Levels	Heavy Metals	Chl- <i>a</i>	Chl- <i>b</i>	T. Chl	Carotenoids
50 µM	100 µM Pb	6.00 ± 0.81 ^{Ab}	1.19 ± 0.72 ^{BCb}	7.19 ± 1.36 ^{Cb}	0.185 ± 0.070 ^{ABb}
80 µM	-	9.26 ± 0.47 ^{Aa}	8.30 ± 0.69 ^{Ba}	17.28 ± 1.50 ^{ABa}	0.59 ± 0.068 ^{Aa}
80 µM	100 µM Cu	6.48 ± 0.90 ^{Ab}	1.98 ± 0.83 ^{Bb}	8.46 ± 1.50 ^{BCb}	0.25 ± 0.087 ^{ABCb}
80 µM	100 µM Pb	6.43 ± 0.39 ^{ABb}	1.32 ± 0.88 ^{BCb}	7.51 ± 1.33 ^{BCb}	0.19 ± 0.071 ^{ABb}
100 µM	-	9.79 ± 0.79 ^{Aa}	8.93 ± 1.39 ^{Aa}	18.56 ± 2.20 ^{Aa}	0.63 ± 0.094 ^{Aa}
100 µM	100 µM Cu	8.05 ± 0.48 ^{ABb}	4.20 ± 0.86 ^{Ab}	11.95 ± 1.28 ^{Ab}	0.36 ± 0.095 ^{ABb}
100 µM	100 µM Pb	7.67 ± 0.53 ^{Bb}	2.80 ± 0.81 ^{ABb}	10.27 ± 1.56 ^{ABb}	0.29 ± 0.087 ^{ABb}
150 µM	-	10.27 ± 0.81 ^{Aa}	8.93 ± 1.13 ^{Aa}	18.98 ± 2.12 ^{Aa}	0.64 ± 0.082 ^{Aa}
150 µM	100 µM Cu	7.85 ± 0.49 ^{Bb}	4.91 ± 0.85 ^{Ab}	13.02 ± 1.29 ^{Ab}	0.42 ± 0.0818 ^{Ab}
150 µM	100 µM Pb	7.57 ± 0.87 ^{Bb}	3.67 ± 0.90 ^{Ab}	11.22 ± 1.39 ^{Ab}	0.33 ± 0.090 ^{Ab}

Table 3. Levels of accumulation of various concentrations of TiO₂ NPs and heavy metal (Cu, Pb) contents in bamboo leaves, stems, and roots. The capital letters show statistically significant differences across different concentrations of TiO₂ NPs alone or in combination with 100 µM Cu and 100 µM Pb, while the lowercase letters show statistically significant differences within each concentration of TiO₂ NPs alone and in combination with 100 µM Cu and 100 µM Pb, according to Tukey's test ($p < 0.05$). They are subscribed on top of the numbers.

Leaves			
Heavy Metal Concentration	TiO ₂ NP Concentration	Heavy Metal Concentration	Ti
µmol/L	µmol/L	µg/L	µg/L
0	0	0	0
100 µmol/L Cu	0	15.10 ± 0.62 ^{Ab}	0
100 µmol/L Pb	0	18.76 ± 0.87 ^{Aa}	0
0	50	0	13.70 ± 0.71 ^{Ca}
100 µmol/L Cu	50	12.36 ± 0.28 ^{Bb}	7.11 ± 0.43 ^{Cb}
100 µmol/L Pb	50	15.59 ± 0.84 ^{Ba}	6.05 ± 0.47 ^{Cb}
0	80	0	14.80 ± 0.46 ^{Ca}
100 µmol/L Cu	80	10.23 ± 0.29 ^{Cb}	9.07 ± 0.51 ^{Bb}
100 µmol/L Pb	80	13.44 ± 0.86 ^{Ca}	6.85 ± 0.46 ^{Cc}
0	100	0	18.59 ± 0.41 ^{Ba}
100 µmol/L Cu	100	8.62 ± 0.29 ^{Db}	10.95 ± 0.32 ^{Ab}
100 µmol/L Pb	100	11.44 ± 0.40 ^{Da}	9.57 ± 0.35 ^{Bc}
0	150	0	21.86 ± 1.32 ^{Aa}
100 µmol/L Cu	150	7.88 ± 0.52 ^{Db}	11.13 ± 0.42 ^{Ab}
100 µmol/L Pb	150	10.83 ± 0.35 ^{Da}	11.05 ± 0.55 ^{Ab}
Stem			
µmol/L	µmol/L	µg/L	µg/L
0	0	0	0
100 µmol/L Cu	0	17.14 ± 0.88 ^{Ab}	0
100 µmol/L Pb	0	21.14 ± 0.60 ^{Aa}	0
0	50	0	16.55 ± 0.44 ^{Ca}
100 µmol/L Cu	50	14.50 ± 0.82 ^{Bb}	8.81 ± 0.49 ^{Cb}
100 µmol/L Pb	50	18.03 ± 0.51 ^{Ba}	6.90 ± 0.54 ^{Cc}
0	80	0	17.63 ± 0.61 ^{Ca}
100 µmol/L Cu	80	12.50 ± 0.65 ^{Cb}	9.47 ± 0.46 ^{Cb}
100 µmol/L Pb	80	15.64 ± 0.31 ^{Ca}	7.40 ± 0.34 ^{Cc}
0	100	0	19.29 ± 0.58 ^{Ba}
100 µmol/L Cu	100	10.70 ± 0.57 ^{Db}	11.40 ± 0.46 ^{Bb}
100 µmol/L Pb	100	13.56 ± 0.56 ^{Da}	10.01 ± 0.45 ^{Bc}
0	150	0	23.00 ± 0.82 ^{Aa}
100 µmol/L Cu	150	9.83 ± 0.73 ^{Db}	14.02 ± 0.84 ^{Ab}
100 µmol/L Pb	150	12.80 ± 0.76 ^{Da}	11.12 ± 0.15 ^{Ac}

Table 3. Cont.

Leaves			
Heavy Metal Concentration	TiO ₂ NP Concentration	Heavy Metal Concentration	Ti
Root			
μmol/L	μmol/L	μg/L	μg/L
0	0	0	0
100 μmol/L Cu	0	20.24 ± 0.49 ^{Ab}	0
100 μmol/L Pb	0	24.56 ± 0.66 ^{Aa}	0
0	50	0	19.39 ± 0.41 ^{Da}
100 μmol/L Cu	50	17.89 ± 0.73 ^{Bb}	9.84 ± 0.52 ^{Db}
100 μmol/L Pb	50	21.43 ± 0.88 ^{Ba}	7.85 ± 0.37 ^{Cc}
0	80	0	22.91 ± 0.85 ^{Ca}
100 μmol/L Cu	80	16.31 ± 0.80 ^{Cb}	11.90 ± 0.58 ^{Cb}
100 μmol/L Pb	80	19.13 ± 0.78 ^{Ca}	9.56 ± 0.61 ^{BCc}
0	100	0	25.17 ± 1.11 ^{Ba}
100 μmol/L Cu	100	14.12 ± 0.50 ^{Db}	15.61 ± 0.45 ^{Bb}
100 μmol/L Pb	100	16.42 ± 0.45 ^{Da}	12.06 ± 0.59 ^{Ac}
0	150	0	30.84 ± 0.71 ^{Aa}
100 μmol/L Cu	150	13.77 ± 0.51 ^{Db}	17.85 ± 0.57 ^{Ab}
100 μmol/L Pb	150	16.30 ± 0.67 ^{Da}	13.08 ± 0.58 ^{Ac}

Table 4. Increase in bamboo shoot and root DW and shoot length at different concentrations of TiO₂ NPs combined with 100 μM Cu and 100 μM Pb compared to those of the control treatment.

TiO ₂ NP Concentration	Heavy Metal Concentration	DW (Shoot)	DW (Root)	Shoot Length
μm	μm	g	g	cm
50	0	12.1%	12.1%	11.5%
50	100 μM Cu	30.5%	46.1%	48.5%
50	100 μM Pb	37.3%	34.6%	35.9%
80	0	21.8%	14.9%	20.12%
80	100 μM Cu	38.8%	81.7%	56.6%
80	100 μM Pb	46.3%	55.1%	57.6%
100	0	44.1%	41.6%	24.01%
100	100 μM Cu	72.2%	128%	62.4%
100	100 μM Pb	91.1%	128%	95.4%
150	0	49.4%	57.2%	26.5%
150	100 μM Cu	95.1%	158%	92.9%
150	100 μM Pb	95.9%	135%	96.8%

Figure 3. Cont.

Figure 3. Impact of the combination of TiO₂ NPs with 100 μM Cu and 100 μM Pb on shoot dry weight (DW) (a), root dry weight (DW) (b) and shoot length (c) in *A. pygmaea*. Each data point is the mean $\pm$ SE of five replicates. The treatments contained various levels of TiO₂ NPs alone or in combination with 100 μM Cu and 100 μM Pb. The capital letters show statistically significant differences across various levels of TiO₂ NPs alone or in combination with 100 μM Cu and 100 μM Pb (the bars with the same colors), while the lowercase letters show statistically significant differences within each level of TiO₂ NPs alone or in combination with 100 μM Cu and 100 μM Pb (the bars with different colors), according to Tukey's test ($p < 0.05$).

4. Discussion

Titanium dioxide nanoparticles (TiO₂ NPs) are one form of titanium found in the environment and 88,000 tons are produced worldwide annually [36]. This amount of titanium nanoparticles can be a source of pollution but, with proper management, they can be dispersed and distributed throughout the vast terrain so that the pollutant can become a microelement for plant nutrition. Titanium has many uses, including in plant protection and environmental remediation [37]. Heavy metal accumulation in plants may lead to alterations in many biochemical and physiological processes and to a disturbance in vital structures such as plant cell membranes [38], which can impact the translocation and accumulation of nutritional elements by plants [39,40]. To resolve this problem, plants increase defense mechanisms by stimulating antioxidant activities. Antioxidants scavenge ROS compounds, such as H₂O₂, and reduce the extent of lipoperoxidation in cell membranes, along with some chemical cycles including glutathione peroxidase, ascorbate-glutathione, and water in plant organs, such as mitochondria, cytosol, apoplast, chloroplasts, and peroxisomes [41,42]. Antioxidant enzymes, such as SOD, GR, APX, CAT, and POD [43], protect plant cell integrity during stress. Many studies have reported that TiO₂ NPs increase antioxidant activities [44–46], which are in agreement with our results in this study and our previous study [21]. Indeed, TiO₂ NPs reduced the negative impacts of ROS compounds in bamboo plants by stimulating antioxidant activity, which could increase the capacity of plant defense mechanisms against oxidative stress. This enhancement is likely related to the positive role of TiO₂ NPs in increasing signaling to activate antioxidant enzyme activity [21]. In our study, 150 μmol TiO₂ NPs showed the greatest increase in antioxidant activity, so this may be an optimal increasing bamboo defense system against 100 μmol of heavy metals. On the other hand, TiO₂ NPs lead to a reduction in MDA, H₂O₂, and soluble protein contents in plant cells under heavy metal stress, which is related to the role of TiO₂ NPs in the detoxification of ROS by the stimulation of antioxidant activity.

Chlorophyll pigments are one of the major traits influenced by metal stress in plants [47]. Metal stress has a deleterious impact on chlorophyll contents by: (1) inhibiting chlorophyll biosynthesis [47]; (2) reducing enzyme activities by substituting for co-factors; and (3) destroying the membrane structure by reducing phospholipid functions and disrupting the photosynthetic electron transport chain, thus degrading chlorophyll [48]. In this study, we found that TiO₂ NPs can increase chlorophyll and carotenoid contents in plants exposed

to 100 μM Cu and 100 μM Pb. TiO_2 NPs can increase the photosynthetic efficiency of plants under heavy metal stress by increasing light-harvesting pigments which can, in turn, improve light capture and reduce energy loss, enhancing photosynthetic quantum yield and ultimately raising the levels of chlorophyll *a*, chlorophyll *b* and total chlorophyll. This observation has been reported on wheat (*Triticum aestivum* L.) [49] and on the common bean [50]. TiO_2 NPs with sizes less than 100 nm can influence stomatal and leaf symplasts in plants and can protect cell walls against ROS compounds and free radicals. This process can help to increase chlorophyll and carotenoid efficiency and finally enhance plant photosynthesis. Wang et al. (2013) [51] showed that NPs directly penetrate the leaf symplasts of watermelon through the stomata. Therefore, we conclude that TiO_2 NPs have the ability to adjust stomatal closure and increase chlorophyll and carotenoid contents in plants under heavy metal stress.

The amelioration effect of Ti on the toxicity of heavy metals in plants is related to the decrease in heavy metal content in plant organs, which was shown in the previous studies [52,53]. This reduction in metal accumulation is attributed to titanium-involved mechanisms that can block metal transfer and limit metal uptake in plants [52,53]. Many studies have indicated that TiO_2 NPs can increase Ti levels in plants such as common bean (*Phaseolus vulgaris* L.) [50], corn (*Zea mays* L.) [54], cabbage [55], and pepper (*Capsicum annuum* L.) [56]. As the enhancement in roots is greater than that in stems and leaves because roots transfer only a small amount of Ti to shoots, titanium can accumulate more in roots [57]. This phenomenon was observed in our bamboo root samples (Table 3). On the other hand, TiO_2 NPs could limit the transfer of heavy metals from roots to shoots, which can be one of the mechanisms by which the metal concentration in roots and shoots is reduced. This could be due to TiO_2 NPs immobilizing the metal particles in the roots, as well as due to adsorption effects exerted by TiO_2 NPs, which can impede the absorption of the metals by the plasma membrane in the roots, resulting in the diminished bioavailability of the metals in the shoots. TiO_2 NPs at low concentrations can reduce the negative impact of heavy metals on plants, but the phytotoxicity of TiO_2 NPs at high concentrations is greater than that at low concentrations [58], as also observed in bamboo.

TiO_2 NPs significantly increase the biomass and crop yield of corn [59,60], tomato [61,62], barley [63] and wheat [64]. Our results revealed that TiO_2 NPs have the ability to increase shoot length and plant shoot and root dry weight under heavy metal stress by 68.2%, 63.4% and 95.8% respectively, in comparison with those of the control treatment. This is related to the stimulation of antioxidant activity and the reduction in ROS compound contents that eventually lead to an increase in the photosynthetic index and, finally, plant growth and plant biomass.

5. Conclusions

The observed increases in bamboo plant biomass and growth using TiO_2 NPs may be related to enhanced plant defense mechanisms, reduced metal translocation from the root to the shoot, and improved photosynthetic activity under metal stress. Nevertheless, the benefit of these mechanisms is also related to various factors, including plant species and genotype, growth conditions, and levels of heavy metals. TiO_2 NPs increased the plant antioxidant activity and reduced the levels of ROS compounds, protecting plant cells and cell membranes under oxidative stress. Moreover, TiO_2 NPs limited the translocation process of heavy metals from the roots to aerial parts, which may constitute an important metal detoxification mechanism. Additionally, we found that TiO_2 NPs increased pigment concentrations in plants exposed to 100 μmol heavy metal (Cu and Pb).

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