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Original Research

The Level of Oxytetracycline Uptake in the Presence of Copper Ions and the Physiological Response of *Brassica chinensis* L.

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Abstract

Copper and oxytetracycline (OTC) are two ordinary contaminants in farmlands. In the present study, the effects of oxytetracycline and Cu on growth, photosynthetic pigments, and antioxidant indices of *Brassica chinensis* were investigated. The results showed that treatments with OTC promoted growth of seedlings and roots, which were diminished by the addition of 100 mg/kg Cu. Contents of photosynthetic pigments decreased along with the elevation of OTC concentration. In comparison, their levels increased in the presence of 100 mg/kg Cu, suggesting that OTC might weaken the toxicity of Cu on photosynthesis. Moreover, the accumulation of OTC in either plant or soil was relatively lower in treatments with Cu than those without Cu. Overall, the present results suggested an antagonistic interaction between OTC and Cu on *B. chinensis.*

Keywords: antibiotics, antioxidant, copper, plant growth, photosynthesis

Introduction

Trace metals and antibiotics are two groups of ordinary contaminants. Both of them reveal high risk to environments. With the rapid development of intensive livestock and poultry farming, trace metals and antibiotics were largely accumulated in breeding wastewater due to common use of metals and veterinary medicines as animal food additives [1]. For instance, the metals copper, zinc, lead, cadmium, nickel, and chromium and several antibiotics were detectable in large-scale pig farms [2]. This wastewater was generally used to irrigate agricultural lands, further leading to concomitant input of metals and antibiotics [3]. Co-contamination of antibiotics and metals would pose considerable toxicological risks to soils and crops [4].

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Cu is an essential microelement to plants, but it is toxic to plants at high concentrations. For instance, Cu displayed harmful effects to *Brassica chinensis* L. with EC_{50} of total Cu varying from 0.06 to 0.72 μM and EC_{50} of free Cu^{2+} from 0.06 to 0.19 μM [5]. Treatments with a high level of Cu severely inhibited growth (shoot length, number of roots and leaves, and fresh weight) of *Solanum nigrum* and *Solanum lycopersicum* [6]. Oxytetracycline (OTC) is widely used to prevent diseases in livestock. A high level of OTC was detected in soil [7], water [8], animals [9,10] and plants [11,12]. Although OTC pollution in soil at concentration of 30 mg/kg showed no significant effects on seed germination and plant growth of Chinese cabbage, pollution at the levels of 10 and 30 mg/kg significantly affected root elongation, shoot length, and biomass of seedling [13]. These reports displayed toxicity and environmental behavior of Cu or OTC to organisms. However, they only investigated effects of single pollutants. Combined effects of Cu and OTC on crops are still rarely understood.

Brassica chinensis L. is a common crop in China and also a model plant for toxic assessments. In the present study, *B. chinensis* was employed as the study object and interactive effects of Cu and OTC on growth and physiological properties of *B. chinensis* and chemical properties of soil were investigated. Meanwhile, the accumulation of Cu and OTC in *B. chinensis* and soil was also determined. These results together would contribute to further understanding of toxicity and safety assessments of combined pollution of Cu and OTC.

Experimental

Materials

For cultivation of *B. chinensis*, surface soil (0-20 cm) was collected from an unpolluted area on a hill located at the Zheshan campus of Anhui Normal University (31°20′9.37″N, 118°22′9.14″E) and then mixed with commercial nutrient soil (Biaoyoumei Ecological Engineering Company, Nanjing, China) at a ratio of 3:1. The basic physiochemical parameters of these artificial soils were as follows: pH = 7.24, content of organic matters = 12.5 g/kg, content of available N = 0.63 g/kg, content of available P = 0.37 g/kg, content of available K = 10.06 g/kg, and content of total Cu = 25.46 mg/kg. No fertilization was performed during the whole experiment. Seeds of *B. chinensis* L. were purchased from Anhui Huahe Seeds Company (Hefei, China). OTC (purity 98%) was purchased from Anhui Tiangen Biochemical Science and Technology Company (Hefei, China). $CuSO_4•5H_2O$ (analytical grade) was used as the source of copper.

Sample Treatments

As previously reported, treatments with 20-50 mg/kg OTC significantly inhibited germination of *B. chinensis* and treatments with 5-30 mg/kg OTC significantly stimulated oxidative effects on *B. chinensis* seedlings [14]. Based on these results, three concentrations of OTC (5, 10 and 20 mg/kg) were set in the present study. Wahida et al. [15] showed that treatment with up to 60 mg/kg Cu in Histosol soil significantly suppressed plant height of *Brassica rapa* var. *parachinensis*. Thus, concentration of total Cu was set at 100 mg/kg in soil in the present study.

Before commencing the experiments, OTC with or without 100 mg/kg Cu was added in soil and thoroughly mixed. Treatment without OTC or Cu was also prepared as control. Each treatment was repeated three times.

In each treatment, 100 seeds of *B. chinensis* were sown in each pot (47 cm × 23 cm × 18 cm, volume of soil = 13.5 L) at the end of September, 2017 and then cultured in the greenhouse. After one week, 80 plants were retained in each pot. During the culture, water content in soil was maintained at 60-70% of maximum water-holding capacity by irrigating distilled water. Light cycle was 14 h: 10 h (day: night). During day time, light intensity was approximately 80-100 klux and temperature was 20-25ºC. During night, temperature ranged from 10ºC to 12ºC. During the whole experiment, air humidity was approximately 70%. After two months, all plants were collected, washed with distilled water and dried using filter paper.

Determining Growth Indices

From each treatment, 10 plants were randomly selected. Plant height and root length were measured using a ruler. Aboveground (AGP) and underground (UGP) parts were separated and weighed using an analytical balance.

Determining Content of Photosynthetic Pigments

Contents of photosynthetic pigments were determined according to Zhang and Qu [16]. For each treatment, 0.5 g of fresh leaves were completely homogenized in 2 mL of acetone with 0.5 g of quartz sands, and then total volume was adjusted to 20 mL by adding 80% acetone. The mixture was filtered using filter paper. Next, absorbance at 663 nm, 645 nm and 440 nm was measured to calculate content of chlorophyll a (Chl-a), b (Chl-b) and carotenoids (Chl-cx).

Determining Antioxidant Indices

Activity of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) and level of malondialdehyde (MDA) was measured as described by Zhang, Huang, and Chen [17], Proinoke [18], Zhang and Qu [16], respectively. One unit of SOD activity was defined as the amount of enzyme required for 1 gram of tissue in 1 ml of reaction mixture to achieve SOD inhibition rate of 50%. One unit of POD activity was defined as a change of absorbance at 420 nm for 0.01. Decrease in absorbance at 240 nm for 0.01 represented one unit of CAT. Each assay was repeated three times.

Determining Soil Parameters

Soil pH value was determined by mixing 10 g of soil with 25 mL of CO_2-free H_2O in 50-mL glass beaker for 1 min. After standing for 30 min, pH value of solution was measured using a pH meter.

Content of organic N in soil was determined using the alkali hydrolysis method. Air-dried soil was sieved using a 1 mm pore-sized sieve. Next, 2 g of soil was mixed with 1 g of $FeSO_4$ and then placed in the outer block of nitrogen diffusion dish. In the inner block of dish, 2 mL of 2% H_3BO_3 solution and 1 drop of indicator were added. After 10 mL of 1.6 mol/L NaOH solution was added to soil, the dish was immediately covered by glass slip and completely sealed using glue. When soil was thoroughly infiltrated by NaOH solution, the whole apparatus was kept at 40ºC for 24 hours. Next, the amount of ammonia absorbed by H_3BO_3 was determined by titration using 0.01 mol/L HCl solution. The titration was ended when the color of the solution changed from blue to light red.

To determine content of organic matters [19], 0.3 g of soil was mixed with 10 mL of 0.4 mol/L $K_2Cr_2O_7$ solution in glass test tubes. Tubes were heated at 170 to 180ºC for 5 min and then totally transferred to a 150-mL flask. Content of organic matter was titrated using 0.2 mol/L $FeSO_4$ solution. 1,10-phenanthrolinemonohydrate was used as the indicator and the alteration of color to brick-red was considered as the endpoint.

Determining Cu Contents in Plants and Soil

Plants were washed carefully using distilled water and then completely dried at 80ºC. Afterward, plants were crushed and 1 g of sample was digested in 5 mL of acid mixture (HNO_3: $HClO_4$: H_2SO_4 = 8: 1 :1). After evaporation of acid, samples were dissolved in 7% HNO_3 solution and then diluted to 50 mL using distilled H_2O. Cu content was determined using an atomic absorption spectrometer [20].

To determine content of available Cu, rhizosphere soil (approximately 2 mm to root surface) was collected, completely dried and then crushed into powders. After being sieved through an 80-mesh sieve, approximately 5 g of soil was weighed and placed in a 100-mL glass flask. After 20 mL of 0.1 mol/L HCl solution was added, the flask was shaken at 180 rpm for 30 min to extract available Cu. The mixture was filtered through filter paper and content of Cu in solution (representing available Cu) was determined using an atomic absorption spectrometer [20].

Determining OTC Contents in Plants

Determining OTC contents followed the Chinese Standard GB/T 22259 with slight modification [21]. Briefly, plant samples were ground into powders using liquid nitrogen. 6 g of each sample was extracted in 10 mL of extraction solution (25 % methanol and 0.3 mol/L HCl) by sonication for 30 min. After centrifuging at 4,000 rpm for 40 min, supernatant was collected. This process was repeated 3 times and all supernatants were pooled and purified using Oassis HLB SPE columns and eluted using 15 mL of ethyl acetate. After being dried using a speedvac, the extract was dissolved in 1 mL of methanol.

For high-performance liquid chromatography (HPLC) analysis, 100 µL of sample solution was injected into an LC-20A HPLC (Shimadzu, Japan) equipped with an Ultimate XB-C_{18} column (150 mm × 4.6 mm × 5 µm). The HPLC process was as follows: concentration of acetonitrile (containing 0.1% TFA) increased from 5% to 95% within 60 min and absorbance at 353 nm was monitored. Standard curve was prepared by injecting 100 µL of 100, 50, 20, 10 and 20 mg/L standard OTC solution to HPLC.

Data Analyses

The statistical analyses were performed using SPSS 19.0. The homogeneity for variance was checked first. One-way variance analysis was performed to check the effects of OTC on all tested indicators, followed by Student-Newman-Keuls tests (SNK). Students' t-test was performed to compare the results between treatment with and without Cu. $P<0.05$ was considered a significant statistical difference.

Results

Effects of Cu and OTC on Growth Indices

In the absence of copper, OTC significantly affected plant height and root length, but not fresh weight of total plant, UGP and AGP. Compared with the control, treatments with 5 and 10 mg/kg OTC significantly increased plant height. Treatment with 10 mg/kg OTC significantly affected root length. However, no significant differences in both plant height and root length were detected in treatment with 20 mg/kg OTC (Table 1).

In the presence of 100 mg/kg Cu, treatment with OTC significantly affected root length but not plant height. Compared with the control, treatment with 10 mg/kg OTC significantly reduced root length.

Table 1. Growth indices of *Brassica chinensis* L. in treatments with Cu and OTC (mean±SE).

OTC (mg/kg)	Plant height (cm)	Root length (cm)	Fresh weight (g/plant)		
			Total plant	UGP	AGP
Cu concentration: 0 mg/kg					
0	13.74±1.08[a]	1.90±0.27[a]	0.28±0.03*	0.01±0.002	0.27±0.03*
5	18.55±0.93[b]	2.25±0.2[a]	0.47±0.06*	0.01±0.001	0.41±0.05*
10	19.18±1.56[b]	3.31±0.51[b]	0.49±0.09*	0.01±0.003	0.48±0.09*
20	16.61±0.94	2.33±0.34	0.43±0.05*	0.02±0.003	0.43±0.06*
Cu concentration: 100 mg/kg					
0	16.69±0.59	3.12±0.38[a]	0.59±0.10	0.02±0.003	0.53±0.10
5	17.34±0.76	3.85±0.35[a]	0.39±0.04	0.02±0.004	0.36±0.04
10	17.34±0.56	2.04±0.23[b]	0.45±0.05	0.02±0.003	0.43±0.05
20	17.18±0.72	2.82±0.36[a]	0.43±0.06	0.01±0.002	0.42±0.06

UPG: under-ground part; AGP: above-ground part; the different letters represent significant differences within the same column
*significantly different between treatments with the same concentration of OTC in the presence and absence of Cu (n = 30)

Treatments with 5 and 20 mg/kg OTC showed no effects on root length (Table 1).

In the absence of OTC, treatment with 100 mg/kg Cu significantly increased fresh weight of total plant and AGP compared with the control. At the same concentration of OTC (5-20 mg/kg), all treatments with 100 mg/kg Cu significantly reduced fresh weight of total plant and AGP than those without Cu.

Effects of Copper and OTC on Photosynthetic Pigments

Without the addition of Cu, treatments with 5-20 mg/kg OTC significantly reduced contents of Chl-a, Chl-b and Chl-cx. In comparison, when 100 mg/kg Cu was added, treatment with 20 mg/kg OTC increased the level of Chl-a, and treatments with 10 and 20 mg/kg OTC elevated contents of Chl-b and Chl-cx (Fig. 1).

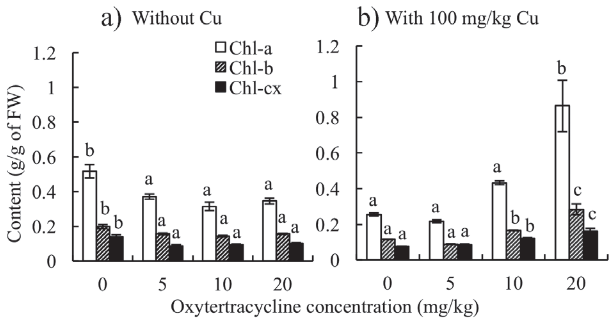

Fig. 1. Effects on Cu and OTC on contents of photosynthetic pigments in *B. chinensis* L (mean±SE). Chl-a: chlorophyll a; Chl-b: chlorophyll b; Chl-cx: carotenoid; the different letters represent significant differences within the same column (n = 3).

Effects of Cu and OTC on Antioxidant Activities

Regardless of Cu, treatments with OTC significantly affected the level of MDA and activities of CAT and POD in *B. chinensis*, but did not affect SOD activity. In comparison to the control, treatments with 10-20 mg/kg OTC significantly elevated activities of CAT and POD. In the absence of Cu, treatment with 10 mg/kg OTC significantly increased the level of MDA, but treatments with 5-20 mg/kg OTC did not affect activity of POD. In the presence of 100 mg/kg Cu, treatment with 20 mg/kg OTC enhanced the activity of POD, but no changes were observed in the level of MDA at all OTC concentrations (Table 2).

Table 2. Effects of Cu and OTC on antioxidant indicators in *B. chinensis* L. (mean±SE).

OTC (mg/kg)	MDA (nmol/g)	CAT (U/g/min)	POD (U/g/min)	SOD (U/g/min)
Cu concentration: 0 mg/kg				
0	1.3±0.1[ac]	82±5[a]	7440±840	0.34±0.03
5	1.4±0.2[ab]	92±8[a]	5232±579[a]	0.38±0.07
10	1.8±0.1[b]	160±15[b]	8295±587[b]	0.44±0.02
20	0.9±0.2[c]	747±4[c]	5994 ±680[a]	0.44±0.02
Cu concentration: 100 mg/kg				
0	1.0±0.2	48±0[a]	4221±558[a]	0.34±0.10
5	1.5±0.1[a]	67±0[a]	6025±542[a]	0.45±0.03
10	1.1±0.2	2016±13[b]	5779±603[a]	0.42±0.04
20	0.8±0.1[b]	1212±6[c]	9547±852[b]	0.44±0.03

Different letters represent significant differences within the same column (n = 3).

Table 3. Chemical properties of rhizosphere soil of *B. chinensis* treated with Cu and OTC (mean±SE).

OTC ($mg \cdot kg^{-1}$)	Organic matter (g/kg)	N (g/kg)	pH
Cu concentration: 0 mg/kg			
0	6.8 ±0.1^{a}	0.6±0.0^{ab}	7.4±0.1
5	7.1±0.1^{a}	1.4±0.0^{c}	7.6±0.1
10	6.3±0.3^{b}	0.5±0.1^{a}	7.6±0.0
20	6.1±0.2^{b}	0.7±0.0^{b}	7.7±0.1
Cu concentration: 100 mg/kg			
0	5.3±0.2^{a}	0.4±0.0^{a}	7.7±0.1^{a}
5	6.8±0.1^{b}	0.6±0.0^{b}	7.6±0.1^{a}
10	5.5±0.1^{ca}	0.8±0.1^{c}	7.0±0.0^{b}
20	5.8±0.1^{dc}	0.4±0.0^{ad}	7.0±0.1^{b}

Different letters represent significant differences within the same column (n = 3).

Effects of Cu and OTC on Soil Parameters

Without extra Cu, treatments with 10-20 mg/kg OTC significantly decreased content of organic matters in rhizosphere soil in comparison to the control. Treatments with 5 and 20 mg/kg OTC significantly increased N content, but no effects of OTC were observed on pH value of soil (Table 3).

With the addition of 100 mg/kg Cu, treatments with 5 and 20 mg/kg OTC increased content of organic matters, treatments with 5 and 10 mg/kg OTC increased content of N, and treatments with 10-20 mg/kg OTC decreased pH value of soil compared with the control (Table 3).

Cu Contents in Plants and Soil

No matter whether extra Cu was added, similar patterns were observed for content of total Cu in plants, total Cu and available Cu in soil, along with increasing concentrations of OTC. Treatments with OTC decreased levels of total Cu but increased available Cu in soil. Without extra Cu, levels of total Cu in plants did not change in response to OTC. However, Cu content in plants increased gradually with increasing levels of OTC when extra Cu was added (Fig. 2).

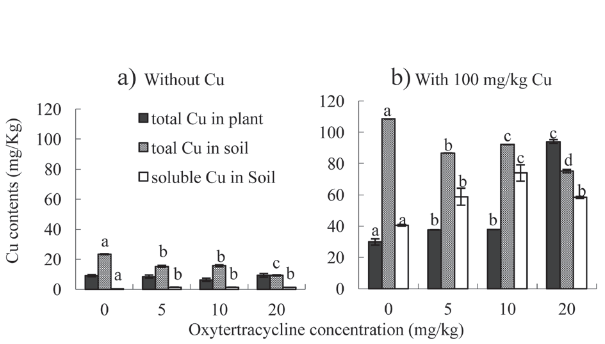

Fig. 2. Cu content in *B. chinensis* and soil in treatments with Cu and OTC (mean±SE).

OTC Contents in Plants

Without the addition of extra Cu, relatively higher levels of OTC were detected in plants compared with those in soil. When extra Cu was added, OTC was not detected in both soil and plants (Table 4).

Discussion

Previously, Tian et al. [22] showed that treatments with 25-50 mg/kg OTC promoted germination and seedling growth of cucumber. Treatment with 1 mg/L OTC inhibited root growth of lettuce, and treatments with OTC higher than 5 mg/L inhibited leaf growth. In the present study, similar results were observed. Treatment with 5 mg/kg OTC increased plant height, and treatment with 10 mg/kg increased root length, but in treatment with 20 mg/kg OTC, no significant differences in plant height and root length were observed. These results suggested that treatments with OTC at low concentrations might facilitate growth of *B. chinensis*, but treatments with OTC at high concentrations might be harmful to its growth, which is consistent with the reports of Tian et al. [22] and Cui [23].

High concentrations of Cu are known to activate oxidative damage and alter cell-membrane properties by lipid peroxidation, thereby demonstrating inhibitory effects on enzymes involved in chlorophyll production [24]. Moreover, exposure to Cu could affect mineral uptake in plants and decrease contents of numerous minerals in leaves, inhibiting synthesis of photosynthetic pigments [25]. In the present study, similar results were observed. Treatment with 100 mg/kg Cu significantly decreased levels of Chl-a, Chl-b and Chl-cx, in comparison to the control (no Cu and no OTC). Regarding underlying mechanisms, oxidative effects of Cu on enzymes might not be the reason, since activities of antioxidant enzymes and MDA level were not significantly higher in treatment with Cu than the control (Table 2). Whether or not changes of mineral contents were involved in this process should be further investigated.

Chen et al. [26] revealed that exposure to 10-200 mg/kg OTC diminished photosynthetic capacity of rape by altering energy distribution in photosynthetic electron transport chain. Similarly, treatments with OTC higher than 25 mg/kg reduced the photosynthetic rate of radish plants [27]. In the present study, treatments with 5-20 mg/kg OTC significantly decreased contents of Chl-a, Chl-b and Chl-cx, which is consistent with previous findings [26, 27]. When Cu was added, levels of photosynthetic pigments increased with elevation of OTC concentration, suggesting interactive effects

Table 4. OTC content in soil and *B. chinensis* after experiments..

OTC treatment (mg/kg)	OTC content (mg/kg)	
	Soil	Plant
Cu treatment: 0 mg/kg		
0	-	-
5	3.72	27.37
10	3.33	87.67
20	7.73	13.66
Cu treatment: 100 mg/kg		
0	-	-
5	-	-
10	-	-
20	-	-

"-": not detected

between Cu and OTC. OTC could form a complex with Cu [23], probably decreasing concentration of available Cu and OTC in plants (Fig. 2 and Table 4) and subsequently weakening the harmful effects of Cu on synthesis of pigments. However, more investigations are required to clarify this viewpoint.

Changes of MDA, CAT, POD and SOD in the presence/absence of extra Cu were rather complicated. Treatment with 20 mg/kg OTC decreased MDA level, but treatment with 20 mg/kg OTC and 100 mg/kg Cu showed no effects on MDA, demonstrating antagonism between OTC and Cu. The tendency of CAT activity with increasing level of OTC was similar between treatments with and without extra Cu, suggesting no interactions between them. In the absence of Cu, no significant effects were detected between treatments with OTC and the control, but a significant difference was observed between treatment with 20 mg/kg OTC and the control in the presence of Cu, indicating synergism between Cu and OTC. Moreover, no changes were detected in SOD activity in all treatments. These results were hard to explain, but implied a complicated mechanism underlying the interactive effects of Cu and OTC on the antioxidant process in *B. chinensis.*

Contents of organic matters and N in soil were mainly influenced by root and microorganism activities. Roots could secrete organic acids to soil [28] and autotrophic bacteria could transform inorganic chemicals to organic matters. These two processes should increase content of organic matters in rhizosphere soils. In comparison, methane generation by microorganisms would decrease content of organic matters due to biogas production [29]. In the present study, regardless of Cu, the level of organic matter and N showed a similar tendency, increasing at low concentrations of OTC but decreasing afterward. In treatment with 5 mg/kg OTC, although no significant differences in root length were observed from the control, the average values of root length were higher than those in the control, suggesting that more organic matters might be secreted by roots into soils. Besides, treatments with OTC might decrease biogas production due to inhibition of methane generation by microorganisms [29], which could accumulate more organic matters in soil. This hypothesis required more evidence to support. When concentration of OTC further increased, only treatment with 10 mg/kg OTC and 100 mg/kg Cu significantly reduced root length. These results suggested that inhibition of root growth might contribute to decreased organic content in soil, but it must not be the sole reason. Alternatively, the activity of microorganisms might be severely inhibited by OTC and less organic matters could be generated by microorganisms.

Certain metals could play a "bridge" role in promoting the removal of antibiotics in flocculation. For example, Cu^{2+} improved the removal of coexisted antibiotic molecules [30] and could accelerate photolysis of OTC [23]. In the present study, the residual level of OTC was relatively lower in plants and soil when Cu was added than those without Cu, probably also due to the promotive effects of Cu on OTC degradation.

When 100 mg/kg Cu was added, content of total Cu in soil decreased, but content of available Cu in soil and content of total Cu in plant increased along with elevated level of OTC, suggesting that treatment with OTC might enhance bioavailability of Cu in soil and promote absorption of Cu by plants. These results might be due a systematic ecological process. As previously reported, dissolved organic matters (DOC) significantly bind heavy metals such as Cu [31, 32]. This kind of Cu-DOC complex could enhance mobility and bioavailability of Cu [33]. In the present study, contents of organic matters and N in soil increased first and then decreased with elevating concentrations of OTC, probably resulting in the similar tendency of changes in available Cu content in soil. The higher level of available Cu content in soil, the more Cu absorbed by plants, and the lower the total Cu retained in soil.

Conclusions

Treatments with OTC promoted growth of seedlings and roots in *B. chinensis*, and these promotive effects could be diminished by the addition of 100 mg/kg Cu. Treatment with OTC decreased contents of photosynthetic pigments, but their levels increased in the presence of 100 mg/kg Cu, suggesting that OTC might weaken toxicity of Cu on photosynthesis. Moreover, the presence of Cu reduced the accumulation of OTC in either plant or soil. Overall, OTC might antagonistically interact with Cu in *B. chinensis.*

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Conflict of Interest

The authors declare no conflict of interest.

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