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Original Article

Phytotoxic effects of copper oxide nanoparticles on two *Brassica* species during the seedling stage

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Abstract

Nanotechnology has the potential to increase global food production. However, the widespread application of nanoparticles (NPs) in agriculture is relatively slow due to concerns on accumulation, translocation, and toxicity in food crops. One such NP is the Cu-based NPs since Cu is a micronutrient and exhibits antimicrobial and antifungal activities. In this study, the uptake of CuONP (vs. bulk CuO) and their toxicological effects in cabbage (*Brassica oleracea* L. var. *capitata*) and pechay (*B. rapa* L. var. *chinensis*) were investigated. High concentrations of CuONP and bulk CuO resulted in the overproduction of ROS, causing elevated catalase, ascorbate peroxidase, and malondialdehyde, which are consistent with the observed inhibition of seedling growth. All treatments, however, had no significant effect on levels of chlorophyll and carotenoids. AAS analysis confirmed that the phytotoxic symptoms may be attributed to the accumulation of Cu. Overall, results showed that CuONP is more toxic than bulk CuO in both crops, particularly in cabbage. The estimated TC_{50} values are 29.06 ± 1.75 mg/L for CuONP and 353.58 ± 16.41 mg/L for bulk CuO in cabbage, and 71.72 ± 3.03 mg/L for CuONP and 371.52 ± 22.79 mg/L for bulk CuO in pechay. This study demonstrates that Cu-based NPs can be taken up by crop seedlings, indicating a potential pathway for entry into the food chain.

Keywords: *Brassica* species, Copper oxide nanoparticles, Oxidative stress, Phytotoxicity, Pigments

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Introduction

Nanotechnology offers a key solution in Agriculture 4.0 for addressing challenges in the current agricultural model and improving food security. It has the potential to develop new innovative products to increase global food production and feed the growing world population (De Clercq et al., 2018). In plants, the beneficial impacts of NPs include increased photosynthesis, root volume, nutrient uptake, enzyme activities, and biomass (Ahmed et al., 2021). However, these responses vary with plant species and genotype. Therefore, the commercial application of NPs in agriculture requires comprehensive screening and optimization adapted to each plant species (Fatima et al., 2020; Feigl, 2023). The practical application of nanoparticles under field conditions remains limited due to concerns regarding their accumulation, translocation, transformation, and potential toxicity in food crops. Risk assessment and nano-toxicological evaluations of NPs are therefore necessary for their successful commercial utilization (Ali et al., 2021). The reported toxic impacts of NPs on plants include reactive oxygen species (ROS) production leading to intracellular oxidative stress, interaction with genetic material of plant cells, and disruptive impacts on plants leading to reduced biomass and water transpiration (Ahmed et al., 2021).

Copper (Cu), an essential micronutrient widely distributed in plants, plays a vital role in numerous physiological processes and exhibits broad-spectrum antimicrobial and antifungal properties. Consequently, Cu-based nanofertilizers and nanopesticides, such as copper oxide nanoparticles (CuONP), are promising for agricultural use due to their nanoscale size, which enhances plant absorption. Studies that investigated the phytotoxicity of Cu-based NPs suggest that the metal's toxicity depends on plant species and genotype, growth conditions, exposure time, application mode, concentration, and size (Ayoub et al., 2018). Therefore, it is essential to establish the maximum allowable doses for specific plants, particularly the economically important crop species.

This study aimed to investigate the uptake and toxicological effects of CuONP in two economically important vegetables in the Philippines: *Brassica oleracea* L. var. *capitata* (cabbage) and *B. rapa* L. var. *chinensis* (locally called pechay). Varieties of these two green leafy vegetables commonly cultivated in the Philippines were chosen. The Scorpio F1 hybrid is a type of white cabbage and is the country's standard for round head cabbage due to its taste, shelf-life, and tolerance to black rot resulting in a high market price (ATI, 2014). Another high-value crop in the Philippines is pechay – a green leafy vegetable that is widely consumed by Filipinos and is always available in the market. Black Behi is one of the preferred varieties by Filipino farmers due to its short duration harvesting (25 – 30 days) and can also be grown year-round (Jimenez et al., 2000). The following were the specific objectives: (1) assess the phytotoxic impacts of CuONP in terms of morphological, physiological, and biochemical parameters; (2) evaluate plant uptake and distribution of CuONP; and (3) compare the phytotoxicity of CuONP and bulk CuO.

Xiong et al. (2017) and Deng et al. (2020) reported phytotoxicity of CuONP on 3-week-old *B. oleracea* L. through foliar exposure and on *B. rapa* L. through soil application, respectively. Di et al. (2023) studied the short-term toxicity of CuONP in *B. rapa* L. grown under hydroponic conditions. However, studies on the impact of CuONP on seed germination and seedling growth for both crops are still lacking. In addition, there is no existing data on the median toxic concentrations (TC_{50}) for CuONP in any *Brassica* species, which were estimated in the present study. Only Xiong and Wang (2005) reported median lethal concentrations (LC_{50}) for bulk Cu^{2+} [as $Cu(NO_3)_2$] in *B. pekinensis* Rupr. (Chinese cabbage) in terms of seed germination.

Material and Methods

Chemicals and plant materials

All chemicals used in this study were of reagent grade. Bulk CuO was obtained from Loba Chemie (India), while hydrochloric acid and acetone were purchased from RCI Labscan (Thailand). Sodium ascorbate, trichloroacetic acid, 2-thiobarbituric acid, and CuONP were sourced from Sisco Research Laboratories (India). Potassium phosphate buffer (1.0 M, pH 7.4) and hydrogen peroxide were acquired from Sigma-Aldrich (USA).

The seeds of *B. oleracea* L. var. *capitata* (Scorpio F1 hybrid cabbage by Sakata Seeds, Japan; Lot No. 03-21-36-D00094, Batch No. 49204) and *B. rapa* L. var. *chinensis* (Black Behi pechay by Condor Seed Production, USA; Lot No. 02-24-35-D00211, Batch No. 46406) were purchased locally. Healthy seeds that were relatively uniform in size were selected for the experiments. The seeds were stored in a dry place, kept in the dark at room temperature prior to use.

Characterization of copper oxide nanoparticles

The size, shape, and composition of CuONP were analyzed using field emission transmission electron microscopy (FE-TEM, JEOL JEM-2100F) with an energy dispersive X-ray spectroscopy detector (EDS, Oxford Instruments X-Max 80T) at the Industrial Technology Development Institute of the Department of Science and Technology (ITDI, DOST). The analysis used an ultrathin carbon film supported by a lacey carbon film on a 300-mesh gold grid (product no. 01824G, Ted Pella Inc.).

Preparation of copper oxide nanoparticle suspension

A 100 mg/L stock solution of CuONP was prepared by dissolving in distilled water. To reduce aggregation, the solution was sonicated for 1 hour and stirred with a magnetic stirrer for 30 min. Working solutions were made by diluting the stock. Similar solutions of bulk CuO were prepared for comparison.

Seed germination and seedling growth assay

To investigate the effect of CuONP and bulk CuO on seed germination and seedling growth, the USEPA OPPTS 850.4200 (1996) for the testing of chemicals was followed. Validity of the test for each vegetable crop was also assessed based on the criteria set by USEPA (2012). Preliminary experiments were conducted (in triplicate) to determine the incubation periods for each crop and to estimate the range in which the TC_{50} will fall. Based on the results, the concentration ranges for the definitive tests are as follows: 10^0 – 10^2 mg/L for CuONP and 10^1 – 10^3 mg/L for bulk CuO.

For each Cu test solution, 20 seeds were placed at an equal distance in petri dish lined with fitted-size filter paper (Whatman No. 1). Five (5) mL of each treatment, i.e., 5 concentrations (T1 – T5) and control (T0), was added per dish. Treatments were arranged in a completely randomized design with 15 petri dishes for each concentration. All petri dishes were sealed with parafilm and incubated at room temperature (26.8 ± 3.4 °C) in the dark for 10 (cabbage) or 7 (pechay) days.

The samples were prepared in batches where each batch contains 15 petri dishes per treatment. Each treatment required 6 batches to collect the required mass of seedlings for all succeeding experiments (i.e., biochemical parameters and NPs uptake) resulting in a total of 90 petri dishes/treatment or 1,800 seeds/treatment.

Assessment of phytotoxic effects on morphological parameters

For the morphological parameters of the seedlings, 12 petri dishes/treatment were randomly selected, where each petri dish is a replicate. The radicle and plumule lengths were measured using the ImageJ program. A dose-response curve was then generated to obtain the values for TC_{50}, no observed effect concentration (NOEC), and lowest observed effect concentration (LOEC). Specifically, the inhibition of seedling growth is the ‘effect’ that was measured.

Assessment of phytotoxic effects on physiological and biochemical parameters

Fresh samples of treated and control seedlings from the seedling growth assay were analyzed for catalase and ascorbate peroxidase activities following the methods of Aebi (1984) and Nakano and Asada (1981), respectively. The determination of lipid peroxidation was done by measuring the concentration of malondialdehyde as previously described by Heath and Packer (1968). Then, the levels of pigments (chlorophyll a, chlorophyll b, and carotenoids) were measured based on the procedure of Lichtenthaler and Wellburn (1983). All assays were done in triplicates.

Nanoparticles uptake in shoot and root of plant seedlings

The uptake of Cu by the treated and control seedlings was determined using an atomic absorption spectrometer (AAS, Varian SpectrAA 55B) in triplicate. Oven-dried plant tissue (at least 100 mg) was placed in a porcelain crucible, digested for at least 24 h in a muffle furnace at 500 °C, dissolved in 10.0 mL of 1 M HCl solution, filtered, and diluted to 50 mL with deionized water. Method blanks and spiked samples were similarly processed for quality assurance and quality control. The Cu concentration, in mg/kg, in roots and shoots was calculated, as well as the translocation factor to evaluate the translocation of Cu from roots to shoots.

Statistical analysis

The statistical analysis was performed using the open statistical software Jamovi©. Prior to analysis of variance (ANOVA), the homogeneity of error variances and normality of errors were checked using

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Levene's and Shapiro-Wilk tests, respectively. Then, ANOVA was conducted to identify significant differences between treatments. If significant differences were found, Tukey's Honest Significant Difference test (HSD) was applied. Differences were considered significant at $\alpha = 5\%$.

Results and Discussion

Characterization of copper oxide nanoparticles

Commercially available CuONP was used in this study. TEM analysis showed that CuONP, with an average size of 46.5 ± 8.5 nm, formed clusters of spherical particles. In addition, dynamic light scattering analysis revealed that the average size of bulk CuO particles is >8 µm. The signal for carbon in the EDS spectra is an impurity, which may be due to the support film used in the analysis.

Ahmed et al. (2019) reported that the zeta potential of CuONP from Sisco Research Laboratories is -29.8 ± 2.1 mV, which indicates that the NPs form a relatively stable suspension in water since the high negative charge on the surface prevents aggregation of the particles. NPs with zeta potentials of ±10 mV are neutral that tend to coagulate, while NPs with zeta potentials equal or more positive than +30 mV or more negative than -30 mV are considered stable (Ibrahim et al., 2022).

Influence of copper oxide nanoparticles on seed germination and seedling growth

The inhibitory effect of CuONP and bulk CuO on seed germination, root length, and shoot length of cabbage and pechay seedlings is presented in Figure 1. Measurements of seed germination and seedling growth are important in the assessment of the toxicity of substances because the success of germination and the vigor of seedlings determine crop yield (Wang et al., 2020).

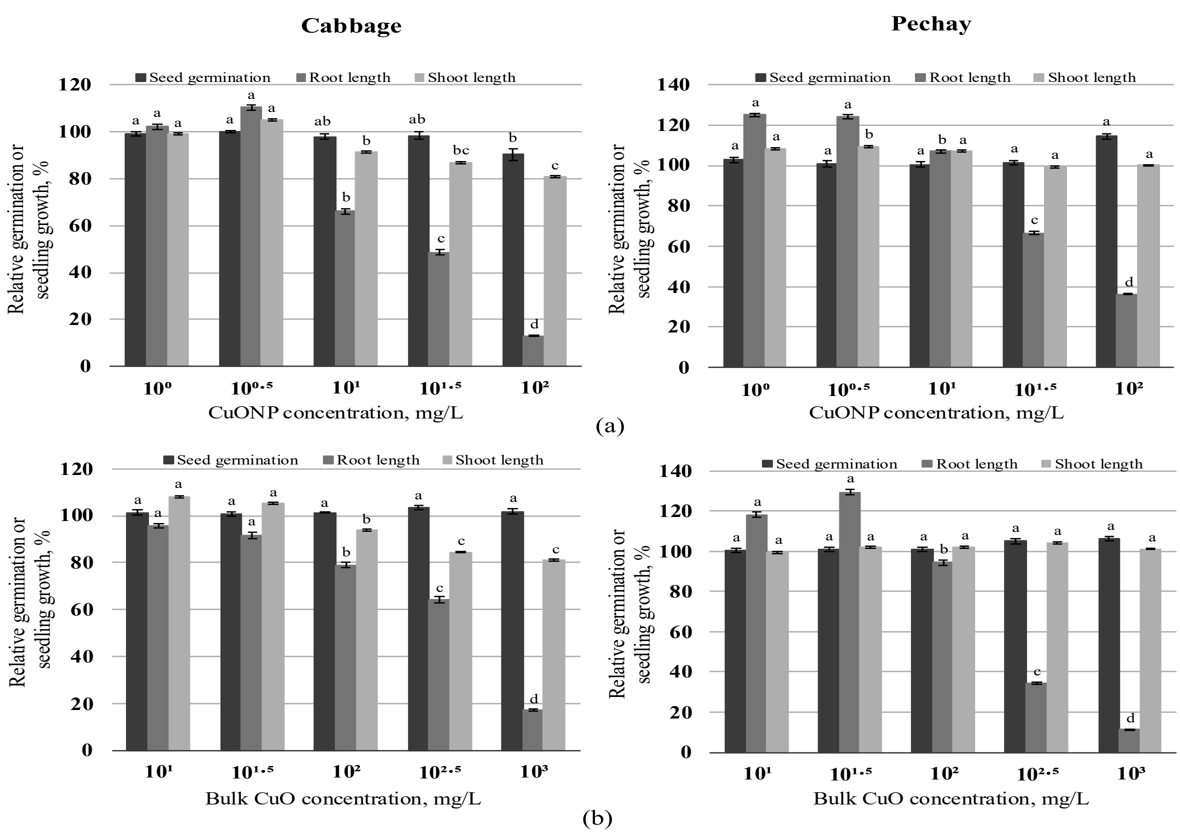

Figure-1. Inhibition of seed germination, root elongation, and shoot elongation of cabbage (Scorpio) and pechay (Black Behi) seedlings exposed to different concentrations of (a) CuO nanoparticles and (b) bulk CuO. Data are mean ± standard error of n = 12. Means of the same letters are not significantly different (HSD, $\alpha = 5\%$).

Seed germination

Seed germination is the first stage of plant development and is one of the parameters commonly evaluated in phytotoxicity studies. A significant decrease in germination was observed for cabbage seeds exposed to the highest concentration of CuONP as compared to the control seeds. All CuONP concentrations did not affect pechay seed germination. However, the observed inhibitory effect in cabbage seeds is relatively negligible since the % germination is ≥90% (da Costa and Sharma, 2016). Bulk CuO also did not exhibit an inhibitory effect on the germination of both cabbage and pechay seeds. The lack of sensitivity to the Cu test solutions in terms of seed germination may be attributed to the protection provided by the seed coat via selective permeability. The embryo is protected from the harmful effects of the NPs by the seed coat until the radicle emerges (da Costa and Sharma, 2016).

Similarly, Ahmed et al. (2019) reported that ≤5000 mg/L CuONP and bulk CuO had no significant effect on the seed germination of radish, which is a member of Brassicaceae family and a close relative of *Brassica*. However, Singh et al. (2017) observed that $\geq 10^2$ mg/L CuONP negatively affected the germination of cauliflower (*B. oleracea* var. botrytis). While Xiong and Wang (2005) reported that as low as 0.5 mM/L bulk Cu^{2+} (approximately 40 mg/L CuO) significantly reduced the germination rate of Chinese cabbage. These results show that the effects of Cu on seed germination vary among species.

Root and shoot lengths

Cabbage and pechay grown in different concentrations of CuONP and bulk CuO exhibited differences in root and shoot lengths after incubation. Measurements of seedling growth parameters are indicators of the early impact of toxicants (Rajput et al., 2021). Root lengths of treated cabbage and pechay seedlings decreased as the concentration of each Cu test solution increased, in a dose-dependent manner. This trend was not observed for the differences in shoot lengths among control and treated seedlings (Figure 1).

Root is the first target tissue exposed to toxicants, resulting in toxic symptoms being more visible in roots than in shoots (Adhikari et al., 2012). As soon as the radicles emerge and rupture the seed coat, the roots will be in direct contact with the growth medium. The high surface area to volume ratio of roots means that they can rapidly absorb nutrients, water, and toxicants, resulting in a dose-dependent response for the differences in root lengths among control and treated test crop seedlings. Thus, root elongation is considered a better measure of phytotoxicity of chemical substances than seed germination and shoot elongation (Adhikari et al., 2012; da Costa and Sharma, 2016). Reduction of root lengths as the Cu concentration increases may be due to the accumulation of Cu in root tissues, resulting in higher levels of Cu ions released, causing oxidative damage to cells (da Costa and Sharma, 2016).

Similar responses for root and shoot lengths were exhibited by CuONP-treated turnip (*B. rapa* ssp. *rapa*) (Chung et al., 2019), cauliflower (Singh et al., 2017), and radish (Wu et al., 2012; Ahmed et al., 2019). The same response was also reported for Chinese cabbage (Xiong and Wang, 2005) exposed to bulk Cu^{2+}.

Copper is essential in completing the plant life cycle as it plays important roles in photosynthesis, respiration, and hormone signaling. However, being a micronutrient, the positive or negative effects of Cu on plants depend on concentration. Copper can either be a nutrient at low concentrations or a toxicant at higher concentrations (Xu et al., 2024). In this study, only the lower concentrations of CuONP ($<10^1$ mg/L) and bulk CuO ($<10^2$ mg/L) had significant positive effect on the root lengths of pechay seedlings. The same observation was reported for radish exposed to CuONP (Ahmed et al., 2019).

The toxicity parameters calculated based on the germination index for both cabbage and pechay exposed to CuONP and bulk CuO are summarized in Table 1. Based on the LOEC and TC_{50} values obtained, both CuONP and bulk CuO exhibited higher phytotoxicity in cabbage than in pechay. In comparison, the reported median effective concentration (EC_{50}) of CuONP in other plants is 480 mg/L in *Elsholtzia splendens* (Shi et al., 2013), 13 mg/L in lettuce (Wu et al., 2012), 398 mg/L in radish (Wu et al., 2012), 228 mg/L in cucumber (Wu et al., 2012), 335 mg/L in mung bean (Lee et al., 2008), and 570 mg/L in wheat (Lee et al., 2008). Ibrahim et al. (2022) recently reported a different EC_{50} for wheat at 0.94 mg/L. Thus, the relative toxicity of CuONP in selected plants is lettuce > cabbage > pechay >> mung bean > radish > *E. splendens*. In these studies, the 'effect' that was measured is the inhibition of seedling growth. The only reported EC_{50} of bulk CuO is 0.37 mg/L in wheat (Ibrahim et al., 2022). Xiong and Wang (2005) estimated the LC_{50} on seed germination of Chinese cabbage to be 0.348 mM/L bulk Cu^{2+} which is approximately equivalent to 27.68 mg/L bulk CuO.

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Table-1. No observed effect concentrations, lowest observed effect concentrations, and median toxic concentrations (mg/L) of cabbage (Scorpio) and pechay (Black Behi) seedlings exposed to different concentrations of CuO nanoparticles and bulk CuO. TC_{50} data are mean ± standard deviation of n = 12.

Crop/Cu test solution	NOEC	LOEC	TC_{50}
Cabbage in CuONP	3.16	10	29.06 ± 1.75
Cabbage in bulk CuO	31.62	100	353.58 ± 16.41
Pechay in CuONP	10	31.62	71.72 ± 3.03
Pechay in bulk CuO	100	316.23	371.52 ± 22.79

The concentration at which phytotoxicity in terms of root growth suppression depends greatly on the type of Cu as well as the physical and chemical nature of the plant species. Even different genotypes of the same species can exhibit different sensitivities to the same type of NP (Feigl, 2023). In both test crops, the dosage of bulk CuO required to cause toxicity in cabbage and pechay seedlings in terms of seedling growth was higher than CuONP. Thus, CuONP is more phytotoxic than bulk CuO. Ahmed et al. (2019) also found CuONP to be more phytotoxic in radish than bulk CuO.

Due to their small size, NPs may be able to cross (or damage) cell wall, then enter root cells and even the nucleus, causing oxidative damage (Rajput et al., 2021). Their small size also means that NPs exhibit higher reactivity or catalytic properties as compared to their bulk counterparts, thus making NPs more toxic. Copper is an essential micronutrient. However, exposure to excess Cu can cause toxicity in plants (Mosa et al., 2018).

Wu et al. (2012) studied if the biological effects of CuONP in aqueous solutions are closely related to the concentration of released metal ions. They concluded that the phytotoxicity of CuONP is not only from dissolved ions but also from their interactions with the seed and root surfaces. Once inside plant tissues, dissolution of CuO occurs due to reduced pH and interaction with organic acids and proteins reducing CuO to Cu^{+} and Cu^{2+} (Wang et al., 2012; Shi et al., 2013). Due to the redox property of Cu, the free ions can enhance the production of ROS causing oxidative stress damaging lipids, proteins, and nucleic acids then eventually cell death (Mosa et al., 2018; Chung et al., 2019). Copper toxicity also results in enzyme inhibition and protein dysfunction due to interactions between the metal and the sulfhydryl groups of proteins (Ibrahim et al., 2022).

The typical symptoms of roots under Cu stress are discoloration, reduced primary root elongation, and deformation (Xu et al., 2024). These issues are believed to be related to the rupture of root epidermis and exodermis (Chen et al., 2022). Such symptoms have been observed in cabbage and pechay seedlings treated with high concentrations of CuONP and bulk CuO. The brown discoloration of the seedlings is attributed to Cu accumulation in the roots and the subsequent translocation to the shoots, as confirmed by the AAS results. Excess Cu is also reported to cause auxin, abscisic acid, and/or cytokinin imbalance, resulting in lignin deposition and remodeling of the root system architecture. This specifically results in inhibition of primary root growth and stimulation of lateral root formation (Chen et al., 2022; Xu et al., 2024), which were observed in some seedlings at T4 and T5.

Influence of copper oxide nanoparticles on physiological and biochemical parameters

Catalase and ascorbate peroxidase

ROS are free radicals and non-radical molecules which include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$), and singlet oxygen ($^1O_2$). ROS are normally produced in chloroplasts, mitochondria, and peroxisomes as byproducts in plant metabolic pathways (Sofo et al., 2015; Xu et al., 2024). They are then scavenged by the plant's antioxidant defense system, which can either be enzymatic or nonenzymatic (Sofo et al., 2015). Under Cu stress, enhanced production of ROS occurs in plants. One of the main mechanisms by which plants maintain the optimal level of Cu and ROS homeostasis is by the production of different types of antioxidants (Chen et al., 2022).

Two of the main enzymatic H_2O_2 scavenging mechanisms in plants involve catalase and ascorbate peroxidase. Catalase, which mainly occurs in

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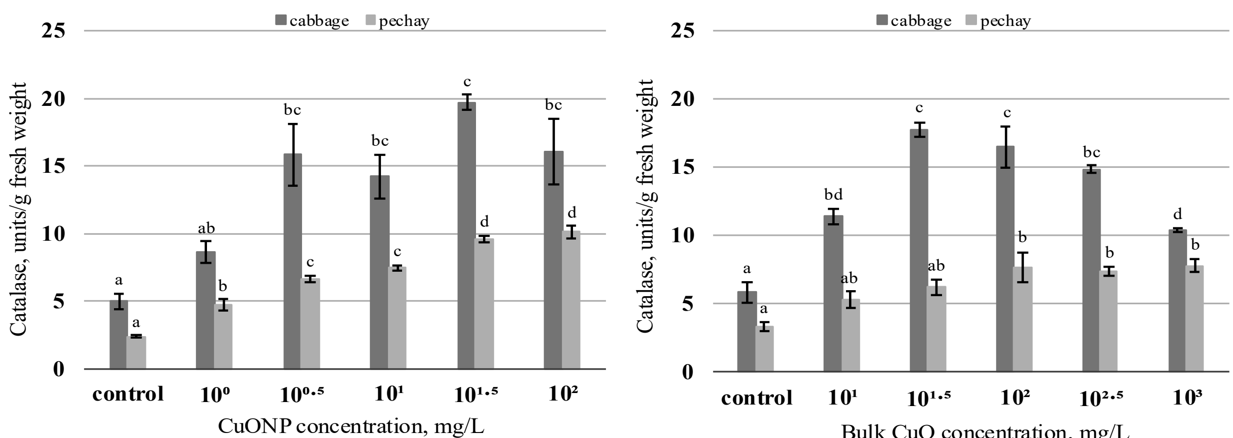
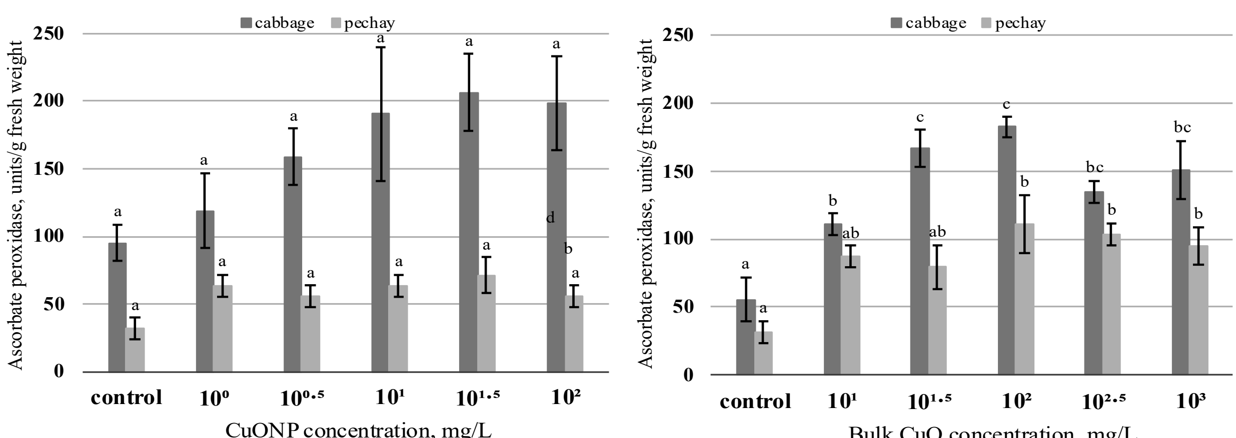
peroxisomes, directly acts on H_2O_2. While ascorbate peroxidase, which is abundant in chloroplasts, cytosol, mitochondria, and peroxisomes, has a higher affinity to H_2O_2 but requires ascorbate as a reducing agent (Sofo et al., 2015).

Results from catalase and ascorbate peroxidase assays are presented in Figure 2. Generally, all treatments of Cu test solutions increased the amount of antioxidant enzymes compared to the control seedlings. The increased expression of these two enzymatic antioxidants in plants suggests a protective mechanism against the development of oxidative stress (da Costa and Sharma, 2016; Singh et al., 2017). The upregulation of catalase in crops treated with CuONP was also reported by Singh et al. (2017) in cauliflower and Chung et al. (2019) in turnip. The decline in catalase activity in cabbage exposed to 10^3 mg/L bulk CuO is probably due to enzyme damage from Cu accumulation as well as excessive ROS generation (Wang et al., 2020).

According to Sofo et al. (2015), changes in the balance of these antioxidant enzymes result in compensatory mechanisms, such as the upregulation of ascorbate peroxidase when catalase activity is reduced and vice versa. This is evident in cabbage exposed to CuONP and bulk CuO, where levels of ascorbate peroxidase are low due to the relatively high catalase activity. In addition, the higher levels of catalase and ascorbate peroxidase in cabbage exposed to CuONP and bulk CuO than in pechay are consistent with the LOEC and TC_{50} values, indicating that both types of CuO are more phytotoxic in cabbage than in pechay.



(a)



(b)

Figure-2. (a) Catalase and (b) ascorbate peroxidase activities in cabbage (Scorpio) and pechay (Black Behi) seedlings exposed to different concentrations of CuO nanoparticles and bulk CuO. Data are mean ± standard error of n = 3. Means of the same letters are not significantly different (HSD, $\alpha = 5\%$).

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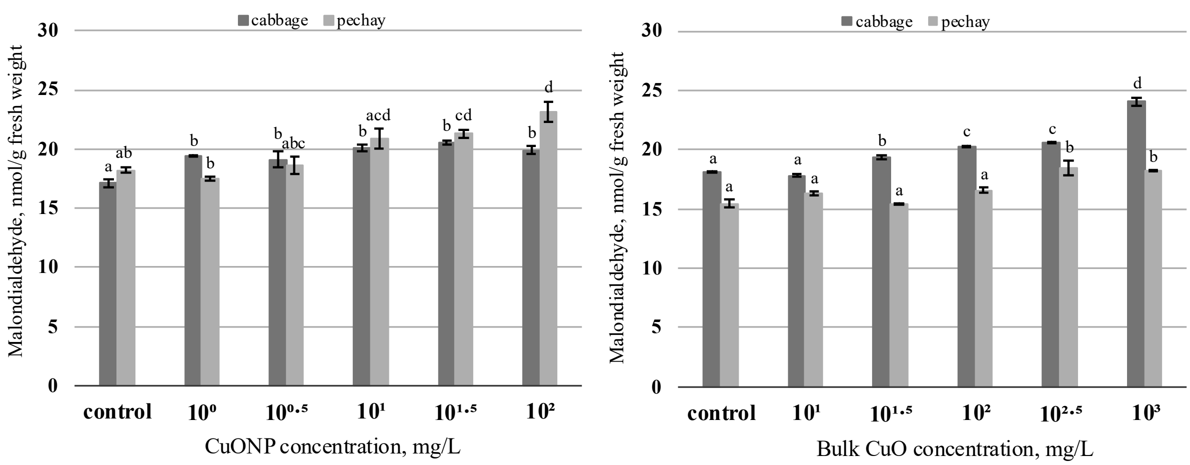
Lipid peroxidation

Even with elevated activities of antioxidant enzymes, incomplete ROS scavenging may still occur when the enzyme's maximum catalytic activities are exceeded, resulting in continuous ROS generation (Roy et al., 2022). Copper ions catalyze the overproduction of ROS, such as the formation of •OH from H_2O_2, via Fenton and Haber-Weiss reactions (Mosa et al., 2018). Among ROS, •OH is the most reactive and can initiate lipid peroxidation of plasma and organelle membranes by abstracting H atoms from methylene groups of polyunsaturated fatty acids (Roy et al., 2022). One of the major products of lipid peroxidation is malondialdehyde, which is an indicator of the extent of cell damage due to ROS (Singh et al., 2017; Chung et al., 2019). Lipid peroxidation can alter membranes that may have negative impacts on membrane permeability and normal cellular metabolic processes, resulting in cell death (Xu et al., 2024).

Figure 3 shows the degree of lipid peroxidation in control and treated cabbage and pechay seedlings. The malondialdehyde levels in cabbage significantly increased at concentrations of $\geq 10^0$ mg/L CuONP and $\geq 10^{1.5}$ mg/L bulk CuO. In contrast, pechay showed increased malondialdehyde at higher concentrations: $\geq 10^{1.5}$ mg/L CuONP and $\geq 10^{2.5}$ mg/L bulk CuO. This indicates that CuONP is more phytotoxic than bulk CuO in terms of membrane damage, and both types of CuO are more phytotoxic in cabbage than in pechay. These results are consistent with the observations from the enzyme assays and the influence on seedling growth.

When compared to other *Brassica* species, significantly high malondialdehyde levels were measured in turnip at ≥250 mg/L CuONP (Chung et al., 2019) and cauliflower at ≥50 mg/L CuONP (Singh et al., 2017). These results further emphasize that the toxic effects of Cu (and other metals) depend on plant species and type of Cu (Feigl, 2023).



(a) (b)

Figure-3. Malondialdehyde levels in cabbage (Scorpio) and pechay (Black Behi) seedlings exposed to different concentrations of (a) CuO nanoparticles and (b) bulk CuO. Data are mean ± standard error of n = 3. Means of the same letters are not significantly different (HSD, α = 5%).

Photosynthetic pigments

About 30% of Cu in plants can be found in chloroplasts, particularly in thylakoids, where it serves an important role in several photosynthetic proteins and complexes, including those involved in the biosynthesis of pigments. Evidence also shows that photosynthesis is an early target of Cu toxicity since plants exposed to excess Cu have decreased chlorophyll and decreased photosynthetic rate. These toxic symptoms are attributed to several factors, such as structural damage to chloroplast and thylakoid membranes, inhibition of photosystem II, downregulation of rubisco, and replacement of cofactors in chlorophyll and other proteins (Xu et al., 2024). Thus, the pigment content of plants is also an important indicator of the phytotoxicity of Cu-based NPs.

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Figure 4 indicates that neither CuONP nor bulk CuO significantly affected the levels of chlorophyll a, chlorophyll b, or carotenoids in cabbage and pechay seedlings. In contrast, notable pigment reductions were reported in turnip (Chung et al., 2019) and cauliflower (Singh et al., 2017) following exposure to varying concentrations of CuONP and/or bulk CuO.

Young and immature seedling leaves contain very few chloroplasts. In *Brassicas*, pigment accumulation typically occurs within 1 – 3 weeks old leaves (Lefsrud et al., 2007). In the present study, both crops were incubated in Cu test solutions in complete darkness. According to Mastropasqua et al. (2020), upregulation of the genes involved in the biosynthesis of chlorophyll and carotenoids occurs in the presence of light. Therefore, the low pigment levels observed in cabbage and pechay seedlings may explain why CuONP and bulk CuO did not have any effect in this study.

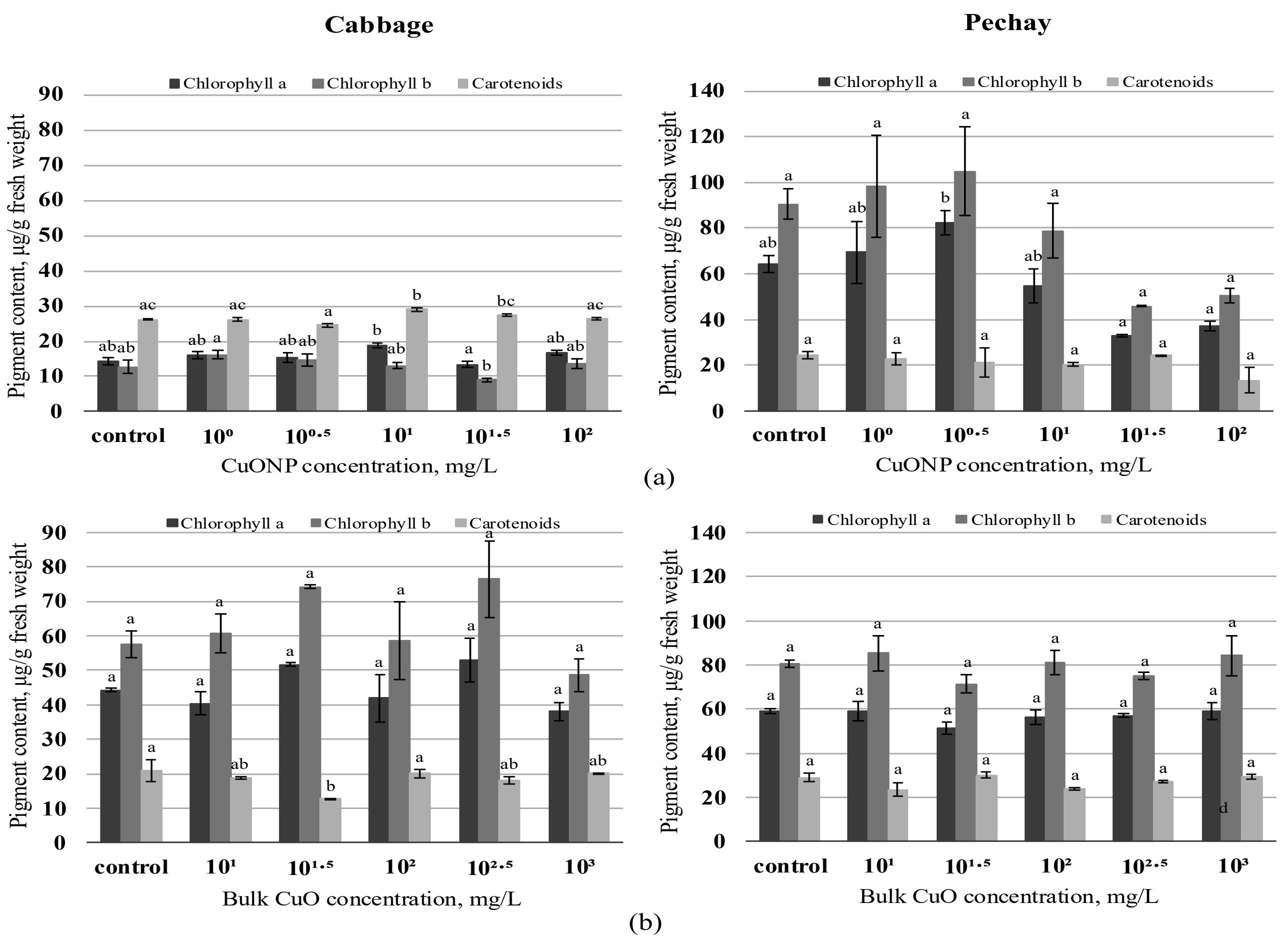

Figure-4. Pigment content of cabbage (Scorpio) and pechay (Black Behi) seedlings exposed to different concentrations of (a) CuO nanoparticles and (b) bulk CuO. Data are mean ± standard error of n = 3. Means of the same letters are not significantly different (HSD, α = 5%).

Uptake and distribution of copper oxide nanoparticles in cabbage and pechay seedlings

The uptake of Cu via root hairs to the vascular system follows both symplastic and apoplastic routes. Then the translocation and distribution of Cu within the plant occur mostly through the xylem. Wang et al. (2012) reported that during the translocation process, CuO are reduced to form Cu ions. In plants, different Cu transporters and Cu chaperones have been identified as responsible for facilitating the transport and entry of Cu into cells and to their target sites (Chen et al., 2022). Internalization of NPs is limited by the size of pores in the cell wall, which is 5 – 20 nm depending on plant species. However, it was observed that NPs can either create new pores or enlarge the existing pores, which enhances their uptake by plants (Fatima et al., 2020; Djanaguiraman et al., 2024). Some studies have shown that NPs with up to 40 – 50 nm diameter were still able to enter plant cells (Djanaguiraman et al., 2024).

To confirm the entry of Cu into the treated cabbage and pechay seedlings, which may have caused the phytotoxic symptoms observed, the concentrations of Cu in roots and shoots were measured using AAS (Figures 5 and 6). For both cabbage and pechay seedlings exposed to each Cu test solution, a dose-dependent increase in the levels of Cu in roots and shoots was observed, which is consistent with the results on the influence of Cu on seedling growth. In all treatments, the amount of Cu in roots is greater than in shoots since roots are the first tissue to be exposed to the growth medium and are therefore exposed to the toxicant over a longer period of time (Adhikari et al., 2012).

Moreover, at the same concentrations of CuONP and bulk CuO, higher levels of Cu were measured in seedlings treated with CuONP as compared to seedlings exposed to bulk CuO, which could be attributed to the small size of NPs allowing their significantly higher uptake by the test crops. Consequently, bulk CuO exhibited less phytotoxic effects in both cabbage and pechay seedlings as compared to CuONP due to its significantly lower accumulation. The same observations have been generally reported in previous studies comparing the uptakes of CuONP and bulk CuO in other crops, such as radish (Ahmed et al., 2019).

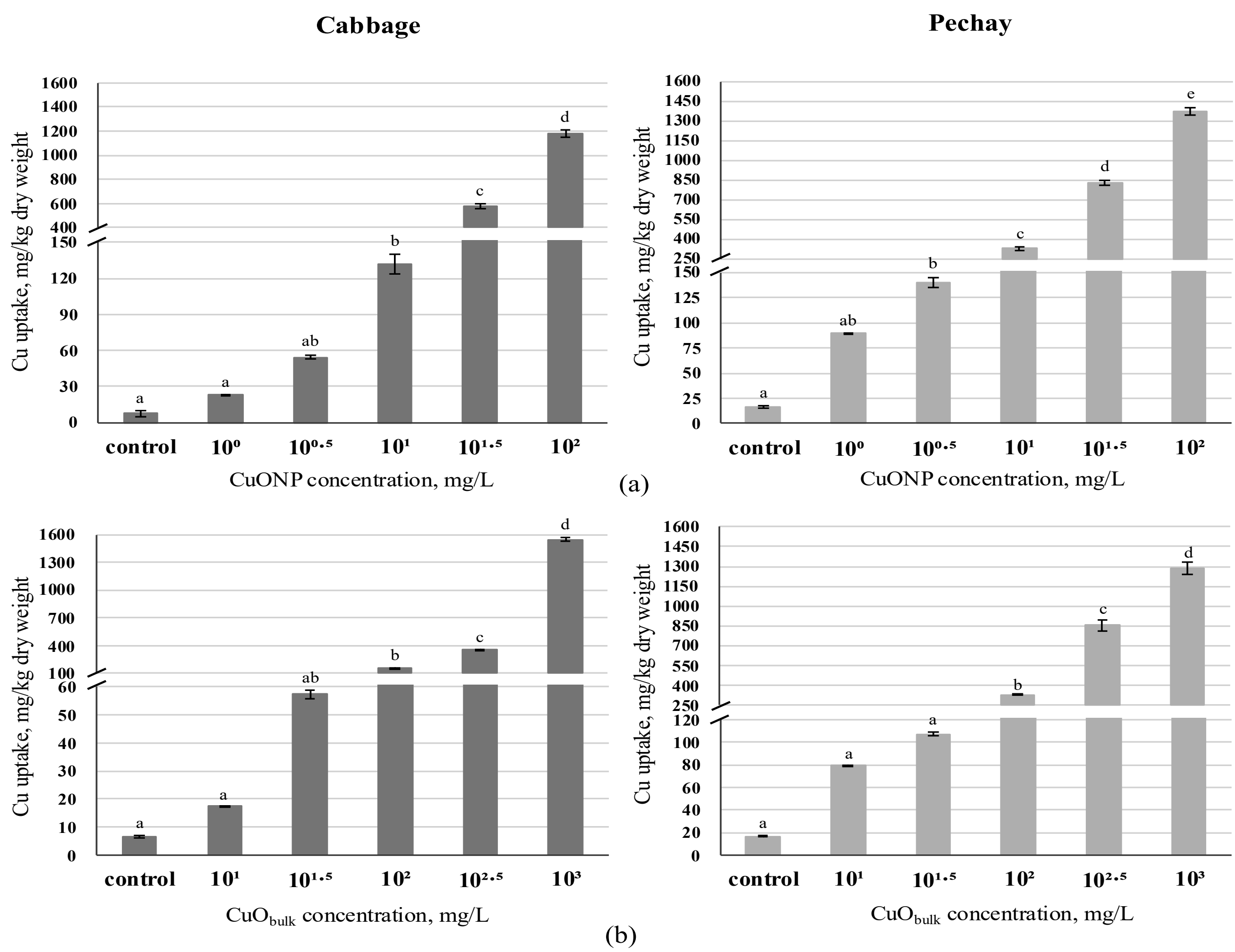

Figure-5. Copper uptake in roots of cabbage (Scorpio) and pechay (Black Behi) seedlings exposed to different concentrations of (a) CuO nanoparticles and (b) bulk CuO. Data are mean ± standard error of n = 3. Means of the same letters are not significantly different (HSD, α = 5%).

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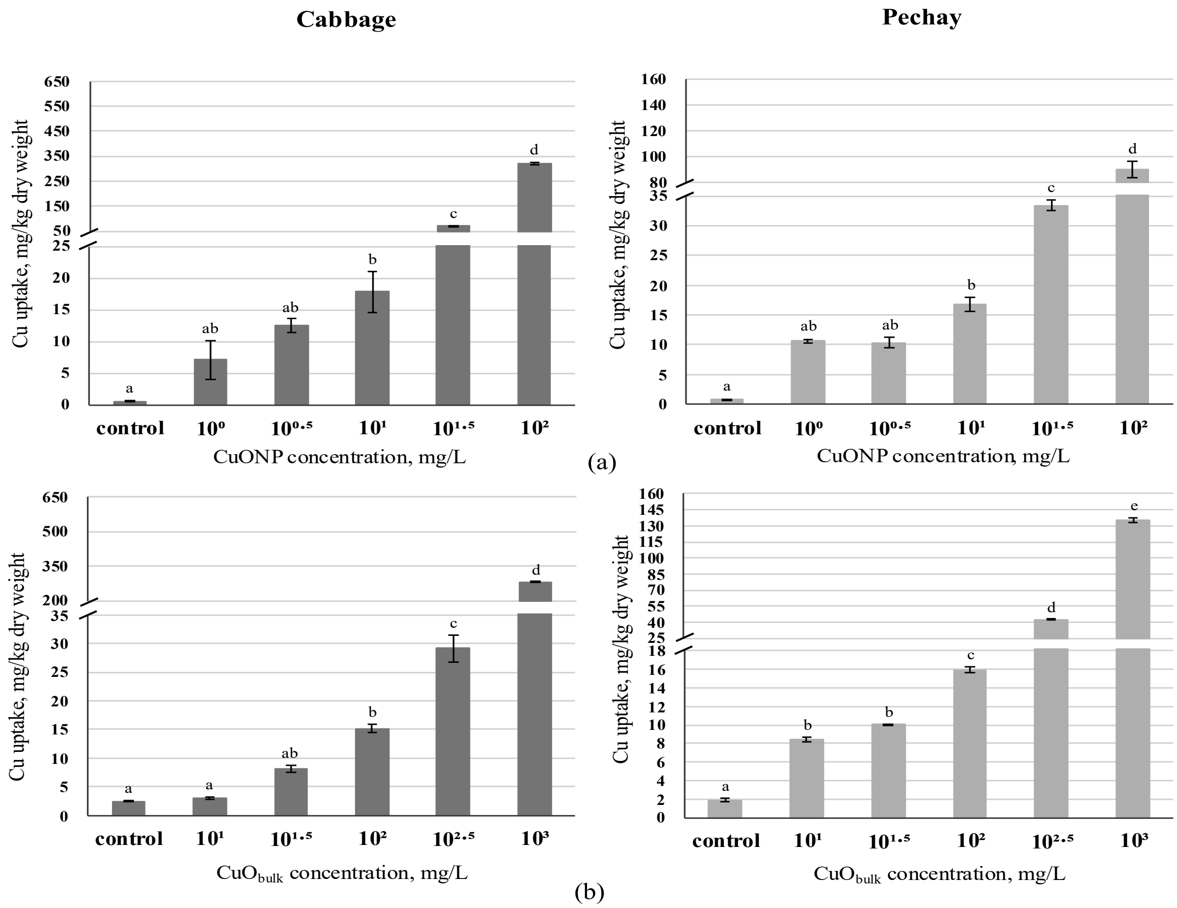

Figure-6. Copper uptake in shoots of cabbage (Scorpio) and pechay (Black Behi) seedlings exposed to different concentrations of (a) CuO nanoparticles and (b) bulk CuO. Data are mean ± standard error of n = 3. Means of the same letters are not significantly different (HSD, α = 5%).

The efficiency of uptake and transport of Cu depends on the unique physiology of the plant species (Fatima et al., 2020; Feigl, 2023). However, the exact mechanism underlying the species-dependent uptake of Cu-based nanoparticles remains poorly understood (Feigl, 2023). Zhang et al. (2019) studied the uptake, translocation, and biotransformation of ceric oxide (CeO_2) NP in four plant species, including cabbage. The authors concluded that different species have different abilities of transforming and translocating Ce, and their mechanisms depend on the chemistry of the surrounding solution (i.e., with or without phosphate), xylem structure, and root exudate compositions. The amount of root exudates also varies among plant species, which may explain the difference between the *Brassica* species studied in terms of the phytotoxic effects of both types of CuO.

Accumulation of higher Cu levels in roots than in shoots results in more significant inhibition of root growth. Peng et al. (2015) reported that CuONP can easily enter the roots' epidermis, exodermis, and cortex, and the formation of lateral roots may provide a pathway for NPs to enter the xylem and phloem. Secretion of root mucilage also increases metal uptake by plants (Singh et al., 2017). It has been suggested that the higher level of metals in roots is due to the plants' defense mechanism to withstand metal toxicity and regulate their transport to shoots, which is the more physiologically active (and usually edible) part (da Costa and Sharma, 2016; da Costa et al., 2020). Storage of excess metals in vacuoles and cell walls as well as sequestration by organic compounds (i.e., citrate, malate) have been reported to prevent metal toxicity (da Costa et al., 2020). When CuONP is translocated to the shoots, they were found bound to cysteine, citrate, and phosphate ligands (Peng et al., 2015). However, exposure to very high concentrations of Cu may exceed the detoxification capacity of organelles or ligands, resulting in damage to the

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plants' antioxidant defense system, thus allowing the metal to exhibit its toxic effects.

The translocation factors for all treatments of cabbage and pechay seedlings are <1, indicating low root-to-shoot translocation of Cu regardless of the particle size. The same pattern was also reported by Singh et al. (2017) in cauliflower. Translocation of cations in dicots, such as cabbage and pechay, is slower since their xylem is more negatively charged than in monocots (Zhang et al., 2019).

The permissible limit of Cu in plants is up to 10 mg/kg (WHO, 1996 as cited by Rajput et al., 2021). In both cabbage and pechay shoots, this maximum allowable limit set by WHO is exceeded at $\geq 10^{0.5}$ mg/L CuONP and $\geq 10^{2}$ mg/L bulk CuO. Although minimal, the translocation of Cu-based NPs from roots to shoots indicates that plants can serve as significant pathway for entry into the food chain and ecosystem (Wang et al., 2012). This finding calls for further investigation in mature plants and field settings.

The findings suggest that the safe application of CuONP in agricultural systems requires precise dosage optimization for each crop species. For example, lower CuO concentrations can improve cabbage productivity compared to pechay. Furthermore, the use of CuONP as fertilizers demands substantially lower concentrations than bulk CuO, reducing environmental and health risks (Feigl, 2023). However, these results are based on short-term controlled laboratory experiments and may not directly translate to field conditions, where factors such as soil microbial interactions, weather variability, and nutrient dynamics were not replicated. Nevertheless, the study provides essential baseline data on CuONP impacts that can inform future field research.

Conclusion

Increasing concentrations of CuONP and bulk CuO have significant inhibitory effects on seedling growth of cabbage and pechay, particularly in root elongation. High treatment concentrations also resulted in upregulation of antioxidant enzymes (catalase and ascorbate peroxidase) and increased lipid peroxidation. No significant effect on pigment content (chlorophyll a, chlorophyll b, and carotenoids) was observed. In all experiments conducted, bulk CuO showed to exhibit less phytotoxicity in both test crops as compared to CuONP. AAS analysis confirmed that the observed phytotoxic symptoms may be attributed to the accumulation of Cu, which increased with treatment concentration. Moreover, the translocation of Cu from roots to shoots was low for all treatments in both crops.

Therefore, cabbage and pechay uptake and accumulate CuONP, causing oxidative stress with adverse effects on plant growth and development. The findings indicate that CuONP is more phytotoxic than bulk CuO in both crops, with both types of CuO being more harmful to cabbage than to pechay. The TC_{50} values are 29.06 ± 1.75 mg/L for CuONP and 353.58 ± 16.41 mg/L for bulk CuO in cabbage, while in pechay, the TC_{50} values are 71.72 ± 3.03 mg/L for CuONP and 371.52 ± 22.79 mg/L for bulk CuO. This study demonstrates that Cu-based NPs are absorbed by crop seedlings, suggesting a potential pathway for their entry into the food chain. Further research involving mature plants and field conditions is recommended.

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Contribution of Authors

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Arreola SLB & Recuenco MC: Conceptualization and writing – review and editing.

Fernando-Corpuz LM: Conceptualization, analysis and writing – review and editing.

Hizon-Fradejas AB: Conceptualization, resources, supervision and writing – review and editing.

All authors read and approved the final draft of the manuscript.

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