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The Effects of Copper Oxide Nanoparticles on the Photosynthesis Characteristics and Antioxidant Enzymes of Radish (Raphanus Sativus L.) Plants

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ABSTRACT

The increase in metal nanoparticles (NPs) in the vegetable cultivation environment may cause their contamination through foliar absorption. This study investigated the effect of different concentrations of copper (Cu)-NPs (0, 50, 100, 200, 300, and 400 mgL^{-1}) on the early growth stages of radish plants and evaluated their physiological and chemical characteristics. The study also examined the toxic effects of Cu oxide (CuO)-NPs, as a source of air stress, on plant growth, photosynthesis pigments, and antioxidant systems in radish plants. The reduction in chlorophyll a, b and carotenoid content were not significant at the 50 and 100 mgL^{-1} concentrations of CuO. However, plants treated with 300 mgL^{-1} and 400 mgL^{-1} demonstrated a significant decrease in chlorophyll pigments. Based on the results, the Cu content of radish leaves increased with increasing Cu concentration, so that the highest Cu content of leaves was observed at a concentration of 400 mgL^{-1}. Thus, CuO nanostructures had a negative effect on photosynthesis pigments and antioxidant enzymes. The results revealed that the uncontrolled release of nanostructured materials into the atmosphere in an agricultural environment may reduce physiological processes.

List of Abbreviations: APX: Ascorbate Peroxidase; POD: Peroxidase; CAT: Catalase; SOD: Superoxide Dismutase; MDA: Malondialdehyde and H_2O_2: Hydrogen Peroxide

1. INTRODUCTION

One of the key properties of radish phytochemicals is their composition. Anthocyanin pigments make radish roots red. Moreover, their strong ability to synthesize isothiocyanates gives them their distinctive flavor, which is highly popular in some countries, including Japan, the Philippines, and Hawaii [1]. A number of metals, such as copper (Cu), zinc, nickel, molybdenum, manganese, and iron, are essential trace elements that participate in normal growth, reduction reactions, electron transfer, and many other oxidation-metabolic processes. However, their excess in soils causes metabolic disorders and growth inhibition in most plant species [2]. Cu oxides (CuO) have gained extensive importance among metal oxide NPs, considering their distinctive properties and diverse applications in different fields [3]. CuO-NPs are increasingly used in various agricultural sectors, including pesticides, herbicides, and fertilizers. Nonetheless, there are some major concerns about the role of these substances in human health [4]. Therefore, it is recommended that more Cu measurements in different vegetable species be performed to determine the potential risks of Cu-NPs [5]. At the cellular level, Cu is a structural and catalytic component of many proteins involved in a wide range of metabolic processes [6]. Plant cells frequently encounter a wide variety of harmful environmental factors, limiting plant growth and agricultural output. Ecological sensors (e.g., plants) are extremely vulnerable to the fast advancement of nanotechnology and the haphazard release of NPs into the environment that contain metals. Various physical, chemical, biological, or hybrid processes can be employed to create engineered NPs [7]. Evaluating the effects of nanopollutants on the environment requires knowledge of the intricate processes through which plants react to these tiny particles. It is unknown how NPs harm plant leaves; however, plants can absorb or excrete them directly from

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surfaces. Physiological studies demonstrated that 100 and 1000 mg/L concentrations of CuO-NPs had detrimental effects on plant development, net photosynthesis, chlorophyll content, carotenoids, reactive oxygen species (ROS) buildup, and antioxidant system activity [8]. The application of CuO-NPs to annual ryegrass (*Lolium rigidum* L.), radish (*Raphanus sativus* L.), and perennial ryegrass (*Lolium perenne* L.) could induce mutagenic DNA lesions and oxidative damage, as shown by the aggregation of 4,6-diamino-5-formamidopyrimidine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 7,8-dihydro-8-oxoguanine [9]. Although different studies have been conducted, there is no clear picture of the role of metal NPs in plants. Some NPs demonstrate positive effects, whereas others display negative impacts, depending on the type of NP size and the concentration of NP [10]. Accordingly, this study aims to investigate the effect of different concentrations of CuO-NPs (i.e., 0, 50, 100, 200, 300, and 400 mgL^{-1}) on phenotypic aspects, photosynthetic pigments, antioxidant enzymes, and micronutrient levels in radish seedlings.

2. MATERIALS AND METHODS

This experiment was conducted in greenhouse conditions in Iranshahr (2024) to evaluate the effect of Cu-NPs on the characteristics of radish plants. The seeds utilized in this study (hybrid F1 "Dolphin") were taken from the gene bank of the Iran National Forestry and Pasture Research Institute. They were then surface sterilized with a 1% potassium permanganate ($KMnO_4$) solution for 20 min and thoroughly cleansed 5–6 times with distilled water. Next, the sterilized seeds were planted in plastic pots that were filled with potting mix consisting of peat moss and vermiculite (1:1). Subsequently, the seeds were manually planted and daily watered until the appearance of the first two true leaves. The plantlets were then preserved in a growth chamber under controlled conditions (250 µmolm− 2 s− 1 (photon), relative humidity of 60–70%, photoperiod of 16 hours of light/8 hours of darkness, and at a temperature of 21 ± 2°C).

A completely randomized design with three replicates and six concentrations of Cu-NPs (i.e., 0, 50, 100, 200, 300, and 400 mgL^{-1}) were considered for this study. Moreover, non-treated radish plants were considered the control. Furthermore, Cu-NPs were obtained from the Pioneers of Iranian Nano Materials Company (Figure 1). Additionally, the aqueous solution of CuO-NPs (nanopowder with a particle size of <40 nm) prepared in Milli-Q water was employed for the spray. The CuO-NPs were sprayed two times a week (50 mL for each pot; four times in total) in order to observe the proper impacts of NPs on plants. The intended radish plants were harvested 15 days after treatment to determine estimate the impact of CuO-NPs on physiological factors.

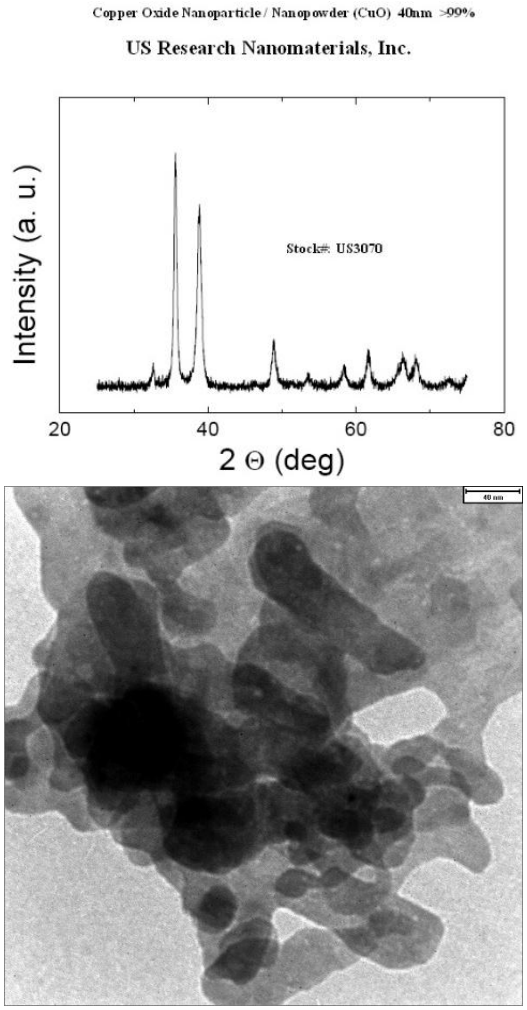

Fig. 1. Copper oxide nanoparticle / Nano powder (CuO), TEM; Purity: 99%; Color: black; APS: 40 nm; SSA: ~20 m^2/g; Morphology: nearly spherical; Bulk density: 0.79g/cm^3; True density: 6.4 g/m^3

2.1. Measurement of chlorophyll and carotenoid content

One gram of fresh radish leaves was crushed and ground, and a homogenous mixture was prepared using 10 ml of 80% acetone. One ml of the homogenous mixture was mixed with nine ml of 80% acetone and centrifuged for 15 minutes at 8000 rpm. Then, the supernatants were separated to measure total chlorophyll and carotenoids. The experiments were performed spectrophotometrically using a VIS/UV spectrophotometer and absorbance values of chlorophyll a, b and carotenoids were determined at wavelengths of 663, 645 and 480 nm, respectively. 80% acetone was used as a blank [11]. The amount of chlorophyll a, b and carotenoid were using the below formulas.

(1): Chl a = 12.21 (A663) – 2.81 (A646)
(2): Chl b = 20.13 (A646) – 5.1 (A663)
(3): Car = (1000 A470 – 3.27 [Chl a] – 104 [Chl b])/227

2.2. Determining the activity of antioxidant enzymes:

About 200 mL of extraction buffer was prepared by dissolving 2.428 g of polyvinyl pyrrolidine in 190 mL of distilled water. The obtained solution was stored in the refrigerator and away from light. This buffer was used to extract soluble proteins and antioxidant enzymes. For extracting, the first 0.25 g of leaf sample was completely crushed in liquid nitrogen, and then 1 mL of the previously prepared buffer was added to it. It was kept for 2 h in the refrigerator and vortexed for 30 seconds. Again, it was placed in the refrigerator 12 h and 30 seconds of vortexing. Then, it was centrifuged for 15 minutes at 13,000 rpm at 4°C. Finally, the upper phase was separated to read the content of soluble proteins and the activity of enzymes. Catalase enzyme activity was calculated using the method of Dazy [12]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH=7) and 15 mM hydrogen peroxide. Immediately after adding hydrogen peroxide, the light absorption was read by a spectrophotometer with a wavelength of 240 nm and after 1 minute the absorbance was read again. The change in absorbance obtained in 1 minute was divided by the molar extinction coefficient of this reaction, which is 36 millimicrons/cm. For measuring the Ascorbate peroxidase (APX) enzyme absorbance, the enzyme reaction was read at a wavelength of 290 nm at the start and after one minute of the reaction. Peroxidase activity was measured by the method of [13] and superoxide dismutase activity was measured by spectrophotometry using the method of [14].

2.3. Determination of soluble protein content

To extract soluble proteins in leaves, 1 gram of frozen leaf sample was completely powdered at -80°C in a porcelain mortar using liquid nitrogen, and then 4 ml of extraction buffer with the composition: 5% 1M Tris-hydrochloric acid (pH=7.5) + 0.2% 1M Na2EDTA + 0.04% 2-mercaptoethanol in distilled water was added to the sample and homogenized. The resulting mixture was transferred to capped tubes, followed by centrifugation at 13,000 rpm for 20 minutes, and the clear extract was separated from the solution and stored at -20°C. The quantitative measurement of soluble proteins was performed according to the Bradford method (1976) using Bio-Rad reagent in a spectrophotometer with a wavelength of 595 nm and then determined by plotting the resulting curve from the reading of protein standards prepared from bovine serum albumin at certain concentrations on graph paper [15].

2.4. H_2O_2 measurement

To prepare 10 mM phosphate buffer, 1 ml of the phosphate buffer prepared in the previous section was made up to a volume of 100 ml. One gram of leaf sample was crushed and 5 ml of 1% (w/v) trichloroacetic acid solution was added to it. 0.5 ml of the centrifuged supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer solution (pH = 7) and 1 ml of 1 M KI solution. H_2O_2 solutions were prepared at concentrations between 2-10 mM and a standard graph was drawn. The absorbance was measured using a UV-VIS spectrophotometer at a wavelength of 390 nm [16].

2.5. Measurement of MDA

Measurement of plant malondialdehyde: To measure the amount of malondialdehyde, 1 gram of fresh leaf tissue was ground in a mortar containing 20% trichloroacetic acid (TCA) for 5 minutes at 0.05 rpm. The solution was centrifuged for 4.000 rpm. To 1.5000 rpm, 5 ml of the supernatant solution was added 20% trichloroacetic acid solution. After being exposed to a temperature of 100°C for 30 minutes, then it was immediately cooled in crushed ice. The cooled mixture was centrifuged again for 10 minutes at 5000 rpm and the light absorption intensity at a wavelength of 523 was determined per gram of fresh weight [17].

2.6. Determination of ionic leakage

Measurement of ionic leakage and membrane stability index was performed by the bain-marie bath method on the leaves [18]. 0.2 g of healthy and fresh plant leaf tissue was placed in a screw-top test tube after washing with distilled water to wash away possible ions from the plant surface and 10 ml of deionized water was added to it. Then, the test tubes were placed in a hot water bath at 32°C for 2 hours and the electrical conductivity of the samples (EC1) was measured using a Metrhom EC meter (Swiss-made). After the tubes were cooled to 25°C, the electrical conductivity of the samples (EC2) was measured again, and the percentage of ion leakage was calculated using the following formula. Percentage of ion leakage = Ec1/Ec2×100.

2.7. Measurement of the RWC

Sampling was done using scissors from the last developed leaf of all the experimental treatments and immediately placed on ice. Next, their weight was measured in the laboratory with an accurate scale (the leaves should not be broken and torn). RWC is obtained by putting the numbers obtained from weighing with a scale with an accuracy of 0.001 in the following formula:

RWC = Fw – Dw / Sw – Dw × 100

Fw: Leaf fresh weight immediately after sampling

Dw: Dry weight of the leaf after placing it in the oven

Sw: Saturated leaf weight after immersion in distilled water [19].

2.8. Measurement of copper content

The amount of copper absorption was calculated from the product of the concentration of copper in the amount of dry matter and the transfer factor of copper, by dividing the concentration of copper in the aerial part by the concentration of copper in the root of corn [20].

2.9. Statistical analysis

The obtained data underwent statistical analysis by SPSS software (version 16.1) using the analysis of variance (ANOVA), and means were calculated by Duncan's test ($P \leq 0.01$).

3. RESULTS

3.1. The effects of copper nanoparticles on the photosynthesis characteristics

The results of the variance analysis of the data in the radish plant showed that application of the concentrations of copper nanoparticles on the chlorophyll a, b and carotenoid content were significant at the one percent level (data not shown). Radish plants treated with different concentrations of copper nanoparticles (50, 100, 200, 300, and 400 mgL^{-1}) for seven days showed a decrease in chlorophyll. According to these results, the lowest amount of chlorophyll a was observed in the leaves under the influence of copper nanoparticles at a concentration of 400 mgL^{-1}, and with increasing the concentration of the copper nanoparticle solution, the amount of chlorophyll a decreased in all treated plants. This performance was measured as photosynthetic performance by applying concentrations of CuO-NPs. However, due to copper's toxicity (Figure 2), it decreased and reached its lowest value. At the lowest levels (50 and 100 mgL^{-1}), the reduction in chlorophyll b content was not significant. However, plants treated with 300 mgL^{-1} and 400 mgL^{-1} showed a significant decrease in chlorophyll b content (Figure 2). The highest amount of carotenoids was observed under the influence of copper nanoparticles at a concentration of 50 mgL^{-1}, and with increasing the concentration of the copper nanoparticle solution, the amount of carotenoids decreased. The average amount of chlorophyll and carotenoids in the leaves was significantly different from the control plants (Figure 2).

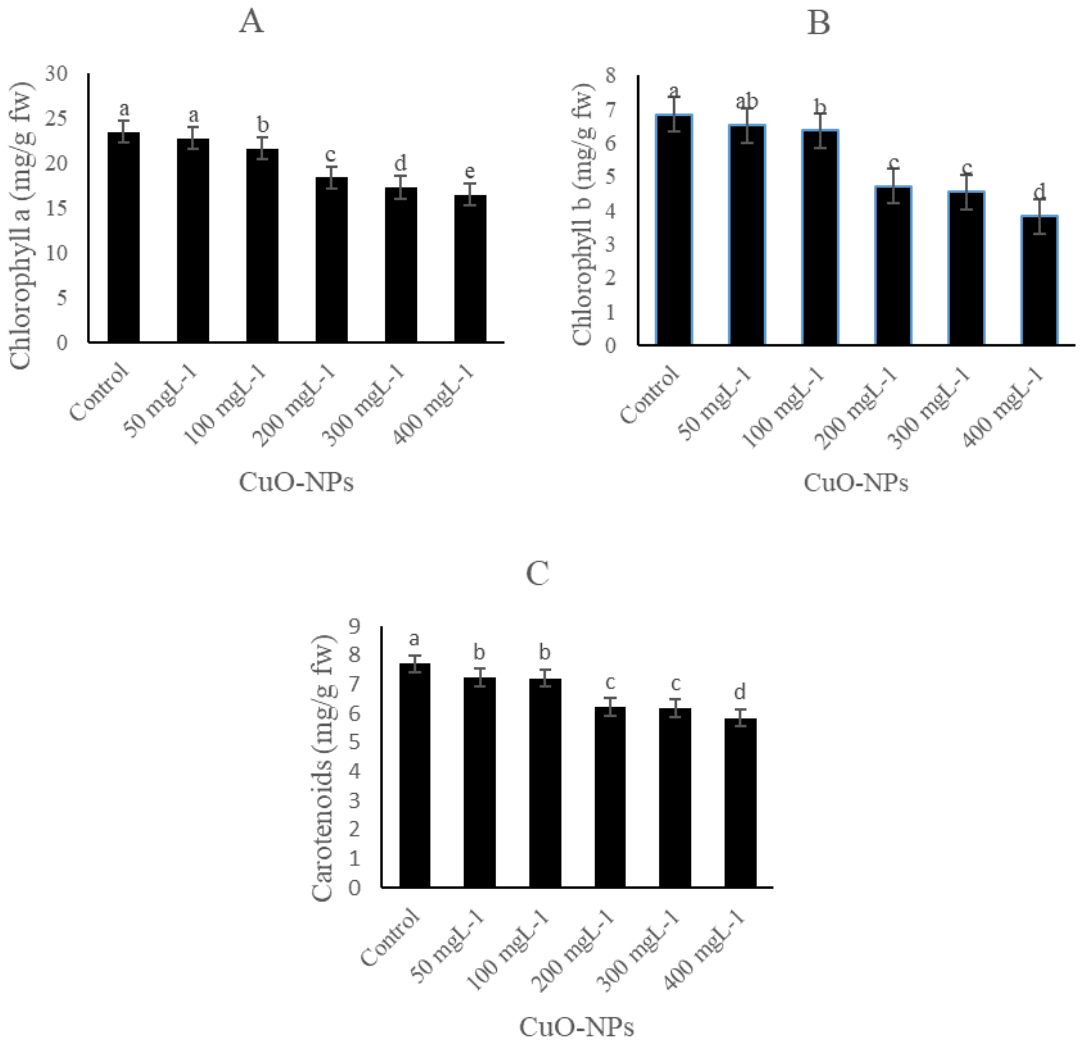

Fig. 2. Mean comparison of CuO nanoparticle on the A) chlorophyll a; B) chlorophyll b; C) carotenoids

3.2. The effects of copper nanoparticles on the antioxidant enzymes

The results of the variance analysis of the data in the radish plant showed that application of the concentrations of copper nanoparticles on the antioxidant enzymes content were significant at the one percent level (data not shown). The ascorbate peroxidase and peroxidase activity increased significantly with CuO nanoparticle concentrations. In this respect, the most induction (3.12 and 0.858 ΔAbs min-1 mg protein, respectively) was related to 400 mgL^{-1} CuO-NPs (Figure 3). Catalase and superoxide dismutase enzymes were also affected by different concentrations of copper nanoparticles, and with increasing concentration of

nanoparticles, the levels of these enzymes also increased, so that the highest levels of catalase and superoxide dismutase enzymes (0.927-2.045 ΔAbs min-1 mg protein, respectively) were observed at a concentration of 400 mgL^{-1} of copper nanoparticles (Figure 3).

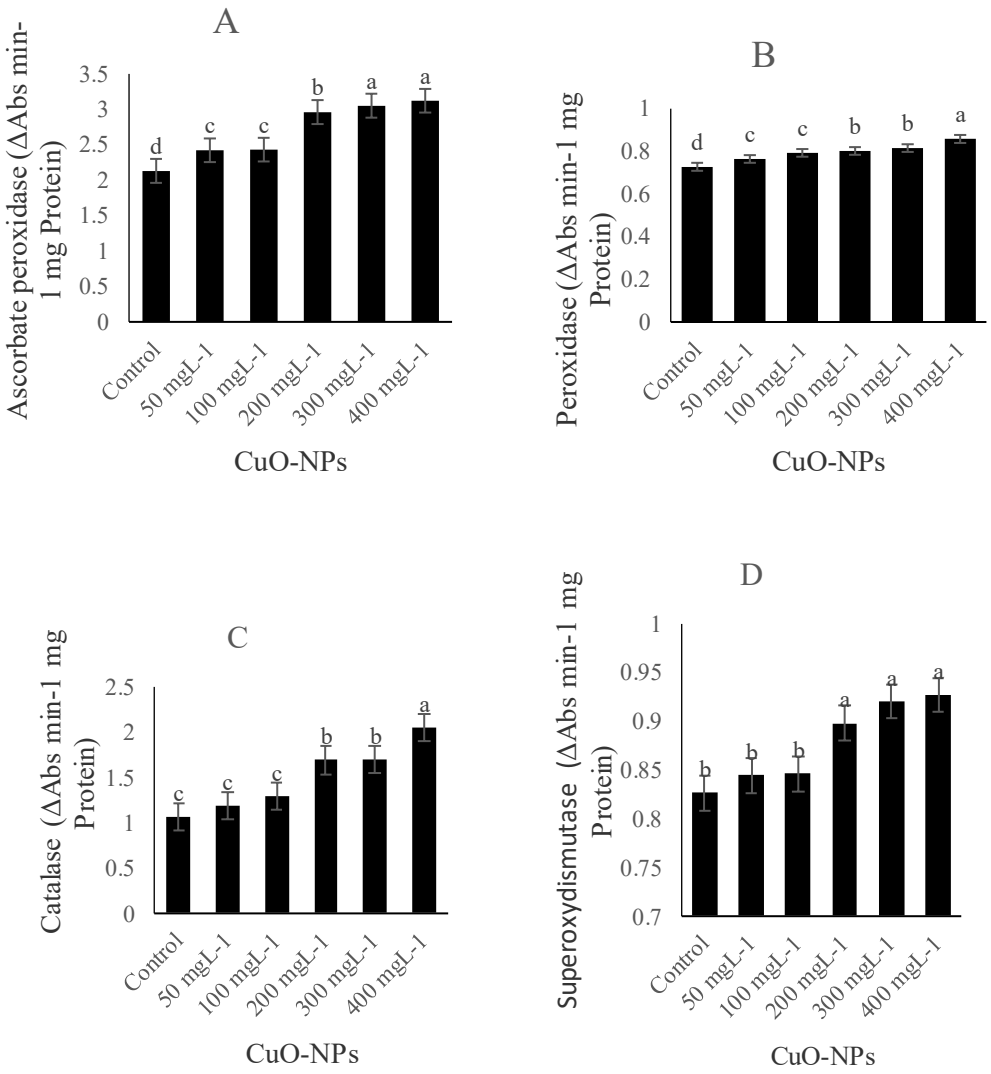

Fig. 3. Mean comparison of CuO nanoparticle on the A) ascorbate peroxidase; B) peroxidase; C) catalase; D) superoxy dismutase

3.2. The effects of copper nanoparticles on the protein, H_2O_2, malondialdehyde and electrolyte leakage content

The results of the variance analysis of the data in the radish plant showed that application of the concentrations of copper nanoparticles on the protein, H_2O_2 and malondialdehyde content were significant at the one percent level (data not shown). In this study, the protein contents of radish leaves were remarkably increased by 38.9% and 40% following treatment with 300 and 400 mgL^{-1} CuO-NPs (Figure 4). Based on the results, Cu-NPs increased the content of H_2O_2 and malondialdehyde in radish plants (Figure 4). ANOVA results related to the radish plant revealed that the application of the concentrations of Cu-NPs had a significant effect on the electrolyte leakage content at the 1% level (data not shown). More precisely, radish plants demonstrated high sensitivity to Cu-NPs and increased electrolyte leakage under different NP treatments (Figure 4).

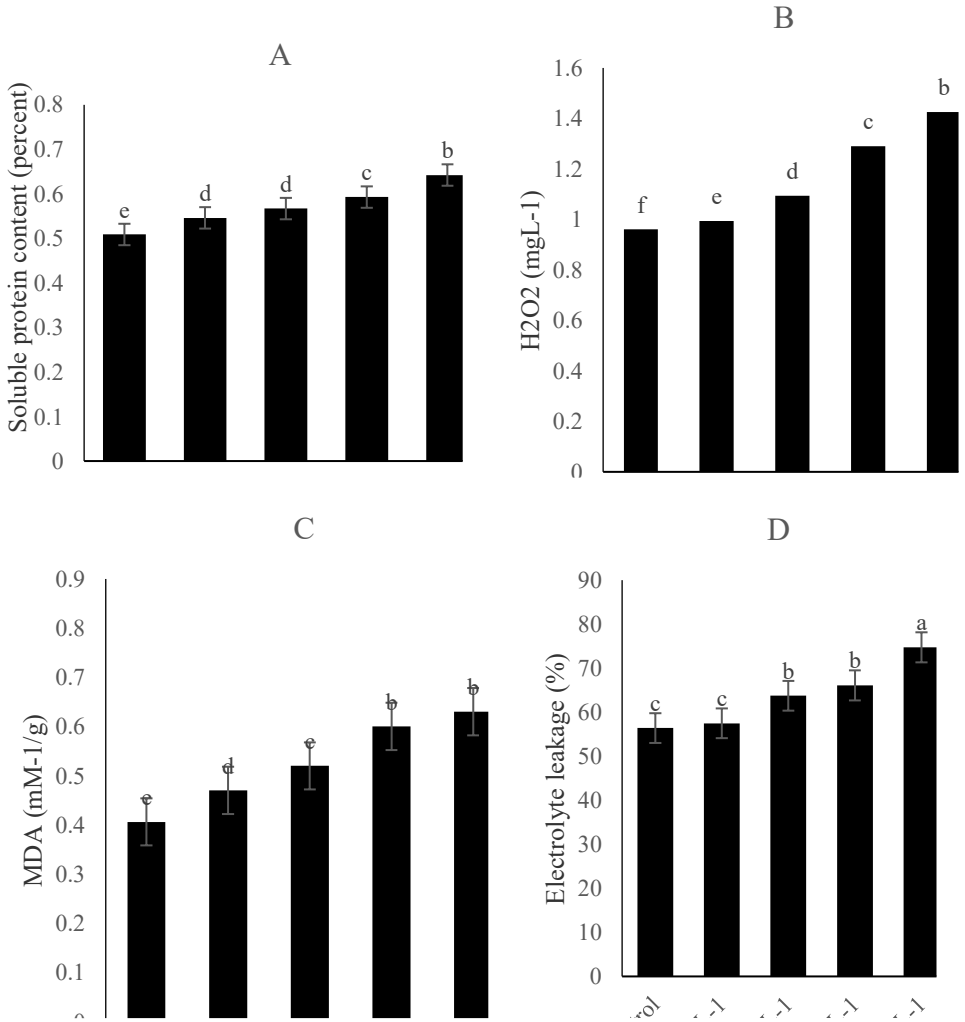

Fig. 4. Mean comparison of CuO nanoparticle on the A) soluble protein content; B) H_2O_2; C) MDA; D) electrolyte leakage

3.2. The effects of copper nanoparticles on the RWC and Cu content

The relative water content of the leaves was affected by different concentrations of copper, and with the increase in the concentration of copper nanoparticles, the relative water content also decreased. Therefore, the highest relative water content of radish leaves was observed in the control (Figure 5). Also, the copper content of radish leaves increased with increasing copper concentration, so that the highest copper content of leaves (7.26 ppm) was observed at a concentration of 400 mgL^{-1} (Figure 5).

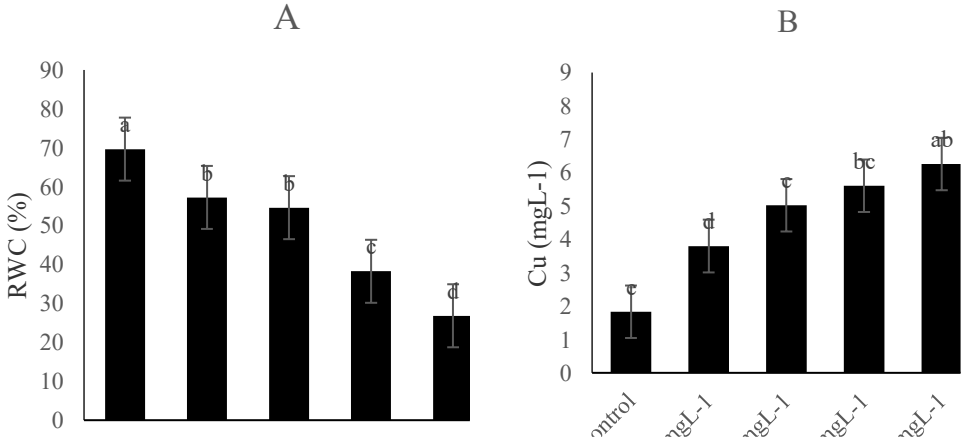

Fig. 5. Mean comparison of CuO nanoparticle on the A) RWC content; B) cu content

4. DISCUSSION

A decrease in chlorophyll a, chlorophyll b, and carotenoid was observed in the treated plants compared to controls. Nevertheless, this response was different according to the concentration of NPs. However, when the concentrations were increased (300 and 400 mgL^{-1}), clear phototoxic effects were observed (Figure 2). This result may be due to excessive lipid peroxidation under higher oxidative stress (OS), which changes leaf thickness and anatomy. It might further result from the reduced availability of mineral elements leading to iron depletion because of the antagonism between Cu and iron absorption [21]. A significant decrease was found in the photosynthetic activity after the foliar application of CuO-NPs [22]. In addition, the photosynthetic rate and pigment contents declined with a complete loss of PSII photochemical quenching at 1000 mgL^{-1} CuO-NPs [23]. Additionally, reducing pigment contents in plant leaves could affect the photosynthetic rate [24]. The reduction of chlorophyll in plants upon exposure to CuO-NPs may be directly attributed to OS [25]. Published studies have shown that high amounts of Cu decrease photosynthesis due to altered photochemical reactions in photosystem II (PSII) and damage to plant growth [26]. CuO-NPs affected chlorophyll fluorescence in *Lemna gibba*, causing a decrease in the maximal PSII yield and the operational quantum yield of PSII [27]. Likewise, the reduction in ΦPSII and ETR characterizes a decrease in the photochemical efficiency of PS II, resulting in excess electron accumulation, ROS production, and subsequent photodamage to the plant [28]. Increasing the concentration of CuO-NPs had a substantial impact on the photosynthetic rate in this investigation [24]. Plants have several antioxidative defense systems to scavenge toxic radicals to protect themselves from OS. The first class includes non-enzymatic antioxidants, such as glutathione (GSH), ascorbic acid (AsA), phenolic compounds, proline (Pro), flavonoids, and carotenoids. The second consists of antioxidative enzymes, which comprise SOD, ascorbate peroxidase (APX), CAT, and glutathione reductase (GR) [29]. Among these antioxidants, peroxidases are involved in the intracellular detoxification of H_2O_2 by oxidizing distinct chemical substrates [30]. In addition, SOD is a typical enzyme with significant antioxidant potency, as its activity directly modulates the amount of $O^{2-}$and H_2O_2. [31]. The leaves of *Annona muricata* L. displayed notable changes in flavonoids, anthocyanins, phenylalanine ammonia lyase, and POD activity after being exposed to Cu-NPs at concentrations of 250, 500, 750, and 1000 ppm over 120 hours [32]. Plants with robust antioxidant systems that can remove harmful ROS from cells and tissues can better withstand metal stress [33]. The O^{2-} and H_2O_2 are considered primary ROS. Furthermore, the generation O_2^- is mainly associated with electron transport chains; thus, chloroplast and mitochondria in complexes I and III, and PSI, and PSII are the major sources of O_2^- within plant cells [29].

5. CONCLUSION

This study showed that radish plants are sensitive to copper nanoparticles and the degree of sensitivity depends on the concentration used. This plant undergoes extensive morphological and physiological changes under the influence of copper nanoparticles solution, changes such as a decrease in the amount of photosynthetic pigments and a decrease in the relative amount of leaf water are among its destructive effects. The concentration of copper in the cell needs to be kept at a lower level, because this element is highly toxic due to its redox properties.

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7. Conflict of interest disclosure

The authors declare that there are no conflicts of interest regarding this article

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