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Research Article

Foliar Application of Copper Oxide Nanoparticles Increases the Photosynthetic Efficiency and Antioxidant Activity in *Brassica juncea*

Ahmad Faraz,[1,2] **Mohammad Faizan**,[3] **Shamsul Hayat**,[2] **and Pravej Alam**[4]

[1]*School of Life Sciences, Glocal University, Saharanpur, UP 247122, India*
[2]*Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh 202002, India*
[3]*Botany Section, School of Sciences, Maulana Azad National Urdu University, Hyderabad 500032, India*
[4]*Biology Department, College of Science and Humanities, Prince Sattam Bin Abdul Aziz University, Alkharj 11942, Saudi Arabia*

Correspondence should be addressed to Ahmad Faraz; ahmadfaraz53@gmail.com

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In the last few decades, use of copper oxide nanoparticles (CuO NPs) has been increased significantly that eventually included as a growth stimulator. This makes it essential to examine their impact on several plants. In the study detailed here, we investigated the effects of CuO NPs on the growth, physiological efficiency, biochemical assays, and antioxidant system in the mustard plant. Varying concentrations (0, 2, 4, 8, and 16 mg/L) of CuO NPs were applied at 25 days after sowing (DAS), and sampling took place at 30 and 45 DAS. The results indicate that CuO NPs-treated plants registered an increase in the growth and biomass over their respective control. Among different concentrations of CuO NPs (0, 2, 4, 8, and 16 mg/L), 8 mg/L proved to be the optimum foliar spray treatment and increase the chlorophyll content, net photosynthetic rate, leaf proline content, and antioxidant enzymes activity. We concluded that CuO NPs interact with meristematic cells triggering biochemical pathways conductive to an enhancement of the growth attributes. Further studies are needed to investigate the mechanisms of CuO NPs in mustard.

1. Introduction

Nanotechnology is one of the most fascinating and emerging fields to explore new things. The role of NPs has been increased for making commercial products, and they are also heavily used in industrial applications, causing health concerns by entering into the food chain. In literature, both effects (positive and negative) of NPs have been reported in plants as well as in animals. The most commonly used nanoparticles in agriculture fields are Ag, Cu, ZnO, Al, Si, Ce, Ti, and Au [1]. A number of research investigations have been conducted to evaluate the impact of NPs in agriculture. Increasing demand for NPs in various fields raises the concern about their impact on the environment and food chain. Phytotoxicity of ZnO NPs had been reported in diverse crop plants such as *Brassica napus*, *Raphanus sativus*, and *Lolium perenne* by Lin and Xing [2]. The positive result of metal oxide NPs has been reported in wheat seeds, which show evidence of enhanced nutrient use efficiency, photosynthetic activity, grain quality, and increased yield [3]. Application of SiO_2 NPs and TiO_2 NPs in soybean crop increased the germination, improved growth, and nitrate reductase activity [4]. The positive impact of NPs in plant growth raises hope for farmers as they can be used as an alternative for harmful chemical fertilizers, but still, extensive research needed in this field.

Among different nanoparticles, CuO NPs are extensively used in various fields, like in superconductors, batteries, gas sensors, etc. [5]. In agriculture, CuO NPs have been used as pesticides, herbicides, fertilizers, additives for soil remediation, and growth regulators [6]. Both positive and negative impacts of CuO NPs have been reported in plants. Shende et al. reported the positive effect of biogenic CuO NPs on the growth of pigeon pea [7]. A positive effect of CuO NPs also reported in *Vigna radiata* [8] and *Cajanus cajan* [9]. In contrast to this, the toxic effect of CuO NPs at higher concentrations has also been reported. Reduced photosynthetic rate, transpiration rate, and photosynthetic pigment have been observed by 1000 mg/L of CuO NPs in rice [10]. Moreover, inhibitory effects of CuO NPs in a dose-dependent manner are also found in mustard [11]. Similarly, the toxic effect of CuO NPs is also reported in pea [12] and *Hordeum vulgare* [13]. In comparison to other NPs, further investigation needed to find out the impact of CuO NPs in plants at the physiological and biochemical levels.

Mustard (*Brassica juncea*) is cultivated all over the world as an oilseed crop. In India, mustard is the second most important crop for oil production. Across the world, India covered 13% of area for the mustard cultivation. India is the third largest producer of the oilseed crops [14].

From the published data, it was hypothesized that nanoparticles can also be used as micronutrients to increase plant growth performances and their yields. Cu works as a micronutrient in plants, so it was assumed that nano form of Cu could also be beneficial in the same way. Following all these data, a study was designed to investigate and explore the impact of CuO NPs on the improvement and overall physiology of mustard plants. Foliar sprays of CuO NPs with concentrations (0, 2, 4, 8, or 16 mg/L) were given on mustard plants. Characterization of nanoparticles was performed using a scanning electron microscope. To our knowledge, very meagre literature is available for CuO NPs, which mediates the positive response in plants. In our study, CuO NPs show a positive effect on *B. juncea* at a lower concentration in comparison to control.

2. Material and Experimental Methods

2.1. Plant Materials. The seeds of mustard (*B. juncea*) were procured from New Delhi (Indian Agriculture Research Institute), India. The seeds selected for sowing were healthy and uniform in size. Surface sterilization of the seeds was performed using 1% sodium hypochlorite solution for the duration of 10 min than repeatedly washed with double-distilled water.

2.2. Nanoparticles Sources. Nanoparticles were purchased from Sigma-Aldrich Division Pvt. Limited. Stock solutions of 16 mg/L of CuO NPs were prepared by dissolving the CuO NPs in double-distilled water, and the final volume was made up to 500 mL in a volumetric flask. After then, required quantities, i.e., 2, 4, 8, or 16 mg/L of CuO NPs, were prepared from the initial stock solution. A surfactant, Tween-20 (purchased from Sigma-Aldrich), was added to the CuO NPs solution before spraying to get a maximum attachment of NPs on foliage.

2.3. Experimental Design and Pattern of Treatment. An experiment was conducted in earthen pots with a randomly set design. Surface sterilized seeds were sown in pots. The soil used in this experiment was sandy loam and equally mixed with green manure in the ratio of 6 : 1. Seeds were germinated in natural circumstances in the net house of the Department of Botany, Aligarh Muslim University, Aligarh, India. Total twenty-five pots were required for this experiment. These twenty-five pots were differentiated into five sets, and every set consists of five pots. Each of the 5 sets was representing one treatment with five replicates. When plants were 25 days old, spraying of CuO NPs water (control), 2, 4, 8, or 16 mg/L was given. After 30 and 45 days of sowing (DAS), plants were collected to assess the various parameters.

2.4. Microscopic Observations of the Nanoparticles. Microscopic studies had been completed using the scanning electron microscope. For this, the leaf samples were fixed in glutaraldehyde (2.5%) and in 0.05 M potassium phosphate buffer at pH 7.1 for 8 h. After this. sample was dehydrated in an ethanol series. CuO NPs were characterized by scanning electron microscopy (SEM) on JEOL JSM–6360 at 15 kV.

2.5. Measurement of Growth Characteristics and Leaf Area. Growth characteristics such as root length and shoot length, fresh and dry mass of root and shoot were measured at 30 and 45 DAS. The method followed by Khan et al. [15] taken into account to measure the growth parameters of plants. For measuring the area of leaves, a portable instrument was used know as a leaf area meter. In our experiment, ADC Bioscientific (UK) leaf area meter had been used.

2.6. Measurement of Chlorophyll Content (SPAD Value). Chlorophyll content was measured in the intact leaves of plants with the help of a SPAD chlorophyll meter (SPAD-502; Konica, Minolta sensing, Inc., Japan).

2.7. Leaf Gas Exchange Parameter. Photosynthetic characters, viz., rate of net photosynthesis (P_N), stomatal conductance (g_s), transpiration rate (E), and internal carbon dioxide concentration (C_i) of leaves were measured as performed by Khan et al. [15], when leaves were entirely stretched during 11 : 00 and 12 : 00 h by using portable instruments known as infrared gas analyzer (IRGA). The model of IRGA used in our study was LI-COR 6400 (Lincoln, NE, USA). The instrument was stabilized at 25°C air temperature, 85% relative humidity, 600 μmol mol^{-1} CO_2 concentration, and PPFD 800 μmol mol^{-2} s^{-1}.

2.8. Analysis of Biochemical Parameter

2.8.1. Enzyme Assay. To measure the carbonic anhydrase (CA) activity method given by Dwivedi and Randhawa was used [16]. The activity of CA was measured in the fresh leaves of plants. Leaves were chopped into minute pieces and transferred into the solution of cysteine-HCl. This sample was then incubated at 4°C for the time of 20 min. After 20 min, these small pieces of leaves were blotted and transferred to the test tube containing phosphate of pH 6.8. To the test tube, 0.002% solutions of bromothymol blue indicator and bicarbonate (HCO_3) were added followed by incubation of the test tube at 4°C for twenty minutes. In last, this reaction mixture was titrated against 0.5 N hydrochloric acid solution (HCl). In this solution, 0.2 mL of methyl red was added as an indicator.

Protocol given by Jaworski was applied to determine nitrate reductase (NR) activity [17]. For NR, the fresh leaves were taken and cut into small pieces. Pieces of the leaves were transferred to vials made up of plastic, which had 1.25 mL phosphate buffer with a pH of 7.5. In the plastic vials, potassium nitrate solution and isopropanol solution were added. This reaction mixture was incubated for 2 h at 30°C. Then, 0.02% N-1 naphthyl ethylenediamine dihydrochloride and sulphanilamide were added to the mixture. The absorbance of this mixture was measured with the help of a spectrophotometer (Elico model No. SL 171) at 540 nm wavelength.

To measure the activity and levels of diverse antioxidant enzymes like catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD), the method used by Khan et al. was followed [15].

2.8.2. Endogenous Proline Level. Levels of proline was measured in the fresh leaves of the plants, and for this, the method given by Bates et al. was used [18]. For proline estimation, extraction of the leaf samples was obtained using a sulfosalicylic acid solution. In this extract, the same amount of glacial acetic acid and solution of ninhydrin was added. The final solution was heated at 100°C for the duration of 1 hour, and then, the reaction was terminated by placing the sample into using an ice bath and then to this terminated sample, 5 mL of toluene was added vigorously. Two separate layers were formed by the addition of toluene. The absorbance of the upper layer was taken on a spectrophotometer (Elico model No. SL 171) at 520 nm wavelength.

2.9. Statistical Analysis of Data. Experimental data of our studies were calculated by using analysis of variance (ANOVA) with the help of the SPSS software (SPSS, Chicago, USA). To differentiate between the mean values of treatment, least significant difference (LSD) was calculated at a significance level of $P \leq 0.05$.

3. Results

3.1. Phenotypic Character. Plants grown with CuO NPs showed a positive increase in growth biomarkers (length, fresh mass, and dry mass of shoot and root) in comparison with control plants at both the stages of plant growth (30 and 45 DAS) (Figures 1(a)–1(f)). Moreover, the maximum increase of growth parameters was reported in the plants exposed to 8 mg/L of CuO NPs at 30 and 45 DAS. However, increased with 16 mg/L of CuO NPs were statistically significant to the values of control while at par with that of 2 mg/L of CuO NPs.

3.2. Leaf Area, SPAD Value, and Photosynthetic Traits. Exogenously applied CuO NPs significantly increased leaf area and SPAD chlorophyll levels over the control (Figures 2(a) and 2(b)). However, the maximum leaf area and SPAD values were recorded, when the plants were subjected with 8 mg/L of CuO NPs, and were about 23.15% and 29.73% (leaf area), 23% and 37.5% (SPAD value) at 30 and 45 DAS, respectively.

The photosynthetic parameters (P_N, C*i*, E, and g_s) were increased as the growth progressed from 30 to 45 DAS irrespective of the treatment (Figure 2). However, concentrations (4 and 8 mg/L) increased it further at both stages. Out of the various concentrations, 8 mg/L was more effective and increased photosynthetic parameters at 30 and 45 DAS over their control. However, at higher concentrations, photosynthetic parameters were significantly decreased.

3.3. Activity of NR and CA. It was evident from Figures 3(a) and 3(b) that leaf CA and NR activity were increased with the advancement of the age of the plant. It further increased with the concentration (8 mg/L) of CuO NPs at both stages of growth. Maximum CA and NR activity were noted in the plants at 8 mg/L of CuO NPs which were 34.32% and 29.55% higher as compared to the control, respectively, at 45 DAS.

3.4. Antioxidant Enzymes. Improvement in the activity of the antioxidant enzyme (POX, SOD, and CAT) was noteworthy in every concentration (2, 4, 8 or 16 mg/L) of CuO NPs at both stages of growth (30 and 45 DAS). The minimum activity of the enzyme reported in control plants which were sprayed with distilled water only while CuO NPs (8 mg/L) showed the maximum increased in antioxidant level (Figures 3(c)–3(e)). The percent increase by 8 mg/L of CuO NPs in POX was 21% (30 DAS) and 53.4% (45 DAS), CAT was 23% (30 DAS) and 56% (45 DAS), and SOD was 23% (30 DAS) and 54.79% (45 DAS).

3.5. Proline Content. Leaf proline content was also increased with the advancement of the age of the plant (Figure 3(f)). It was further increased by the concentrations (4 and 8 mg/L) of CuO NPs, whereas maximum proline was recorded in the leaf of the plant grown in the presence of 8 mg/L of CuO NPs at both the stages of growth (30 and 45 DAS). The higher concentration was proved to be inhibitory, and it decreased the level of proline.

4. Discussion

Copper was considered an essential element for plant growth and metabolism. It plays a significant role in the electron transport chain of photosynthesis, mitochondrial respiration, responses in oxidative stress, hormonal signalling, etc. Copper also acts as a cofactor in many enzymes such as cytochrome c oxidase, amino acid oxidase in superoxidase dismutase as reported by Mazhoudi et al. in [19]. Scientists are working on nanoparticles of metal to find out its feasibility in improving the growth and productivity of the crop. Various studies have been performed, but no clear-cut picture of its role in plants has been ascertained. Some of them show positive while others show negative impact. It

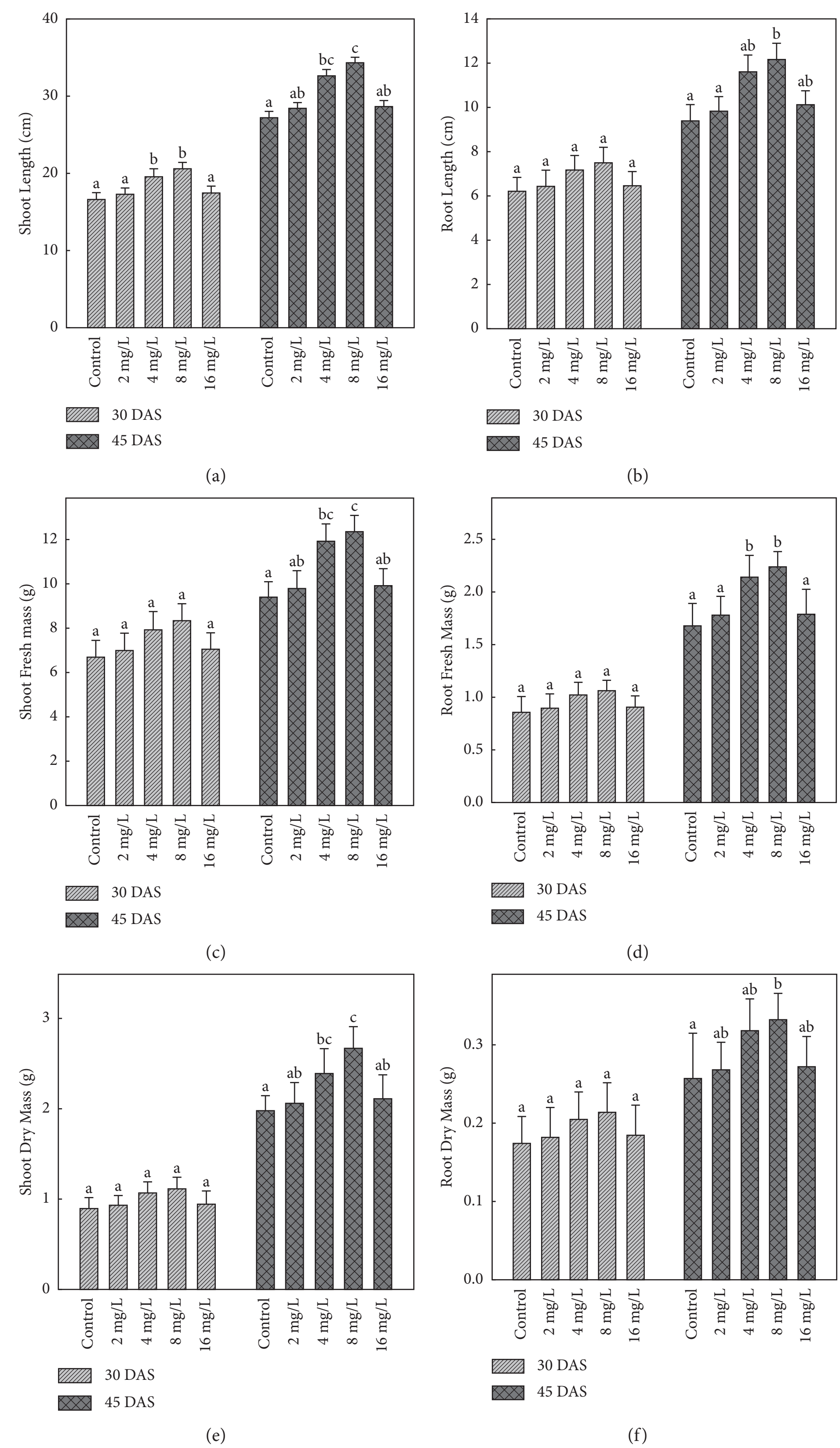


Figure 1: Effect of nanoparticles (NPs) on the length of shoot (a), root length (b), fresh mass of shoot (c), root (d), dry mass of shoot (e), and root (f) of mustard at 30 and 45 DAS. All the data are the mean of five replicates ($n = 5$), and vertical bars shows standard errors (±SE). (Different alphabet represents the significant difference ($p < 0.05$) of treatments compared to the control group).

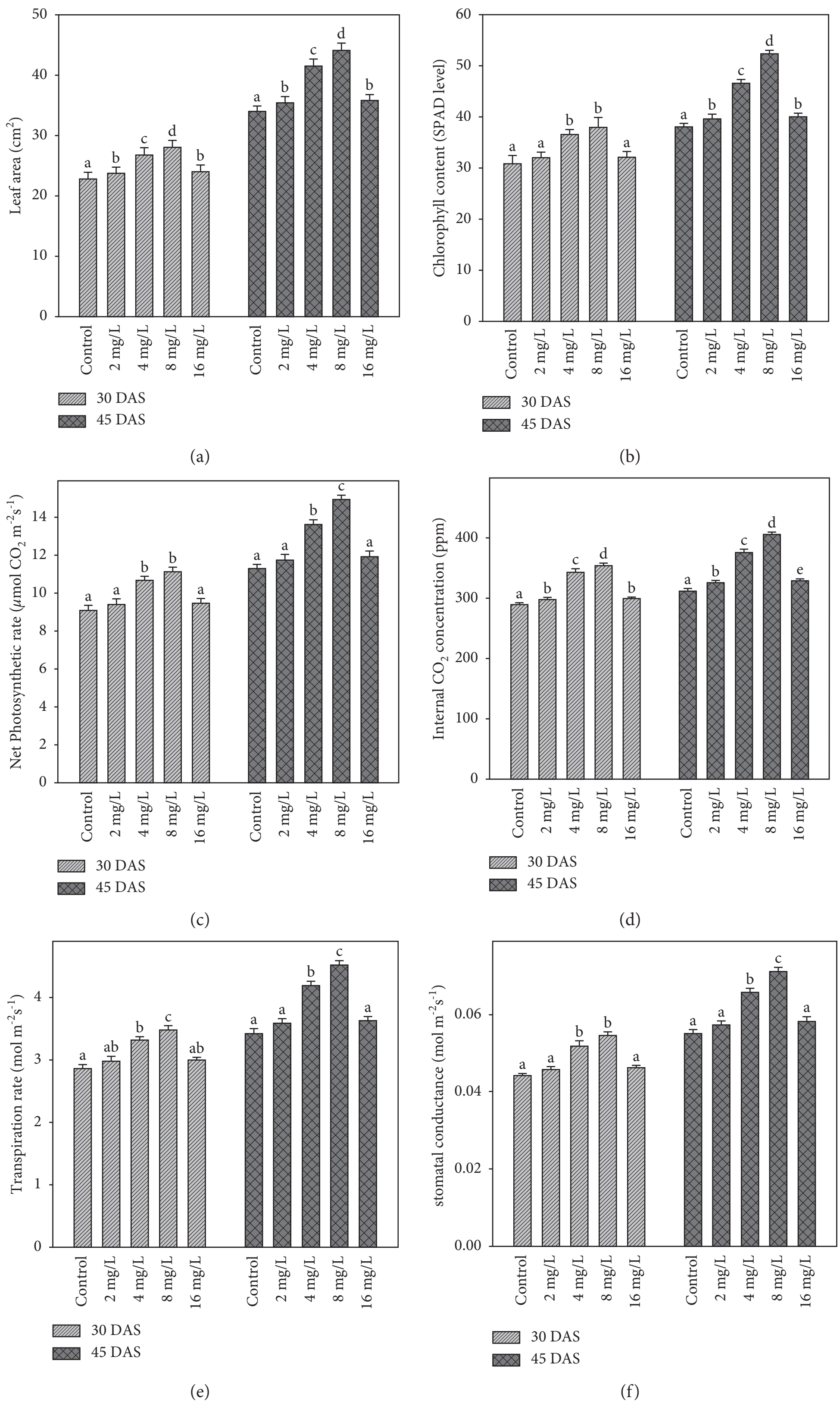


Figure 2: Effect of nanoparticles (NPs) on the (a) leaf area, (b) chlorophyll content, (c) net photosynthetic rate, (d) internal CO_2 concentration, (e) transpiration rate, and (f) stomatal conductance of mustard at 30 and 45 DAS. All the data are the mean of five replicates ($n = 5$), and vertical bars shows standard errors (±SE). (Different alphabet represents the significant difference ($p < 0.05$) of treatments compared to the control group).

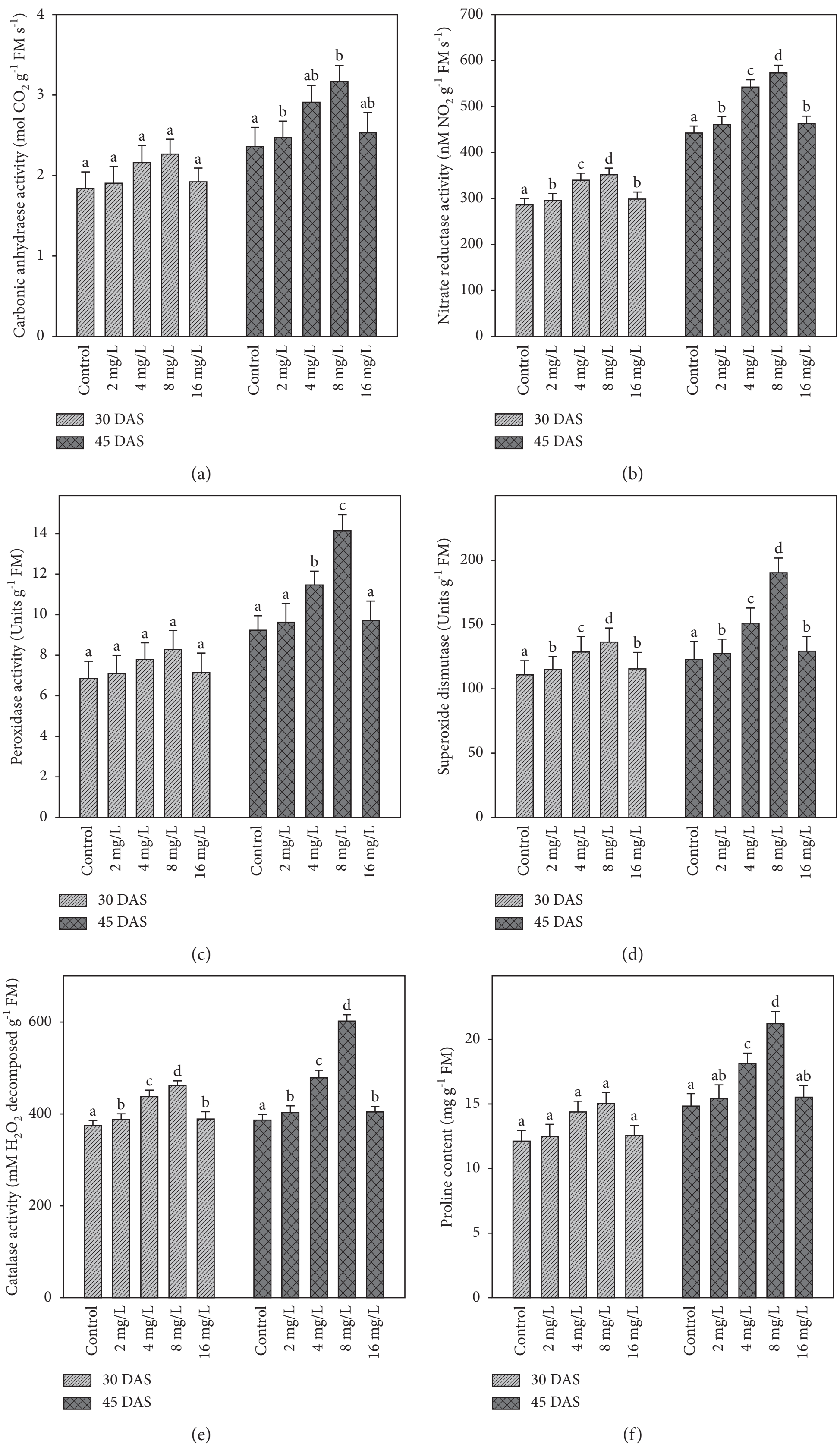


Figure 3: Effect of nanoparticles (NPs) on the (a) carbonic anhydrase activity, (b) nitrate reductase activity, (c) peroxidase activity, (d) superoxide dismutase, (e) catalase activity, and (f) proline of mustard at 30 and 45 DAS. All the data are the mean of five replicates ($n = 5$), and vertical bars shows standard errors (±SE). (Different alphabet represents the significant difference ($p < 0.05$) of treatments compared to the control group).

depends on the type of nanoparticle size and also on the concentration of nanoparticles. The result of nanoparticles also varies from one plant species to another species. In the present observation, it was noted that spraying of CuO NPs on the leaves of mustard seedling leads to a significant change (Figures 1–3). Out of various concentrations of nanoparticles, 8 mg/L shows the maximum increased in the growth, photosynthetic rate, and antioxidant level. Whereas at higher level (i.e., 16 mg/L), the results are less effective but still better than the control (Figures 1–3).

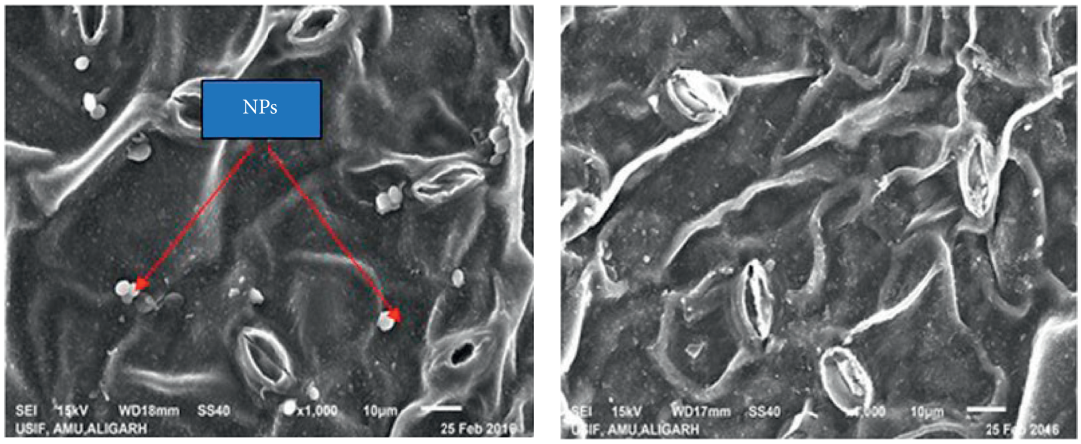
Our finding is also in accordance with the finding of other nanoparticles in which NPs improved the overall growth of plants, so these finding suggested that CuO NPs can be used in plants for enhancing their growth and development. Earlier research conducted on the application of nano-SiO_2 found that when seeds of changbai larch soaked in nano-SiO_2, it enhanced the quality and the growth of seedling and the parameters such as mean height, root length, collar diameter, lateral roots in number, and length of the root [20]. Raliya and Tarafdar reported that ZnO NPs can be useful in plant growth. They reported that when ZnO NPs were given as a foliar spray in cluster bean plants, the treatment improved overall growth of the plant [21]. Arora et al. found that gold nanoparticles (AuNPs) on *Brassica juncea* made improvement in growth and seed yield and increased the number of leaves, area of the leaf, plant height, chlorophyll content which leads to the better crop yield [22]. All these results clearly indicate that nanoparticles act as a growth regulator in plants and defend our finding in the case of CuO NPs.

Photosynthesis, respiration, and transpiration are the indicators of a healthy plant. Out of all radiation coming from the sun, plants utilize only 2–4% of this radiation during photosynthesis. Therefore, there is an immense need to increase these values so that plants can maximize the photosynthetic rate, which ultimately leads to higher biomass production. In our study, foliar-applied CuO NPs increased leaf area (LA), total chlorophyll content (SPAD), leaf net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and internal CO_2 concentration (Figures 2(a)–2(f)). All these gas exchange traits increase gradually with CuO NPs. CuO NPs of 8 mg/L concentration found to be best when it comes to the untreated plant. The results of our experiments are similar to other previous reports where nanoparticles showed improved photosynthetic rate [23]. When carbon nanotubes (CNTs) were embedded in isolated chloroplast of *A. thaliana* leaves, it increases the photosynthetic rate by three times in comparison to plant without SWCNTs [24]. Illumination of TiO_2 NPs to the chloroplast of the spinach plant protect from aging which may be a possible cause for higher photosynthetic rate [25]. Increased activity of Rubisco was reported with TiO_2 nanoanatase [23]. Increased activity of Rubisco enhances the photosynthetic carbon assimilation, so there is increased growth in plants, which also satisfies the finding of our study. Feizi et al. in wheat and Zheng et al. in spinach have also reported the same result of improved photosynthesis by nanoparticles [26, 27]. They found that the use of nano-TiO_2 improves seedling growth or promote germination and photosynthesis in comparison to untreated control plants and stabilized our experimental result of positivity of CuO NPs. Moreover, the size/density of stomata also plays a major role in coordinating the process of diffusion of gases. In the present study, foliar application of CuO NPs increased the density of the stomata (Figure 4) which may be the possible reason behind the increase in gas exchange between the plant tissues and the atmosphere which ultimately increased the photosynthetic attributes. All these findings suggest that our results are good enough to show the positive character of CuO NPs in plants and improve photosynthetic efficiency.

Carbonic anhydrase and nitrate reductase are the features that determine the health of the plants and responsible for growth related physiological reactions. Both these traits improved by the exogenous application of CuO NPs in the form of a foliar spray, which confers the overall plant's growth and development (Figures 3(a) and 3(b)). Our finding is similar to another study where positive result by the application of others nanoparticles has been found on these enzymes. Increased nitrate reductase level has been reported with the use of nano-SiO_2 and nano-titanium dioxide (nano-TiO_2) which finally improve the seed germination in soybean [4].

Plants have adapted two types of defense mechanism, one is enzymatic (superoxide dismutase, peroxidase, and catalase) and another one is non-enzymatic (proline). Both this mechanism, i.e., proline (Figure 3(c)) and antioxidant level such as POX, SOD, and CAT (Figures 3(c)–3(e)) improved by the treatment of CuO NPs when given in the form of a foliar spray, these increased levels mitigate any ill effect caused by these nanoparticles and improve the overall plant's growth. Our result is also similar to that of Costa and Sharma, who used CuO NPs in *Oryza sativa* and reports increased expression of enzymatic antioxidants, APX and SOD at 10 and 100 mg L^{-1} of CuO NPs, respectively in which also protect plants from oxidative stress [10]. Lei et al. work on spinach plants and reported that nanoanatase TiO_2 diminished the accumulation of H_2O_2, MDA content and increased the level of SOD, CAT, and POX thus improved the antioxidant system under abiotic stress [28]. *Brassica juncea* showed improved activity of antioxidant enzyme such as APX, POX, and CAT when treated with Ag NPs. This increased level of antioxidant enzyme reduces the reactive oxygen species activity [29]. Recently, SOD activity was checked under CuO nanoparticles in wheat, and it was found that its activity was increased significantly with 25 mg/L CuO NPs when compared to untreated plants [30].

Proline is an important amino acid that stores in plants as compatible solutes and helps the plant against oxidative stress by removing the reactive oxygen species which had been released during that period of oxidative stress [31]. During oxidative stress accumulation of ROS increased the levels of antioxidative enzymes which are helpful to maintain homeostasis [32]. Accumulation of proline was reported at the highest level in plants treated with NPs with different concentrations as compared to control plants. Plants treated with 8 mg/L of CuO NPs possess 43% higher proline content than control at 45 DAS. Increased proline content was observed upon exposure to different concentrations of CuO NPs [11, 33, 34].



(a) (b)

Figure 4: Scanning electron microscope (SEM) image of treated with CuO NPs (a) and control (b).

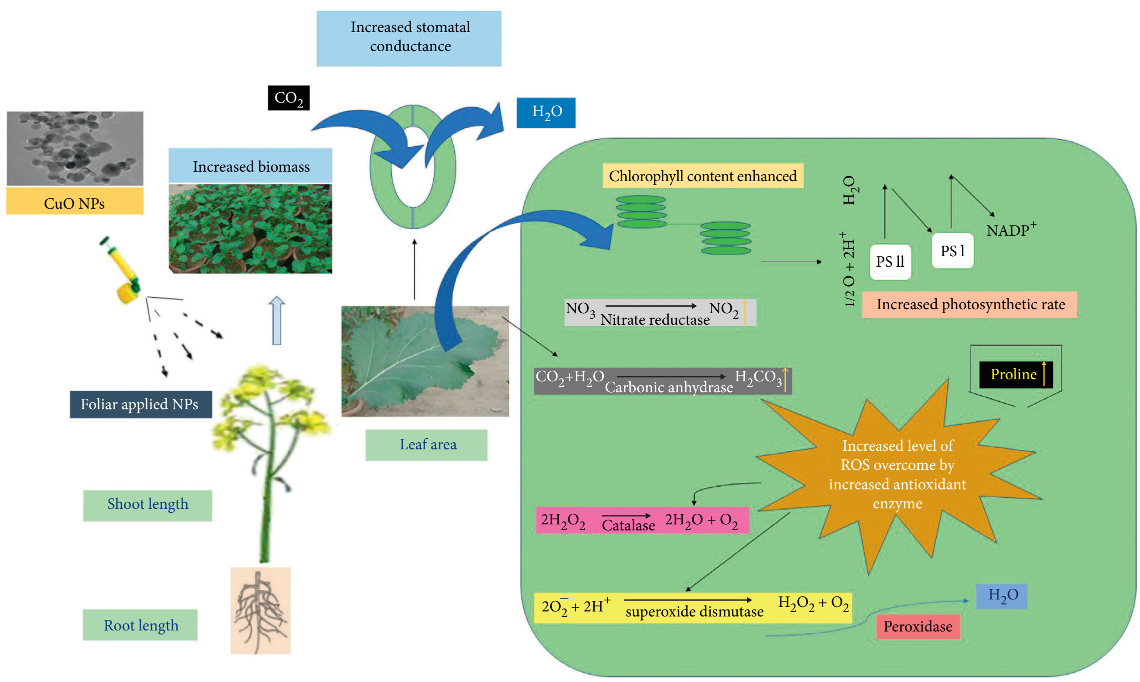


Figure 5: A simplified view of the foliar application of CuO NPs and their effect on the physiological and biochemical parameters in *Brassica juncea*.

Our finding with CuO NPs shows a positive response as we can see in the aforementioned paragraph. It has been reported that CuO NPs at all the concentration, when applied on foliage, enhanced almost all the growth, biochemical, and physiological parameters. This result can be applied in the field to see their further outcome on a large scale before making any recommendation to farmers. CuO NPs then can be utilized as micronutrients to enhance the production of mustard and their growth which ultimately results in higher yields. A simplified view of foliar application of CuO NPs in *B. juncea* has been shown in Figure 5, where we can see how NPs enters into plant cells and affect different growth and physiological parameters in plants.

5. Conclusions

It can be concluded from the present study that CuO NPs-mediated response was concentration-dependent. Moreover, the foliage of mustard plants treated with 8 mg/L showed the most promising response, increased the growth, and enhanced the photosynthetic efficiency of plants. NPs can get attached/entered to the cell surface through the pores and lenticels. This attachment increases the gaseous exchange and causes improved growth and development of *B. juncea*. The physiological parameters were improved by the application of CuO NPs which led to overall growth improvement in mustard plants. Increased stomatal conductance increases the gas exchange rate which further improves photosynthesis and results in the production of higher biomass and crop yield. However, studies at the field level using different crops and soil types are needed before recommendations can be confirmed.

Data Availability

All the related files are included in the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest amongst them.

Authors' Contributions

All the authors contributed to the study conception and design. Ahmad Faraz and Mohammad Faizan prepared the material preparation, collected the data, and analysed the data. Ahmad Faraz wrote the first draft of the manuscript. All authors commented on the previous version of the manuscript and read and approved the final manuscript.

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