Research Article

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Leveraging of mycogenic copper oxide nanostructures for disease management of *Alternaria* blight of *Brassica juncea*

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Abstract: *Brassica* is one of the crops sensitive to low copper supply, leading to *Alternaria* blight. The present study reflects the synthesis of myco-derived copper oxide (M-CuO) nanoparticles (NPs) from *Trichoderma asperellum* and investigates their effect against *Alternaria* blight of *Brassica* in two soil types, alluvial and calcareous. Foliar applications of different treatments were used to treat plants: T1 (mancozeb@0.2%), T2 (propiconazole@0.05%), T3 (*T. asperellum* filtrate), T4 (M-CuO NPs), T5 chemically synthesized (C-CuO NPs), and T6 bulk phase (BP-CuO @25, 50, 100, 150, and 200 ppm) of each in twice such as protectant and curative method under pot experiments. M-CuO NPs in two protective sprays exhibit up to 75% disease suppression in alluvial soil, compared to 68.9% suppression in curative spray at 200 ppm. Maximum seed yield and seed number were obtained, 1.95 g/plant and 850 seeds/plant in alluvial soil, but in calcareous soil, seed yield (1.14 g/plant) and seed number 414 seeds/plant were recorded in plants supplemented with M-CuO NPs as a protectant. In both soils, maximum plant height was increased by protective applications of M-CuO NPs at 200 ppm. Thus, the present study suggested that among foliar sprays of copper nanocompounds, protective activity shows better results as compared to curative activity. Among all the treatments, M-CuO NPs were found to be most effective in suppressing disease and improving productivity and growth-promoting effects of *Brassica*.

Keywords: foliar spray, protective, curative, defense enzyme, plant growth promoter

1 Introduction

Crops require a sufficient, but not excessive supply of essential micronutrients for disease suppression and optimal productivity. An insufficient supply of mineral elements may lead to a limit in plant growth. Deficiency of insufficient micronutrients, such as copper (Cu), is more common in agricultural soils, particularly in calcareous soil. As a result, these elements can be used in both intensive and extensive agricultural systems supplied as fungicides and fertilizers. Copper nanoparticle (NP), for example, has an interesting and complex biological profile in multiple oxidation states (Cu, Cu_2O, CuO) as antimicrobials, agrochemicals, etc. [1,2]. *Brassica* is one of the sensitive crops to inadequate copper supply, which results in *Alternaria* symptoms on leaves, stem, and pod blight [3,4]. Furthermore, Cu works as an essential inducer, activating the plant defense system and enzymes, such as peroxidases, plastocyanins, and multi-Cu oxidases, which boost the crop resistance against plant diseases [5,6]. Plant–pathogen interactions activate the plant defense system by producing reactive oxygen species (ROS), antioxidants, and stress enzymes [7]. Several inducers and strategies have been employed to improve plant resistance toward pathogens [7–9]. To increase plant resistance to infections, a variety of inducers and techniques have been used [7–9]. Because disease-resistant inducers can activate the plant's defense system against fungus. Compounds that induce plant resistance include salicylic acid and benzothiadiazole. Proteins involved in pathogenesis, such as peroxidase, 1,4 glucanase, and chitinase, have been found to be active in resistant plants [10]. To overcome the stress, defense activities, such as deposition of the phenolics,

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ascorbic acid, and the stimulation of antioxidant enzymes, are evoked following an attempted microbial invasion [11].

Copper is a major element in the activation of plant defense system when they are subjected to biotic stress. However, NPs' phytotoxicity results in lower biomass, limited seed germination, reduced root length, and stunted growth [11–13]. The majority of prior studies were conducted in lab-scale trials to evaluate the effect of CuO NPs in soil settings, while its favorable benefits through foliar exposure have not yet been investigated. It has been found that the application route of NPs considerably effect ion dissolution, aggregation, and bioavailability. Furthermore, NPs' foliar spray minimizes agglomeration and increases NP solubility [14–17]. Moreover, the foliar spray of NPs lowers aggregation and increases NP solubility, resulting in less diverse phytotoxic effects. These NPs release Cu^{2+} ions on the surface of leaves, acting as a protective shield that prevents the pathogenic attack while simultaneously increasing the nutritional content of seeds and fruits. As a result, the objective of this study was to examine the biocontrol efficacy of copper-based compounds against *Alternaria* blight, growth dynamics of *Brassica juncea*, and plants' stress response.

2 Materials and methods

2.1 Synthesis of M-CuO NPs

Mycogenic copper oxide NPs were synthesized from the cell-free extract of *Trichoderma asperellum* at specific conditions, which has been reported in our previous paper [4].

2.2 Characterization of M-CuO NPs

Characterization of CuO NPs was done by ultraviolet–visible (UV-Vis) spectrophotomter, scanning electron microscope (SEM), and dynamic light scattering (DLS). The UV-Vis characterization revealed surface plasmon resonance and optical properties, as 10 $mg{\cdot}mL^{-1}$ of M-CuO NPs were suspended in water. An instrument Malvern zeta sizer (Nano ZS90, Noida, India) was used to measure mean particle size and zeta potential was recorded to determine the surface charge on the surface of NPs. Measurements were carried out in triplicate with a temperature equilibration of 1 min at 25°C with an angle of 90°C [18].

2.3 Preparation of NP suspension, stock solution of CuO NPs, and standard check fungicides

Sequential concentrations of both types of CuO NPs from a 1,000 ppm working solution were prepared to 25, 50, 100, 150, and 200 ppm doses before use. Suspension of C-CuO NPs <50 nm particle size, SRL chemicals, and BP-CuO <10 µm of different concentrations was also prepared. The solution of NPs was filtered through 0.22 µm syringe Millipore filters and sonicated for 2 min before use for getting monodispersed population. The commercially available fungicides (1) mancozeb (0.2%) and (2) propiconazole (0.05%) at their standard concentrations were prepared in distilled water and 50% filtrate of *T. asperellum* was also prepared.

2.4 Nutrient analysis of soil

Clay pots of size (12 cm × 12 cm) were purchased from a local store. Five to six kilograms of soil per pot were filled. Two soil samples, i.e., alluvial and calcareous soil, were submitted to the Division of Agronomy, ICAR-IARI, New Delhi, for macro- and micro-nutrient analyses. Nitrogen (N), phosphorus (P), potassium (K), and micro-nutrients, such as iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn), were also quantified.

2.5 Sample collection of leaves

Samples were collected on the 30th day of sowing before infection and after infection on the 60th day. Plants' stress level was estimated by checking the antioxidant enzymes (superoxide dismutase and catalase).

2.6 Superoxide dismutase and catalase assay

Total superoxide dismutase activity was determined by nitroblue tetrazolium photochemical assay according to Misra and Fridovich [19]. The Aebi method was used for determining catalase activity in leaves challenged with *A. brassicae* and CuO NP treatment [20].

2.7 Plants *in vivo* experiment

Seeds of PM-25 variety of *B. juncea* (germination efficiency >98%) were provided by ICAR-IARI, Division of Plant Pathology, New Delhi, India. Pots were divided into six treatments with three replicates each. Seeds were surface sterilized with 0.4% sodium hypochlorite solution followed by repeated washing with distilled water. Surface-sterilized seeds were sown (10 seeds) in sterile soil and pots. Seeds were allowed to germinate for 1 month. After 1 month, pots were divided into six treatments with three replicates each: (1) negative control (healthy plants without infection), (2) positive control (plants infected with *A. brassicae*), (3) plants infected with *A. brassicae* and sprayed with double and single application of mancozeb, (4) plants infected with *A. brassicae* and sprayed with double and single application of propiconazole, (5) plants infected with *A. brassicae* and treated with two and one foliar sprays of M-CuO NPs (dose of 25, 50, 100, 150, and 200 ppm) in volume 2 mL/plant in protective and curative set, (6) plants treated with two and one foliar sprays of C-CuO NPs (25, 50, 100, 150, and 200 ppm) and infected with *A. brassicae* in protective and curative set, and (7) plants treated with two and one foliar sprays of BP-CuO NPs (25, 50, 100, 150, and 200 ppm).

Some of the brassica plants were sprayed before one week with two consecutive foliar applications of CuO NPs in the protective method, while in curative set, after the first symptom of disease, the NPs were sprayed on the rest of the plants. Plants were bagged with a transparent plastic bag and kept for 10 days. Randomly five plants were selected and tagged for taking observations. Five leaves were taken from plants for scoring the disease intensity. Observations for disease severity were taken 75 days after sowing. The overall disease scoring was done at 0–6 rating scale on the basis of the disease assessment key for *Alternaria* blight in rapeseed-mustard [21].

2.8 Statistical analysis

Data were processed using the statistical package WASP 2.0 (Web Agri Stat Package, Indian Council of Agricultural Research, India). One-way analysis of variance and critical differences at a probability level of 0.01 and 0.05 were obtained with mean values of treatment. Treatments were compared using Duncan's multiple-range test.

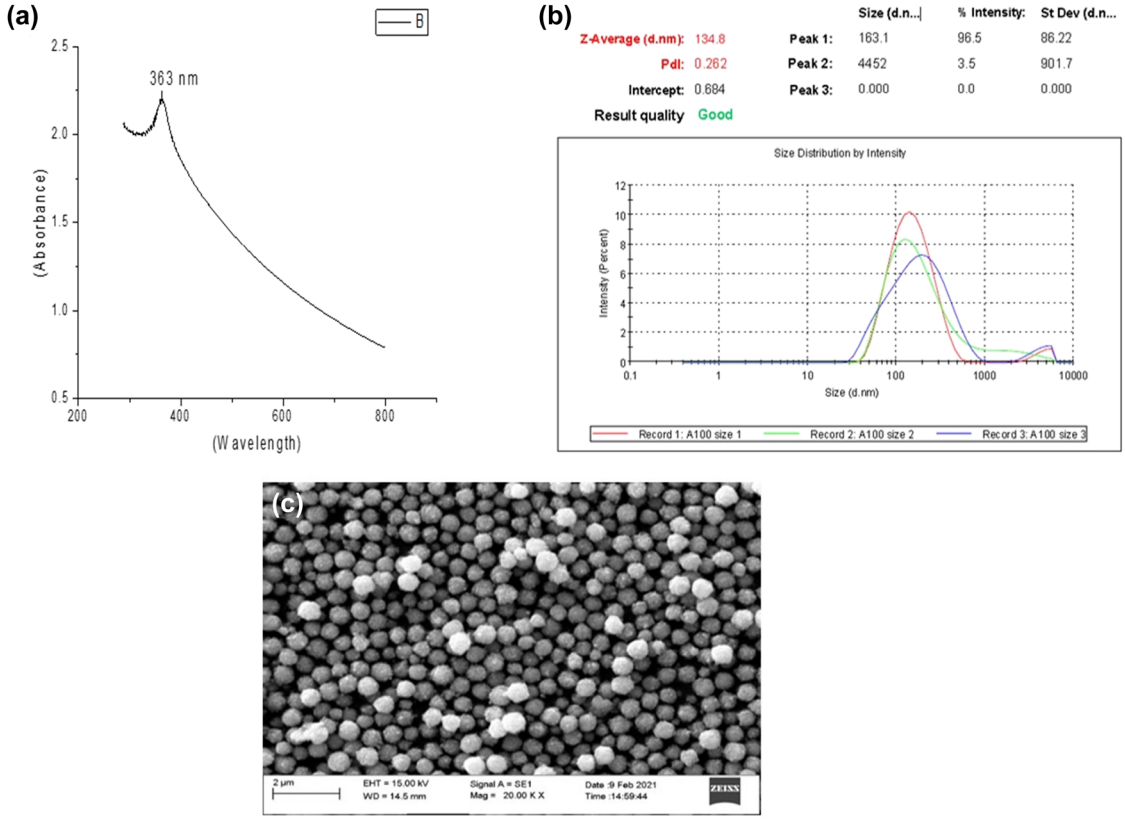

Figure 1: Spectroscopic- and microscopic-based characterization: (a) UV-Vis spectra of M-CuO NPs, (b) particle size of M-CuO NPs, and (c) SEM image of M-CuO NPs.

3 Results and discussion

3.1 Characterization of M-CuO NPs

UV-Vis results provide information regarding the particle size and excitation of electron from the valance band to the conduction band by absorption of light and also calculated the band gap [22–24]. A broad-centered peak revealed at 363 nm indicates the excitation of electrons from the valence band to the conduction band (Figure 1a). Similar peaks in the range of 300–350 nm were recorded for CuO NPs synthesized from cell-free supernatants of *Pseudomonas fluorescens* and *Bacillus cereus*, respectively, by Hesham et al. and Tiwari et al. [25,26].

DLS measurements have confirmed the hydrodynamic diameter of CuO NPs as 134.8 nm as shown in Figure 1b. SEM micrographs indicated the spherical shape and particle size of CuO NPs <50 nm size range (Figure 1c). Image J has been used to estimate the particle size of CuO NPs.

3.2 Disease severity index in *Brassica* plants

In this study, the application of copper nano-forms and bulk CuO was assessed and compared with the efficacy of fungicides mancozeb (2,000 ppm) and propiconazole (500 ppm), and with the biological extract *T. asperellum* (50%) to check its effects on the growth of *B. juncea* and in the suppression of *Alternaria* blight of *Brassica*.

The positive control plants infected with *A. brassicae* have the highest disease severity index to 65%; in conjunction with that, the highest biocontrol efficacy was observed for the plants exposed with M-CuO NPs recorded with very less percentage of blight disease, i.e., 13.8% and 14.8% at 200 ppm concentration in mustard plants grown in alluvial soil, respectively, through protective and curative methods as shown in Figure 2a and Table 1. On the other hand, with C-CuO NPs, the disease recorded was 24.8% and 24.1% through protective and curative methods, respectively. Similarly, with BP-CuO, 23.4% and 20.6%, the disease index was recorded.

Moreover, plants sprayed with mancozeb at 2,000 ppm dose as a protectant and curative agent resulted in 33.6% and 32.6% severity index, respectively. Also, when propiconazole was applied at 500 ppm, 33.6% and 29.6% severity index was observed. The findings of our study revealed a significant decrease in disease severity index when plants were exposed to M-CuO NPs when applied in protective and curative modes.

Abdelkhalek and Al-Askar [27] revealed that biologically synthesized ZnO NPs were assessed against tobacco mosaic virus, which showed both protective and curative activities with better protective activity. Attia et al. [28]

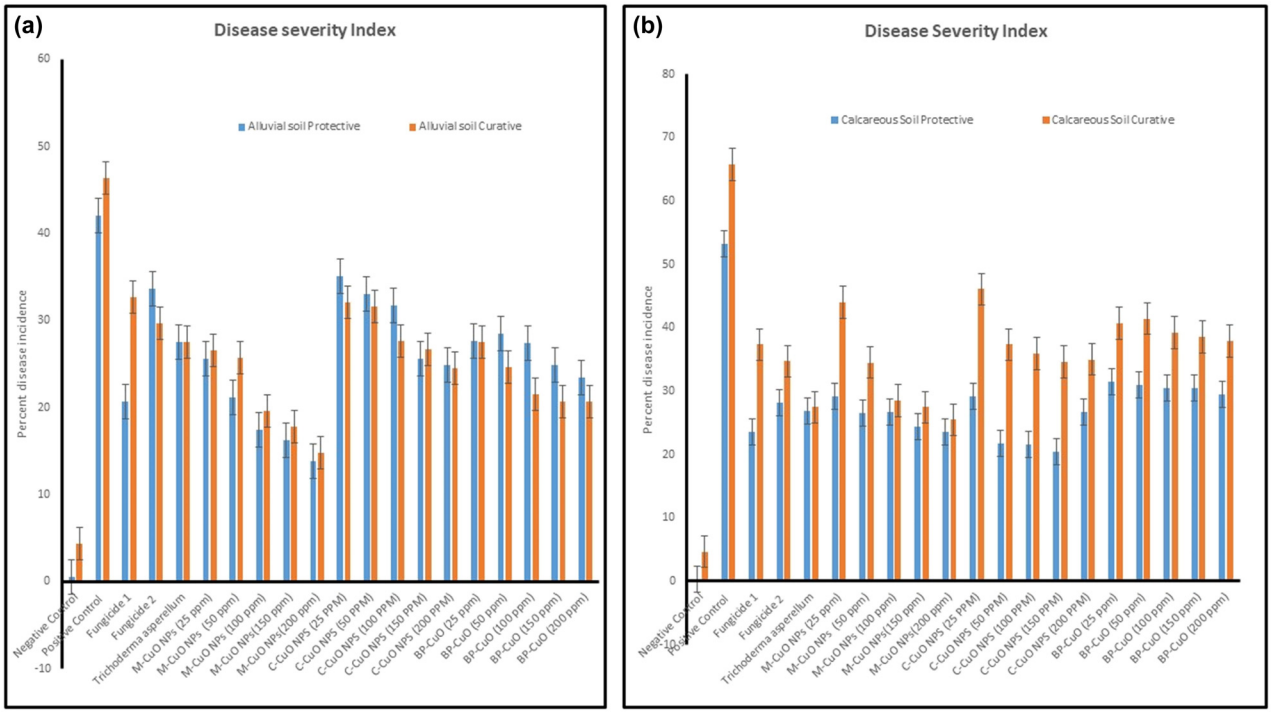

Figure 2: Disease severity index in *Brassica* plants. Protective and curative activities of CuO NPs in alluvial soil (a) and in calcareous soil (b).

have reported a similar influence in the biocontrol efficacy of CuO-streptomycin drug, which was found against potato brown rot disease with 55.8% protection efficiency. Thus, these studies have supported the better protective activity of M-CuO NPs. However, as compared to separately ameliorative effects of M-CuO NPs and C-CuO NPs, BP-CuO occupied the lowest rank in biocontrol efficacy.

On the contrary, for control plants grown in calcareous soil, a 53.2% disease severity was recorded. However, at 200 ppm, with M-CuO NPs, 24.8% and 25.8%, the disease index was recorded with protective and curative methods (Figure 2b). Likewise, with C-CuO NPs at 200 ppm, 26.7% and 34.6% index was recorded. Similarly, with BP-CuO, 29.5% and 37.8% severity index was observed. Some of brassica plants were sprayed with mancozeb has been recorded with disease severity index 23.6 % in protective method and 37.3 %, in curative method, respectively. Also, the propiconazole at 500 ppm dose resulted in a severity index, i.e., 28.1% and 34.7% through protective and curative methods. Thus, these experiments at pot levels suggested that the optimized dose for the suppression of disease was 100–200 ppm (Figure 3).

In a nutshell, the comparison of treatments showed that M-CuO NPs at higher doses (100–200 ppm) efficiently provide resistance to plants before infection, while in a curative way, single spray was less effective. Thus, our study has proved that the application of M-CuO NPs compared with other treatments acts as a better protectant, which significantly increases the disease resistance and efficiently control the blight disease by 73.25% and control equally as a curative method, which reduced the disease by 68%. However, the protectant mode was considered the best treatment for mustard plants grown under normal and stress conditions (nutrient deficiency of Cu) in both soils. It was found that the applications of M-CuO NPs were also effective in stress conditions and were able to provide the optimum Cu^{2+} ions.

3.3 Plant growth-promoting potential of CuO NPs

Plant growth studies revealed a steady increase in plant height between the 30th and 40th days up to 200 ppm dose in the protective method, with more pronounced effects in nutrient-deficient soil. In alluvial soil, as compared to untreated plants where height was 57 cm, the maximum height recorded was 61 cm in plants treated with M-CuO NPs (Figures 4a and 5a and Table 2). However, at higher doses of C-CuO NPs (200 ppm), a negative impact was

Table 1: Disease severity index of *Alternaria* blight on *Brassica* plants infected by *A. brassicae*

Disease severity index at day 75 after sowing on *Brassica juncea* plants grown in alluvial and calcareous soil					
		Alluvial soil		calcareous soil	
S. no.	Treatments	Protective	Curative	Protective	Curative
1.	Negative control	0.523 ± 0.103^{a}	4.33 ± 0.471^{a}	0.30 ± 0.471^{a}	4.66 ± 0.471^{a}
2.	Positive control	42 ± 1.414^{b}	46.3 ± 0.471^{b}	53.2 ± 0.471^{b}	65.7 ± 0.081^{b}
3.	Fungicide 1 (Mancozeb)	20.7 ± 0.45^{c}	32.6 ± 0.169^{c}	23.6 ± 0.169^{c}	37.3 ± 0.974^{c}
4.	Fungicide 2 (Propiconazole)	33.6 ± 1.02^{d}	29.6 ± 0.124^{d}	28.1 ± 0.124^{d}	34.7 ± 0.081^{d}
5.	*Trichoderma asperellum*	27.5 ± 0.21^{e}	27.5 ± 0.047^{e}	26.8 ± 0.041^{e}	27.4 ± 0.329^{e}
6.	M-CuO NPs (25 ppm)	25.5 ± 0.77^{f}	26.5 ± 0.141^{f}	29.0 ± 0.141^{f}	43.9 ± 1.837^{f}
7.	M-CuO NPs (50 ppm)	21.1 ± 0.43^{g}	25.7 ± 0.047^{g}	26.5 ± 0.124^{e}	34.5 ± 0.216^{g}
8.	M-CuO NPs (100 ppm)	17.4 ± 0.44^{h}	19.5 ± 0.169^{h}	26.7 ± 0.169^{e}	28.5 ± 0.169^{h}
9.	M-CuO NPs (150 ppm)	16.2 ± 0.43^{i}	17.7 ± 0.124^{i}	24.3 ± 0.124^{g}	27.4 ± 0.047^{h}
10.	M-CuO NPs (200 ppm)	13.8 ± 0.49^{j}	14.8 ± 0.57^{j}	23.6 ± 0.571^{c}	25.4 ± 0.262^{i}
11.	C-CuO NPs (25 ppm)	35.1 ± 0.45^{k}	32.0 ± 0.89^{c}	29.1 ± 0.899^{f}	46.0 ± 1.268^{j}
12.	C-CuO NPs (50 ppm)	33.0 ± 1.02^{d}	31.6 ± 0.124^{c}	21.6 ± 0.204^{h}	37.3 ± 0.124^{c}
13.	C-CuO NPs (100 ppm)	31.6 ± 0.54^{d}	27.6 ± 0.204^{e}	21.5 ± 0.205^{h}	35.9 ± 0.571^{d}
14.	C-CuO NPs (150 ppm)	25.5 ± 1.43^{f}	26.6 ± 0.124^{e}	20.4 ± 0.124^{i}	34.6 ± 0.124^{d}
15.	C-CuO NPs (200 ppm)	24.8 ± 0.46^{f}	24.5 ± 0.205^{g}	26.7 ± 0.205^{e}	35.0 ± 1.84^{d}
16.	BP-CuO (25 ppm)	27.6 ± 0.82^{e}	27.5 ± 0.047^{e}	31.4 ± 0.047^{j}	40.7 ± 0.081^{k}
17.	BP-CuO (50 ppm)	28.4 ± 0.26^{e}	24.6 ± 0.216^{g}	31.0 ± 0.216^{j}	41.4 ± 0.169^{l}
18.	BP-CuO (100 ppm)	27.3 ± 0.68^{e}	21.5 ± 0.169^{k}	30.5 ± 0.169^{k}	39.2 ± 0.355^{m}
19.	BP-CuO (150 ppm)	24.8 ± 0.88^{f}	20.7 ± 0.163^{k}	30.4 ± 0.163^{k}	38.5 ± 0.249^{n}
20.	BP-CuO (200 ppm)	23.4 ± 0.04^{f}	20.6 ± 0.047^{l}	29.5 ± 0.047^{l}	37.8 ± 0.188^{o}

Different superscript letters indicate statistically significant results among control and experimental groups.

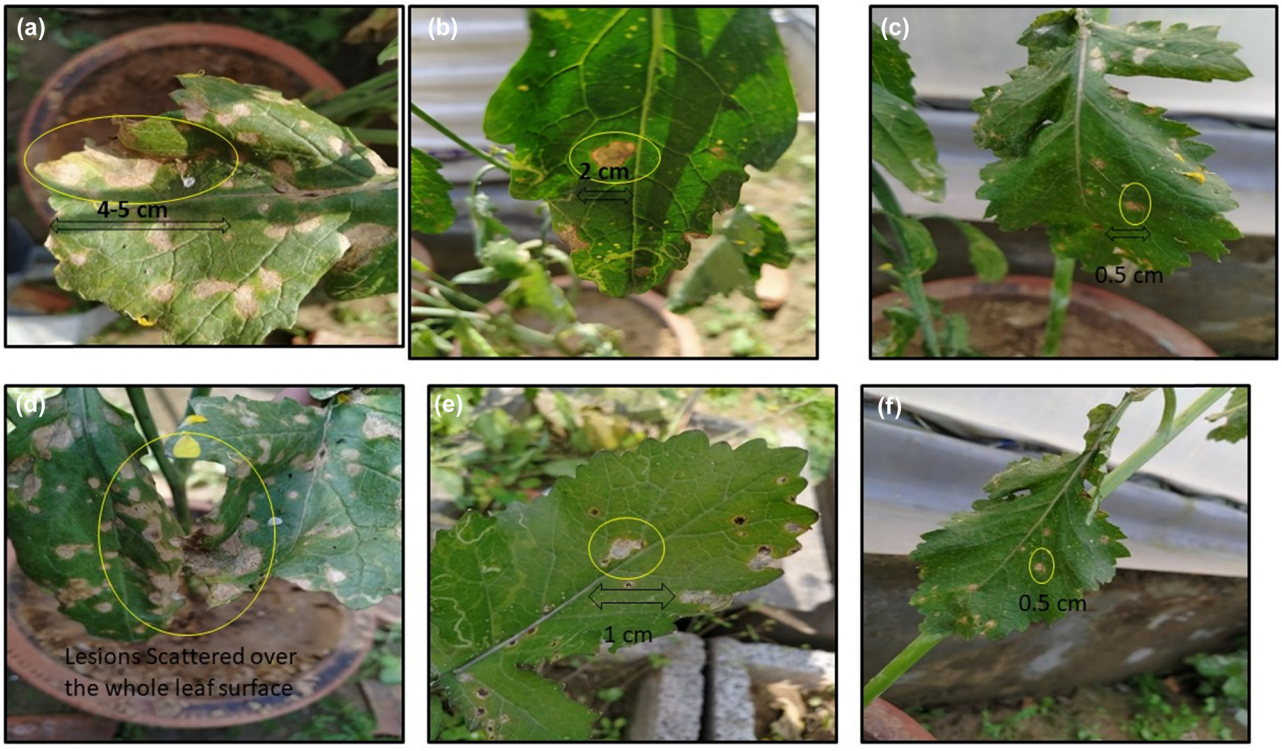

Figure 3: A photograph showing the disease symptoms of *Alternaria* blight on mustard leaves at 75th day post inoculation: (a) plants inoculated with *A. brassicae*, (b) plants treated with two protective foliar sprays of C-CuO NPs (100 ppm), (c) plants treated with two foliar sprays of M-CuO NPs (100 ppm), (d) plants inoculated with *A. brassicae*, (e) plants treated after the first symptom of disease with one foliar spray of C-CuO NPs (100 ppm), and (f) plants treated after the first symptom of disease with one foliar spray of M-CuO NPs (100 ppm).

observed in plant height as 54 and 50 cm height recorded as compared to 57 cm height recorded in untreated plants. Similarly, a minimum plant height of around 48.6 and 54.6 cm in protective and curative modes was observed with BP-exposed plants at 200 ppm. Thus, the trend evident was retarded plant growth with higher doses of C-CuO NPs and BP-CuO as compared to the positive effects of M-CuO NPs. The application of NPs through both the methods suggested no significant effects on plant height in alluvial soil, while significant effect was observed in calcareous soil.

Interestingly, the plant groups grown in calcareous soil have attained maximum plant height of up to 61 and 47 cm, respectively, at 200 ppm dose with M-CuO NPs as compared to untreated plants (33 and 35 cm) in protective and curative treatments. The positive effect in the plant height was due to the uptake of NPs by the plant and significant effects in both modes were observed with M-CuO NPs. Similarly, with C-CuO NPs, maximum plant height of 61.6 and 38.6 cm was recorded, respectively, as compared to untreated plants (33 and 35 cm) (Figures 4b and 5b). However, the plant groups exposed to bulk copper have no significant effects on plant height through both modes. Thus, the analysis indicated that in calcareous soil, the protectant mode worked better with superior performance of M-CuO NPs; moreover, no phytotoxicity effects were observed with M-CuO NPs and C-CuO NPs, up to 200 ppm dose.

Despite the fact, the toxicity issue was observed with C-CuO NPs and BP-CuO at higher doses (150 and 200 ppm) in alluvial soil. Among five treatments conducted in a study, it has been found that both of the fungicides mancozeb and propiconazole has no significant effects on plant height in alluvial soil. Thus, the overall analysis has suggested that among all the treatments, M-CuO NPs have a positive effect on plant height even up to higher dose of 200 ppm, while C-CuO NPs have also given promising results up to lower doses at 100 ppm. The experiments revealed the minimum concentration of copper also leads to stimulate plant growth. Their mode of supply, dose and nature varies. It has been supported by several studies that minimum dose of 50-100 ppm promoting the growth in several crops such as wheat, maize, and *Brassica* [29–31]. Thus, a positive effect in the plant groups supplied with a 200 ppm dose of M-CuO NPs supported by previous findings was evident in both soils, with more pronounced effects in calcareous soil, where nutrient deficiency of Cu was fulfilled by two sprays of CuO NPs.

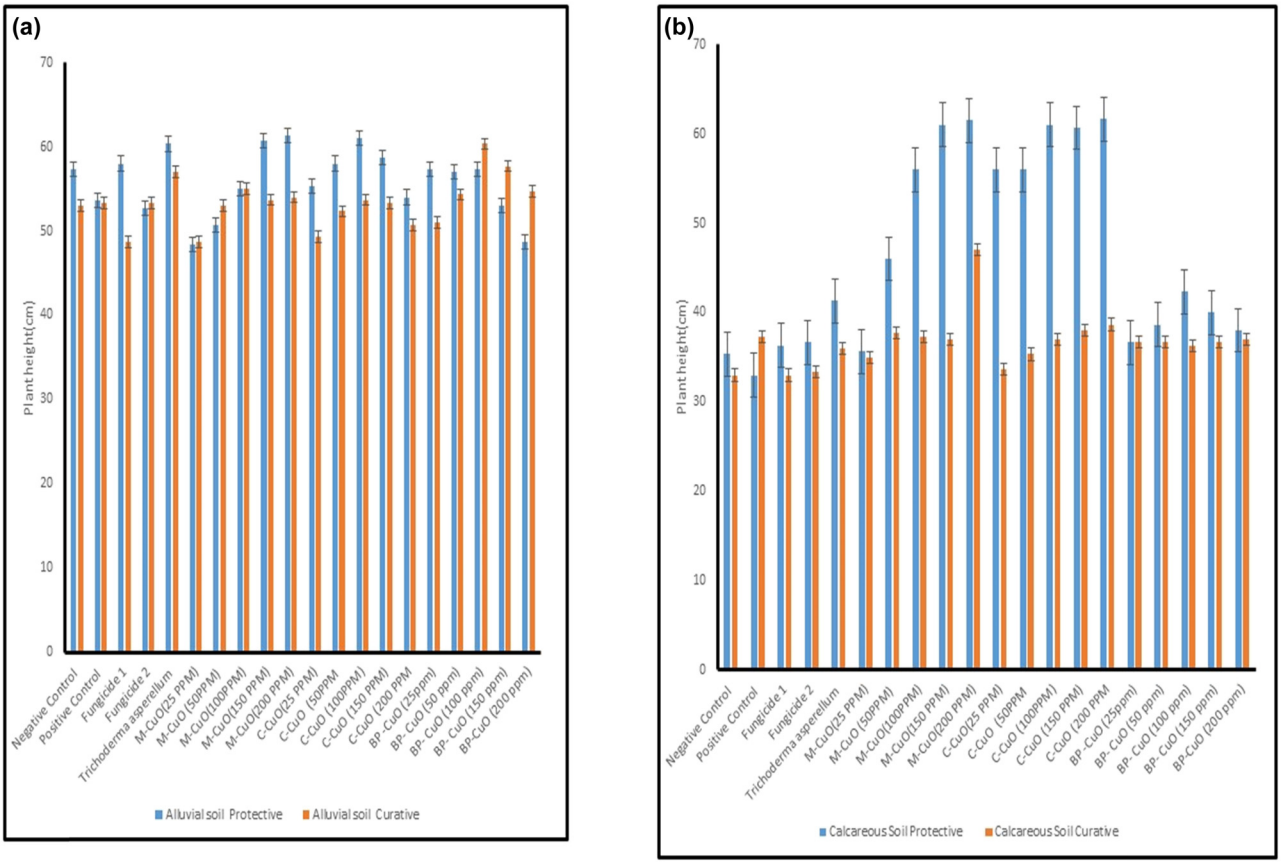

Figure 4: Plant growth-promoting effects of CuO NPs: (a) plant height in alluvial soil and (b) plant height in calcareous soil.

3.4 Productivity of *Brassica* plants

Pods perform a developmental role in seed encapsulation and protection against biotic and abiotic stress. Their count is a crucial parameter for yield quantification [32,33]. It was observed that plants exposed to M-CuO NPs increased yield by 1.95 and 1.24 g/plant at 200 ppm through protective and curative modes as compared to untreated (control) plants where 0.72 g/plant yield was recorded. However, in calcareous soil with mycogenic nanoparticles, the seed yield was 0.92 and 0.77 g/plant in protective and curative treatments as compared to control (0.67–0.633 g/plant). Thus, the seed yield analysis revealed that mycogenic NP treatment resulted in the maximum yield in alluvial soil with the protectant mode. Similarly, the exposed plants at 200 ppm with C-CuO NPs increase the seed yield by 1.84 and 0.86 g/plant through protective and curative methods as compared to the control (0.72 g/plant). Moreover, with BP-CuO, the seed yield was 1.66 and 1.55 g/plant, respectively in protective and curative treatments as compared to control (0.72 g/plant).

However, the results in calcareous soil were less significant near around 0.83 and 0.76 g/plant, in protective and curative methods as compared to control (0.67 g/plant) with mycogenic NP treatment. Also, C-CuO NPs increase the yield around 0.93 and 0.806 g/plant in protective and curative treatments as compared to control (0.67 g/plant). Thus, the overall analysis has also suggested that the BP-CuO, somehow, worked well for the increment of seed yield in calcareous soil (Table 3). However, the maximum yield was 1.95 g/plant with mycogenic NPs, which outcompeted the rest of the treatments.

Furthermore, the fungicides mancozeb and propiconazole in protective and curative modes have no significant effect on the seed yield as compared to control plants (0.72 g/plant). However, the mancozeb at 500 ppm resulted in a seed yield of around 0.86 and 0.77 g/plant for plants grown in alluvial soil. Likewise, in calcareous soil, a significant reduction in seed yield was observed, which was around 0.439 and 0.53 g/plant in mancozeb-exposed plants through both modes, respectively. Thus, it could be concluded that in calcareous soil, the mancozeb treatment leads to stress through both modes.

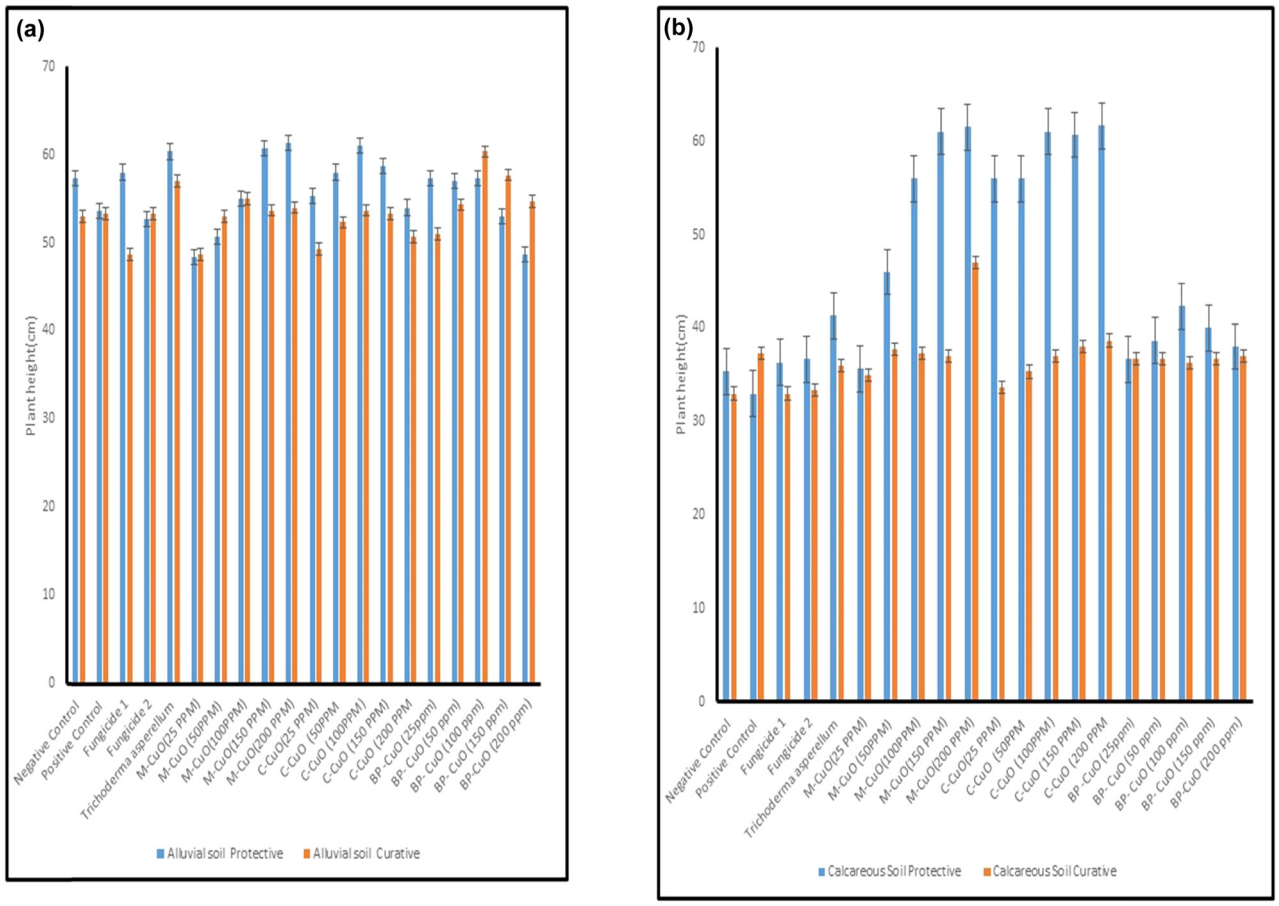

Figure 5: Effect of CuO NPs on seed yield/plant: (a) alluvial soil and (b) calcareous soil.

Moreover, the plants treated with propiconazole have no significant increase in seed yield as compared to control plants. Likewise, in calcareous soil, a decline in seed yield with 0.478 and 0.563 g/plant through both modes was obtained as compared to control plants (0.67 g/plant). On the contrary, with the filtrate of *T. asperellum*, in calcareous soil, nearly equal results were 0.63 and 0.60 g/plants obtained as compared to the control (0.63 g/plant). While in alluvial soil, a one-fold decrease (0.67 and 0.61 g/plant) as compared to control plants (0.72 g/plant) was obtained. Thus, the analysis of all treatments suggested that mycogenic NPs doubled the seed yield, which outcompeted the rest of the treatments.

3.5 Defense enzyme estimation in *Brassica* plants

ROS are the major factors responsible for tissue deterioration during abiotic stress and biotic stress. These are known to disintegrate nucleic acids and alter protein structures, which ultimately cause mutation in gene and delay the process of cell division, thereby affecting the plant growth. Tian et al. [34] reported that higher accumulation of ROS can lead to cell death, which can be either necrotic or programmed. In the present study, the production and accumulation of O^{2-} in control and treated mustard plants were recorded by spectrophotometry (Figure 6 and Table 4). The probable reasons for O^{2-} accumulations are the oxidative burst and reduced efficiency of antioxidant enzymes occurred during NP exposure. In our study, it has been found that M-CuO NP exposure and C-CuO NPs at higher doses (150 and 200 ppm) lead to the accumulation of O_2 radicals; results are comparable with the study of Roy et al. [35] who have reported the comparative impact of bulk phase CuO and nanoforms of CuO in maize seedlings, separately which showed more pronounced and higher SOD and CAT activities in BP-CuO-exposed maize seedlings [35]. The mustard leaves challenged with BP-CuO has shown poor performance of bulk particles which could be explained by high tissue Cu accumulation, comparatively higher membrane injury, marked decline in carotenoids level and higher Cu ions dissolution.

Table 2: Shoot length of *Brassica* plants treated with CuO NPs

Shoot length of mustard plants grown in alluvial and calcareous soil					
		Alluvial soil		calcareous soil	
S. no.	Treatments	Protective	Curative	Protective	Curative
1.	Negative control	57.3 ± 1.77^{a}	53.0 ± 2.16^{a}	35.3 ± 1.24^{a}	33.0 ± 1.63^{a}
2.	Positive control	53.6 ± 2.27^{b}	53.3 ± 2.27^{a}	33.0 ± 4.32^{b}	37.3 ± 1.41^{b}
3.	Fungicide 1 (mancozeb)	58.0 ± 0.00^{c}	48.6 ± 4.61^{b}	36.3 ± 0.94^{c}	33 ± 2.49^{c}
4.	Fungicide 2 (propiconazole)	52.6 ± 0.40^{d}	53.3 ± 0.33^{a}	36.6 ± 0.47^{d}	33.3 ± 0.47^{c}
5.	*Trichoderma asperellum*	60.3 ± 2.8^{e}	57.0 ± 1.06^{c}	41.3 ± 1.24^{e}	36 ± 2.82^{d}
6.	M-CuO NPs (25 ppm)	48.3 ± 1.08^{f}	48.6 ± 1.08^{b}	35.6 ± 0.94^{f}	35 ± 2.16^{e}
7.	M-CuO NPs (50 ppm)	50.6 ± 0.40^{g}	53.0 ± 3.24^{a}	46 ± 0.81^{g}	37.6 ± 0.47^{f}
8.	M-CuO NPs (100 ppm)	55 ± 1.41^{h}	55.0 ± 1.87^{h}	56 ± 2.16^{h}	37.3 ± 0.47^{g}
9.	M-CuO NPs (150 ppm)	60.6 ± 1.47^{e}	53.6 ± 0.81^{a}	61 ± 0.18^{i}	37.0 ± 0.81^{h}
10.	M-CuO NPs (200 ppm)	61.3 ± 0.43^{e}	54.0 ± 1.41^{g}	61.5 ± 0.44^{i}	47.0 ± 0.23^{i}
11.	C-CuO NPs (25 ppm)	55.3 ± 0.47^{h}	49.3 ± 3.09^{c}	56 ± 2.16^{j}	33.6 ± 1.24^{j}
12.	C-CuO NPs (50 ppm)	58.0 ± 0.81^{c}	52.3 ± 1.69^{d}	56 ± 0.81^{j}	35.3 ± 0.81^{k}
13.	C-CuO NPs (100 ppm)	61.0 ± 0.81^{e}	53.6 ± 1.69^{d}	61 ± 0.81^{k}	37.0 ± 3.09^{l}
14.	C-CuO NPs (150 ppm)	58.6 ± 1.24^{c}	53.3 ± 1.69^{d}	60.6 ± 0.47^{l}	38 ± 1.24^{m}
15.	C-CuO NPs (200 ppm)	54 ± 0.816^{b}	50.6 ± 4.49^{c}	61.6 ± 0.47^{l}	38.6 ± 0.92^{n}
16.	BP-CuO (25 ppm)	57.3 ± 2.05^{c}	51.0 ± 0.471^{d}	36.6 ± 0.47^{c}	36.6 ± 0.47^{o}
17.	BP-CuO (50 ppm)	57 ± 0.816^{c}	54.3 ± 0.471^{a}	38.6 ± 0.47^{c}	36.6 ± 1.24^{o}
18.	BP-CuO (100 ppm)	57.3 ± 0.471^{c}	60.3 ± 1.69^{i}	42.3 ± 1.69^{e}	36.3 ± 0.94^{o}
19.	BP-CuO (150 ppm)	53 ± 2.16^{b}	57.6 ± 0.816^{c}	40.0 ± 0.81	36.6 ± 0.47^{o}
20.	BP-CuO (200 ppm)	48.6 ± 1.24^{f}	54.6 ± 0.816^{h}	38.0 ± 0.81	37.0 ± 0.81^{p}

Different superscript letters indicate statistically significant results among control and experimental groups.

Table 3: Seed weight of *Brassica* plants exposed with CuO NPs

Seed weight (1,000 seeds) of mustard plants grown in alluvial and calcareous soil					
		Alluvial soil		calcareous soil	
S. no.	Treatments	Protective	Curative	Protective	Curative
1.	Negative control	0.726 ± 0.012^{a}	0.67 ± 0.089^{a}	0.65 ± 0.056^{a}	0.60 ± 0.047^{a}
2.	Positive control	0.473 ± 0.05^{b}	0.493 ± 0.047^{b}	0.37 ± 0.014^{b}	0.37 ± 0.012^{b}
3.	Fungicide 1 (mancozeb)	0.863 ± 0.012^{c}	0.439 ± 0.019^{b}	0.54 ± 0.04^{c}	0.45 ± 0.169^{c}
4.	Fungicide 2 (propiconazole)	0.773 ± 0.08^{d}	0.478 ± 0.029^{b}	0.38 ± 0.06^{b}	0.56 ± 0.07^{d}
5.	*Trichoderma asperellum*	0.676 ± 0.136^{e}	0.633 ± 0.03^{c}	1.46 ± 0.13^{d}	0.60 ± 0.04^{e}
6.	M-CuO NPs (25 ppm)	1.01 ± 0.03^{f}	0.71 ± 0.035^{d}	0.49 ± 0.047^{e}	0.72 ± 0.04^{f}
7.	M-CuO NPs (50 ppm)	1.37 ± 0.08^{f}	0.71 ± 0.166^{d}	0.88 ± 0.081^{f}	0.72 ± 0.03^{f}
8.	M-CuO NPs (100 ppm)	1.40 ± 0.175^{g}	0.76 ± 0.069^{e}	1.53 ± 0.024^{d}	0.74 ± 0.02^{f}
9.	M-CuO NPs (150 ppm)	1.88 ± 0.014^{h}	0.92 ± 0.044^{f}	1.54 ± 0.07^{d}	0.77 ± 0.07^{f}
10.	M-CuO NPs (200 ppm)	1.95 ± 0.038^{i}	1.14 ± 0.026^{g}	1.58 ± 0.102^{d}	0.78 ± 0.14^{f}
11.	C-CuO NPs (25 ppm)	1.15 ± 0.338^{f}	0.67 ± 0.009^{c}	0.526 ± 0.054^{c}	0.68 ± 0.004^{g}
12.	C-CuO NPs (50 ppm)	1.38 ± 0.286^{f}	0.68 ± 0.004^{c}	0.633 ± 0.08^{a}	0.77 ± 0.012^{h}
13.	C-CuO NPs (100 ppm)	1.59 ± 0.08^{g}	0.76 ± 0.012^{e}	1.61 ± 0.05^{d}	0.92 ± 0.04^{i}
14.	C-CuO NPs (150 ppm)	1.72 ± 0.054^{h}	0.76 ± 0.008^{e}	1.65 ± 0.007^{d}	0.88 ± 0.09^{j}
15.	C-CuO NPs (200 ppm)	1.84 ± 0.047^{h}	0.83 ± 0.024^{f}	1.71 ± 0.004^{e}	0.76 ± 0.16^{f}
16.	BP-CuO (25 ppm)	0.653 ± 0.038^{e}	0.65 ± 0.042^{c}	0.70 ± 0.040^{f}	0.65 ± 0.03^{g}
17.	BP-CuO (50 ppm)	0.67 ± 0.081^{e}	0.68 ± 0.054^{c}	0.51 ± 0.049^{c}	0.73 ± 0.03^{h}
18.	BP-CuO (100 ppm)	0.74 ± 0.352^{d}	0.74 ± 0.021^{e}	0.54 ± 0.098^{c}	0.71 ± 0.08^{h}
19.	BP-CuO (150 ppm)	1.52 ± 0.122^{f}	0.93 ± 0.422^{f}	1.39 ± 0.04^{d}	0.85 ± 0.05^{j}
20.	BP-CuO (200 ppm)	1.60 ± 0.116^{f}	0.55 ± 0.159^{c}	1.50 ± 0.04^{d}	0.80 ± 0.05^{j}

Different superscript letters indicate statistically significant results among control and experimental groups.

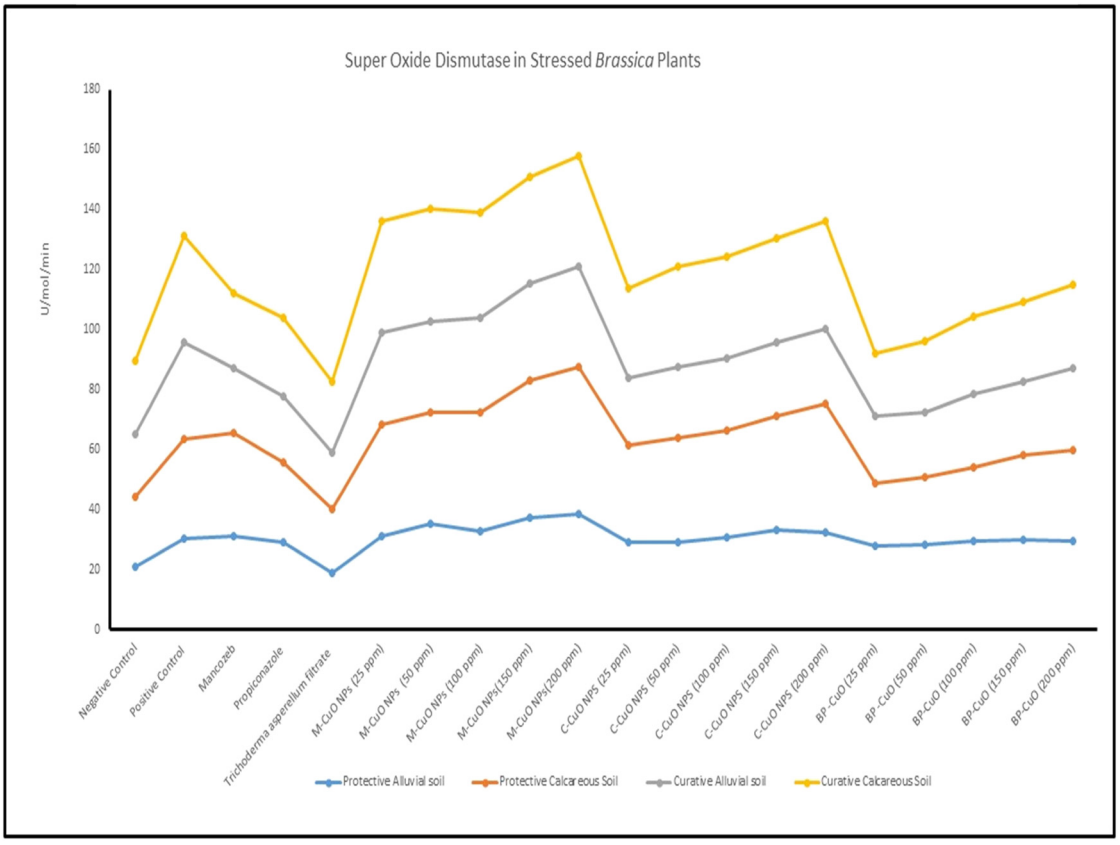

Figure 6: Superoxide dismutase enzyme estimation in *Brassica* plants.

Table 4: Antioxidant enzyme analysis (superoxide dismutase) in *Brassica* plants

Superoxide dismutase activity of mustard plants treated with CuO NPs (mol·min^{-1})					
		Alluvial soil		calcareous soil	
S. no.	Treatments	Protective	Curative	Protective	Curative
1.	Negative control	21.1 ± 0.47^{a}	20.7 ± 0.12^{a}	22.9 ± 0.46^{a}	24.7 ± 0.52^{a}
2.	Positive control	30.3 ± 0.12^{b}	31.7 ± 0.33^{b}	33.2 ± 0.12^{b}	35.5 ± 0.81^{b}
3.	Fungicide 1 (mancozeb)	31.3 ± 0.12^{b}	21.6 ± 0.12^{c}	34.3 ± 0.14^{c}	24.7 ± 0.43^{c}
4.	Fungicide 2 (propiconazole)	29.3 ± 0.36^{c}	22.6 ± 0.57^{d}	26.1 ± 0.40^{d}	26.2 ± 1.13^{d}
5.	*Trichoderma asperellum*	18.7 ± 0.16^{d}	18.5 ± 0.12^{e}	21.4 ± 0.08^{e}	23.9 ± 1.43^{e}
6.	M-CuO NPs (25 ppm)	31.3 ± 0.09^{b}	30.5 ± 0.08^{f}	37.1 ± 0.49^{f}	36.9 ± 1.73^{f}
7.	M-CuO NPs (50 ppm)	35.0 ± 0.44^{e}	30.2 ± 0.16^{f}	37.4 ± 1.29^{f}	37.3 ± 0.97^{g}
8.	M-CuO NPs (100 ppm)	32.9 ± 0.601^{b}	31.4 ± 0.14^{g}	39.3 ± 0.46^{g}	35.3 ± 0.49^{f}
9.	M-CuO NPs (150 ppm)	37.5 ± 0.244^{f}	32.2 ± 0.14^{h}	45.5 ± 0.21^{h}	35.3 ± 2.19^{f}
10.	M-CuO NPs (200 ppm)	38.4 ± 1.06^{g}	33.2 ± 0.12^{i}	49.1 ± 0.43^{i}	37.0 ± 0.33^{g}
11.	C-CuO NPs (25 ppm)	28.9 ± 0.124^{c}	22.2 ± 0.71^{j}	32.4 ± 0.09^{b}	29.8 ± 0.08^{d}
12.	C-CuO NPs (50 ppm)	29.2 ± 0.44^{c}	23.8 ± 1.13^{k}	34.5 ± 0.12^{c}	33.6 ± 0.75^{f}
13.	C-CuO NPs (100 ppm)	30.7 ± 0.08^{c}	23.9 ± 0.40^{l}	35.4 ± 0.16^{c}	34.1 ± 2.46^{f}
14.	C-CuO NPs (150 ppm)	33.2 ± 0.89^{b}	24.8 ± 0.32^{m}	37.8 ± 0.04^{f}	34.7 ± 1.72^{f}
15.	C-CuO NPs (200 ppm)	32.5 ± 0.41^{b}	25.4 ± 0.47^{n}	42.6 ± 0.98^{h}	35.7 ± 1.35^{f}
16.	BP-CuO (25 ppm)	27.9 ± 0.08^{c}	20.2 ± 2.02^{o}	20.7 ± 0.04^{a}	20.8 ± 0.40^{h}
17.	BP-CuO (50 ppm)	28.2 ± 0.12^{c}	21.9 ± 0.46^{p}	22.3 ± 0.20^{a}	23.7 ± 0.99^{c}
18.	BP-CuO (100 ppm)	29.4 ± 0.41^{c}	23.0 ± 2.1^{q}	24.4 ± 0.12^{b}	25.9 ± 0.57^{d}
19.	BP-CuO (150 ppm)	29.8 ± 0.08^{c}	24.0 ± 1.00^{r}	28.5 ± 0.08^{d}	26.5 ± 0.08^{d}
20.	BP-CuO (200 ppm)	29.6 ± 0.08^{c}	26.2 ± 1.72^{s}	30.2 ± 0.12^{d}	27.4 ± 1.00^{d}

Different superscript letters indicate statistically significant results among control and experimental groups.

Table 5: Stress enzyme analysis (catalase) in mustard plants treated with CuO NPs

Catalase activity of mustard plants treated with CuO NPs (mol·min^{-1})					
		Alluvial soil		calcareous soil	
S. no.	Treatments	Protective	Curative	Protective	Curative
1.	Negative control	23.6 ± 0.08^{a}	25.6 ± 0.81^{a}	23.3 ± 0.169^{a}	26.0 ± 0.53^{a}
2.	Positive control	34.3 ± 0.16^{b}	35.2 ± 1.66^{b}	32.5 ± 0.08^{b}	37 ± 0.99^{b}
3.	Fungicide 1 (mancozeb)	34.4 ± 0.12^{b}	24.7 ± 1.86^{c}	34.6 ± 0.09^{c}	27.3 ± 1.16^{c}
4.	Fungicide 2 (propiconazole)	38.3 ± 1.10^{c}	24.2 ± 0.98^{d}	36.5 ± 0.16^{d}	27.7 ± 0.78^{c}
5.	*Trichoderma asperellum*	17.9 ± 0.08^{d}	23.8 ± 0.47^{e}	21.7 ± 0.49^{a}	24.5 ± 1.51^{d}
6.	M-CuO NPs (25 ppm)	25.3 ± 0.47^{e}	31.8 ± 0.80^{f}	37 ± 0.35^{d}	35.6 ± 1.51^{e}
7.	M-CuO NPs (50 ppm)	25.7 ± 0.21^{e}	32.1 ± 0.42^{f}	38.4 ± 0.24^{d}	36.4 ± 0.56^{f}
8.	M-CuO NPs (100 ppm)	31.6 ± 0.16^{f}	33.8 ± 1.01^{f}	39.5 ± 0.43^{d}	37.7 ± 0.12^{f}
9.	M-CuO NPs (150 ppm)	31.5 ± 0.20^{f}	33.7 ± 0.08^{f}	40.8 ± 0.08^{d}	39.7 ± 0.124^{f}
10.	M-CuO NPs (200 ppm)	36.6 ± 0.08^{g}	33.7 ± 0.04^{f}	48.5 ± 0.98^{e}	39.8 ± 0.08^{f}
11.	C-CuO NPs (25 ppm)	21.8 ± 0.88^{h}	27.7 ± 0.78^{g}	32.6 ± 0.12^{b}	25.7 ± 0.29^{a}
12.	C-CuO NPs (50 ppm)	24.8 ± 0.57^{e}	26.7 ± 0.04^{g}	34.6 ± 0.09^{b}	27.6 ± 0.09^{c}
13.	C-CuO NPs (100 ppm)	25.8 ± 1.23^{e}	27.0 ± 0.57^{g}	35.4 ± 0.12^{c}	24.0 ± 0.38^{a}
14.	C-CuO NPs (150 ppm)	29.7 ± 0.21^{f}	27.7 ± 0.81^{g}	37.7 ± 0.21^{d}	27.0 ± 1.37^{c}
15.	C-CuO NPs (200 ppm)	38.2 ± 1.62^{g}	28.0 ± 0.91^{g}	41.7 ± 0.33^{d}	28.4 ± 0.47^{c}
16.	BP-CuO (25 ppm)	21.3 ± 2.1^{a}	23.6 ± 0.08^{c}	20.7 ± 0.12^{a}	24.6 ± 0.82^{d}
17.	BP-CuO (50 ppm)	19.7 ± 0.12^{i}	23.6 ± 0.01^{c}	22.6 ± 0.24^{a}	25.9 ± 1.21^{a}
18.	BP-CuO (100 ppm)	19.6 ± 0.12^{i}	24.2 ± 0.94^{c}	24.7 ± 0.08^{a}	26.6 ± 0.69^{a}
19.	BP-CuO (150 ppm)	19.4 ± 0.35^{i}	25.5 ± 0.81^{d}	28.3 ± 0.37^{a}	27.3 ± 0.46^{a}
20.	BP-CuO (200 ppm)	19.5 ± 0.12^{i}	27.5 ± 0.08^{d}	30.3 ± 0.12^{b}	27.3 ± 0.87^{a}

Different superscript letters indicate statistically significant results among control and experimental groups.

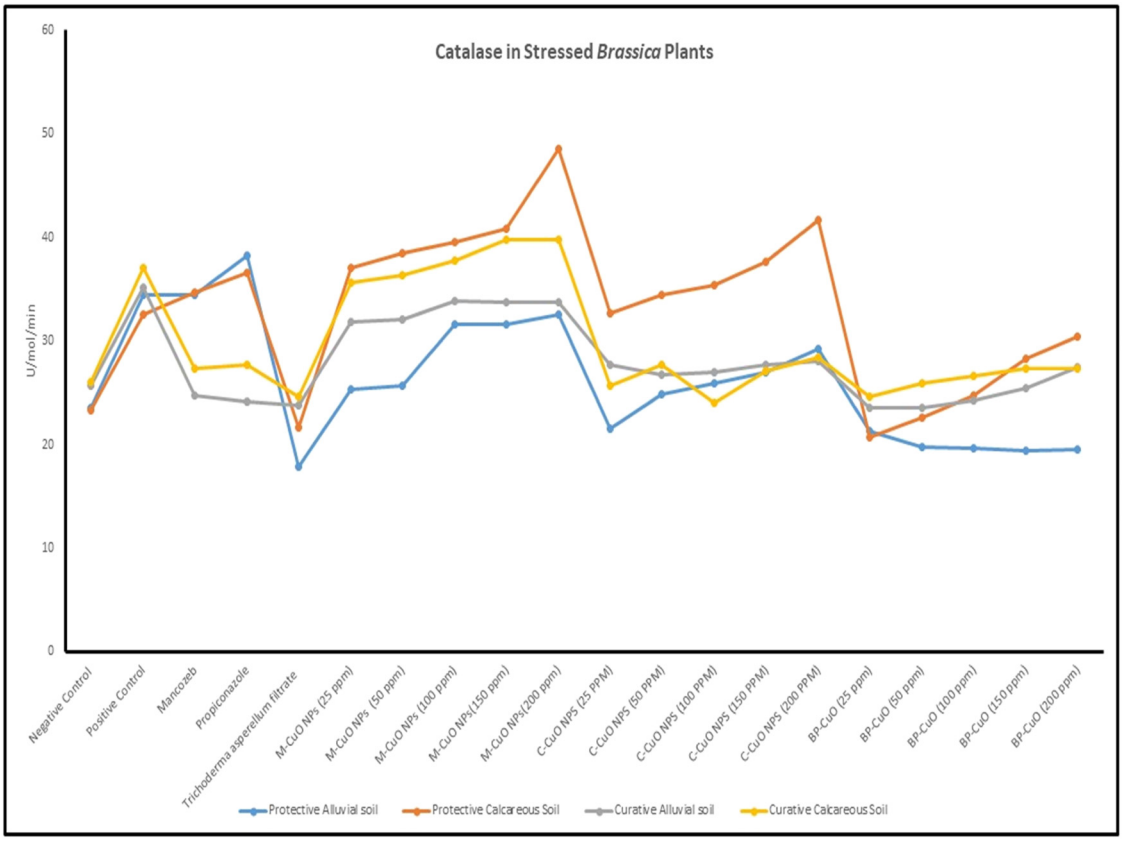

Figure 7: CAT activity in *Brassica* plants exposed to CuO NPs.

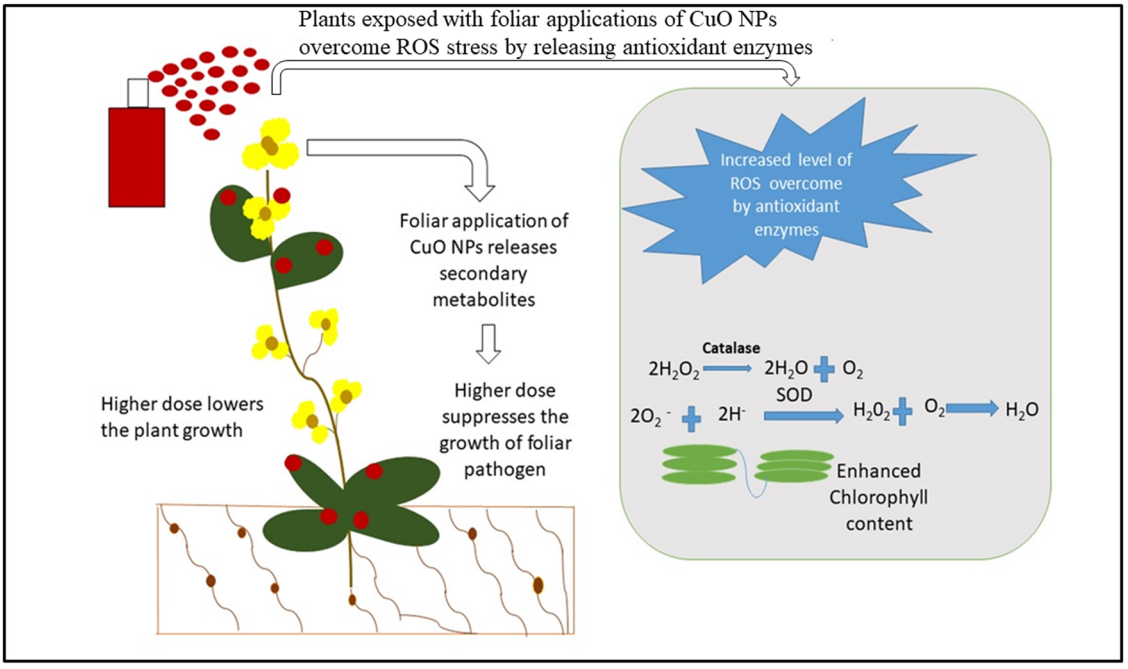

Figure 8: Impact of CuO NPs on disease suppression and biochemical parameters.

Interestingly, in our study, we found that, in protective and curative treatments, plants exposed to M-CuO NPs leads to a lower level of O_2 accumulation, as compared to control plants, which ultimately results in antioxidant enzymes' downregulation, and proved to be an efficient non-toxic molecule for plant system (Figure 6 and Table 5). Also, C-CuO NPs' exposure and BP-CuO at higher doses (150 and 200 ppm) lead to the accumulation of O_2 radicals. It has been suggested that O^{2-} gets converted into H_2O_2 and O_2 by the enzyme SOD. Moreover, it has been found that a significantly higher percentage of SOD activity was recorded in protective treatment for mustard plants grown in calcareous soil, which could be due to the synergistic effect of biotic stress and abiotic stress; the plants grown in calcareous soil and exposed to two foliar applications of CuO resulted in more O^{2-} accumulation. Similarly, CAT activity was increased in BP-CuO plants and C-CuO, and the traditional fungicides when acted as a protectant also lead to the accumulation of H_2O_2, which may be the result of enhanced SOD activity in the same plant groups with protective treatment and plants grown in calcareous soil (Figure 7). Thus, the alterations found in both treatments in both soils concluded that M-CuO NPs worked more proficiently in both soils with both treatments. However, the study needs to be explored more for mustard plants grown in calcareous soil.

Our finding with M-CuO NPs shows a positive response at higher concentrations, when applied on foliage, enhanced almost all the growth, biochemical, and physiological parameters. A simplified view of the foliar application of CuO NPs in *B. juncea* has been shown in Figure 8, where we can see how NPs enter into plant cells and affect different growth and physiological parameters in plants. Based on all the effects observed in the study, NPs promotes disease suppression at higher concentration. Therefore, the release of antifungal compounds is responsible for the inhibition of *Alternaria* blight pathogens.

Plants adapted to two defense systems, one is enzymatic that involves the activation of superoxide dismutase and catalase. 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. Also, biochemical parameters like an increase in photosynthetic pigment and antioxidant enzyme activity upregulation were found in *Brassica* plants.

4 Conclusion and future perspectives

The current investigation reveals that the phytopathogenic fungi *Alternaria brassicae* negatively affected the plant growth, biomass, and productivity of mustard plants. The maximum disease severity index was 42–65% in positive

control plants, whereas plants supplied with mycogenic copper compounds scored with disease suppression by more than 50%. Also, the plant growth characteristics, with enhanced productivity, were obtained having more than 30% increase. The study also revealed that among six treatments tested in a field, M-CuO NPs performed significantly better even from 50 ppm concentration in nutrient-rich and nutrient-deficient soil. As compared with standard chemical fungicides, M-CuO NPs because of their lower particle size and prolonged release of Cu ions resulted in better fertilizing and fungicidal activity.

Also, exogenous supplementation of M-CuO NPs on mustard plants mitigated the detrimental effects by improving the growth characteristics and total chlorophyll content. However, if the plants were exposed with protective applications of C-CuO NPs and BP-CuO leads to the activation of antioxidant enzymes like SOD and CAT, thus indicating the comparatively higher accumulation of ROS but low levels of antioxidant enzymes in M-CuO NPs exposed plants. Therefore, a lesser accumulation of ROS leads to the selection of M-CuO NPs in the field.

The experimental outcomes are based on the controlled condition established through field and pot experiments. Therefore, the *Brassica* plant life cycle monitored with nano-formulations accurately mimics the impacts of CuO NPs as fungicides and fertilizers on plants and their parts to generate environmentally relevant applications. Nanofungicides denote the next generation of traditional fungicides, as well as they will offer more advances such as high efficacy, durability, and less doses of effective ingredients. Preparation of nanofungicides can be done in a simple cost-effective manner, which is found to be appropriate for formulating recent types of biohybrid nanocide constituents and, hence, would be proved as novel environment responsive antimicrobial against diverse fungal pathogens of plants.

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Conflict of interest: The first corresponding author (Ram Prasad) is a member of the Editorial Board of Green Processing and Synthesis.

Data availability statement: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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