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Research Article

EVALUATING THE EFFICACY OF PLANT-MEDIATED COPPER-SILVER NANOPARTICLES FOR CONTROLLING CERCOSPORA LEAF SPOT IN MUNG BEANS

[a]Muhammad Atiq, [a]Laraib Malik, [a]Nasir Ahmed Rajput, [a]Maryam Shabbir, [b]Maryam Ashiq, [a]Muhammad Jahanzaib Matloob, [a]Aisha Waseem, [c]Abdul Jabbar, [a]Asad Ullah, [b]Rida Zawar, [a]Anosh Majeed

[a] *Department of Plant Pathology, Faculty of Agriculture, University of Agriculture, Faisalabad, Pakistan.*
[b] *Department of Botany, Faculty of Sciences, University of Agriculture, Faisalabad, Pakistan.*
[c] *Department of Agronomy, Constituent College Depalpur, Okara, University of Agriculture, Faisalabad, Pakistan.*

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ABSTRACT

Cercospora leaf spot (CLS), caused by *Cercospora canescens*, is a highly destructive disease of mung beans, capable of causing up to 95% yield losses under favorable environmental conditions. Plant-mediated nanoparticles (NPs) have gained significant attention in plant disease management due to their unique properties and potential benefits. This study aimed to evaluate the antifungal efficacy of green-synthesized copper (Cu) and silver (Ag) nanoparticles, both individually and in combination, against *C. canescens*. The nanoparticles were tested at different concentrations (0.25%, 0.50%, and 0.75%) using the poisoned food technique under laboratory conditions. The most effective concentrations were further assessed under field conditions. In the laboratory, the combination of Cu and Ag nanoparticles showed the least mycelial growth (13.29 mm), followed by Ag nanoparticles (18.18 mm) and Cu nanoparticles (22.22 mm), compared to the untreated control. Similarly, under field conditions, the combination of Cu and Ag nanoparticles resulted in the lowest disease incidence (22.45%), followed by Ag (29.94%) and Cu nanoparticles (36.50%). These findings suggest that hybrid Cu-Ag nanoparticles have significant potential as an effective tool for managing CLS in mung beans, offering a sustainable alternative for disease control.

Corresponding Author: Muhammad Atiq
Email: muhammad.atiq@uaf.edu.pk

INTRODUCTION

Mung bean (*Vigna radiata* L.) is a highly nutritious legume crop belonging to the Fabaceae family. It serves as an important source of proteins, antioxidants, and carbohydrates, making it a preferred ingredient in staple diets alongside rice, wheat, and maize (Abbas et al., 2020; Akhtar et al., 2023). Its seeds are widely used to prepare soups, bean paste, beverages, transparent noodles, ice creams, and desserts. Globally, mung bean is cultivated on approximately 7.3 million hectares with an average yield of 721 kg/ha (Ademe, 2023). Major producers include India, Pakistan, Myanmar, Sri Lanka, China, Bangladesh, Thailand, Indonesia, and Australia.

Mung bean has significant ecological importance due to its ability to fix atmospheric nitrogen through symbiotic relationships with rhizobia (Diatta et al., 2020). It contains polyphenols that play a critical role in lipid metabolism, the absorption of anti-inflammatory agents,

and the management of diabetes. Moreover, mung bean has been reported to help prevent hair and nail loss, reduce the risk of hypercholesterolemia and heart disease, and minimize the absorption of toxic substances (Kalim et al., 2021).

Despite its numerous benefits, mung bean is vulnerable to a range of biotic and abiotic stresses (Iqbal and Mukhtar, 2014; Mukhtar et al., 2017, 2021). Key biotic threats include dry root rot, anthracnose, Cercospora leaf spot (CLS), powdery mildew, and yellow mosaic virus. Abiotic stresses such as high temperatures (>35 °C), waterlogging, drought, and heat also significantly impact its yield and quality (Nair et al., 2019; Pratap et al., 2019). Among these, CLS, caused by *Cercospora canescens*, is particularly devastating, with potential yield losses reaching up to 95%. The disease was first reported in Delhi, India (Kumar et al., 2020).

CLS thrives in temperatures between 25 and 30°C and requires high relative humidity (98-100%) for its development and spread (Kumar et al., 2020). It is characterized by reddish-brown margins and chlorotic (yellow) lesions on the leaves, which eventually dry out and fall off (Nair et al., 2019).

The excessive use of harmful chemicals and traditional plant disease management methods presents significant safety and environmental challenges. The widespread application of pesticides can negatively impact human health and disrupt beneficial soil microbes (Khan et al., 2022). To address these growing concerns, the use of nanoparticles has gained attention as a safer and more efficient approach to diagnosing and managing plant diseases (Shahbaz et al., 2022, 2023; Zielińska et al., 2023).

Kumar et al. (2022) highlighted the crucial role of nanoparticles in agriculture, emphasizing their ability to provide direct nutritional benefits and alleviate plant stress. Nanoparticles are valuable tools for the precise detection of phytopathogens, as they interact effectively with biomolecular targets. Among these, green-synthesized nanoparticles have emerged as a sustainable and eco-friendly alternative to conventional plant disease management strategies. Plant parts such as leaves, seeds, and flowers possess unique nanoscale properties that make them suitable for nanoparticle synthesis (Jannat et al., 2022). This plant-based approach utilizes readily available natural compounds, offering minimal risks to human health and the environment (Atiq et al., 2022).

Plant extracts are rich in phytochemicals that act as stabilizing and reducing agents in nanoparticle synthesis (Hossain et al., 2019). In recent years, silver nanoparticles (AgNPs) have gained significant attention due to their production through environmentally friendly methods, known as “green synthesis”. Green synthesis refers to the sustainable production of nanoparticles using natural sources (Moradi et al., 2021). Furthermore, copper nanoparticles (CuNPs) have emerged as a promising treatment option for fungal diseases (Cruz-Luna et al., 2021). Copper, an essential nutrient for plant growth, exhibits minimal toxicity when applied in nanoparticle form (Bakshi and Kumar, 2021).

Recent research efforts have focused on exploring the efficacy of green-synthesized AgNPs and CuNPs using *Moringa oleifera* leaves to combat *C. canescens*, demonstrating their potential as an eco-friendly and effective solution for plant disease management.

MATERIALS AND METHODS

Isolation of the pathogen

A survey was conducted in various localities of district Faisalabad, Punjab, Pakistan, including the Ayub Agricultural Research Institute (AARI), Samundri, and Jaranwala, to collect diseased leaf samples. The fungal pathogen was isolated using Potato Dextrose Agar (PDA) medium. For this, sterilized Petri dishes (90 mm) were filled with PDA medium and allowed to solidify. The infected samples were washed under running tap water to remove dust particles. Subsequently, both infected and healthy portions were cut into small pieces (approximately 5 mm) using sterilized scissors. The samples were surface sterilized with 1% sodium hypochlorite (NaOCl) for 30 sec, followed by three successive rinses with distilled water to eliminate any residual NaOCl. The samples were then dried on sterilized filter paper and transferred to PDA plates using sterilized forceps. The plates were sealed and incubated at 28 ± 1°C.

Identification, purification, and preservation of the pathogen

The fungal pathogen was identified based on morphological characteristics such as colony color, growth pattern, and sporulation type, as well as taxonomic features (Asif et al., 2023). For purification, the hyphal tip technique was employed (Leyronas et al., 2012). A small portion of fungal mycelium was transferred using a sterile needle onto fresh PDA plates, which were incubated at 25 °C for 10-15 days (Atiq et al., 2021).

For preservation, PDA medium was poured into test tubes and slanted to increase the surface area for fungal growth. The pure fungal culture was transferred into these test tubes and incubated in a shaking incubator (REICO) at 25 °C for 24 h. To prepare for long-term storage, Eppendorf tubes (2 ml) were filled with 1 ml of glycerol and 1 ml of fungal growth suspension. The tubes were labeled and stored at -4 °C in a refrigerator (IZ-308SI).

Pathogenicity test

To confirm the presence of the pathogen, a pathogenicity test was performed following Koch's postulates. A susceptible mung bean variety (PML 2005) was grown in plastic pots (30 × 15 cm) under a completely randomized design (CRD). A fungal suspension was prepared at a concentration of 1×10^6 spores/ml using a spectrophotometer (Hitachi U-2001, model 121003) as described by Bashir et al. (2020). The pathogen was inoculated using the syringe method early in the morning, when the stomata were maximally open. Disease symptoms began to appear 6-7 days post-inoculation and were compared with those in the parental plants to confirm pathogen presence.

Preparation of nanoparticles

Fresh moringa leaves (*Moringa oleifera* L.) were collected and shade-dried for 4-5 days. Subsequently, the leaves were sun-dried for 3 days and then placed in a drying oven (108-1AB) at 65 °C for 4 h to remove residual moisture. The dried leaves were ground into a fine powder using a sterilized pestle and mortar.

To prepare the extract, 20 g of this powder was added to 100 ml of methanol in a beaker, which was then covered with aluminum foil and placed in a dark room for 24 h. The solution was filtered using Whatman No. 41 (12.5 cm) filter paper. Separately, 15 g of copper sulfate ($CuSO_4$) and 17 g of zinc oxide (ZnO) were added to the filtrate. The mixture was placed on a magnetic stirrer for 4-5 min to ensure proper mixing, followed by ultrasonic treatment in an ultrasonic cleaner (YJ 5120-1) at 60°C for 30 min to disrupt chemical bonds.

The solution was then transferred to a furnace oven and heated at 80 °C for 24 h. The resulting copper (Cu) and zinc oxide (ZnO) nanoparticles were ground into fine particles using a sterilized pestle and mortar and stored in separate sterilized test tubes for further use. For the preparation of hybrid (Cu-Zn) nanoparticles, the copper and zinc oxide nanoparticles were mixed in appropriate proportions and processed similarly.

Characterization of nanoparticles

Scanning electron microscope (SEM)

The Scanning electron microscope (SEM) was used to analyze the size and morphology of CuNPs and silver AgNPs nanoparticles.

X-ray diffraction (XRD)

X-ray diffraction (XRD) was employed to determine the crystalline structure, composition, and particle size of CuNPs and AgNPs.

Evaluation of green-synthesized Cu-Ag nanoparticles under laboratory conditions

Phyto-nanoparticles of Cu and Ag synthesized from *Moringa* leaves, both individually and in combination, were tested against *C. canescens* under laboratory conditions using the poisoned food technique.

To prepare three nanoparticle concentrations (0.25%, 0.5%, and 0.75%), 0.25 g, 0.5 g, and 0.75 g of Cu-Ag nanoparticle powder were mixed into 500 ml medium bottles containing 100 ml of PDA medium, respectively. Untreated PDA medium served as the control.

The PDA medium was poured into Petri plates and allowed to solidify. A sterilized needle was used to transfer a small piece of *C. canescens* fungal culture to the centre of each plate under a laminar airflow chamber. The plates were sealed with tape and incubated at 28 °C ± 2 °C in a Heraeus incubator for 2-3 days. The experiment followed a CRD with three replications per treatment. Fungal growth was measured at 24, 48, and 72 h post-inoculation.

***In vivo* evaluation of green-synthesized Cu-Ag nanoparticles**

For the *in vivo* experiment, a moderately susceptible mungbean variety, PML 2005, was cultivated in the experimental area of the Department of Plant Pathology, University of Agriculture, Faisalabad. A 2-day-old culture of *C. canescens* was used to prepare a fungal spore suspension at a concentration of 1×10^6 spores ml^{-1}, measured using a spectrophotometer (Hitachi U-2001, model 121003) following the method of Bashir et al. (2020). The spore suspension was applied early in the morning to ensure optimal infection conditions, as stomata were maximally open during this time.

Symptoms of the disease began to appear one week post-inoculation. The most effective concentration of each nanoparticle treatment was then assessed under field conditions. Distilled water served as the control treatment. The experiment followed a Randomized Complete Block Design (RCBD) with three replicates per

treatment to ensure statistical reliability and minimize experimental error.

Data on disease incidence were recorded at intervals of 7, 14, and 21 days after inoculation. Disease incidence was calculated using the following formula:

$$\text{Disease Incidence (\%)} = \frac{\text{No. of infected plants}}{\text{Total no of plants}} \times 100$$

Data analysis

Lab experiments were conducted using CRD, while field experiments followed RCBD. Mean comparisons were performed using the Least Significant Difference (LSD) test (Stahle and Wold, 1989). The collected data were analyzed through Analysis of Variance (ANOVA) using Minitab 18.1 software.

RESULTS

Characterization of AgNPs and CuNPs

SEM was used to capture sub-microscopic images of nanoparticles, revealing that AgNPs exhibit spherical and cubic shapes with a yellow to brown coloration, while CuNPs display spherical, cubic, and hexagonal structures with reddish to dark brown appearance. Moreover, XRD analysis indicated that the average particle size of AgNPs was 31.25 nm, whereas CuNPs had an average size of 26.41 nm, as shown in Table 1. The high surface area-to-volume ratio of the NPs enhances their interaction with targeted fungal cells, leading to leakage of intracellular components and ultimately causing cell death.

Table 1. Characterization of AgNPs and CuNPs.

Characteristics	AgNPs	CuNPs
Average particle size	31.25 nm	26.41 nm
Appearance	Yellow to brown	Reddish to dark brown
Shape	Spherical and cubic	Spherical, cubic and Hexagonal

In vitro* evaluation of green synthesized copper and silver nanoparticles against *C. canescens

Among all treatments, Cu-Ag hybrid nanoparticles exhibited the lowest mycelial growth (13.29 mm), followed by AgNPs (18.18 mm) and CuNPs (22.22 mm), compared to the control (Table 2). The interaction between treatment and concentration (T × C) revealed that Cu-Ag NPs achieved the minimum mycelial growth (15.33, 13.11, and 11.44 mm) at concentrations of 0.25%, 0.5%, and 0.75%, respectively, followed by AgNPs (20.76, 18.37, and 15.41 mm) and CuNPs (24.86, 22.67, and 19.13 mm), as compared to the control (Table 3 and Figure 1).

Furthermore, the interaction between treatment and time indicated that Cu-Ag nanoparticles resulted in the least mycelial growth (11.08, 13.27, and 15.53 mm) after 24, 48, and 72 h, respectively, followed by AgNPs (15.89, 18.01, and 20.65 mm) and CuNPs (19.45, 22.03, and 25.16 mm), compared to the control (Table 4 and Figure 2).

Table 2. *In vitro* evaluation of green synthesized Cu-Ag NPs against *C. canescens*.

Treatments	Mycelial growth (mm)
Cu-AgNPs	13.298 d
AgNPs	18.183 c
CuNPs	22.220 b
Control	47.143 a
LSD	1.3662

Interpretation: Pairwise comparison LSD tests (P ≤ 0.05) showed that mean values within a column sharing the same letters do not differ significantly.

Table 3. Impact of treatment and concentration interaction on the mycelial growth of *C. canescens*.

Treatments	Mycelial growth (mm)		
	Concentrations (%)		
	0.25 %	0.5 %	0.75 %
Cu-AgNPs	15.333 h	13.117 hi	11.444 i
AgNPs	20.761 ef	18.372 g	15.417 h
CuNPs	24.861 d	22.667 de	19.133 fg
Control	43.822 c	46.883 b	50.722 a
LSD	2.3663		

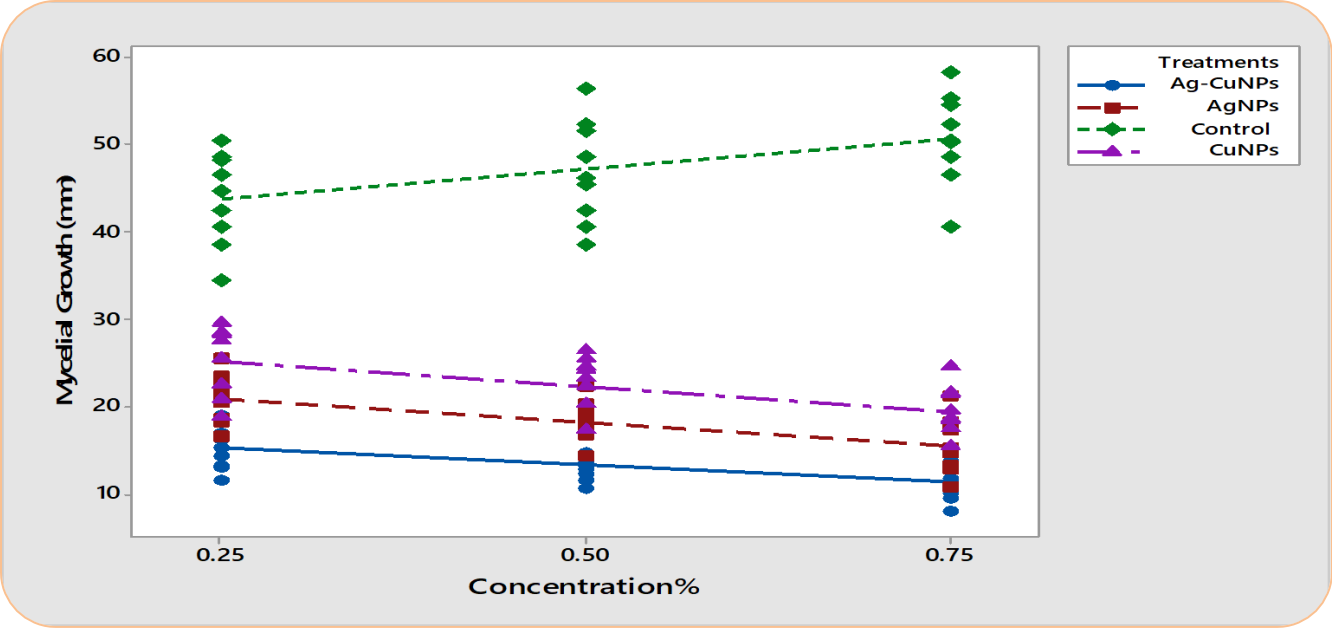

Figure 1. Impact of treatment and concentration interaction on the mycelial growth of *C. canescens.*

Table 4. The impact of treatments and time interactions on the mycelial growth of *C. canescens*.

Treatments	Mycelial growth (mm)		
	Time (hours)		
	24 h	48 h	72 h
Cu-AgNPs	11.083 k	13.278 jk	15.533 ij
AgNPs	15.889h i	18.011 gh	20.650 ef
CuNPs	19.456 fg	22.039 e	25.167 d
Control	41.833 c	47.822 b	51.772 a
LSD		2.3663	

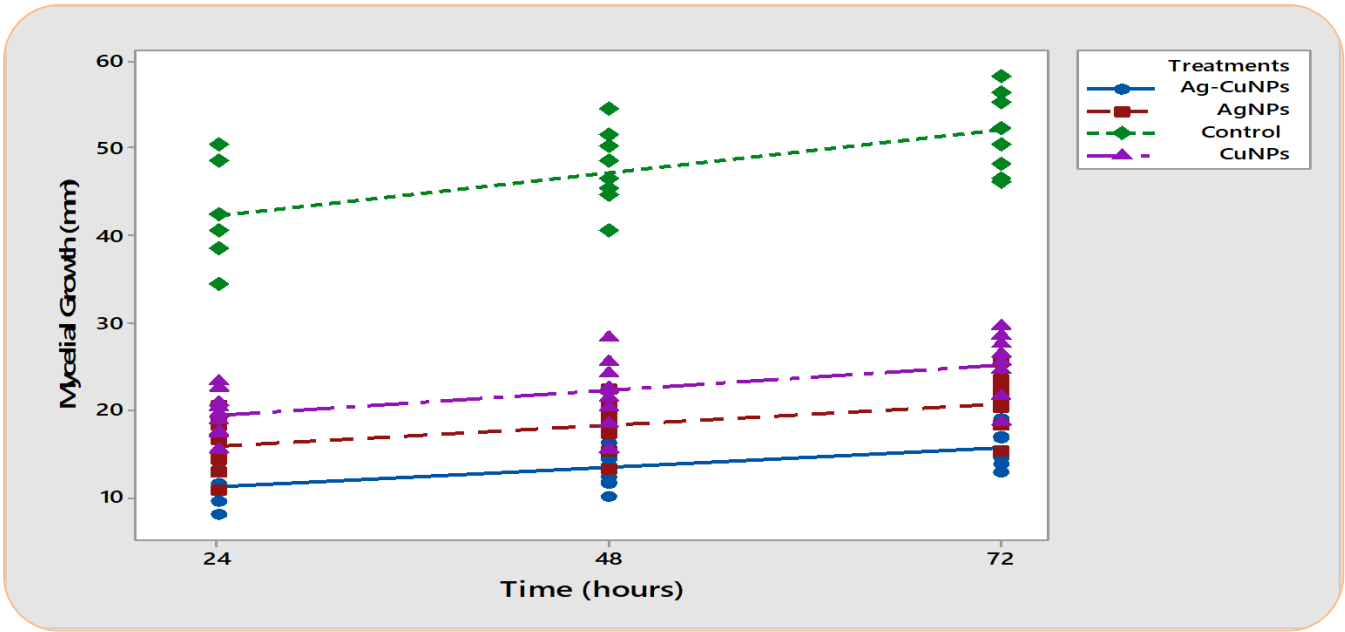

Figure 2. The impact of treatment and time interaction on the mycelial growth of *C. canescens.*

Evaluation of silver and copper nanoparticles against CLS under field conditions

In terms of disease incidence, Cu-Ag (hybrid) NPs exhibited the lowest rate at 22.27%, followed by AgNPs at 29.94%, and CuNPs at 36.44%, respectively, compared to the control treatment (Table 5). The interaction between treatment and duration (T×D) revealed that Cu-AgNPs resulted in the lowest disease incidence, with values of 26.05%, 22.32%, and 18.45% at 7, 14, and 21 days, respectively. This was followed by AgNPs at 36.67%, 30.52%, and 22.63%, and CuNPs at 42.33%, 36.45%, and 30.55% at the same intervals (Table 6 and Figure 3).

Table 5. *In vivo* evaluation of green-synthesized Cu-Ag hybrid nanoparticles on the incidence of CLS.

Treatments	Disease incidence (%)
Cu-AgNPs	22.273 d
AgNPs	29.943 c
Cu-AgNPs	36.443 b
Control	53.244 a
LSD	3.8755

Interpretation: As determined by pairwise comparison using LSD tests (P ≤ 0.05), mean values in a column that share the same letters indicate no significant difference.

Table 6. The interaction between treatments and days affects the incidence of CLS.

Treatments	Disease incidence (%)		
	Days		
	After 7 days	After 14 days	After 24 days
Cu-AgNPs	26.050 fg	22.320 gh	18.450 h
AgNPs	36.667 de	30.523 ef	22.630 gh
CuNPs	42.330 cd	36.450 de	30.550 ef
Control	46.500 bc	52.650 b	60.583 a
LSD	6.7126		

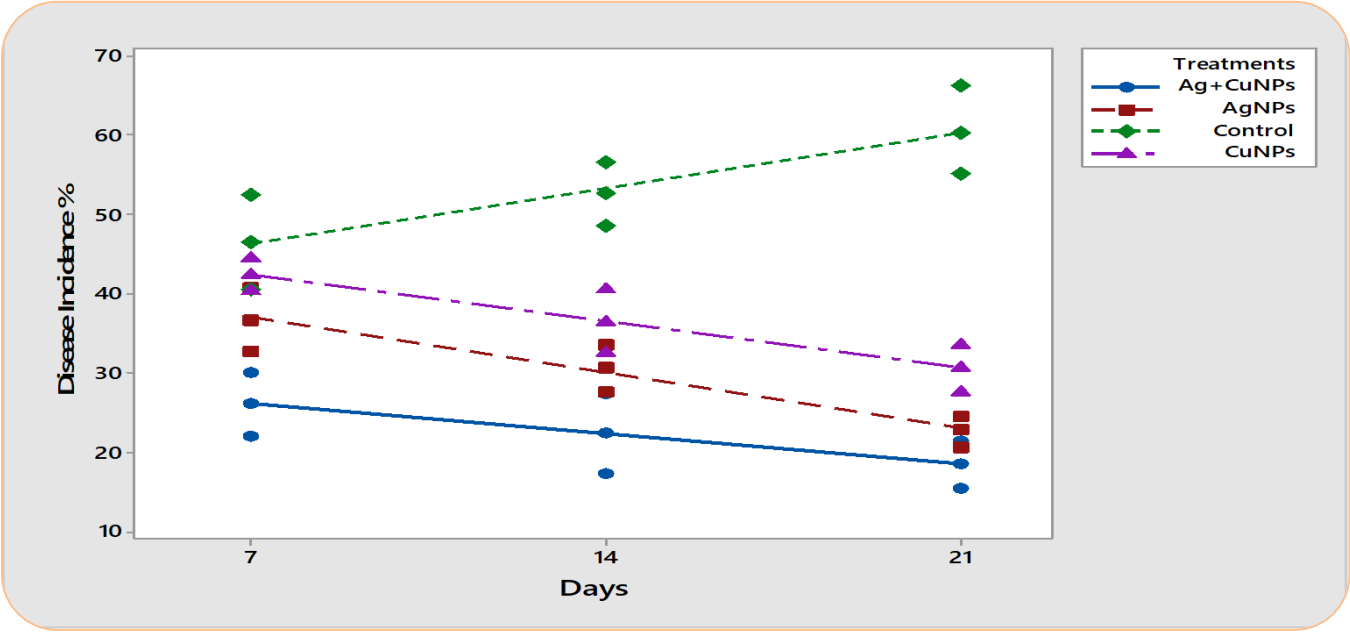

Figure 3. Interaction between treatments and days on the disease incidence of CLS.

DISCUSSION

Researchers are increasingly turning to advanced technologies for the safe and environmentally friendly management of various pathogens. A prominent application of nanotechnology in this context is the use of nanoparticles (Duhan et al., 2017; Nazir et al., 2019; Khan et al., 2021; Jabbar et al., 2022). Metal nanoparticles, including AgNPs, ZnNPs, CuNPs, and

magnesium oxide (MgO NPs), have proven effective in controlling fungal and bacterial activity (Baker et al., 2017). What distinguishes this approach is its eco-friendly and cost-efficient nature, as it utilizes the biosynthesis of nanoparticles rather than relying on traditional physical and chemical methods (Parveen et al., 2016). In real-world agricultural applications, this approach could protect crops from fungal pathogens, offering a sustainable alternative to chemical treatments. Plant-mediated nanoparticles significantly reduced disease incidence compared to the application of plant extracts alone. These nanoparticles stimulate the activity of antioxidant enzymes (SOD, POD, and CAT) and pathogenesis-related proteins (such as chitinases and glucanases), enhancing the defense mechanisms of plants and acting as antimicrobial agents (Castillo-Henríquez et al., 2020; Flieger et al., 2021; Satti et al., 2022). In the current study, we explored the potential of Moringa-based AgNPs and CuNPs for managing *C. canescens*, both *in vitro* and *in vivo*. Under controlled *in vitro* conditions, Cu-Ag hybrid nanoparticles exhibited the most effective inhibition of mycelial growth, while in the field, Cu-Ag hybrid NPs showed the lowest disease incidence. Our findings are consistent with previous research by Babar et al. (2022), who used AgNPs to combat CLS, a fungal disease affecting mung bean plants. Their study focused on the antifungal activity of AgNPs against *C. canescens* under both greenhouse and laboratory conditions. Moreover, our results align with the findings of Oloyede et al. (2016), who demonstrated the antifungal potency of AgNPs against *C. canescens*, *Fusarium oxysporum*, and *F. solani*. Furthermore, our research supports the study by Jenish et al. (2022), which highlighted the fungicidal potential of silver nanoparticles derived from *M. oleifera* against *Pestalotiopsis mangiferae*. Finally, our results are corroborated by Lamsal et al. (2011), who assessed the antifungal activity of AgNPs and demonstrated their effectiveness against *Colletotrichum falcatum*. This comprehensive evaluation underscores the potential of green-synthesized nanoparticles as a safe and sustainable alternative for managing plant diseases, contributing to both agricultural and environmental sustainability. Moreover, our findings are in agreement with the results of Priya et al. (2022), who evaluated CuO NPs and demonstrated their antifungal efficacy against *Aspergillus flavus*, *A. niger*, and *Penicillium frequentans*.

The interaction between nanoparticles and microbial cell membranes occurs primarily through electrostatic forces. These forces cause an attraction between the negatively charged microbial membrane and the nanoparticles, regardless of whether the nanoparticles carry a positive or negative charge. This interaction disrupts the structural integrity of the microbial membrane, leading to depolarization and increased permeability. As a result, essential respiratory processes are impaired, causing damage to cellular structures and, ultimately, cell death. This disruption also triggers the release of intracellular contents, including proteins, enzymes, DNA, and metabolites. Furthermore, nanoparticles can induce irregularities or pits on the microbial cell wall, facilitating their entry into the periplasmic space and enabling further penetration into the cells (Lemire et al., 2013).

CONCLUSION

Under controlled *in vitro* conditions, the combination of Cu-Ag NPs exhibited minimal mycelial growth. In the field, these nanoparticles showed the lowest disease incidence, followed by AgNPs and CuNPs. Copper and silver nanoparticles possess unique physicochemical properties, making them an emerging tool against fungal pathogens. Therefore, it is concluded that the hybrid form of copper and silver nanoparticles is strongly recommended to farmers as an effective approach for managing Cercospora leaf spot.

AUTHORS CONTRIBUTIONS

MA, and LM designed, formulated and laid out the study; NAR, MS, and MA conducted the experiments; MA, MJM, and AW collected, arranged and analyzed the data; MA, and NAR provided technical assistance; LM, MA, and NAR supervised the work; LM, AJ, and AU wrote the manuscript; MJM, RZ and AM proofread the paper.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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