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In vitro antifungal evaluation of silver nanoparticles against *Colletotrichum gloeosporioides* (Penz.)

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Abstract

Anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) is a major field and postharvest disease of *Citrus*, leading to severe reductions in fruit yield and quality. The use of conventional fungicides for its management is increasingly limited due to resistance development, chemical residues and associated environmental risks. In the present study, silver nanoparticles (AgNPs) were synthesized, followed by their characterization through UV-Vis spectroscopy, FTIR, XRD, EDX and SEM analysis. The synthesized AgNPs exhibited particle sizes ranging from approximately 50 to 100 nm. *In vitro* antifungal assays showed that the AgNPs exerted significant, concentration-dependent inhibition of *C. gloeosporioides*, achieving a maximum mycelial suppression of 92.8% at 120 µg/ml. Smaller-sized nanoparticles (~50 nm) consistently demonstrated superior antifungal activity compared to larger ones (~100 nm). These results highlight the strong antifungal potential of AgNPs as eco-friendly nanofungicides for *Citrus* anthracnose management.

1. Introduction

Citrus is one of the world's most important fruit crops, valued for both its nutritional benefits and economic importance. In India, *Citrus nobilis* × *C. deliciosa* is a prominent cultivar, especially in the northwestern states, where it plays a vital role in supporting farmers' livelihoods. However, its yield and fruit quality are often limited by various biotic stresses, among which anthracnose, caused by *C. gloeosporioides*, is one of the most destructive. The disease manifests as wither tip, dieback, fruit rot, and stem-end rot, ultimately reducing marketable yield and compromising postharvest storability. Nanotechnology has recently emerged as a promising frontier in agriculture, offering novel solutions for plant disease management. Among metal-based nanomaterials, silver nanoparticles (AgNPs) have gained significant attention due to their well-documented antimicrobial broad-spectrum properties. AgNPs have been shown to inhibit several phytopathogenic fungi, including *Fusarium* sp. and *Alternaria* sp. (Mishra *et al*., 2021; Rani *et al*., 2023). Importantly, smaller particles often demonstrating higher inhibitory potential due to greater surface reactivity (Siddiqui *et al*., 2022).

Several studies explored plant-mediated of AgNPs against different phytopathogens (Gurunathan *et al*., 2020; Hernández-Castillo *et al*., 2022), while others investigated their synergy with conventional fungicides (Meena *et al*., 2021). Despite these advances, reports on AgNPs against *C. gloeosporioides* in *Citrus*, particularly *Citrus nobilis* × *C. deliciosa*, remain limited. Given the economic importance of *Citrus nobilis* × *C. deliciosa* and the increasing demand for residue-free produce, it is imperative to evaluate AgNPs as alternative nanofungicides against *C. gloeosporioides*.Therefore, the present study deals with synthesis and characterization of silver nanoparticles and their evaluation against *C. gloeosporioides* causing anthracnose in *Citrus*. The investigation further assessed the influence of nanoparticle concentration and size on *C. gloeosporioides*, thereby providing insights into their potential application in eco-friendly disease management strategies.

2. Materials and Methods

2.1 Pathogen isolation and identification

Fruits and twigs of *Citrus nobilis* × *C. deliciosa* showing typical anthracnose symptoms (wither tip, fruit rot, dieback) were collected from Kinnoworchard of Kharia Village of Sirsa District in Haryana. Samples were washed, surface sterilized with 0.1% mercuric chloride (HgCl,) for 30-40 seconds, rinsed thrice with sterile distilled water, and blotted dry. Small sections (2 mm) from lesion margins were placed on potato dextrose agar (PDA) medium and incubated at 25 ± 2°C. Emerging colonies were purified by single-spore isolation. Pathogenicity was confirmed under laboratory conditions. Morphological identification was carried out based on cultural features (colony colour, texture, pigmentation) and microscopic observations of conidia, conidiophores, and setae, following standard procedures described by Rangaswami and Mahadevan (1999).

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2.2 Synthesis of silver nanoparticles (AgNPs)

Silver nanoparticles were synthesized using sodium borohydride ($NaBH_4$) as a reducing agent and sodium dodecyl sulfate (SDS) as a stabilizer. In brief, 10 ml of 0.02 M $NaBH_4$ was mixed with 3and 5 ml of 0.02 M SDS in separate beakers and stirred for 25 min. Subsequently, 10 ml of 0.001 M $AgNO_3$ solution was added dropwise under continuous stirring, followed by one h of additional stirring. The appearance of a brownish-black colour indicated nanoparticle formation. The resulting AgNPs were stored in foil-covered containers at 4^0C until further use.

2.3 Characterization of silver nanoparticles

The synthesized nanoparticles were characterized using UV-Vis, FTIR, XRD, EDX spectroscopy and scanning Electron Microscopy.

2.4 Antifungal assay

The antifungal activity of AgNPs was evaluated against *C. gloeosporioides* using the poisoned food technique (Grover and Moore, 1962). PDA medium was amended with AgNPs (50 nm and 100 nm) at concentrations of 15, 30, 60 and 120 µg/ml, while unamended PDA served as the control. A 5 mm mycelial disc from a 7-day-old culture was placed at the center of each Petri plate and incubated at 25 ± 2^0C. Radial mycelial growth was measured at 2-day intervals up to 8 days. Per cent inhibition of mycelial growth over control was calculated using Vincent's formula (1947):

$$\text{Percent inhibition of mycelial growth} = \frac{\text{Colony diameter in control} - \text{Colony deameter in treatment}}{\text{Colony diameter in control}} \times 100$$

2.5 Experimental design and statistical analysis

The experiment was conducted in a completely randomized design (CRD) with three replications. Data were subjected to analysis of variance (ANOVA), and treatment means were compared at a 5% level of significance. Critical difference (CD) at 5% and standard error of mean (SEm) were calculated according to the methods of Panse and Sukhatme (1985).

Figure 1: Symptoms of *C. gloeosporioides* on Kinnow (A) Stem-end rot of peduncle (B) Styler-end rot of fruit (C) Wither-tip of twigs (D) Physiological fruit drop in Kinnow (E) Pathological fruit drop in Kinnow.

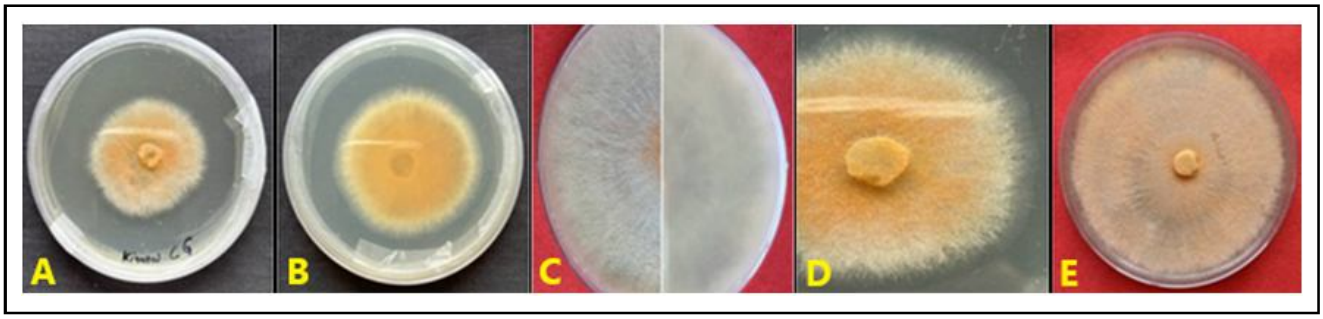

Figure 2: Cultural characteristics of *C. gloeosporioides* (A) Colony colour is white with orange conidial mass in center (B) Pigmentation (C) Dark black colour of colony on above and below (D) Raised and cottony growth of mycelium with irregular margins (E) Dense growth of mycelium.

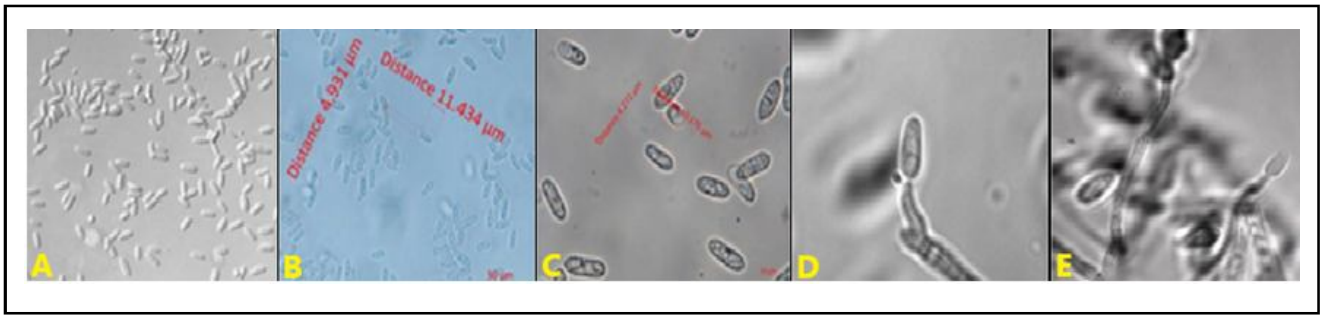

Figure 3: Morphological characteristics of *C. gloeosporioides* (A) Conidia, hyaline, aseptate (B) Conidium dimension (11.34×4.93µm) (C) Conidium dimension (10.17×4.27 µm) (D) Conidiophore (E) mycelium Septate and hyaline.

3. Results

3.1 Culture characterization

Cultures obtained on PDA from symptomatic *Citrus nobilis* × *C. deliciosa* tissues produced initially white, rapidly growing colonies that turned dark black at advanced stages; pigmentation was recorded on day 8 after incubation (Figures 1A-E and 2A-E). Asexual morphology was typical of *C. gloeosporioides*: acervular conidiomata; brown, smooth, 2-3 septate setae (50-120 µm) with conical/inflated bases; hyaline, septate, branched conidiophores (~50 µm); and hyaline, aseptate, cylindrical conidia with rounded ends. Conidial dimensions measured 11.34 × 4.93 µm and 10.17 × 4.27 µm (Figures 3A-E). Similar colony and conidial characteristics have been reported earlier for *C. gloeosporioides* infecting *Citrus* in India.

3.2 Silver nanoparticle characterization

The synthesized AgNPs yielded a characteristic brownish-black colloid. The UV spectra exhibited single, symmetric SPR bands at 392 nm confirming Agz reduction to Agp . Particle-size distribution analyses indicated average sizes of ~50 nm and ~100 nm with different concentration of SDS. FTIR (Fourier Transform Infrared) showed bands at 3329, 2108, 1635, 1215, and 1015 cm^{-1}, consistent with O–H/N–H stretching and C=O/C–O functionalities implicated in reduction/stabilization . XRD (X-ray Diffraction) patterns displayed distinct peaks corresponding to the facecentered cubic (fcc) phase of metallic Ag while EDX (Energy Dispersive X-Ray Spectroscopy) spectra showed strong Ag signals with no notable impurities. SEM (Scanning Electron Microscopy) revealed predominantly triangular nanoparticles having less than 100 nm size (Figures 4-6). These results are consistent with earlier reports on AgNPs, where characteristic absorption peaks between 390-410 nm and crystalline face-centered cubic structures were considered diagnostic of successful nanoparticle formation (Bhattacharya and Mukherjee, 2008; Lee *et al*., 2020).

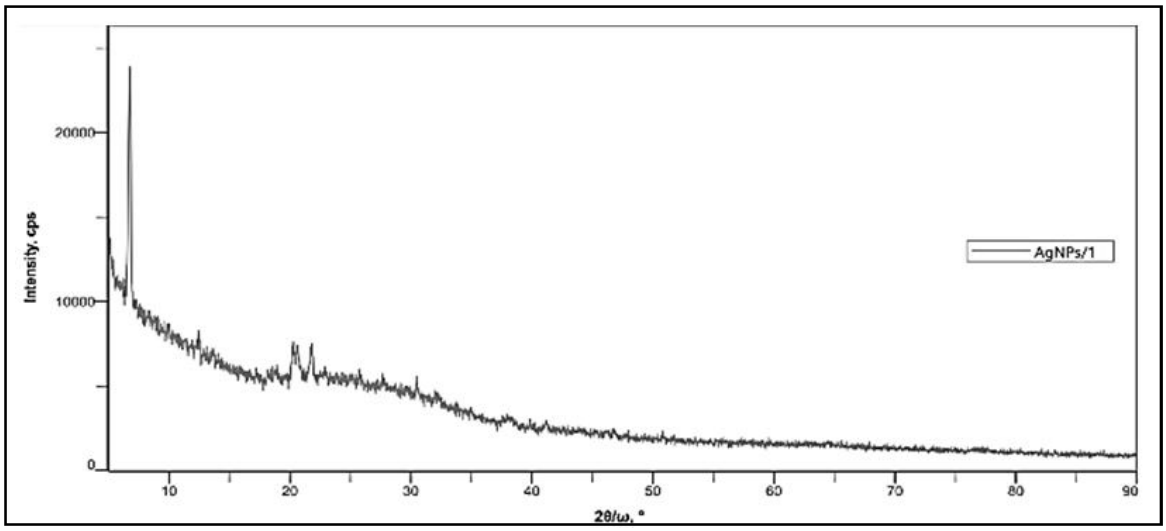

Figure 4: X-ray diffraction (XRD) chromatogram of AgNPs.

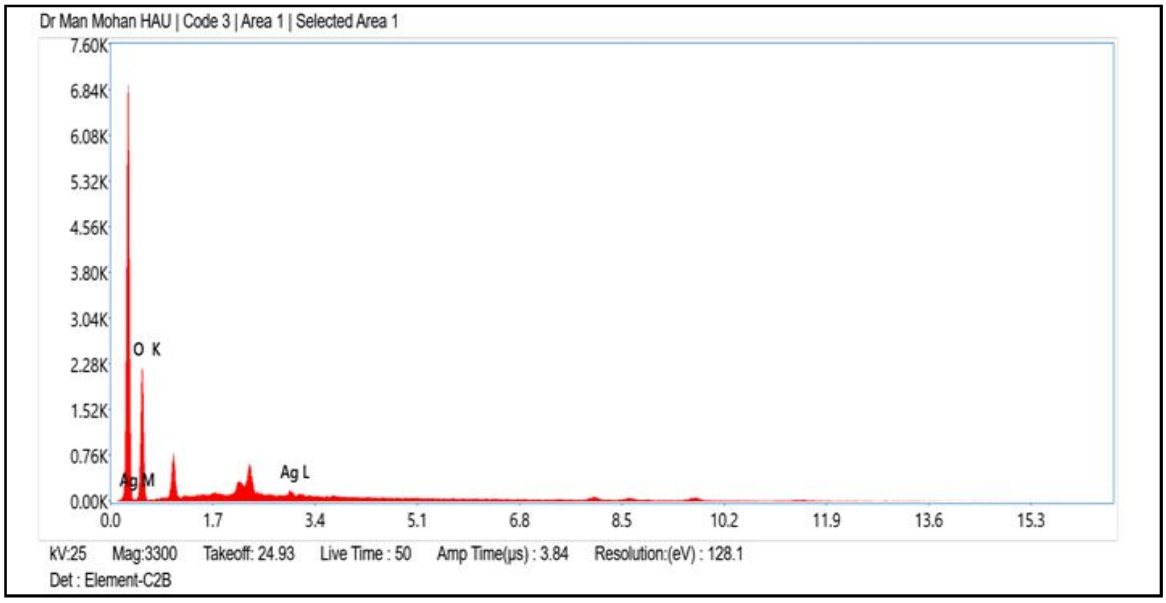

Figure 5: Energy dispersive X-ray (EDX) pattern of AgNPs nanoparticles.

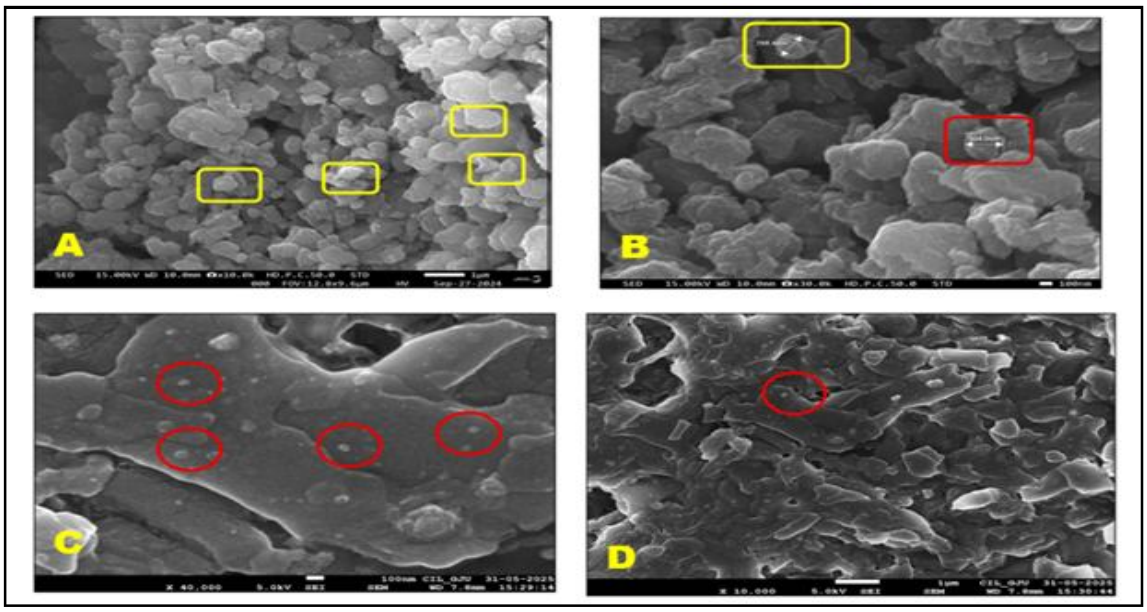

Figure 6: SEM images of silver nanoparticle (A-D):AgNPs highlighted in yellow and red circles.

3.3 Antifungal efficacy

The synthesized AgNPs (~50 nm size) significantly inhibited mycelial growth of *C. gloeosporioides* in dose-response manner (Table 1). After 8th day 92.8% mycelial growth inhibition at 120 µg/ml, followed by 82.2% (at 60 µg/ml), 70.6% (at 30 µg/ml), and 61.8% (at 15 µg/ml) was observed. Radial growth was found minimal at 120 and 60 µg/ml throughout incubation, and cumulative daily growth declined with increasing concentration. CD@5% for inhibition was found 5.46 at 8th day and corresponding SE(m) was1.65. The difference between 120 and 60 µg/ml remained statistically significant by 8th day, corroborating stronger efficacy at the highest dose (Table 1).

Table 1: Effect of silver nanoparticles (~50 nm) on the inhibition of mycelial growth of *C. gloeosporioides*

Concentration of ~50 nm size AgNPs (µg/ml)	Mycelial growth inhibition (%)			
	2 DAI	4 DAI	6 DAI	8 DAI
15	15.4	28.6	45.8	61.8
30	18.2	32.3	50.4	70.6
60	20.1	40.5	65.7	82.2
120	20.8	45.7	70.8	92.8
CD @ 5%	1.93	2.47	3.0	5.46
SE(m)	0.58	0.75	0.91	1.65

DAI= Days after inoculation, AgNPs= Silvernanoparticles, CD= Critical difference, SE(m)= Standard error

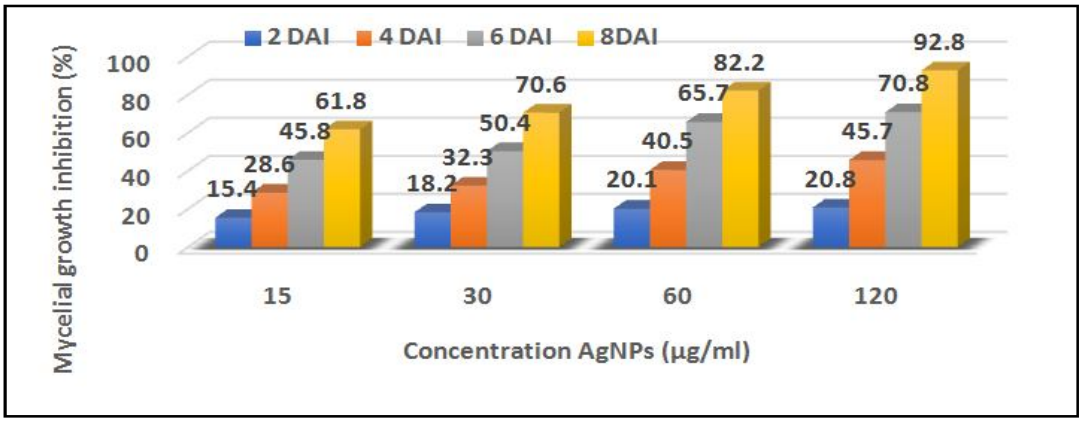

Figure 7: Effect of silver nanoparticles (~50 nm) on the inhibition of mycelia growth of *C. gloeosporioide.*

The size dependence antifungal efficacy of ~50 and ~100 nm sized AgNPs were also and shown in Table 2. The ~50 nm AgNPs showed 92.8 % MGI in comparison to ~100 nm AgNPs with 80.9 % MGI at a concentration of 120 µg/ml.

Table 2: Effect of various sizes AgNPson mycelial growth of *C. gloeosporioides*

AgNPs concentration (µg/ml)	Mycelial growth inhibition (%) AgNPs size	
~100 nm	**~50 nm**	
15	56.40	61.80
30	68.20	70.60
60	76.80	82.20
120	80.90	92.80
CD @ 5%	3.07	5.46
SE(m)	1.31	2.33

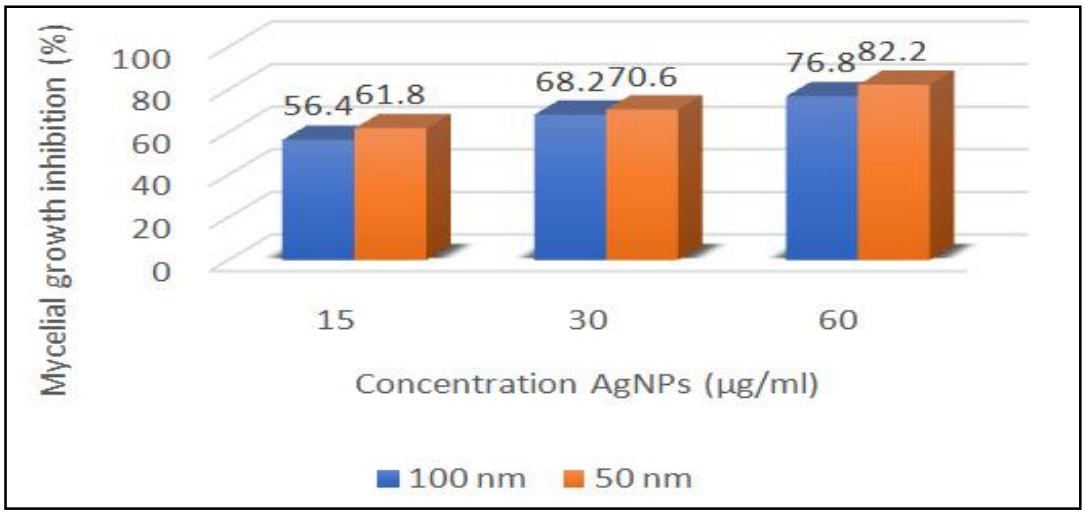

Figure 8: Effect of ~100 nm and ~50 nm AgNPs on mycelial growth of *C. gloeosporioides.*

4. Discussion

The present investigation demonstrated that synthesized silver nanoparticles significantly inhibited *C. gloeosporioides*, the causal agent of anthracnose in Kinnow, in a concentration and size-dependent manner. The findings are in agreement with earlier studies that reported strong antifungal effects of AgNPs against a wide range of phytopathogens, including *Colletotrichum musae*, *Alternaria alternata*, *Fusarium oxysporum* and *Botrytis cinerea* (Mishra *et al*., 2021; Rani *et al*., 2023; Gurunathan *et al*., 2020). The superior efficacy of smaller size AgNPs (~50 nm) compared to larger particles (~100 nm) can be attributed to their higher surface area-to-volume ratio, which enhances reactivity and increases the probability of interactions with fungal cell structures (Siddiqui *et al*., 2022).

Although, *in vitro* results clearly demonstrate the potential of AgNPs as antifungal agents, translation to field application requires further validation. In addition, biosafety evaluations are essential, as several studies have reported that excessive or uncontrolled use of AgNPs may negatively affect soil microbial diversity, enzymatic activity, and nutrient cycling. Therefore, careful optimization of application rate, timing, and formulation is required to maximize disease suppression while minimizing non-target effects.

5. Conclusion

The present study established *C. gloeosporioides* as the causal agent of anthracnose, fruit rot, and dieback in Kinnow, which remains a major constraint to *Citrus* production in Haryana. The synthesized AgNPs displayed potent antifungal activity, with mycelial growth inhibition increasing in a concentration dependent manner. Maximum inhibition (92.8%) was recorded at 120 µg/ml, while smaller-sized particles (~50 nm) were consistently more effective than larger ones (~100 nm). These findings confirm AgNPs as promising candidates for development as nanofungicides in the management of citrus anthracnose.While the *in vitro* results are encouraging, translation into practical disease management requires further research. Greenhouse and field evaluations are necessary to validate the efficacy of AgNPs under natural environmental conditions and to compare their performance with conventional fungicides. Overall, the study demonstrates that AgNPs can serve as a valuable component of next-generation disease management strategies in citrus. These findings support their potential role as nanofungicides in horticulture. Future work should focus on formulation development, field efficacy validation, and comprehensive biosafety assessments to facilitate their integration into citrus IPM strategies.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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