

Antifungal activity of zinc oxide nanoparticles against *Phytophthora capsici* and their phytotoxicity in *Piper nigrum*

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Received: 4 May 2026 / Accepted: 2 September 2026

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Abstract

Phytophthora capsici is a destructive oomycete pathogen responsible for severe yield losses in black pepper (*Piper nigrum* L.), particularly in tropical production systems. Limitations associated with conventional fungicides, including resistance development and environmental concerns, necessitate alternative disease management strategies. This study evaluated the antifungal activity of zinc oxide nanoparticles (ZnO NPs) of two particle sizes (~50 nm and ~100 nm) against *P. capsici* isolates from Sarawak, Malaysia, and assessed their phytotoxicity on black pepper leaves. Both nanoparticle sizes significantly inhibited mycelial growth in a concentration-dependent manner, with smaller particles exhibiting greater efficacy. The minimum effective concentration ranged from 0.4 to 0.5 mg/mL for ~50 nm ZnO NPs and 0.5–0.6 mg/mL for ~100 nm ZnO NPs. At 1.0 mg/mL, growth inhibition reached 72.2% for ~50 nm ZnO NPs and 47.9% for ~100 nm ZnO NPs. Nanoparticle treatment also delayed colony expansion and altered growth characteristics. Recovery assays indicated that the antifungal effect was primarily fungistatic. No visible structural damage to hyphae was observed; however, reduced aerial mycelial formation suggested interference with fungal development. No phytotoxic effects were detected on detached black pepper leaves under the tested conditions. These findings demonstrate the in vitro inhibitory potential of ZnO NPs against *P. capsici* and support their further evaluation under whole-plant and environmentally relevant conditions.

Keywords Antifungal activity · *Phytophthora capsici* · Phytotoxicity · *Piper nigrum* · Plant protection · Zinc oxide nanoparticles

1 Introduction

Phytophthora capsici is a destructive soilborne oomycete pathogen responsible for severe diseases in a wide range of economically important crops, particularly under tropical conditions [1]. It infects numerous solanaceous crops, such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), and pepper (*Capsicum annuum*) [2], as well as tropical crops including black pepper (*Piper nigrum*) [3], rubber (*Hevea brasiliensis*), and

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cacao (*Theobroma cacao*) [4]. The pathogen is highly aggressive and capable of infecting multiple plant tissues, making it difficult to manage once established in the field [1, 5].

Infection of *P. capsici* usually begins as water-soaked lesions that rapidly enlarge into slimy dark patches [3, 5–7]. Under favorable environmental conditions, *P. capsici* can cause yield losses of up to 100%, posing a significant threat to agricultural productivity and food security [8]. The epidemiological success of *P. capsici* is largely attributed to its complex life cycle and adaptive survival strategies. The pathogen produces motile zoospores that facilitate rapid dispersal in water-saturated soils, enabling rapid infection under humid and waterlogged conditions [1]. In addition, the formation of long-lived survival structures such as oospores allows the pathogen to persist in soil for extended periods, even in the absence of a host [8, 9]. These biological characteristics contribute to recurrent disease outbreaks and complicate long-term management efforts, particularly in tropical agricultural systems where environmental conditions are highly favorable for the pathogen [1, 6, 10].

In Malaysia, black pepper (*Piper nigrum*) is predominantly cultivated in Sarawak, which accounts for approximately 98% of the total pepper cultivation area in the country and contributes majority of the national production [11]. The crop plays a vital role in supporting local smallholder farmers and represents a key component of the agricultural economy in the region. However, pepper production in Sarawak is severely constrained by foot rot disease caused by *P. capsici*, which remains one of the most destructive diseases affecting this crop [3, 5]. The warm and humid tropical climate of Sarawak provides ideal conditions for rapid disease development and epidemic spread. Despite its economic importance, effective and sustainable management strategies specifically targeting *P. capsici* in Sarawak remain limited.

Current management practices of *P. capsici* rely heavily on the application of chemical fungicides, as alternative approaches such as biological control and cultural practices often provide inconsistent or limited efficacy [2, 12]. While these compounds can provide short-term disease suppression, their prolonged and intensive use has led to several critical challenges. These include the development of fungicide-resistant pathogen populations, accumulation of chemical residues in the environment, and potential risks to human health [13–15]. Furthermore, the efficacy of chemical control is often inconsistent under field conditions, particularly in tropical regions where high rainfall and humidity can reduce pesticides persistence and effectiveness [15, 16]. Increasing regulatory restrictions on agrochemical usage further limits the long-term feasibility of reliance on synthetic fungicides. Collectively, these challenges highlight the urgent need for alternative, effective and environmentally sustainable disease management strategies.

In the past few decades, nanotechnology has emerged as a promising approach in agricultural disease management. Nanoparticles refer to particles with size ranges between about 1–100 nm, which exhibit unique physical, chemical, and biological properties compared to their bulk counterparts, including increased surface area, enhanced reactivity, and improved interaction with microbial cells [17, 18]. These properties have been shown to enhance antimicrobial efficacy of nanoparticles, making them attractive candidates for plant disease control. In previous studies on agricultural application, nanoparticles have been explored as nanopesticides, and as carriers for agrochemicals, with the potential to improve efficacy while reducing overall chemical usage [17, 19–21].

Zinc oxide nanoparticles (ZnO NPs) are among the most extensively studied metal oxide nanoparticles on their antimicrobial properties [22]. ZnO NPs have demonstrated significant inhibitory effects against a wide range of plant pathogens, including *Pyricularia oryzae* [23], *Fusarium oxysporum* [18], and *Alternaria alternata* [22]. The antimicrobial mechanisms of ZnO NPs are generally attributed to multiple mechanisms, including the generation of reactive oxygen species (ROS), disruption of cell membrane integrity, release of zinc ions, and interference with cellular metabolism [24, 25]. These complex mechanisms reduce the likelihood of resistance development compared to conventional fungicides. Importantly, the antifungal efficacy of ZnO NPs is influenced by several factors, including particle size, concentration, and the characteristics of target pathogen [18, 23]. Smaller nanoparticles are generally associated with higher antimicrobial activity due to their greater surface area-to-volume ratio [26, 27].

Despite the growing body of research on ZnO NPs, several important gaps remain. Variability among pathogen isolates and regional differences are often overlooked. Previous reports have highlighted the importance of evaluating multiple isolates when assessing antifungal agents as reliance on a single isolate may not accurately represent field conditions [23, 28]. In the case of *P. capsici*, significant variability exists among isolates in terms of virulence, growth characteristics and potential sensitivity to control measures [3, 5]. Such variability may influence the effectiveness of nanoparticle-based treatments, yet it remains poorly understood, particularly for isolates affecting black pepper in Sarawak. Moreover, limited attention has been given to the potential phytotoxic effects of ZnO NPs on host plants, which may raise concerns regarding their practical applications in agricultural systems. Recent work by [21] demonstrated strong antifungal activity of ZnO NPs against *P. capsici* isolated from infected pepper plant, showing complete growth inhibition at relatively low concentrations using nanoparticles averaging 26.1 nm in size. However, their study was conducted on different isolates and nanoparticle sizes from the present study. Also, the study did not address potential phytotoxic effects on host plants.

Given these considerations, there is a clear need to evaluate the efficacy of ZnO NPs against *P. capsici* isolates relevant to local agricultural contexts, as well as to assess their safety on the host plant. Therefore, this study aimed to evaluate the in vitro antifungal activity of ZnO NPs with two different particle sizes (~50 nm and ~100 nm) against three *P. capsici* isolates obtained from two different divisions in Sarawak, Malaysia. In addition, the phytotoxic effects of ZnO NPs on *Piper nigrum* were evaluated to determine their potential as a candidate material for further development in sustainable disease management. The findings of this study are expected to provide a more comprehensive understanding on the potential of ZnO NPs as a candidate for further evaluation as part of integrated management for managing *P. capsici* in Sarawak, Malaysia.

To the best of our knowledge, this study represents the first evaluation of ZnO NPs against *P. capsici* isolates associated with black pepper in Sarawak, incorporating phytotoxicity assessment.

2 Materials and methods

2.1 Characterization of zinc oxide nanoparticles

Two separate commercially supplied ZnO NPs with nominal sizes of ~50 nm and ~100 nm were purchased from Sigma-Aldrich (St. Louis, MO, USA). The particle sizes of the ZnO NPs were observed using scanning electron microscope (SEM) (JEOL, JSM-6390 LA) with 50,000x magnification. One hundred particles were selected randomly for each particle size and measured using SmileView software to determine the particle size distribution. The measured distributions were subsequently compared with the nominal particle sizes provided by the manufacturer.

2.2 Preparation of *Phytophthora capsici*

Three isolates of *P. capsici* maintained in the culture collection of the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, were used in this study. Their isolate codes, geographical origins, and GPS coordinates are presented in Table 1. The isolates were routinely maintained on potato dextrose agar (PDA) in the dark at room temperature (25 ± 1 °C). Among the selected isolates, isolate MRI1 and MBI1 have been previously reported to be pathogenic towards black pepper (*Piper nigrum*; Kuching cultivar) [3].

Table 1 Details of three *Phytophthora capsici* isolates used in this study

Isolate	Division	GPS coordinates
NGD4	Betong	1.6302292, 111.7299839
MRI1	Sarikei	1.8958314, 111.9339715
MBI1	Betong	1.632106, 111.733093

2.3 Pathogenicity test of *Phytophthora capsici* isolate NGD4

The pathogenicity of *P. capsici* isolate NGD4 was assessed in this study following the protocol described by [3] with modifications. Healthy young black pepper shoots were obtained from the same vine of Kuching cultivar (from black pepper farmer recommended by Malaysian Pepper Board), known to be highly susceptible to *Phytophthora* foot rot disease. The shoots were surface sterilized with 10% commercial bleach solution (ca. 0.5% v/v sodium hypochlorite) for 1 min, then rinsed thrice with sterile distilled water under aseptic conditions. The surface sterilized shoots were then air-dried on sterile paper towels. After that, the cut end of the shoots was wrapped with moist tissue paper and placed in a surface-sterilized transparent plastic container (20 × 20 cm) containing a moistened sterile tissue ball to maintain a humid environment and reduce desiccation throughout the observation period. A mycelium plug (6 mm in diameter) obtained from a 7-day-old *P. capsici* isolate NGD4 cultured on PDA was inoculated onto the first node from the base of each shoot. Shoots inoculated with fresh PDA plugs were served as the control. The test was repeated once with five replicates. The shoots were incubated in the dark at room temperature (25 ± 1 °C) and observed daily for symptom development. Lesion development was the indicator of pathogenicity. Upon symptom development, a small piece from the advancing margin of the lesion was excised and surface sterilized to eliminate surface contaminants. It was then placed on PDA plates for pathogen re-isolation. Colony and hyphal characteristics of the re-isolated pathogens were recorded to affirm consistency with the original isolate.

2.4 Antifungal properties of zinc oxide nanoparticles

A stock suspension of ZnO NPs was prepared at a concentration of 100 mg/mL by dispersing ZnO NPs of two particle sizes (~ 50 nm and ~ 100 nm) in sterile distilled water under aseptic conditions. To improve particle dispersion, the suspension was sonicated for 10 min prior to use. The stock suspension was added into molten PDA to obtain final concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mg/mL. PDA without ZnO NPs served as the control. Approximately 20 mL of PDA was poured into sterile Petri plates and allowed to solidify under aseptic conditions. A 6 mm mycelium plug was aseptically excised from the actively growing margin of a 7-day-old pure culture and placed at the center of each Petri plate. The inoculated plates were incubated at room temperature (25 ± 1 °C). To standardize colony growth measurements, two perpendicular lines were drawn on the underside of each plate. The endpoints of the lines were labelled A, B, C, and D, serving as reference points for radial growth measurement [23, 29]. The colony growth was measured and recorded when the fungal culture in control plates had nearly reached the edge of the plate.

The average colony growth was calculated as:

$$\text{Average colony growth} = \frac{(A + B + C + D) \text{ cm}}{4}$$

The growth inhibition percentage was determined using the following equation:

$$\text{Growth inhibition (\%)} = \frac{(D_0 - D_a)}{D_0} \times 100$$

Where D_0 represents mean radial growth of the control group and D_a represents the mean radial growth of the treatment group, respectively.

The effects of ZnO NPs of different sizes (~ 50 nm and ~ 100 nm) were assessed in separate experiments. For each experiment, treatments were conducted with five replicates per isolate, and the complete experiment was conducted twice. Prior to analysis, data were assessed for normality. As assumptions of normal distribution were not met, non-parametric tests were employed to ensure robustness of statistical interpretation. Data were analyzed using Kruskal-Wallis test followed by pairwise comparisons performed using Dunn's post hoc test with

Bonferroni correction to determine significant differences among treatments. A significance level of $p < 0.05$ was applied. Statistical analyses were performed using IBM SPSS Statistics 30 software.

Additionally, a recovery assay was performed to determine whether the antifungal effect of ZnO NPs was fungistatic or fungicidal. Mycelial plugs were excised from the margins of colonies grown on PDA treated with the highest effective concentration of ZnO NPs, as well as the control plates. The plugs were then subcultured onto fresh PDA plates without ZnO NPs and incubated under the same conditions as the antifungal assay. Colony growth and morphology were observed and compared between treated and control groups.

2.5 Phytotoxicity test

The phytotoxic effects of ZnO NPs at different concentrations were evaluated on black pepper (*Piper nigrum*; Kuching cultivar) leaves using a detached leaf assay. Healthy, fully expanded leaves were collected and surface sterilized following the procedure described in the pathogenicity test. Subsequently, the leaves were rinsed with sterile distilled water and air-dried under aseptic conditions. After that, each leaf petiole was wrapped with sterile moist tissue paper and placed in sterile transparent plastic containers (20 × 20 cm) containing a moistened sterile tissue ball throughout the 7-day incubation period to reduce leaf desiccation. The tissue ball was placed at the beginning of the experiment and was not re-moistened during the incubation period. The leaf lamina was divided into five marked sections to designate treatment sites. Each marked section represented an individual treatment site. Treatments were distributed across the required number of detached leaves. The use of localized treatment sites minimized variation associated with differences in leaf age, size, and physiological condition while allowing treatment responses to be assessed under comparable conditions.

A drop (30 µL) of ZnO NP suspension at varying concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mg/mL) was carefully applied onto each designated site using a micropipette. Control treatments consisted of an equal volume of sterile distilled water applied to separate marked sites on the same leaf. The application approach was designed to ensure localized exposure and uniform droplet deposition on the leaf surface, which is critical for assessing nanoparticle-leaf interactions and potential phytotoxic responses [30, 31]. Each treatment was conducted with three replicates. The treated leaves were incubated at room temperature (25 ± 1 °C). Observations were recorded daily and phytotoxicity symptoms were assessed seven days post-treatment. Phytotoxic effects were evaluated based on the visible symptoms at treatment sites, including necrotic lesions (dark spots), chlorosis, or water-soaked areas [32]. The experiment was conducted separately for ZnO NPs of two particle sizes (~ 50 nm and ~ 100 nm), with one repetition performed for each nanoparticle size.

3 Results

3.1 Characterization of zinc oxide nanoparticles

Scanning electron microscopy analysis revealed that ZnO NPs exhibited heterogeneous morphologies (Fig. 1). The ~ 50 nm ZnO NPs consisted predominantly of small, irregular to near-spherical particles, while the ~ 100 nm ZnO NPs displayed more readily distinguishable angular, polyhedral, and short rod-like morphologies. These morphological characteristics are consistent with those reported in previous studies [21, 23, 33]. In addition, clustering of particles were observed in both ZnO NPs size groups (~ 50 nm and ~ 100 nm), which is commonly reported for metal oxide nanoparticles due to their high surface energy. For ~ 50 nm ZnO NPs, particle size distribution analysis indicated that majority of particles ranged between 34 and 50 nm, with highest frequency (59%) observed within this size range (Fig. 1). For the ~ 100 nm ZnO NPs, the dominant particle size range was 81–92 nm (25%), followed by 70–81 nm (17%) and 92–103 nm (17%) (Fig. 1). Overall, both nanoparticle groups were consistent with the nominal sizes provided by the manufacturer, confirming their suitability for subsequent experiments.

Fig. 1 Scanning electron microscopy images of zinc oxide nanoparticles (ZnO NPs) with nominal particle sizes of (a) ~50 nm and (b) ~100 nm at 50,000 $\times$ magnification, and their corresponding particle size distribution profiles for (c) ~50 nm and (d) ~100 nm ZnO NPs

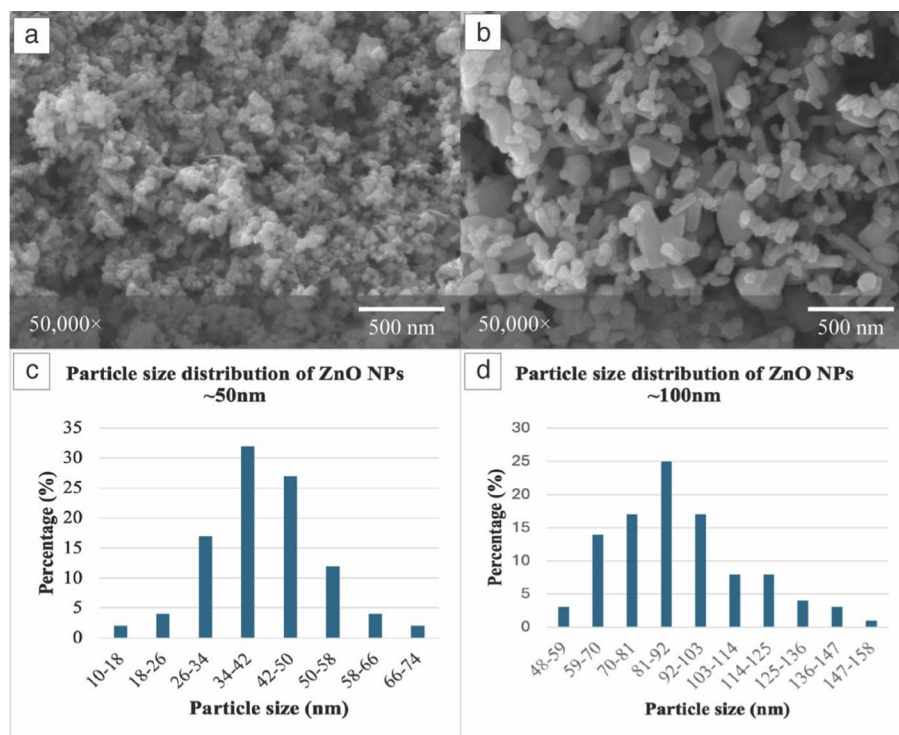
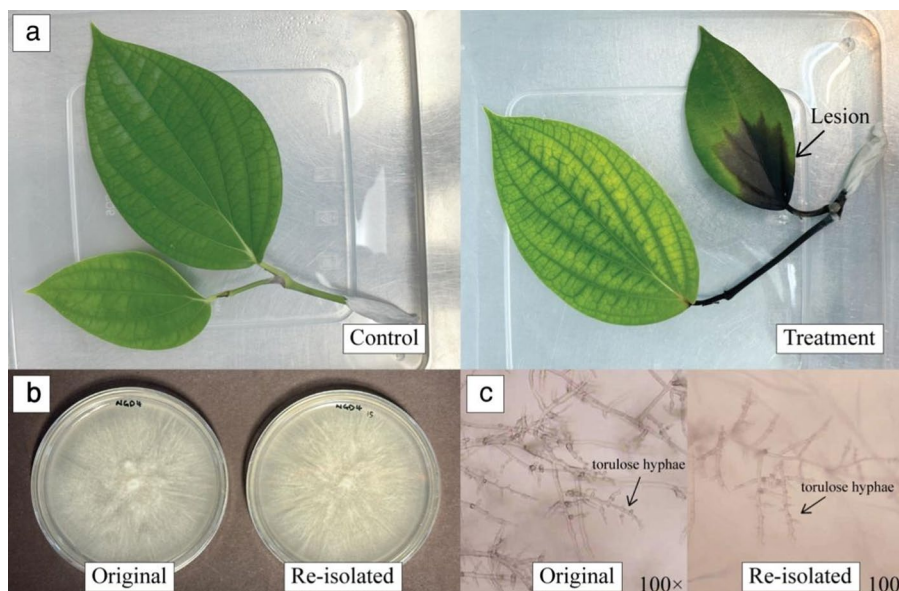


Fig. 2 Verification of pathogenicity of *Phytophthora capsici* isolate NGD4. **a** Representative of detached black pepper shoots 5 days post-inoculation, **b** pure culture of *P. capsici* isolate NGD4, **c** Light microscopy images showing torulose hyphae of *P. capsici* isolate NGD4 at 100 $\times$ magnification



3.2 Pathogenicity test

Typical disease symptoms were observed on black pepper shoots inoculated with *P. capsici* isolate NGD4. Dark brown to black lesions developed on both stem and leaves of the shoots, whereas no visible symptoms were observed in the control group (Fig. 2). Initial symptoms appeared as water-soaked lesions on the leaves at 2 days post-inoculation, which is consistent with the symptom progression described for naturally infected black pepper plants [3, 5]. The pathogen was successfully re-isolated from the infected leaves and cultured on PDA. The

re-isolated colonies exhibited identical morphological characteristics with those of the original inoculum (Fig. 2), thereby fulfilling Koch's postulates and confirming the pathogenicity of isolate NGD4.

3.3 Antifungal effect of zinc oxide nanoparticles against *Phytophthora capsici*

Zinc oxide nanoparticles reduced the mycelial growth rate of all three *P. capsici* isolates (NGD4, MRI1, and MBI1) in a concentration-dependent manner, where the magnitude of inhibition differed among isolates and between the two nanoparticle size groups (Table 2). Both nanoparticle sizes showed significant inhibitory effects on mycelial growth across all tested isolates, with increasing inhibition effect as concentration increased.

For the ~50 nm ZnO NPs, significant growth inhibition ($p < 0.05$) was observed at 0.4 mg/mL for isolate NGD4, at 0.5 mg/mL for isolates MRI1 and MBI1, as compared to the control. At the highest concentration tested (1.0 mg/mL), growth inhibition ranged from 57.7% to 72.2% (Table 2). In addition to reduced radial growth, ZnO NPs treatments delayed colony expansion. Compared to the control, the time required for colonies to reach full plate was extended by approximately 1–5 days at the lowest effective concentration and up to 12–26 days at the highest concentration (Table 3). For the ~100 nm ZnO NPs, significant inhibition was first observed at 0.5 mg/mL for isolates NGD4 and MBI1, at 0.6 mg/mL for isolate MRI1, as compared to the control. At the highest concentration, growth inhibition ranged from 40.5% to 47.9% (Table 2). Similarly, colony growth was delayed relative to the control, with a delay of approximately 2–4 days at the lowest concentration and 6–8 days at the highest concentration (Table 3).

To further evaluate whether the antifungal effect of ZnO NPs was fungistatic or fungicidal, mycelial plugs from the control and the highest effective ZnO NPs treatment were subcultured onto fresh PDA without nanoparticles. No observable differences in colony morphology or growth rate were detected between treated and control

Table 2 Fungal growth rate (mean $\pm$ SE) and growth inhibition percentage in different concentrations of zinc oxide nanoparticles (ZnO NPs)

Treatment (mg/mL)	Fungal growth rate (Inhibition %)		
	~50 nm ZnO NPs		
	NGD4	MRI1	MBI1
Control	3.75 ^a $\pm$ 0.025	3.78 ^a $\pm$ 0.043	3.71 ^a $\pm$ 0.023
0.05	3.64 ^{ab} $\pm$ 0.017 (3.1%)	3.11 ^{ab} $\pm$ 0.088 (17.8%)	3.59 ^{ab} $\pm$ 0.023 (3.4%)
0.10	3.62 ^{ab} $\pm$ 0.015 (3.5%)	3.05 ^{abc} $\pm$ 0.093 (19.6%)	3.56 ^{ab} $\pm$ 0.021 (4.0%)
0.20	3.57 ^{abc} $\pm$ 0.013 (4.9%)	2.92 ^{abc} $\pm$ 0.061 (23.0%)	3.50 ^{abc} $\pm$ 0.065 (5.7%)
0.30	3.15 ^{abcd} $\pm$ 0.039 (16.0%)	2.65 ^{abcd} $\pm$ 0.055 (30.1%)	3.35 ^{abcd} $\pm$ 0.053 (9.8%)
0.40	2.64 ^{bcd} $\pm$ 0.108 (29.7%)	2.59 ^{abcd} $\pm$ 0.069 (31.8%)	2.87 ^{abcde} $\pm$ 0.082 (22.6%)
0.50	2.43 ^{bcd} $\pm$ 0.079 (35.2%)	2.34 ^{bcd} $\pm$ 0.081 (38.3%)	2.28 ^{bcd} $\pm$ 0.190 (38.6%)
0.60	2.14 ^{cde} $\pm$ 0.159 (43.1%)	2.21 ^{cde} $\pm$ 0.087 (41.7%)	2.14 ^{cde} $\pm$ 0.166 (42.5%)
0.70	2.04 ^{de} $\pm$ 0.084 (45.7%)	2.09 ^{de} $\pm$ 0.058 (44.8%)	1.98 ^{def} $\pm$ 0.093 (46.8%)
0.80	1.95 ^{de} $\pm$ 0.041 (48.0%)	1.98 ^{de} $\pm$ 0.053 (47.8%)	1.69 ^{def} $\pm$ 0.111 (54.5%)
0.90	1.76 ^{de} $\pm$ 0.082 (53.1%)	1.87 ^{de} $\pm$ 0.113 (50.8%)	1.52 ^{ef} $\pm$ 0.090 (59.1%)
1.00	1.17 ^e $\pm$ 0.086 (68.7%)	1.60 ^e $\pm$ 0.059 (57.7%)	1.03 ^f $\pm$ 0.124 (72.2%)
~100 nm ZnO NPs			
Control	3.79 ^a $\pm$ 0.028	3.76 ^a $\pm$ 0.044	3.86 ^a $\pm$ 0.012
0.05	3.77 ^{ab} $\pm$ 0.047 (0.6%)	3.64 ^a $\pm$ 0.050 (3.1%)	3.84 ^a $\pm$ 0.018 (0.7%)
0.10	3.71 ^{abc} $\pm$ 0.058 (2.2%)	3.42 ^{ab} $\pm$ 0.122 (9.3%)	3.81 ^{ab} $\pm$ 0.015 (1.4%)
0.20	3.55 ^{abc} $\pm$ 0.041 (6.4%)	3.30 ^{abc} $\pm$ 0.127 (12.6%)	3.77 ^{ab} $\pm$ 0.023 (2.5%)
0.30	3.34 ^{abcd} $\pm$ 0.018 (12.0%)	3.26 ^{abc} $\pm$ 0.125 (13.5%)	3.69 ^{abc} $\pm$ 0.050 (4.7%)
0.40	2.90 ^{abcde} $\pm$ 0.025 (23.4%)	2.78 ^{abcd} $\pm$ 0.067 (26.2%)	3.33 ^{abcd} $\pm$ 0.103 (13.9%)
0.50	2.73 ^{bcd} $\pm$ 0.018 (27.9%)	2.64 ^{abcde} $\pm$ 0.041 (29.6%)	3.15 ^{bcd} $\pm$ 0.065 (18.5%)
0.60	2.67 ^{cdef} $\pm$ 0.042 (29.5%)	2.49 ^{bcd} $\pm$ 0.037 (33.7%)	3.01 ^{bcd} $\pm$ 0.103 (22.2%)
0.70	2.35 ^{def} $\pm$ 0.075 (38.0%)	2.41 ^{cde} $\pm$ 0.043 (35.7%)	2.74 ^{cd} $\pm$ 0.150 (29.2%)
0.80	2.22 ^{ef} $\pm$ 0.057 (41.4%)	2.34 ^{de} $\pm$ 0.044 (37.5%)	2.71 ^{cd} $\pm$ 0.154 (30.1%)
0.90	2.19 ^{ef} $\pm$ 0.061 (42.3%)	2.16 ^{de} $\pm$ 0.027 (42.4%)	2.53 ^d $\pm$ 0.102 (34.7%)
1.00	1.97 ^f $\pm$ 0.065 (47.9%)	2.00 ^e $\pm$ 0.046 (43.7%)	2.30 ^d $\pm$ 0.125 (40.5%)

*Different letters indicate a significant difference in fungal growth rate in different concentrations of ZnO NPs based on Dunn's post hoc test with Bonferroni correction ($p < 0.05$)

Table 3 Number of days for fungal colony to reach full plate

	NGD4		MRI1		MBI1	
	No. of days to full plate	No. of days delayed	No. of days to full plate	No. of days delayed	No. of days to full plate	No. of days delayed
~ 50 nm ZnO NPs						
Control	8.15	–	7.53	–	9.18	–
Lowest effective concentration	11.49 ¹	3.34	12.12 ²	4.59	10.96 ²	1.78
Highest effective concentration	26.65 ⁴	18.50	19.60 ⁴	12.06	35.14 ⁴	25.96
~ 100 nm ZnO NPs						
Control	8.45	–	7.46	–	9.33	–
Lowest effective concentration	11.72 ²	3.27	11.27 ³	3.81	11.46 ²	2.14
Highest effective concentration	16.40 ⁴	7.96	13.39 ⁴	5.93	16.08 ⁴	6.76

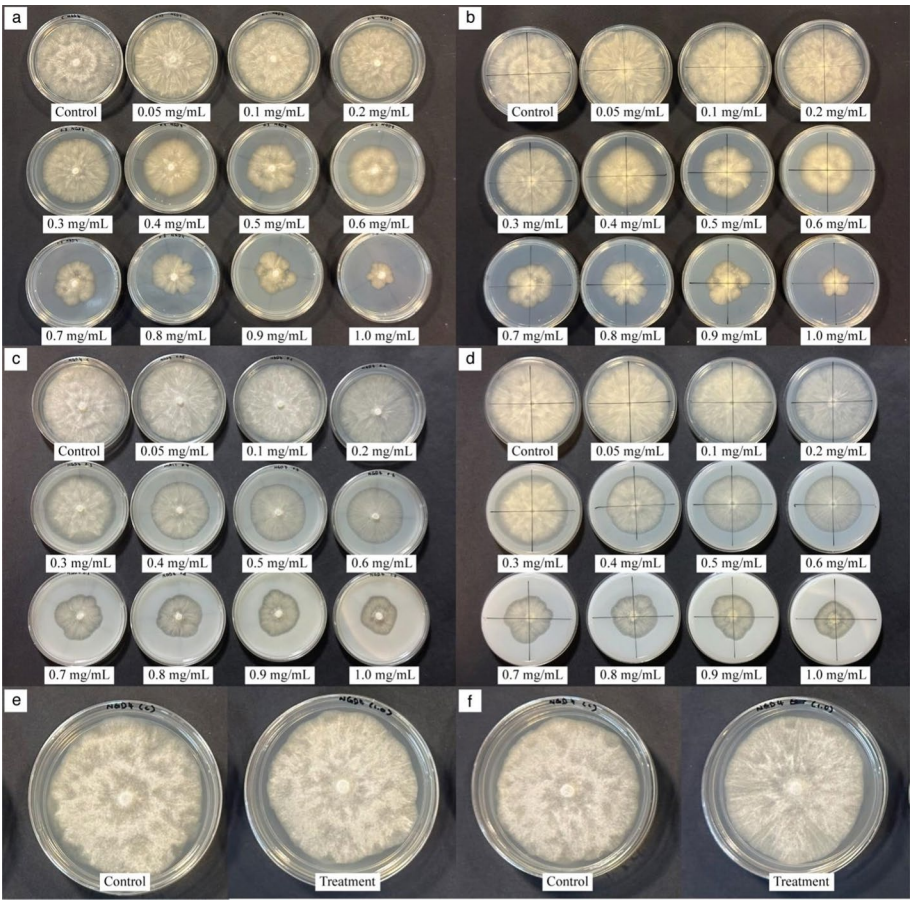
¹at concentration 0.4 mg/mL

²at concentration 0.5 mg/mL

³at concentration 0.6 mg/mL

⁴at concentration 1.0 mg/mL

Fig. 3 Growth response of *Phytophthora capsici* isolate NGD4 treated with different concentrations of zinc oxide nanoparticles (ZnO NPs). Representative colony growth of ~ 50 nm ZnO NPs is shown in (a) front view, (b) reverse view, while ~ 100 nm ZnO NPs are shown in (c) front view, (d) reverse view. Recovery assay following treatment is shown in (e) ~ 50 nm and (f) ~ 100 nm ZnO NPs



cultures after subculturing, indicating that the inhibitory effect of ZnO NPs was reversible and primarily fungistatic under the conditions tested (Fig. 3).

Scanning electron microscopy observations comparing the control and ZnO NP-treated samples at the highest effective concentration revealed no obvious structural deformation or damage in the hyphal structure of all tested *P. capsici* isolates for both nanoparticle sizes (Fig. 4). Hyphal morphology appeared to be similar between treated

Fig. 4 Scanning electron microscope images of *Phytophthora capsici* hyphae following treatment with zinc oxide nanoparticles (ZnO NPs) at 1000× magnification. Panels (a–c) show isolate NGD4, (d–f) isolate MRI1, and (g–i) isolate MBI1. Within each isolate, the images show the untreated control, ~50 nm ZnO NP treatment, and ~100 nm ZnO NP treatment from left to right. Arrows indicate the agar medium where hyphae are embedded

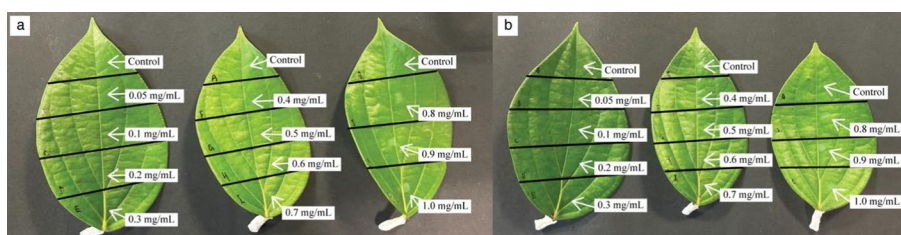
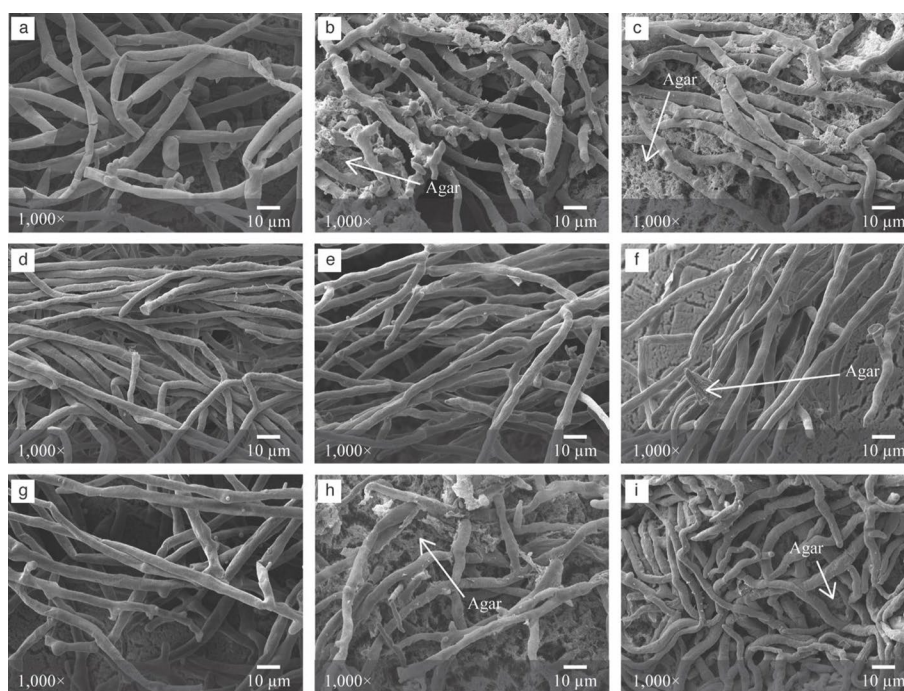


Fig. 5 Phytotoxicity assessment of black pepper leaves 7 days after zinc oxide nanoparticles (ZnO NPs) treatment. Leaves were treated with (a) ~50 nm ZnO NPs and (b) ~100 nm ZnO NPs at different concentrations. Arrows indicate the locations where ZnO NP droplets were applied

and untreated samples, with no signs of deformation, collapse, or fragmentation. However, clear differences in growth patterns were observed. In the control group, abundant aerial hyphae were present, with mycelia forming a dense and fluffy network on the surface of the medium. In contrast, cultures treated with ZnO NPs at the effective concentrations exhibited reduced aerial growth, with most hyphae tending to remain embedded within the agar medium (Fig. 3a, c). This altered growth behavior was consistently observed across all tested isolates and for both nanoparticle sizes, suggesting that ZnO NPs in this experiment primarily affect fungal growth dynamics rather than directly disrupting hyphal structure.

3.4 Phytotoxicity test

No visible phytotoxic symptoms were observed on detached black pepper leaves treated with ZnO NPs at any tested concentrations for both nanoparticle sizes (~50 nm and ~100 nm) (Fig. 5). The treated leaves remained comparable to the control throughout the observation period of 7 days post-treatment. No visible signs of necrosis, chlorosis, or tissue collapse were observed at the application sites. These results indicate that ZnO NPs did not exert detectable phytotoxic effects on black pepper leaves at the tested concentrations under experimental conditions.

4 Discussion

The present study demonstrates the antifungal potential of ZnO NPs (~ 50 nm and ~ 100 nm) against all tested *P. capsici* isolates from Sarawak, with inhibitory effect increased in a concentration-dependent manner (Table 2). The pattern is consistent with previous reports describing dose-dependent antifungal activity of ZnO NPs against a range of phytopathogenic fungi [23, 27]. Although the precise mode of action of ZnO NPs was not directly investigated in this study, several mechanisms have been proposed in previous reports. ZnO NPs are known to induce the generation of ROS, disruption of cell membrane integrity, and interference with enzymatic activities of fungal pathogens [24, 25, 34]. These mechanisms may collectively explain the inhibitory effects observed against *P. capsici* in the present study. The recovery of fungal growth following transfer to nanoparticle-free medium indicates that the observed antifungal activity of ZnO NPs is primarily fungistatic rather than fungicidal under the tested conditions. This suggests that ZnO NPs primarily suppress and delay the development process of *P. capsici* rather than complete pathogen eradication. From a disease management perspective, such suppression may still be highly relevant, as it can delay pathogen establishment and may potentially reduce disease progression during early stages of infection. Consequently, the practical value of ZnO NPs may lie in their use as one component of an integrated preventive disease management strategy, subject to further validation under greenhouse and field conditions.

The minimum effective concentration of ZnO NPs observed in this study ranged from 0.4 to 0.5 mg/mL for ~ 50 nm ZnO NPs and 0.5–0.6 mg/mL for ~ 100 nm ZnO NPs, with variations detected among isolates. At the highest effective concentration tested (1.0 mg/mL), growth inhibition reached up to 72.2% for ~ 50 nm ZnO NPs and 47.9% for ~ 100 nm ZnO NPs (Table 2), indicating a size-dependent effect. These findings differ from those reported by [21], who observed complete inhibition of *P. capsici* using smaller ZnO NPs (~ 26.1 nm) at substantially lower concentrations. This discrepancy is likely attributable to differences in nanoparticle size and physicochemical properties [18, 27]. Smaller nanoparticles possess higher surface area-to-volume ratio, which is associated with enhanced surface reactivity and interaction with microbial cells [18, 21, 27]. In addition, reduced particle size has been associated with increased generation of ROS, leading to oxidative stress, lipid peroxidation, and impairment of cellular components [34]. In contrast, the larger nominal particle sizes (~ 50 nm and ~ 100 nm) used in the present study may have provided less available surface area for interaction with the pathogen than the smaller ZnO NPs reported by [21]. Particle morphology and the close association or agglomeration of some particles, particularly in the ~ 50 nm product, may also have influenced their effective surface contact and antifungal activity. However, particle dispersion and aggregate size were not quantitatively assessed, thus their contribution to the observed differences cannot be determined from the present results. Nevertheless, both nanoparticle sizes (~ 50 nm and ~ 100 nm) demonstrated substantial inhibitory activity. The consistently greater inhibition produced by the ~ 50 nm ZnO NPs than by the ~ 100 nm ZnO NPs across all tested isolates supports particle size as an important factor influencing antifungal performance under the conditions evaluated.

In addition to reducing radial growth, ZnO NPs treatment delayed colony expansion. Delays of up to 12–26 days for ~ 50 nm ZnO NPs and 6–8 days for ~ 100 nm ZnO NPs were observed at the highest concentrations. The prolonged growth period indicates that exposure to ZnO NPs causes physiological stress on *P. capsici*, likely affecting cell division and hyphal extension rather than causing rapid cell death. Such growth delays are particularly relevant in agricultural contexts, as reduced pathogen growth rates may lower infection pressure and provide a wider window for host defense or intervention strategies. The greater delay associated with smaller nanoparticles further supports the role of particle size in modulating antifungal efficacy [18, 21, 27].

Interestingly, despite significant inhibition of fungal growth, no visible structural damage to hyphae was observed under SEM. Hyphal morphology, including diameter and surface integrity, remained comparable between treatment and control groups. This suggests that the antifungal activity of ZnO NPs in the present study is unlikely to be primarily associated with direct physical disruption of hyphal structure. Instead, the observed reduction in aerial mycelial formation and delayed colony expansion indicate interference with fungal

developmental processes. These effects may be associated with intracellular or subcellular mechanisms, such as disruption of metabolic pathways, cellular signaling, or enzymatic activity [34]. Although previous studies have reported visible morphological deformities in fungal hyphae after ZnO NP exposure [35], the absence of visible structural damage in the present study suggests that the effects may occur at levels not detectable by SEM. Similar observations have been reported by [23], where significant growth inhibition occurred without visible morphological deformation, highlighting that antifungal activity does not necessarily involve observable structural damages. However, higher-resolution techniques such as transmission electron microscopy, confocal microscopy, and fluorescent viability staining would be required to evaluate these possibilities directly.

The reduction in aerial mycelia development observed in this study provides further insights into the mode of action of ZnO NPs. Aerial hyphae formation is a regulated developmental process typically associated with nutrient availability, internal physiological factors, environmental cues, and intracellular signaling pathways [36–38]. Studies have also suggested that nanoparticle exposure can affect mitochondrial function and enzyme activity [34]. Disruption of these processes may reflect underlying metabolic stress or impaired cellular coordination. It is therefore plausible that ZnO NPs exposure interferes with regulatory pathways associated with fungal development, leading to altered growth patterns. Such effects may potentially influence pathogenicity, as proper hyphal development is often associated with host colonization and disease progression.

Importantly, no phytotoxic effects were observed on detached black pepper leaves treated with ZnO NPs at any of the tested concentrations for both nanoparticle sizes. The absence of visible necrosis, chlorosis, or tissue collapse suggests that the tested ZnO NP concentrations did not cause detectable short-term visible injury to detached black pepper leaves under the experimental conditions. However, the assay did not assess whole-plant growth, physiological responses, zinc accumulation, or long-term exposure. Therefore, broader conclusions regarding plant safety cannot yet be made.

Despite the concentration-dependent inhibition observed in this study, several limitations should be acknowledged. Although the antifungal assay was complemented by recovery assay, SEM observations, and a detached-leaf phytotoxicity assessment, these experiments do not establish the precise cellular or molecular mechanism of the inhibition under whole-plant conditions. In addition, the antifungal evaluation focused on mycelial growth and did not assess other biologically important stages of the *P. capsici* life cycle, including sporangial formation, zoospore release and motility, and cyst germination. Also, whether prolonged exposure, repeated application, or concentrations above the tested concentrations could produce irreversible inhibition remains unknown. Furthermore, the experiments were conducted under controlled in vitro conditions, which may not fully reflect the complexity of field environments. Possible interactions between ZnO NPs and PDA constituents, as well as factors such as aggregation, dissolution, soil composition, organic matter, and microbial interactions, may influence nanoparticle availability and efficacy. Detailed physicochemical properties, including hydrodynamic size, surface charge, dissolution behaviour, and suspension stability, were not determined. Although the ZnO NP suspensions were sonicated before incorporation into PDA, quantitative assessment of dispersion stability was not performed. These analyses would provide additional information regarding nanoparticle behaviour and should be considered in future studies. Moreover, the detached-leaf assay provided only an initial assessment of short-term visible injury following a single exposure and does not establish whole-plant or long-term safety. Plant physiological responses, zinc uptake and accumulation, repeated exposure, nanoparticle persistence, and effects on non-target organisms were not evaluated. As no pot-culture, greenhouse, or field experiments were conducted, the present findings demonstrate only the in vitro inhibitory potential of ZnO NPs, and a definitive application method cannot yet be recommended. Future studies should therefore evaluate whole-plant disease suppression, application strategy, nanoparticle behaviour, plant responses, and environmental safety under greenhouse and field conditions.

Overall, the findings of this study indicate ZnO NP exposure suppressed the growth of *P. capsici* without causing visible structural damage detectable by SEM. The observed reversible inhibition and altered aerial mycelial development further supports that nanoparticle exposure affects fungal developmental processes. However, these mechanisms were not directly investigated in the present study. The consistent inhibitory effects observed across isolates obtained from different locations in Sarawak highlight the potential applicability of ZnO NPs for

managing local pathogen populations. Given the economic importance of black pepper cultivation in this region, such findings provide valuable insights into the development of alternative disease management strategies relevant to local pathogen populations.

5 Conclusions

ZnO NPs demonstrated significant antifungal activity against *P. capsici* isolates from Sarawak in a size- and concentration-dependent manner. Smaller nanoparticles (~50 nm) exhibited increased efficacy compared to larger nanoparticles (~100 nm). However, the antifungal effect observed under the present experimental conditions was primarily fungistatic and associated with altered fungal growth behavior with no obvious structural damage detected by SEM, indicating suppression and delayed development of *P. capsici* rather than complete pathogen eradication. No visible phytotoxic symptoms were observed on black pepper leaves at effective concentrations under the conditions tested. These findings support further evaluation of ZnO NPs as potential components of integrated disease management for black pepper. Greenhouse and field efficacy, application strategy, environmental persistence, plant accumulation, and non-target effects must be evaluated before practical or sustainability-related conclusions can be made.

Acknowledgements The authors would like to acknowledge Universiti Malaysia Sarawak for the facilities provided.

Author contributions All authors contributed to the study concept and design. Main research: EJYK; Material preparation and data collection: EJYK, FKS; Data analysis: EJYK, FKS, SFC, BSW, WD, SP, LSL, THW, AES; Manuscript writing: EJYK. All authors reviewed and approved the final manuscript.

Funding No funding was received for conducting this study.

Data availability All data and materials used and/or analyzed during this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate The plant materials used in this study were cultivated and collected in compliance with relevant institutional, local, and national guidelines. No specific permits or licenses were required for the collection of plant materials.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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