GREEN SYNTHESIS OF ZINC OXIDE NANOPARTICLES USING *MORINGA OLEIFERA* LEAF EXTRACT AND ITS ANTIFUNGAL EFFECT AGAINST *COLLETOTRICHUM GLOEOSPORIOIDES* IN AVOCADO

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ABSTRACT
Avocado, one of the most commercially important fruits, is susceptible to several postharvest diseases, including anthracnose caused by *Colletotrichum gloeosporioides*, which renders the fruit unpalatable. Prochloraz, a synthetic postharvest chemical treatment, is currently used to control anthracnose in avocados. However, several studies have reported adverse effects of Prochloraz on human health due to its metabolite. Thus, there is an imposed ban on this fungicide on fruits destined for the European Union, which is the primary target and a lucrative market for the South African avocado industry. The present study evaluated the efficacy of *Moringa oleifera* leaf extract (MLE) on the green synthesis of zinc oxide (ZnO) nanoparticles (NPs) (ZnO NPs) and its antifungal effect against *C. gloeosporioides* on 'Hass' avocado fruit. Green synthesis of ZnO NPs was done using zinc acetate dihydrate (0.2 M) as a source of zinc ions and moringa leaf extract (5%) as a reducing and stabilizing agent. The antifungal activity of the synthesized ZnO NPs was tested against *C. gloeosporiodes* in potato dextrose agar (PDA) amended with ZnO NPs (0.25, 0.5, 1, and 2%) and observing the mycelial growth on a two-day basis for nine days. The antioxidant radical scavenging activity of the ZnO NPs formed was found to be dependent on concentrations, with activity increasing with increasing concentrations. Moreover, compared to the control, all the tested concentrations (0.25, 0.5, 1, and 2%) of moringa-based ZnO NPs significantly inhibited the radial growth of *C. gloeosporioides* isolates in the *in vitro* study. The highest inhibition percentage (72%) of mycelial growth was observed in isolates treated with 1% ZnO-NPs. The findings from this study showed a strong effect of ZnO NPs against *C. gloeosporioides*. Therefore, this could be recommended as an effective antifungal agent to control anthracnose in avocados.

Keywords
Prochloraz; anthracnose; nanotechnology; mycelial growth; antioxidants; *in vitro*

INTRODUCTION
A significant amount of avocado fruit is lost during postharvest due to diseases and the perishable nature of this fruit. This impedes the economy since the avocado producing industry in South Africa is export orientated; thus, the quality of this fruit is crucial. Maintaining avocado fruit quality throughout the export process is still challenging. *Colletotrichum gloeosporioides*, the causal agent of anthracnose, is one of the most economically devastating postharvest pathogens that is responsible for massive spoilage of fresh produce (Freeman *et al.*, 1996). Although it starts developing pre-harvest while the fruit is still attached to the tree, the symptoms are commonly observed in storage during fruit ripening. This pathogen compromises the quality of fruit, which results in a decline of market value, and, in the case of severe infection, the fruit becomes inedible. The control

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of anthracnose has depended almost entirely upon Prochloraz, especially for South African avocado fruit. However, there is an imposed ban on Prochloraz by some of the important international markets (Sivakumar *et al.*, 2021). This follows from the US Environmental Protection Agency (EPA) associating some of these fungicide metabolites with elevated chronic diseases, such as cancer, and marking them as a priority pollutant (Shimshoni *et al.*, 2020). In fact, the adverse effects of Prochloraz on human health and the environment are documented as well (Xu *et al.*, 2018; Obianom *et al.*, 2019; Sivakumar *et al.*, 2021; Lin *et al.*, 2023). Moreover, various pathogens have developed resistance to most chemical treatments. This, therefore, necessitates continued research aiming to develop or improve environmentally safe organic postharvest treatments to replace synthetic fungicides.

Nanotechnology has gained popularity for its wide applications in different fields, including renewable energy, environmental remediation, surface disinfection, medicine, and agriculture (Sharma *et al.*, 2020; Jobe *et al.*, 2022). This is due to unique properties, such as crystallinity, size, shape, and morphology resembled by nanoparticles (NPs) (Shinde, 2015). Various metals and metal oxides, such as copper (Cu), copper oxide (CuO), zinc oxide (ZnO), titanium dioxide (TiO_2), silver (Ag), palladium (Pd), and iron (Fe) and its oxides have been used as source of NPs and featured in horticultural commodities postharvest preservative studies as well (Suryavanshi *et al.*, 2017; Iliger *et al.*, 2021; Sharifan *et al.*, 2021; Wang *et al.*, 2023). Commonly, the resulting NPs maintain the quality and increase the shelf life of most horticultural produce (de Oliveira Filho *et al.*, 2021; de Oliveira Filho *et al.*, 2022). However, most authors have demonstrated great potential for ZnO NPs, mainly due to their high piezoelectric properties, excellent biocompatibility, large binding energy, non-toxicity, and low cost (Jiang *et al.*, 2018; Jain *et al.*, 2020). Furthermore, ZnO NPs have unique chemical and physical properties, giving these compounds strong antibacterial and antifungal activity at low concentrations, owing to their high surface area to volume ratio (Espitia *et al.*, 2013).

Different techniques, such as chemical, physical, and biological methods, have been used to synthesize NPs (Rane *et al.*, 2018). However, some methods, such as chemical and physical, are not sustainable for the environment and consumers. For example, the chemical method involves the use of toxic chemicals, which makes this approach unfriendly, whereas, on the other hand, there is more energy, area, and time required by the physical approach (Thema *et al.*, 2015). There is consistently increasing interest in the biological synthesis of nanoparticles, and more focus has been put on optimizing green chemistry technology to synthesize such materials. Biological synthesis involves using plant extracts and other microorganisms with biomedical applications. Various authors have presented the potential of different plant parts, such as stems, roots, leaves, and the actual fruit, in the synthesis of nanoparticles (Elia *et al.*, 2014; Ghaffari-Moghaddam *et al.*, 2014; Niraimathee *et al.*, 2016; Kumar *et al.*, 2017; Santhoshkumar *et al.*, 2017; Behravan *et al.*, 2019). These plant parts produce the phytochemicals that act as stabilizing and reducing agents during synthesis (Ramesh *et al.*, 2015; Thema *et al.*, 2015). Thus, synthesizing nanoparticles by such an approach raises no concerns as it is an environmentally friendly, safe, cost-effective, and biocompatible green approach.

Moringa oleifera is one of the most widely cultivated crops for its nutritional and medicinal properties (Matthew, 2016). There has been extensive research on the antimicrobial and antioxidant activity of different parts of this crop (Tesfay *et al.*, 2011; Kumar *et al.*, 2012; Ndhlala *et al.*, 2014; Mohammed *et al.*, 2019; Tshabalala *et al.*, 2020). Moreover, extracts from different parts of this crop have featured in research on different pre- and postharvest fruit treatments with positive responses (Adetunji *et al.*, 2013; Kubheka *et al.*, 2020; Mahmoud *et al.*, 2020; Nasir *et al.*, 2020). However, little research has been conducted to optimize the synthesis of nanoparticles using this crop. This work, therefore, focused on the synthesis of ZnO-NPs using *Moringa oleifera* leaf extract following a greener approach and evaluating its morphological, structural, and antifungal properties against *C. gloeosporioides* on 'Hass' avocado fruit.

MATERIALS AND METHODS

Materials

The fresh moringa leaf powder used in this study was purchased from the Agricultural Research Council (ARC), located in Pretoria, South Africa. All the chemicals and reagents were purchased from Merck Life Science (Pty) Ltd, Johannesburg, South Africa.

Plant extract preparation and synthesis of zinc oxide nanoparticles

The plant extract was prepared following a slightly modified method previously described by Bhuyan *et al.* (2015) and Hassan *et al.* (2021). Briefly, the extract was prepared by mixing 10 g of dried moringa leaf powder with 100 mL of distilled water in a 250 mL Erlenmeyer flask. The mixture was after that heated at 75 °C for 15 minutes. This was followed by allowing the mixture to cool at room temperature and filtering through Whatman No.1 filter paper. The filtrate was collected and stored in a refrigerator at 4 °C until further use.

Zinc acetate dihydrate (0.2 M) was prepared in 100 mL deionized water under constant stirring on a hot plate magnetic stirrer at 85 °C for 60 minutes. After 20 minutes, 25 mL of 2 M sodium hydroxide was slowly added until the pH of the solution was 12. After 1 hour, 25 mL of moringa leaf extract was slowly added under continuous stirring for 2 hours using a magnetic stirrer. The resulting deep yellow coloured paste was collected, oven-dried overnight at 60 °C, transferred to a ceramic crucible cup, and heated in a furnace at 460 °C for 2 hours. The resulted powder

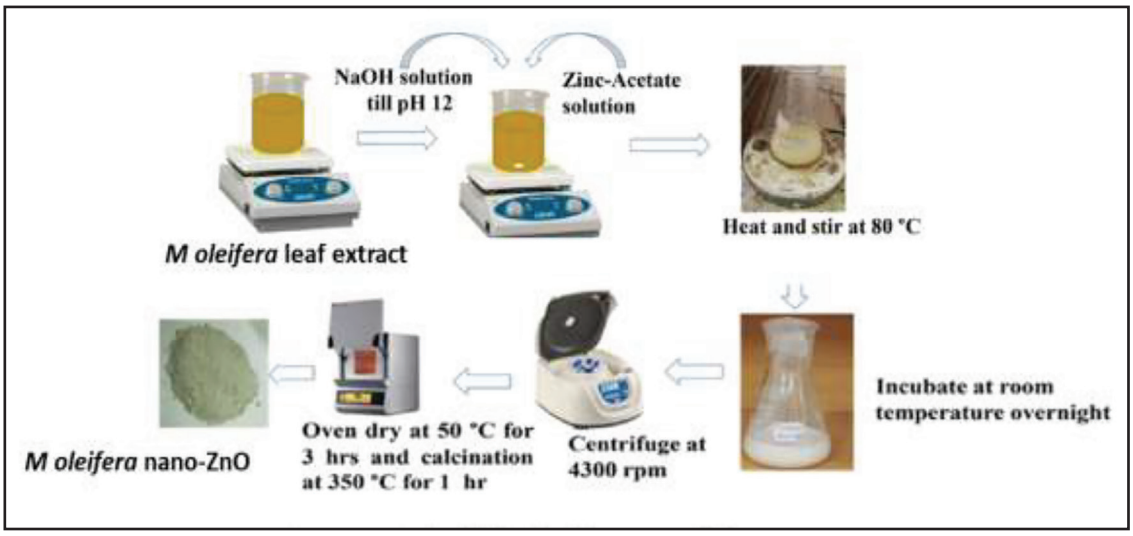

Figure 1: The schematic diagram of the stages involved in the biosynthesis of zinc oxide nanoparticles using moringa extract.

material was collected, put in an air-tight bottle, and stored at room temperature until further use.

Characterization of zinc oxide nanoparticles

The synthesized ZnO NPs were confirmed by evaluating the size, structure, and shape of the crystal using a Zeiss EVO scanning electron microscopy (Zeiss, Oberkochen, Germany) (SEM) and a JEOL JEM 1400 transmission electron microscopy (JEOL, Beijing, Shanghai, China) (TEM). For TEM analysis, the ZnO NPs were suspended in absolute ethanol and sonicated for 15 minutes for clear dispersion of particles. A drop of the resulting sonicated solution was cast onto a carbon-coated copper grid, dried in a mercury lamp for 10 minutes, and examined under TEM. For SEM analysis, the ZnO NPs were mounted onto stubs and coated three times with gold deposited by a quorum sputter (Q150R ES) under a vacuum using argon gas. This was followed by viewing the NPs under the Zeiss EVO SEM in high vacuum mode.

Antioxidant activity of zinc oxide nanoparticles

The scavenging activity of ZnO NPs was evaluated using 2,2-diphenyl-1-picrylhydrazyl assay following a method previously described by Safawo *et al.* (2018), with slight amendments. Briefly, 1 mL of different concentrations of ZnO NPs (0.25, 0.5, 1, 2% v/w) dissolved in ethanol were separately mixed with 1 mL of methanolic DPPH (0.1 mM) and vortexed for 1 minute to mix the solution thoroughly. Thereafter, the mixture was incubated in the dark for 30 minutes at room temperature. The absorbances for the prepared solutions were read at 520 nm wavelength using a UV-1800 Spectrophotometer (Shimadzu Scientific Instruments INC., Columbia, USA), and the experiment was repeated three times. The control contained only methanol and DPPH solution. The scavenging activity was estimated using Eq. 4.1 below.

$$\% \text{ Inhibition} = \frac{\textit{Control absorbance} - \textit{Sample absorbance}}{\textit{Control absorbance}} \times 100\% \quad (4.1)$$

Isolation of pathogen and media preparation

The pathogen, *C. gloeosporioides*, was isolated from avocado fruit by aseptically cutting internally infected tissues using an alcohol-sterilized scalpel. The tissues were surface sterilized for 30 seconds in 70% ethanol, followed by rinsing twice in distilled water, placed on potato dextrose agar (PDA) plates, and incubated for seven days at 28 °C to allow for fungal growth. To prepare the PDA media, 15.5 g of potato dextrose was mixed with 400 mL of distilled water and autoclaved at 121 °C for 15 minutes. The mixture was allowed to cool to 50 °C in water and poured into 90 mm Petri dishes. The pure culture was obtained by subculturing the isolated colonies into fresh PDA plates.

Confirmation of pathogen isolates

The isolates were confirmed by evaluating their morphological structures under a light microscope at 40x and 100x magnifications based on their hyphal orientation and the shape of their spores. Based on visual observation, the pathogen had fast-growing white to off-white colonies with dense whitish mycelia. On the reverse side of the Petri dishes, the colour was yellowish towards the centre. Light microscopy confirmed the formation of cylindric and aseptate conidia with obtuse ends (Fig. 2). All these observed characteristics match the ones reported for *Colletotrichum gloeosporioides* in a study by Hassan *et al.* (2018).

Preparation of ZnO-based treatments and *in vitro* assay

The antifungal activity of the synthesized ZnO NPs was tested at different concentrations (0, 0.25, 0.5, 1, and 2% v/w) against *C. gloeosporioides* isolates in Petri dishes. The used concentrations were amended from those previously tested by Le *et al.* (2021) on avocado. Different treatments were obtained by sep-

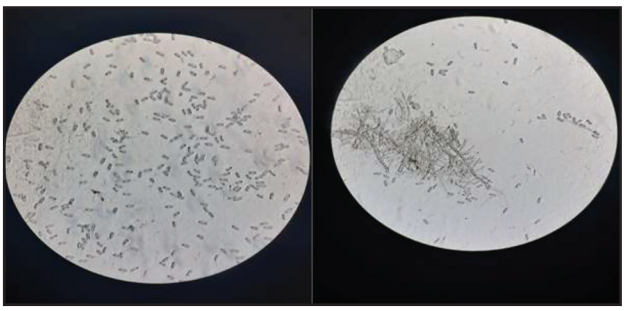

Figure 2: Morphology of *C. gloeosporioides* confirmed under light microscope.

arately amending the prepared PDA agar with ZnO NPs (0.25, 0.5, 1, and 2%), and the pure (unamended) PDA served as a control. After cooling, about 20 mL of the amended PDA was poured into 90 mm sterile Petri dishes, and a disc of mycelium (3 mm diameter) excised from the pure culture was used for inoculations. This was followed by incubating the Petri dishes for nine days at 25 °C. During this time, mycelial growth was evaluated by measuring the diameter of the colony on a two-day basis during the incubation period. The treatments were completely randomized, and each treatment had three replicate cultures with two measurements recorded per replicate and per measuring day. The inhibition percentage was determined using the following Eq. 4.2 (González-Merino *et al.*, 2021).

$$\% \text{ Inhibition} = \frac{MGC\ (mm) - MGT\ (mm)}{MGC\ (mm)} \times 100\% \quad (4.2)$$

where MGC = mycelial growth in the control; MGT = MGC (mm) mycelial growth in the ZnO NPs treatment

Statistical analysis

A completely randomized design was used for this experiment with three replicates. The GenStat statistical software (GenStat Twentieth Edition, VSN International Ltd, UK) was used for the analysis of variance (ANOVA) of the data analysis. The means separation was performed using Duncan's Multiple Range Test (DMRT) at a 5% significance level.

RESULTS AND DISCUSSION

Confirmation of ZnO nanoparticles through SEM and TEM

The formation of ZnO NPs was first confirmed by the yellow colour development during biosynthesis, which is regarded as the most reliable colour indicating the formation of NPs (Barzinjy and Azeez, 2020). Further evaluations using the SEM revealed amorphous ZnO NPs, while the TEM showed weakly agglomerated nanoparticles with a uniform spherical shape (Fig. 3, Fig. 4). The agglomeration occurred due to the narrow space between particles caused by the aqueous MLE and the high surface energy and electrostatic attraction of ZnO NPs (Ramesh *et al.*, 2015; Darvishi *et al.*, 2019). However, the weak agglomeration indicated the high potential of MLE to act as a stabilizing agent, thereby preventing the aggregation of the NPs (Holmes *et al.*, 2003). This study reports the formation of ZnO NPs with a diameter estimated to range between 35 and 78 nm, as indicated in Figure 4. The overlap of NPs contributed to the increased reported size. Various factors, such as the selected plant species, plant extract concentration, precursor concentration, reaction time, pH value, and calcination time, have been reported to influence the morphology of synthesized NPs (Xu *et al.*, 2021). Although this study did not compare the effect of different pH and calcination temperatures on the resulting NPs, increasing the pH to 12 and the high calcinating temperature (350 °C) might have influenced the shape, size, and aggregation of the resulted NPs. Ochieng *et al.* (2015) previously reported a formation of weakly agglomerated homogeneous NPs at alkaline pH compared to acidic using *Spathodea campanulate* P. Beauv leaves extract. Another study by Karam and Abdulrahman (2022) reported that NPs are formed as non-orientation nanorods at low calcination temperatures and only join together and form various shapes at temperatures between 250 and 450 °C using thyme plant leaf extract (Karam and Abdulrahman, 2022). Similarly, Zhu *et al.* (2021) observed a directly proportional trend between pH and ZnO NPs synthesized using *Cinnamomum camphora* (L.) Presl leaf extracts; the smallest average size was obtained at pH 7 (13.92 nm) while the highest (21.13 nm) was obtained at pH 9. Furthermore, this could be the reason for the ZnO NPs sizes reported in this study, given that the pH was raised to 12; however, it is important to mention that they were still within nanoscale (>100 nm). There are, however, still some arguments about the effect of pH on the particle size of nanoparticles. Some authors claim that particle sizes decrease with increased pH (Alias *et al.*, 2010), while others claim the opposite (Jay Chithra *et al.*, 2015). This study has no conclusive results about the effect of pH on the green synthesis of ZnO NPs using moringa. Further studies still need to be conducted to determine various factors affecting the synthesis of these particles, following a similar approach. However, in general, the size of the ZnO NPs increased with increased reaction period and calcination temperature and decreased with increased precursor and plant extract concentrations (Xu *et al.*, 2021).

Antioxidant activity of zinc oxide nanoparticles

The potential antioxidant activity of moringa leaf extract-based nanoparticles was evaluated using the DPPH method. Various authors have considered the DPPH method the most reliable and cheap method for determining antioxidants' radical scavenging activity (Hossain *et al.*, 2015; Ningsih *et al.*, 2016). Figure 5 shows the results of the tested ZnO NPs at different concentrations against DPPH. The inhibition percentage increased with increasing ZnO concentrations. The antioxidants present in these NPs were due to the MLE used in the biosynthesis. This could also indicate that the method used to synthesize moringa-based ZnO NPs was very effective and

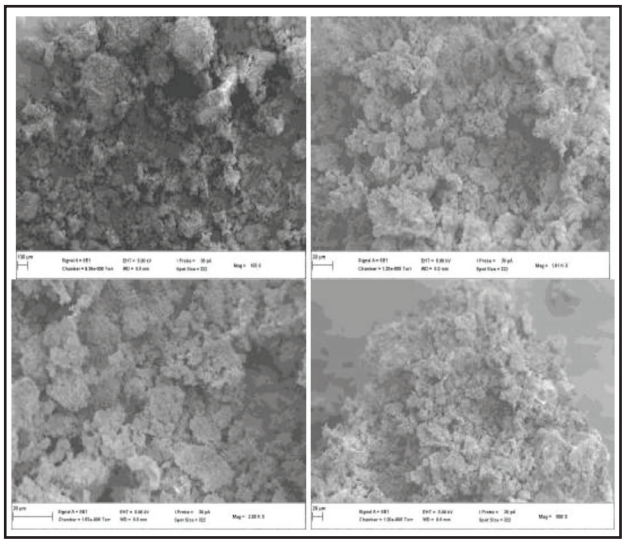

Figure 3: Scanning electron microscopy images of moringa-based ZnO nanoparticles at different magnifications.

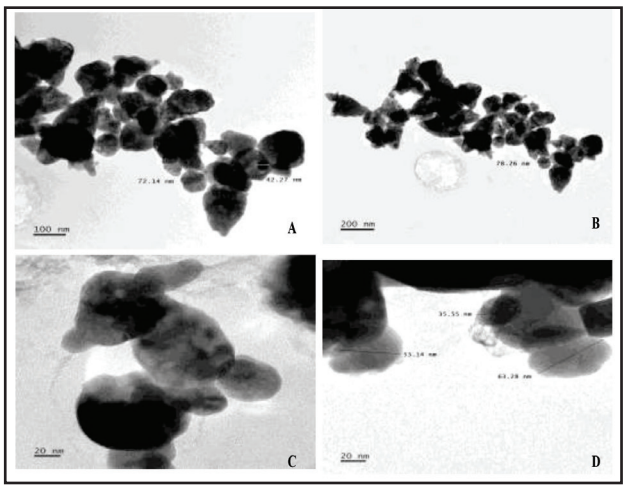

Figure 4: ZnO nanoparticles synthesized using moringa leaf extract confirmed under transmission electron microscopy at different magnifications.

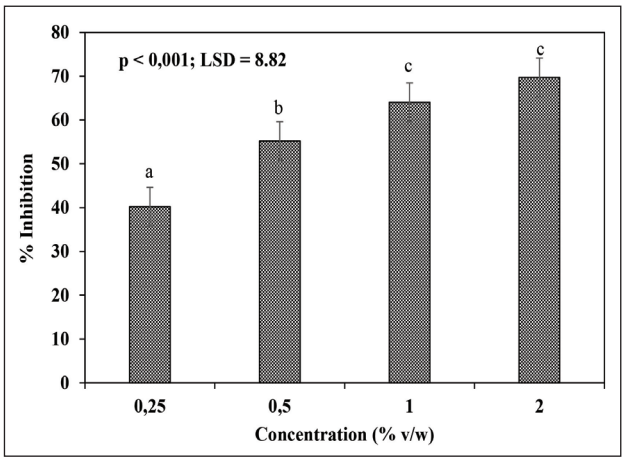

Figure 5: Scavenging activity of ZnO NPs at different concentrations against DPPH. The vertical bars represent standard error (SE) at n = 3; means sharing the same letter are not statistically significant according to Duncan's Multiple Range Test (DMRT) (P = 0.05).

did not negatively affect the antioxidants of moringa and the resulting NPs. This observed moringa-based ZnO NPs antioxidants trend is similar to that generally displayed by ascorbic acid at different concentrations when used as a standard to determine antioxidants. The results in this study corroborate those by Zhu *et al.* (2021), who reported an increase in antioxidant activity with increased ZnO NPs synthesized with *Cinnamomum camphora* (L.) Presl leaf extracts. Jobe *et al.* (2022) also reported that the lowest concentration of ZnO and Ag/ZnO NPs showed the lowest radical scavenging activity. Similarly, Mthana *et al.* (2022) demonstrated increased inhibition percentage with increasing concentrations of ZnO NPs synthesized following a green or conventional method. Generally, the antioxidant activity of greenly synthesized nanoparticles depends on the chemical composition of the extract. Therefore, the resulting nanoparticles will have a higher scavenging activity if the extract exhib-

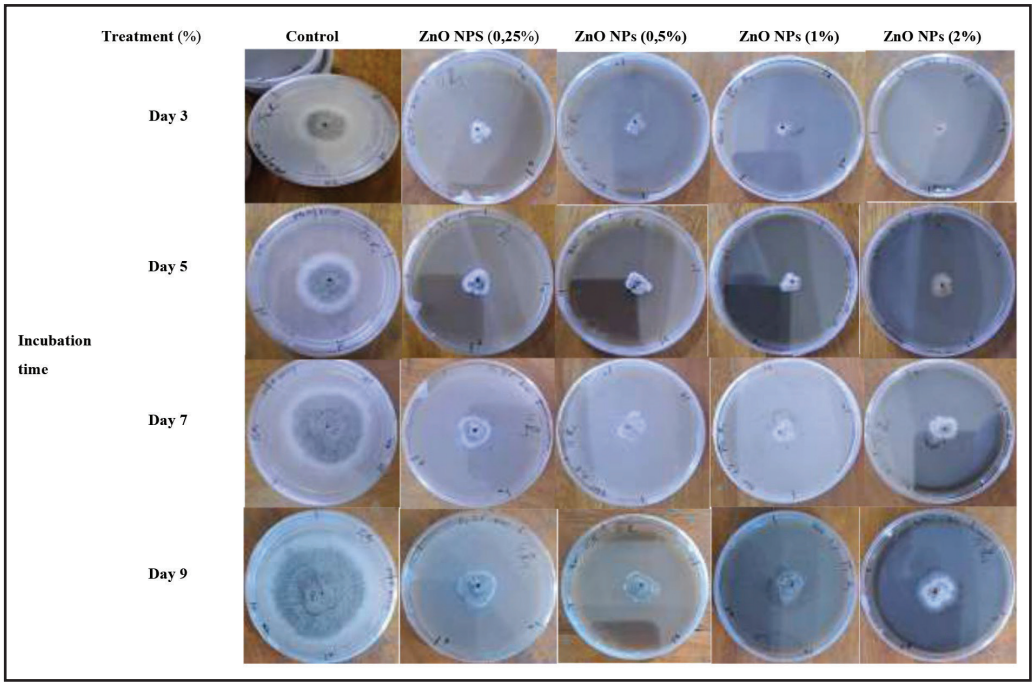

Figure 6: The mycelial growth of *Colletotrichum gloeosporioides* isolate during storage as influenced by the application of ZnO NPs at 0, 0.25, 0.5, 1, and 2% concentrations.

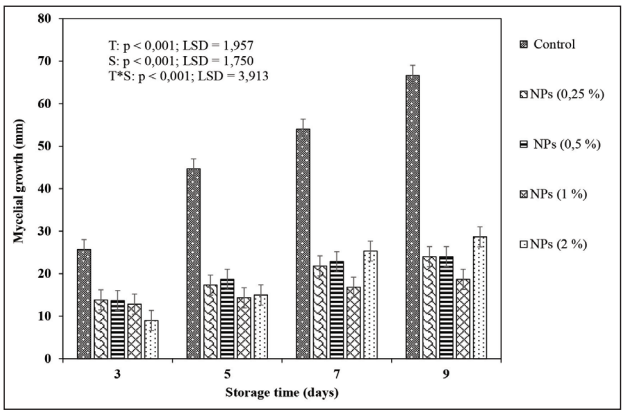

Figure 7: The mycelial growth of *Colletotrichum gloeosporioides* isolate as influenced by ZnO NPs and storage period. The vertical bars represent standard error (SE) at n = 3; T, treatment; S, storage period.

its higher phenolics and flavonoids. Thus, this depicts that moringa leaves are a good source of antioxidants.

Effect of moringa-based ZnO NPS on the mycelia growth of *Colletotrichum gloeosporioides*

Figure 7 shows that the ZnO NPs significantly ($p < 0.001$) affected the mycelia growth of *C. gloeosporioides*. The growth of mycelia reached 24, 24, 17, 29, and 67 mm for *C. gloeosporioides* cultures in 0.25, 0.5, 1, 2, and 0% (control) concentrations of ZnO NPs, respectively. This marked a decrease of 64, 64, 71, and 56% in the mycelial growth of the *C. gloeosporioides* cultures exposed to 0.25, 0.5, 1, and 2%, respectively, compared to the control. Various authors have reported the antibacterial effect of green synthesized ZnO NPs (El-Kady *et al.*, 2023; Ihsan *et al.*, 2023; Mushtaq *et al.*, 2023; Yilma *et al.*, 2023), while few have examined their antifungal effect. In this study, ZnO NPs were synthesized using moringa leaf extract and evaluated for their potent antifungal effect against avocado *C. gloeosporioides*. The enhanced ZnO NPs efficacy presented in this study could be attributed to their unique characteristics, including their large surface area. Similar results were reported by Pariona *et al.* (2020), where all the concentrations used for ZnO NPs significantly inhibited the mycelial growth of *Fusarium solani*, *Fusarium oxysporum* f. sp. *lycopersici*, and *Colletotrichum gloeosporioides* with variable effectivity. However, the efficacy of NPs may also be affected by variation in shape, size, and fungal species. It was found that increasing the concentration of ZnO NPs to 2% resulted in the highest mycelial growth on day nine compared to the other concentrations used, except the control. Still, based on a visual perspective, it is important to mention that this treatment inhibited the pathogen from sporulating. Similarly, Arciniegas-Grijalba *et al.* (2017) reported a high antifungal effect of ZnO NPs against *Erythricium salmonocolor* (Berk. & Broome) on day 10, which was, thereafter, reduced over time on day 22. Various authors have reported different levels of cellular damage caused by different concentrations of NPs to other pathogens, including *Colletotrichum gloeosporioides* (la Rosa-García *et al.*, 2018). Although there were no cellular evaluations in the present study, the results obtained could indicate the ability of ZnO NPs to inhibit fungal growth by distorting and damaging its conidia (He *et al.*, 2011). Furthermore, ZnO NPs are associated with the development of reactive oxygen species (ROS), which damage the cell (Lipovsky *et al.*, 2011). A study by Espitia *et al.* (2012) further demonstrated that the resulting ROS on the cell wall of the fungi, due to the presence of ZnO NPs, causes protein denaturation and consequently destroys the DNA and the cell wall.

CONCLUSION

The biological approach for the synthesis of ZnO NPs using moringa leaf extract has been proven to be a simple, environmentally friendly, and effective approach. Using plant-based materials as reducing and stabilizing agents minimizes the usage of synthetic harmful and toxic materials. Furthermore, given the potential of these particles in controlling the postharvest pathogen *Colletotrichum gloeosporioides*, they can be recommended as an environmentally friendly substitute for the currently used fungicide at packhouses. However, further *in vivo* assessment is still required since this study was only done *in vitro*. Nanotechnology has great potential, and more research needs to be done on the mechanism of the formation of nanoparticles to have complete control of the characteristics of the resulting product, such as size and shape. This is necessary due to the relationship between the morphology and the properties of the nanoparticles; therefore, it is much more important to synthesize the NPs with the morphology that best suits the aim intended.

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