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Original Research

Potential Antifungal Effects of *D. malabarica* Assisted Zinc Oxide and Silver Nanoparticles against Sheath Blight Disease of Rice Caused by *Rhizoctonia solani*

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Abstract

The plant based synthesis of nanoparticles (NPs) has become a promising substitute to the conventional chemical synthesis methods that involved the environmental hazardous chemicals. This study is designed to evaluate the antifungal potential of *Diospyros malabarica* leaves assisted zinc oxide (ZnO) and silver (Ag) NPs as an eco-friendly green route against *R. solani* causing sheath blight disease of rice. The physicochemical characteristics of ZnO and Ag-NPs were studied using the UV-Vis spectroscopy, scanning electron microscopy (SEM) and energy dispersive x-ray (EDX) spectroscopy. The Ag-NPs showed a phenomenal peak at 416 nm. The morphology of ZnO and Ag-NPs was observed using SEM as they were spherical in shape with a few agglomeration and smooth surface. The EDX elemental detection spectrum showed that both the ZnO and Ag-NPs were in pure form. The antifungal *in vitro* assay was performed using poison food technique against *R. solani*. The findings of growth inhibition assay have shown that *R. solani* was more sensitive to the Ag-NPs (61.8%) as compared to ZnO-NPs (51.1 %). It can be concluded that plant assisted NPs could be possible alternative for the inhibition of fungal plant disease contrary to the synthetic fungicides in biological domains. Overall, this study suggested that plant assisted ZnO and Ag-NPs can be an attractive and green candidate to control rice fungal disease. This study may perhaps strengthen a new green chemistry approach for environmental science and biomedical applications.

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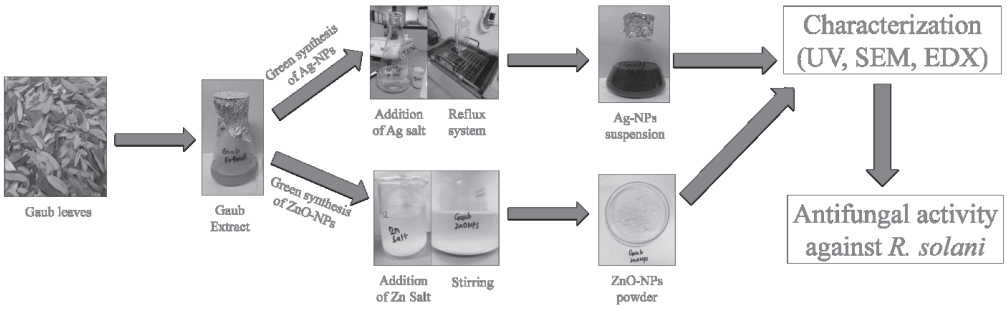

Graphical representation for the *D. malabarica* leaves assisted synthesis of zinc oxide (ZnO) and silver nanoparticles (Ag-NPs) and its application.

Keywords: Nanoparticles, green synthesis, poison food technique, *D. malabarica*, antifungal efficacy, SEM, EDX

Introduction

It is estimated that total food production will only be sufficient for 60% of the world population, as by 2050 it is expected to reach over 9 billion [1-2]. This predicted growing population also requires a significant increase in crop yields to meet the requirements of the expanding global demand for food. The yield and quality of crops are gradually decreasing annually caused of various microbial diseases which may in turn food decline [3]. Rice (*Oryza sativa* L.) belongs to the family *Graminae* is the world's most widely consumed cereal crop particularly in Asian countries [4-5]. The leading producer of rice is China followed by India. It is the 2nd major crop in Pakistan subsequent to wheat and the main source of foreign trade after cotton [6].

Several pathogens affect the rice crop among which *Rhizoctonia solani* (*R. solani*) is causing sheath blight (ShB) disease that is responsible for up to 45% of yield loss [7].The disease cycle starts with the development of abrasion leading to suppleness and lodging of the sheath, which finally restrains the grain filling [8]. The ShB pathogen, *R. solani* (sclerotia) remains alive for 2-3 years in the soil and water that multiplies quickly through contact of plant parts with each other [9]. It is challenging to manage the *R. solani* due to the extensive host range of the pathogen and perseverance of sclerotia even in unfavorable environmental conditions [10-11]. Chemically synthesized fungicides offer rapid control but have adverse effects on the environment in addition to the immunity of fungi to chemical fungicides [12].

Thus, an environment-friendly, efficient, and economical alternative is needed to control the phyto pathogenic fungus, which is accomplished by nanotechnology [13]. The nanotechnology provides a sustainable and eco-friendly solution against the plant fungal pathogens contrary unlike conventional fungicides [14-15]. More efficient bio-fungicides made up of metals i.e. silver, zinc, copper, gold, and iron are being used against plant pathogens [16]. Several studies have been reported on the synthesis of metal oxide/metal nanoparticles (NPs) showing significant antifungal effects due to the large surface area-to-volume ratio and other physicochemical properties [17-18]. Zinc oxide (ZnO) NPs have received attention due to their strong antifungal potential [19], improved durability, and low toxicity [20-22]. Silver (Ag) NPs have also attracted researchers, attention due to their salient characteristics, i.e. chemical stability, high catalytic activity, and antifungal efficiency [23]. Due to the antifungal potential of ZnO and Ag-NPs, they are considered an alternative to synthetic fungicides against plant fungal pathogens.

There is always a need to develop an inexpensive, non-toxic and dynamic synthesis route that synthesizes the NPs without using toxic chemicals or production of harmful by-products. The green approach is an eco-friendly practice for the synthesis of NPs of precise size and shape [24-25]. The advantage of these eco-friendly methods is to generate NPs with active biological roles (i.e., antimicrobial). The green approach deals with the use of safe microorganisms and plant extracts that replace the hazardous reducing agent used in chemical methods [26-27]. Plants extracts mediated synthesis is comparatively faster and a preferred choice than other biogenic routes due to the easy accessibility of raw material, simplicity, and efficiency of the method [28-29]. The plant extracts are enriched with various phyto constituents that have the ability to act as capping and reducing agents for the synthesis of metal oxide/metal NPs [30-31].

The presence of alkaloids, saponins, glycosides, steroids, flavonoids, and amides etc. in the plant extract causes the reduction of Zn ions from salt into ZnO-NPs [32], the reduction of Ag^{+} to Ag° and stabilize the Ag-NPs [33-34]. *Diospyros malabarica* (*D. malabarica*) is commonly known as gaub and belongs to *Ebenaceae* family. It is an important plant having

various bioactive compounds like tannins, gallic acid, triterpenes, saponin, vitamin C, anthocyanin, flavonoid, alkaloid, sitosterol, and betulinic acid which are responsible for their antioxidant, antimicrobial, Anti-diarrheal, antiviral and anticancer potential [35-37]. In view of the significance of plant-assisted synthesis of NPs and various therapeutic potentials of *D. malabarica*, it has been used in the present study to synthesize ZnO and Ag-NPs. Both the NPs were applied (*in vitro*) as antifungal agents against *R. solani*, a causal agent of ShB disease in rice.

Material and Methods

Collection of Plant Material and Extract Preparation

Fresh leaves of *D. malabarica* were collected from the Punjab Forest Department, Faisalabad, Pakistan. The collected fresh leaves were washed firstly with tap water followed by distilled water to remove surface debris. Then the sample leaves were air-dried and chopped in uniform size. The plant extract was prepared using 3 g of *D. malabarica* leaves and boiled in 100 mL of distilled water for 15 minutes at 40ºC. The extract was cooled and filtered using Whatman No. 1 filter paper and stored at 4ºC for further use [38].

Synthesis of Zinc Oxide Nanoparticles

All the glassware was autoclaved prior to use. For biosynthesis of ZnO-NPs, 60 mL of zinc acetate dihydrate salt solution (0.1 M) was stirred with 40 mL of *D. malabarica* leaves extract for 45 minutes. While stirring, NaOH (0.2 M) was added dropwise until precipitates formed. The suspension mixture was then allowed to reside overnight at room temperature. The suspension was filtered and the resultant precipitates were washed thrice with distilled water: ethanol (3:1) solution to remove ionic impurities and dried in a hot air oven at 40ºC. The pale yellow dried powder was then calcinated at 500ºC in a muffle furnace for 2 hours. The final white powder sample was grinded to obtain a fine nano sized ZnO-NPs. The prepared ZnO-NPs were stored in a dark container at 4ºC [39].

Synthesis of Silver Nanoparticles

D. malabarica leaves extract (5 mL) with 1 mM of silver nitrate (95 mL) was refluxed for 1 hour at 100ºC Fig. 1. The resultant Ag-NPs suspension was stored at 4ºC in dark conditions to use later [40].

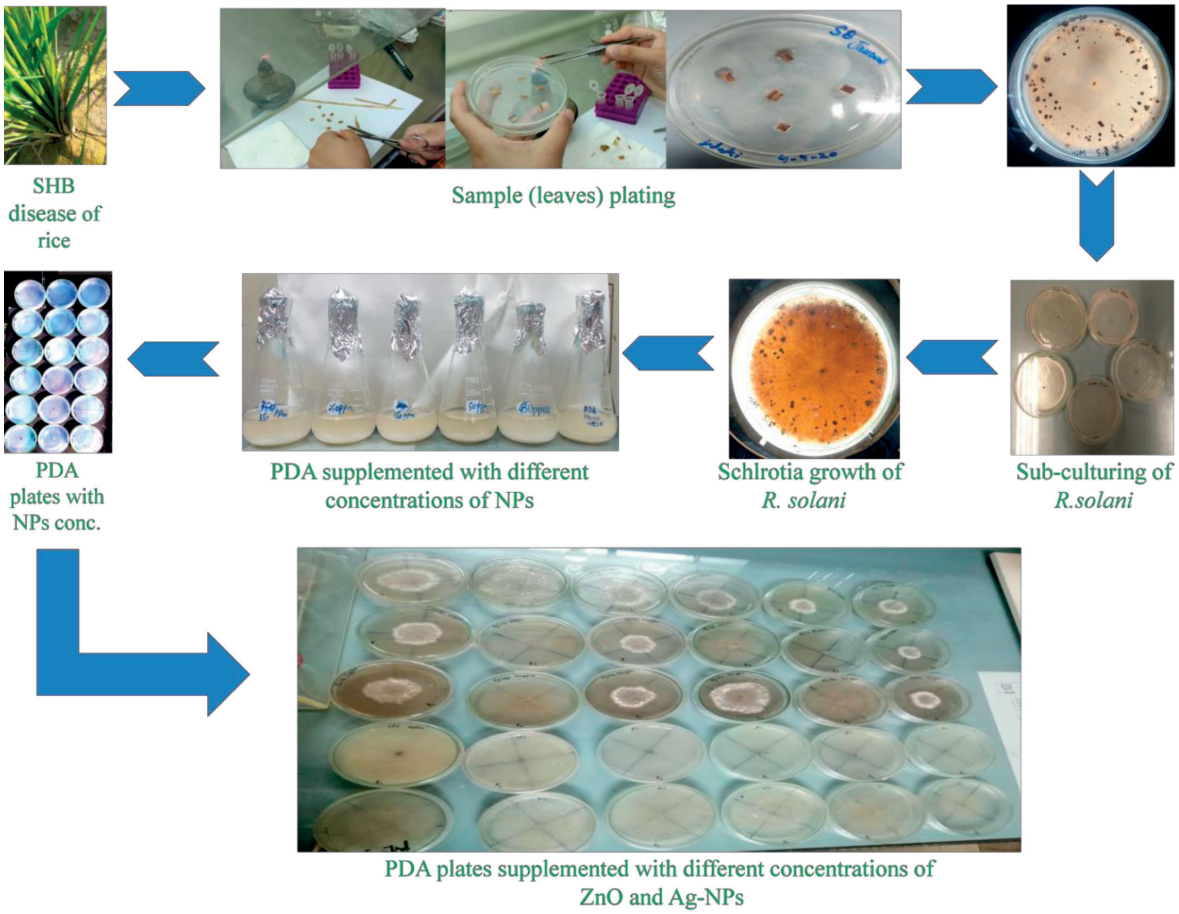

Fig. 1. The representation of antifungal (*in vitro)* assay of *D. malabarica* assisted ZnO and Ag-NPs against *R. solani.*

Characterization of Nanoparticles

D. malabarica leaves assisted ZnO and Ag-NPs were characterized through UV-Visible spectrophotometer, Scanning Electron Microscope (SEM), and Energy Dispersive X-ray (EDX) analysis.

Antifungal Efficacy of *D. malabarica* Assisted Zinc Oxide and Silver Nanoparticles

R. solani was isolated from infected sheaths of rice plants. The isolated fungus was maintained on potato dextrose agar (PDA) plates at 30ºC for 7 days till the growth reached to edges. The culture was purified via sub-culturing the *R. solani* by inoculating the agar plugs containing mycelia in the center of PDA plates and incubated at 30ºC. The poison food technique was used to test *in vitro* efficiency of *D. malabarica* assisted ZnO and Ag-NPs against *R. solani*. Different concentrations of ZnO and Ag-NPs (0.05, 0.15, 0.25, 0.35 and 0.45 mg/mL) were prepared using stock solutions of both, mixed in 150 mL of PDA media. The autoclaved media supplemented with each concentration of NPs was transferred into 6 Petri plates as one plate/replicate. Fifteen days old culture of *R. solani* maintained on PDA plates was used in this experiment. Agar plugs of 5 mm dia containing mycelia and sclerotia were placed or inoculated at the center of petri plates after solidifying the transferred PDA media and were incubated the plates at 30ºC. The data of fungal mycelia growth was recorded after every 24 hours till the growth of *R. solani* reached to edges of control plates [41]. The experiments with both ZnO and Ag-NPs were performed in triplicates. The schematic representation of the antifungal assay is presented in Fig. 1. Inhibition in mycelia and sclerotia growth of *R. solani* was determined based on the area of fungal growth, calculated using the formula as proposed [42].

Inhibition (%) = (average growth of control-average growth of treatment)/average growth of control*100

Statistical Analysis

All the data was accessed by taking the average of means. The data was evaluated by computing standard errors (SEs) of means in triplicates [43].

Results and Discussion

The current study reported the biosynthesis of ZnO and Ag-NPs using *D. malabarica* leaves extract which was a convenient, eco-friendly, and cost-effective technique. It did not involve any external stabilizing and accelerating agents. The leaves extract used in the current study provided biomolecules to the system for the biosynthesis of ZnO and Ag-NPs. The leaves extract of *D. malabarica* has been preceded as both a reducing and capping agent in the synthesis of NPs. It successfully stabilized the Zn and Ag ions from the salt solution into ZnO and Ag-NPs, respectively without using any hazardous chemicals. An efficient, eco-friendly, and cheaper (plant assisted reflux) technique has been employed in the current research study for the synthesis of Ag-NPs. It is evident from the literature that the reflux method induces a fast reduction of Ag ions into Ag metal NPs that accelerates the formation of Ag-NPs with less aggregation [40]. The lesser time consumption and uniform-sized NPs synthesis was the main attraction of this method [44]. The primary indication of ZnO and Ag-NPs synthesis was the color change observed in the extract shown in Fig. 2. The color change was might be due to the reduction of extract and salt solution into the ZnO [45] and Ag-NPs [46]. This reduction was also supported by UV-Visible spectroscopy results.

Characterization of *D. malabarica* Assisted ZnO and Ag-NPs

UV-Visible Spectroscopy

Further, the synthesis of ZnO and Ag-NPs was confirmed by UV-Visible spectroscopy. The UV-Vis

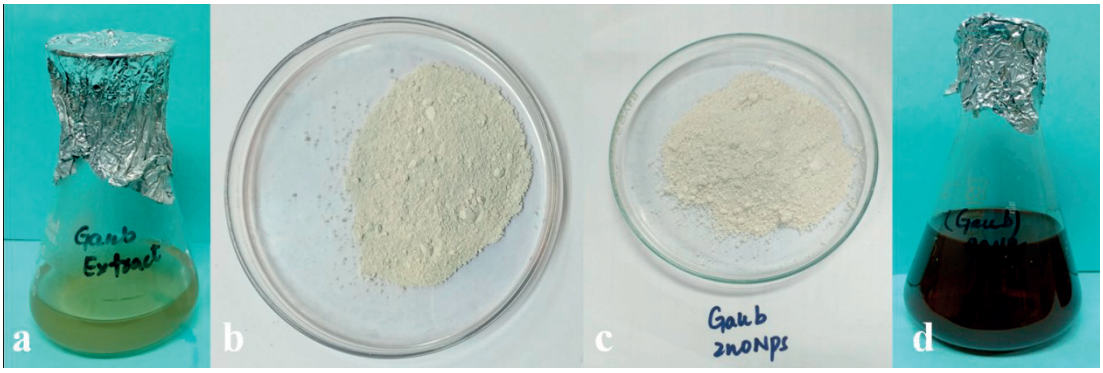

Fig. 2. The yield images a) *D. malabarica* extract, b) *D. malabarica* assisted ZnO-NPs w/o calcination, c) calcinated ZnO-NPs and d) *D. malabarica* assisted Ag-NPs suspension.

spectrum of Ag-NPs suspensions was recorded at different reflux time intervals and centrifugation times for pellets and supernatants. The optical density of the reaction mixture shown in Fig. 3a) represents the detailed overview of reflux time optimization of biosynthesized Ag-NPs using a UV-Vis spectrophotometer (Thermo Scientific: Multiskan GO). The Fig. 3a) explained the absorbance of Ag-NPs suspensions at different reflux times (such as 0.5, 1, 1.5, and 2 hours). At 0.5, 1.5, and 2 hours the broad peaks appeared at the lower absorbance tend to recommend that the Ag-NPs at those reflux times are larger in size. The spectrophotometric analysis expressed a characteristic surface plasmon resonance (SPR) band at 1 hr reflux that is due to the vibrations in electromagnetic light waves with the nanostructures in the colloidal suspension. This peak represented the formation of Ag-NPs in the extract and the reduction of metal salt ions into Ag-NPs. At 1 hour of reflux, the Ag-NPs suspension showed a sharp or narrow peak and maximum absorbance at 416 nm which may be due to the smaller size of NPs. The symmetry of SPR band represented that the Ag-NPs suspension (1-hour reflux) has not contained many aggregated particles. Moreover, the UV- Visible spectrum showed that the *D. malabarica* leaves extract has significant potential to synthesize the Ag-NPs. Another factor that has been under study in this section is the optimization of centrifugation time. Fig. 4b) showed the UV-visible

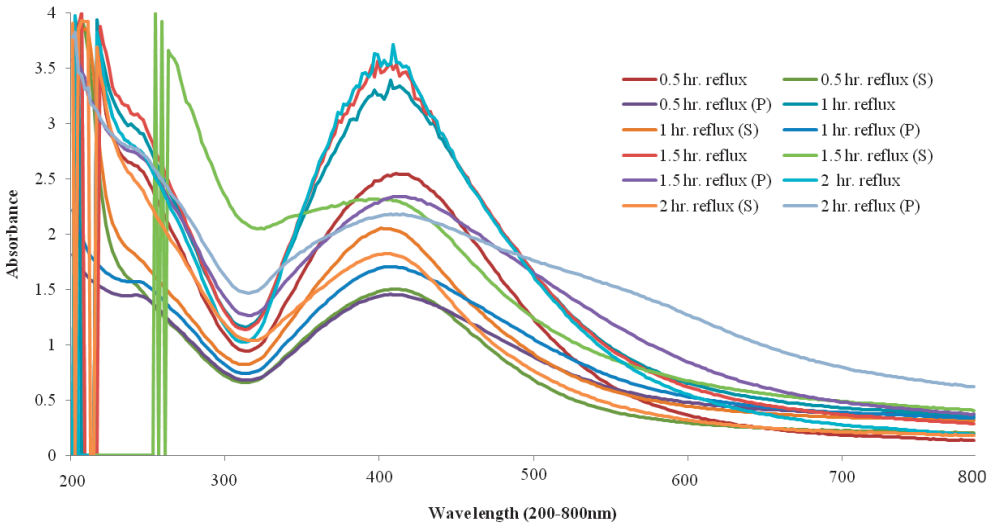

Fig. 3a). UV-Visible absorption spectrum of *D. malabarica* assisted Ag-NPs synthesized by reflux method. Note: Here S represents the supernatant and P is for the pellet.

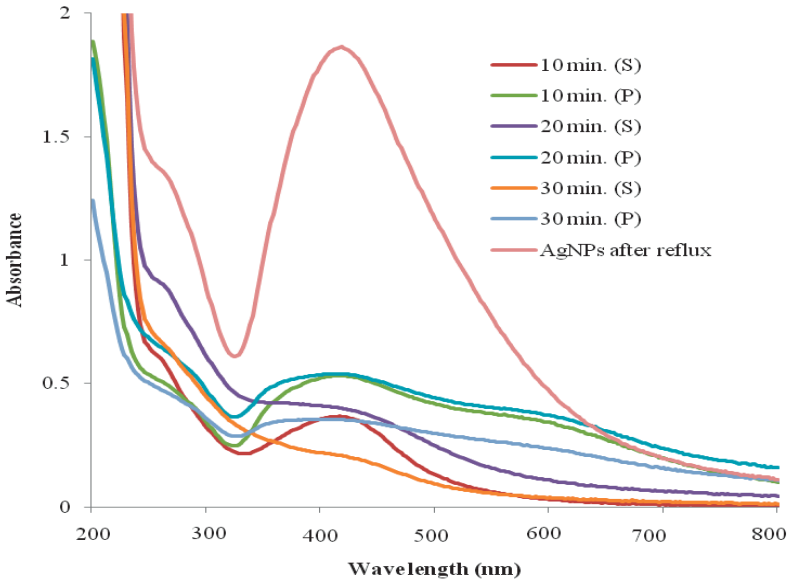

Fig. 3b). UV-Vis absorption spectrums of *D. malabarica* assisted Ag-NPs with different centrifugation times. Note: Here S represents the supernatant and P is for the pellet.

spectrum peaks of Ag-NPs suspensions centrifuged for 10, 20, and 30 minutes at 14000 rpm. All the peaks were broad and wide except the peak with 1-hour reflux without centrifugation which represented the existence of agglomerated nanobodies in the Ag-NPs suspensions. It is concluded that the centrifugation for a longer time may increase the particle size and agglomeration in suspensions Fig. 3b).

The UV-Visible spectrum used to examine the formation and stabilization of Ag-NPs that is due to the existence of metal SPR in Ag-NPs suspensions [47]. A sharp peak in the visible region (416 nm) of the spectrum was the confirmatory sign of Ag-NPs synthesis [48]. In addition, the region of 425-800 nm observed no SPR band indicating the absence of agglomeration in particles further explaining the stability of Ag-NPs. The results of the current study were supported by the previous reports that represented the 410-450 nm as the indication range of Ag-NPs synthesis and might be attributed to the spherical shape [49].

SEM and EDX Analyses

The SEM micrographs shown in Fig. 4 exhibited the morphology of both the ZnO and Ag-NPs. Fig. 4(d-f) shows the SEM images of ZnO-NPs at several magnifications (i.e. 50000,10000, 100000x). The images were recorded at different scales of 1 µm, 5 µm, and 500 nm. The images Fig. 4(d-f) confirmed the formation of spherical and rod-shaped ZnO-NPs having smooth surfaces. By a close look at images, it was clearly observable that some individual crystals were also present. The ZnO-NPs have low-level agglomerations in small clusters. The SEM images Fig. 4(g-i) represented that the Ag-NPs were well defined and predominantly spherical in shape with very few agglomerations. Both the ZnO and Ag-NPs were spherical in shape with smooth surface suitable for the antifungal potential against plant fungal pathogens [41, 50]. The SEM results were in accordance with the previous study [51].

The elemental analysis of the *D. malabarica* assisted ZnO and Ag-NPs was carried out by EDX analysis. The EDX characterization suggested that the ZnO nanopowder has good purity and it had high zinc content (53.26%) along with oxygen (28.2%) and carbon (13.68%), as shown in Fig. 5b). Sulphur (0.19%) and copper (4.67%) were also found in trace amounts, which pointed towards the involvement of phytochemical groups of the *D. malabarica* leaves extract as

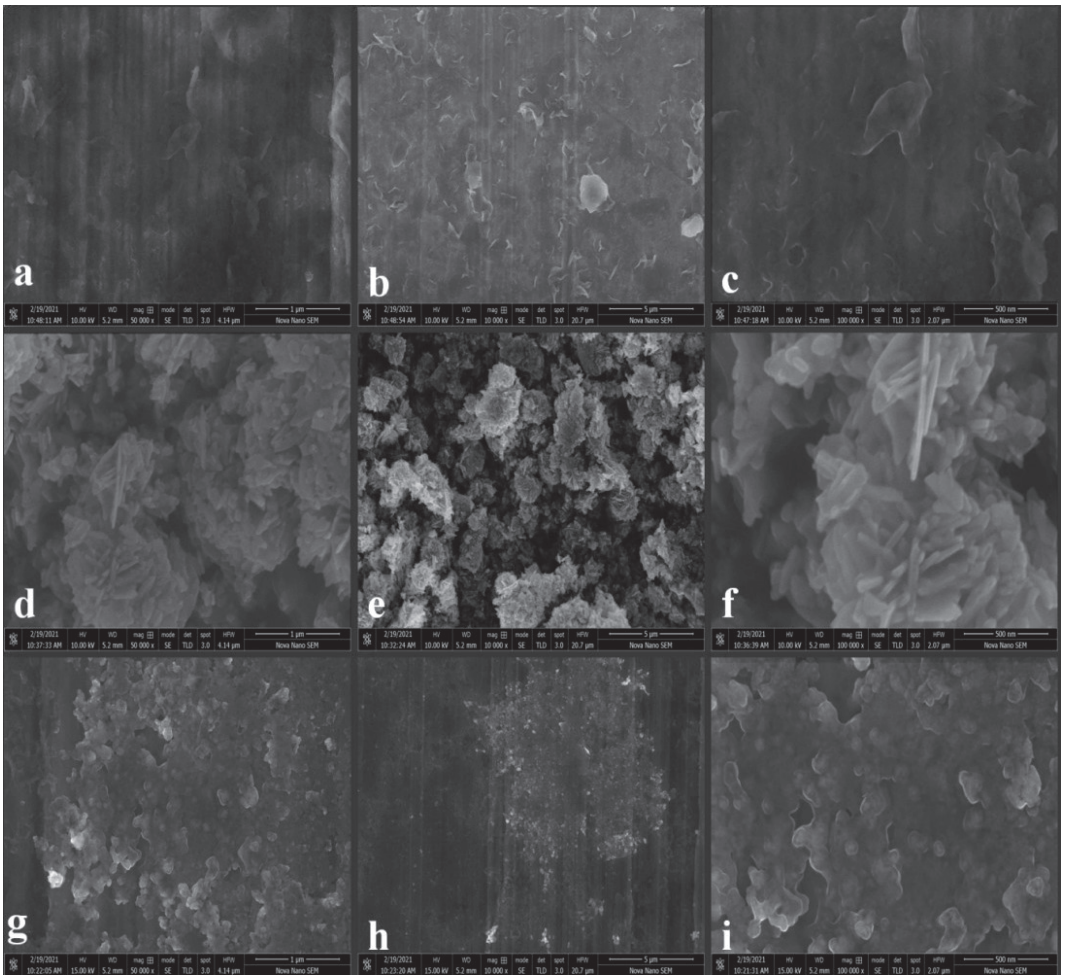

Fig. 4. SEM images of (a-c) *D. malabarica* extract (d-f) ZnO-NPs and (g-i) Ag-NPs at different magnifications.

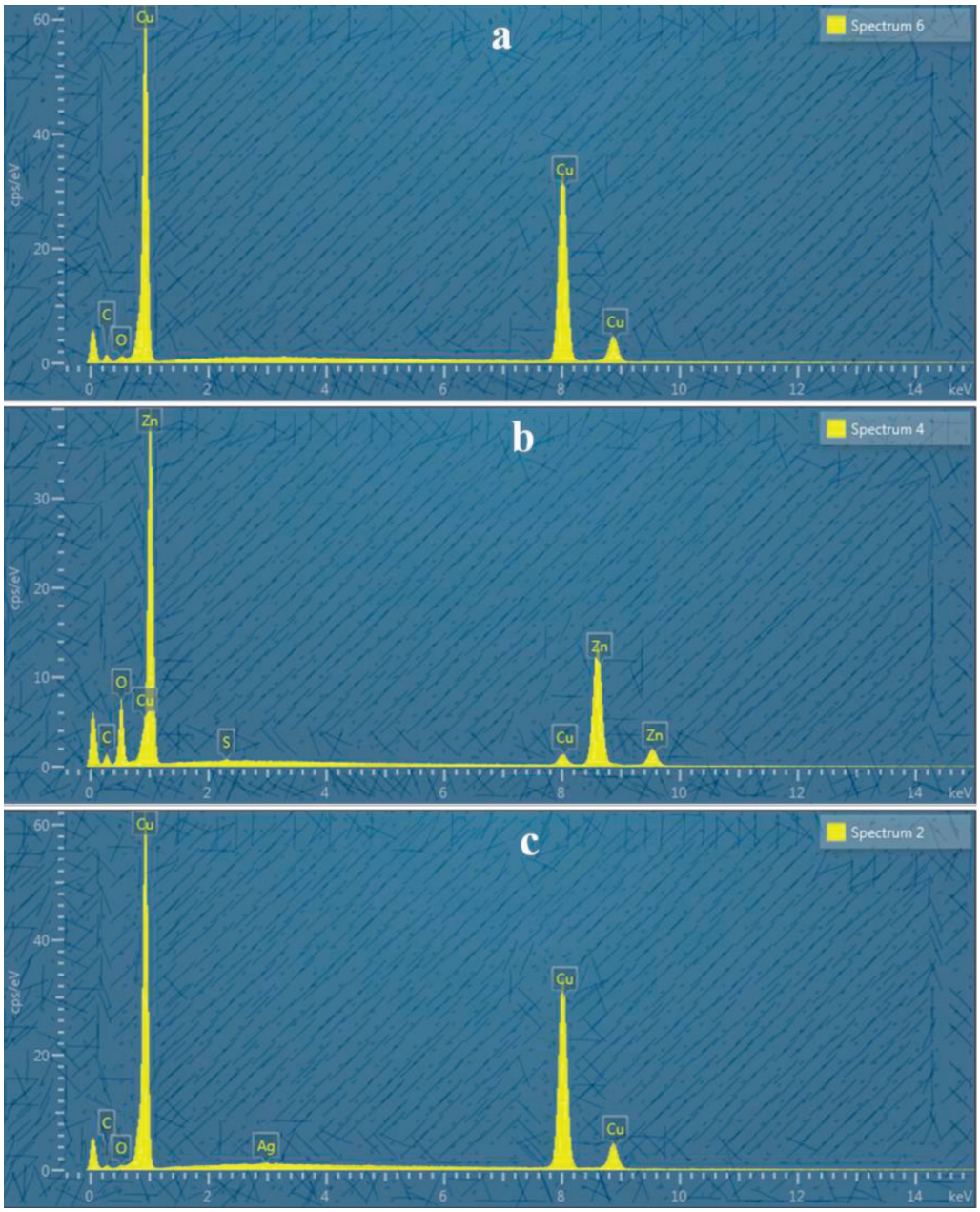

Fig. 5. Elemental composition analyses of (a) *D. malabarica* extract (b) ZnO-NPs and (c) Ag-NPs using EDX.

the reducing and capping agents in the synthesis of ZnO-NPs. A strong spectral signal in the silver region has been observed at 3 KeV with 0.55% Fig. 5c). This pronounced peak strongly supported the formation of Ag-NPs. The copper was present in high content identical to the EDX spectrum of extract. Although elemental carbon and oxygen were also observed that was originated from the phytometabolites present in *D. malabarica* leaves extract adjacent to the core of Ag-NPs as potential stabilizing agents.

These phytometabolites assisted as reducing and capping agents in the synthesis of Ag-NPs. The EDX spectrum leaves extract of *D. malabarica* has copper element along with oxygen and carbon content that stabilized and reduced both ZnO and Ag-NPs also acted as capping agents Fig. 5a). The results of the EDX spectrum were supported by the already reported work that specifies the position of the Zn peak at 8-10 keV [52]. The metallic Ag nano-crystals generally show a typical optical absorption peaks at 3 keV due to SPR [53-55]. Other minor peaks might be due to the presence of phytochemicals in *D. malabarica* leaves extract responsible for the stabilization of both the ZnO and Ag-NPs. This piece of information was in good agreement with the earlier report [13].

Antifungal Efficacy of *D. malabarica* Assisted Zinc Oxide and Silver Nanoparticles

It was observed from the mycelia growth (recorded after 24 hours till 96 hours) that ZnO-NPs showed 17.1, 31, 40, 48.7 and 51.1 % growth inhibition of *R. solani*

Table 1.Growth inhibitions (%) of *R. solani* using different concentrations of ZnO and Ag-NPs.

Sr. number	Concentrations (mg/mL)	*R. solani* growth Inhibition (%)	
		ZnO-NPs	Ag-NPs
1	0.05	17.1	28.8
2	0.15	31	38.3
3	0.25	40	49.3
4	0.35	48.7	50.2
5	0.45	51.1	61.8

at 0.05, 0.15, 0.25, 0.35 and 0.45 mg/mL respectively. The results showed the maximum growth inhibition (*R. solani*) at highest concentration of ZnO-NPs (0.45 mg/mL) as while; the lowest inhibition (%) was observed at lower concentration (0.05 mg/mL). Comparatively, Ag-NPs showed 28.8, 38.3, 49.3, 50.2 and 61.8 % growth inhibition of *R. solani* at 0.05, 0.15, 0.25, 0.35 and 0.45 mg/mL respectively. It was found that the maximum growth inhibition (61.8%) was observed at 0.45 mg/mL and the lowest inhibition (28.8%) was observed at 0.05 mg/mL.

Consequently, it is concluded that *R. solani* growth was inhibited by ZnO and Ag-NPs (0.05, 0.15, 0.25, 0.35 and 0.45 mg/mL) in a dose-dependent manner Fig. 6. Descending growth pattern was observed with an increase in the concentration of ZnO and Ag-NPs from 0.05 to 0.45 mg/mL (Table 1). The obtained results narrated that 0.45 mg/mL for both the ZnO and Ag-NPs has a better capacity to inhibit the growth of *R. solani*. Less than 50 % growth inhibition was observed at lower concentrations (0.05-0.35 mg/mL) for both the ZnO and Ag-NPs. Fungal growth inhibition was effective on selective plant pathogenic fungi (*R. solani)* and was more effective by *D. malabarica* assisted Ag than ZnO-NPs. Accordingly, it is considered by the results that *D. malabarica* assisted ZnO and Ag-NPs at higher doses are competent antifungal agents against ShB pathogen (*R. solani).*

The poison food technique using five different concentrations (mg/mL) of ZnO and Ag-NPs showed significant *in vitro* antifungal efficacy against *R. solani* (ShB pathogen). The larger surface area of the NPs had a major contribution to the penetration of NPs into the cell wall of the targeted pathogen and cause damage to the regular function of cells by losing their ability to replicate. In support of our findings, antifungal activities of several bio-based inorganic metal/metal oxides NPs including ZnO and Ag-NPs have been investigated previously [56-60]. These studies supported the greenly synthesized ZnO and Ag-NPs as antifungal agents that protected the plants from fungal pathogens in an eco-friendly, cheaper, and proficient manner. In a previous study, the use of ZnO and Ag-doped ZnO-NPs against *Fusarium* spp. and *Rosellinia necatrix* clearly showed that Ag-doped ZnO-NPs were more efficient than the ZnO-NPs alike our results [61].

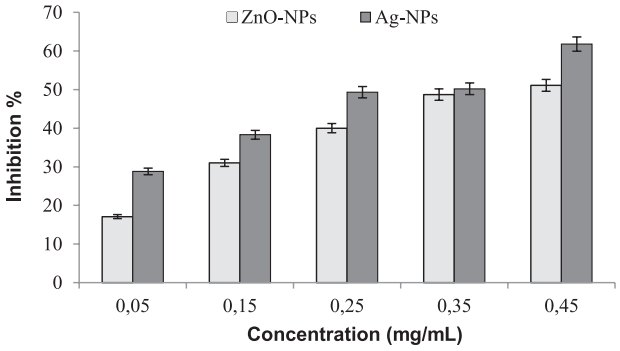

Fig. 6. Inhibition growth (%) of *R. solani* using different concentrations of ZnO and Ag-NPs, values are the mean of six replicates.

Again, it's important to reveal that the antifungal potential of ZnO-NPs was not as outstanding as that stimulated by Ag-NPs in the current study. In our study, *R. solani* was more sensitive to Ag-NPs (61.8%) as compared to ZnO-NPs (51.1%). In another study *in vitro* antifungal potential was investigated against *Colletotrichum gloeosporioides, Colletotrichum capsici, Curvularia lunata* and *Botrytis cinerea* using *G. applanatum* based Ag-NPs. It was concluded that Ag-NPs inhibitory action was increased by increasing the concentration [62]. The same trend was followed by our study in which both the ZnO and Ag-NPs were dose-dependent. This study established the concept of plant-assisted NPs and their use against plant fungal pathogens. Besides, this green synthesis may possibly be scaled up for industrial applications to maximize the yield of ZnO and Ag-NPs and further studies are required to use them against phytopathogens in control and field conditions.

Conclusions

ZnO and Ag-NPs were synthesized using *D. malabarica* leaves extract as reducing and stabilizing agents with no hazardous chemicals involved in the synthesis. Regarding biological application of both the ZnO and Ag-NPs, we found the significant antifungal

efficiency against *R. solani* beyond a 50% reduction in growth. However, *D. malabarica* assisted Ag-NPs showed maximum growth inhibition against *R. solani* as compared to ZnO-NPs at higher concentrations. This potential, compatible and eco-friendly plant-assisted synthesis can be encouraged as a trustworthy substitute for hazardous chemical techniques. Furthermore, future practices should be carried out to use such greenly synthesized bio-fungicides in field conditions to defeat the chemically synthetic fungicides.

Acknowledgments

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Declaration of Funding Statement

This research study has not received any funding.

Conflict of Interest

There is no conflict of interest among all authors.

Data Availability Statement

The whole data is available in this manuscript.

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