An In Vitro Study of The Antifungal Activity of Silver/Chitosan Nanoformulations Against Important Seed Borne Pathogens.

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Abstract- This research is reports the fungicidal properties of nano-size silver/chitosan nanoformulations (NFs) used as an agent for antifungal treatment of various seed borne plant pathogens. Fungal phytopathogens, especially seed borne disease causing species, Rhizoctonia solani, Aspergillus flavus, Alternaria alterneta were isolated form chickpea seeds. Differences were observed in the antifungal activity of the silver nanoparticle, chitosan nanoparticles and silver /chitosan nanocomposite, upon the mycelial growth and zone of inhibition of the fungi. Extent of fungal inhibition was derived using the above information, in order to evaluate the antifungal efficacy of these nanoformulations against pathogens. Tests for the fungal growth revealed that the NFs showed significant inhibition effectiveness. This study suggests the possibility to use silver/chitosan NFs as an alternative to fungicides for controlling seed borne phytopathogens.

Keywords: Antifungal, Chitosan, mycelium, nanoformulations, phytopathogens, seed borne, silver.

1 INTRODUCTION

Fungi are significant destroyers of foodstuffs and grains during storage, rendering them unfit for human consumption by retarding their nutritive value and often by producing mycotoxins [1-2]. A significant portion of the agricultural produce in the country and the world over becomes unfit for human consumption due to mycotoxin contaminations, especially those produced by species of Aspergillus [3]. More than 300 fungal metabolites are reported to be toxic to man and animals [4]. Rhizoctonia solani, one of the sclerotia forming microbe is widespread in the world and causes many important diseases in a wide host range of plants. For example, sheath blight caused by R. solani is one of the destructive diseases of rice (Oryza sativa L.), causing significant yield losses in all rice growing countries [5]. A close correlation between disease incidence and sclerotia density was demonstrated by Dillard and Grogan (1985), indicating that sclerotia are major factors for disease dissemination and initiation [6]. As primary survival structures of the pathogens, sclerotia exhibit longevity in soil and resistance to unfavorable abiotic factors such as heat, drought, and fungicide [7]. Also, Alternaria alternata can have a certain pathogenic effect; it has been recorded as a saprophytic or a weak pathogen causing so-called "indefinite or opportunistic disease" on a number of crops [8].

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Even though effective and efficient control of seed borne fungi of seeds can be achieved by the use of synthetic chemical fungicides, the same cannot be applied to grains for reasons of pesticide toxicity [9]. Thus, there is a need to search for alternative ecofriendly and less capital intensive approaches to store grains/cereals for human consumption without toxicity problems. As an alternative to chemically manufactured pesticides, use of silver nanoparticles as antimicrobial agents has become more common as technological advances have made their production more economical [10]. One of the potential applications of silver is in management of plant diseases. Silver displays multiple modes of inhibitory action against microorganisms [11]; therefore, it may be used with relative safety for control of various plant pathogens, compared to synthetic fungicides [12]. Numerous studies on antifungal activity of chitosan against plant pathogens have been carried out [13-15] and reviewed [16-17]. Chitosan's inhibition was observed on different development stages such as mycelial growth, sporulation, spore viability and germination, and the production of fungal virulence factors. It has been commonly recognized that antifungal activity of chitosan depends on its molecular weight, deacetylation degree, pH of chitosan solution and, of course, the target organism. Mechanisms proposed for the antifungal activity of chitosan focused mainly on its effect on fungal cell wall [18] and cell membrane [19]. Reports on the antimicrobial effects of silver/chitosan nanoformulations (NFs) are primarily focused on bacterial pathogens and to some extent on viral pathogens, however work on fungal pathogens is scarce and hence we decided to work on this aspect. Here we evaluated the antifungal activity of silver/chitosan NFs against various commercially important plant-pathogenic fungi in vitro.

2 MATERIALS AND METHODS

All chemical Chitosan, Tri-sodium citrate, Silver nitrate and Acetic acid were obtained from S.d fine-chem Limited, India. Amphotericin-B antibiotic was purchased from Hi-media, India.

2.1 Silver nanoparticles

Silver/chitosan NFs were prepared and characterized as in our previous work [20] according to Du et. al., (2004) [21] with some modification. We found all nanoparticles were 10-20

nm in size. All nanoformulations were used by dissolving in distilled water for experimental study.

2.2 Test Fungi

Seed samples (chickpea) were plated on potato dextrose agar, and subjected to Standard Blotter Method (SBM) to isolate the frequently occurring important seed-borne phytopathogenic fungi and storage fungi associated with these seeds. Eight species were isolated and among these, three species i.e. Aspergillus flavus, Rhizoctonia solani and Alterneria alterneta were maintained on PDA medium and incubated for 7 days at 25^{o}C, which served as the test fungi for antifungal activity assay.

2.3 Assessment of antifungal assay

2.3.1 Agar well diffusion method

Nanoformulations of different nanoparticles were screened for antifungal activity by agar well diffusion method with sterile cork borer of size 6.0mm according to Bobbaralal et. al., (2009) [22]. 72 hours old cultures grown on potato dextrose agar (PDA) were used for inoculation of fungal strain on PDA plates. An aliquot (0.02 ml) of inoculums was introduced to molten PDA and poured in to a petri dish by pour plate technique. After solidification, the appropriate wells were made on agar plate by using cork borer. In agar well diffusion method 500 µl of Chitosan/metal nanocomposites solution, homogenized using an ultrasonic cleaner, filled in deep blocks. Incubation period of 48-72 hours at 25^{o}C was maintained for observation of antifungal activity. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the plant extracts. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicates.

2.3.2 Mycelium Growth Inhibition

In vitro assay was performed on growth medium treated with 100µg/ml concentrations of all nanoformulations. One ml of stock solutions containing the different NFs was poured into the growth media prior to plating in a petri dish (90 × 15 mm). Media containing NFs was incubated at room temperature. After 48 hr of incubation, agar plugs of uniform size (diameter, 8 mm) containing fungi were simultaneously inoculated at the centre of each petri dish containing the various NFs, followed by incubation at 25 ± 2^{o}C for 14 days. After incubation of fungi on culture medium containing NFs, radial growth of fungal mycelium was recorded. Radial inhibition was calculated when growth of mycelia in the control plate reached the edge of the petri dish. The toxicity of the extracts to growth of fungi, in terms of percentage inhibition of mycelial growth was calculated by using the formula % inhibition = dc – dt/dc x 100
Where dc = Average increase in mycelial growth in control, dt = Average increase in mycelial growth in treatment [23]. Synthetic fungicides i.e. Amphotericin-B were also tested at their recommended dosage (20µg/100 ml) for antifungal activity.

2.3.3 Statistical calculations

For statistical analysis, XLSTATE ver. 2012. 2.4. Software used and all tests were performed in triplicate, and the results were expressed as the mean ± the standard errors of the mean. P values lower than 0.05 were considered significant.

3 Results and Discussion

3.1 Silver/Chitosan NFs used

Transmission electron microscopy (TEM) was used to investigate the surface morphology of Ag/Ch NFs. Micrograph in fig. 1 shows that nanoparticles have colloidal shapes with an average size of 10-20 nm. There is no agglomeration of nanoparticles (may be due to presence of the chitosan as capping agent), and the surface was somewhat rough. It is noteworthy that the particles are uniformly embedded in chitosan matrix.

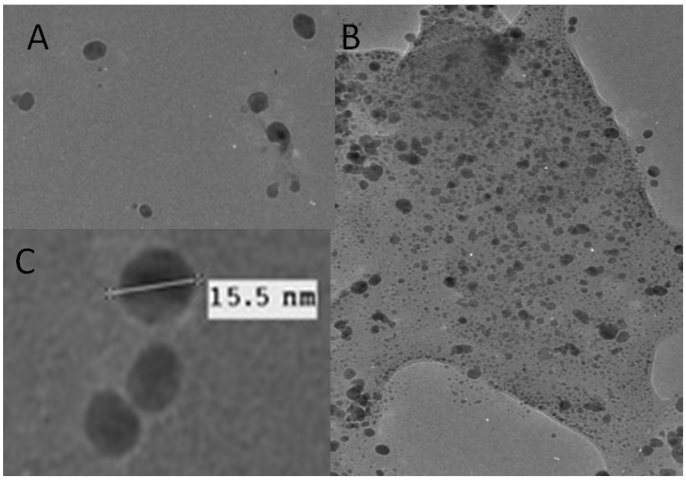

Fig. 1.a) Ag NPs in solution, b and c) AgNPs embedded in chitosan matrix and their Size

3.2 Assessment of Antifungal assay

3.2.1 Agar well diffusion method

All fungi tested were inhibited to various extents by different nanoformulations (Fig. 2, Table 1). Ag/Ch NFs showed higher antifungal activity than silver and chitosan nanoparticles (Fig. 2). Ag/Ch exhibited highest inhibition against Aspergillus followed by Alternaria and Rhizoctonia species, as shown by the zone of inhibition 19.66 ± 0.28, 16.33 ± 0.29, 12.66 ± 0.76 against Aspergillus, Alternaria and Rhizoctonia, respectively. This was nearly equal to the test antifungal drug, i.e. Amphotericin –B.

3.2.2 Mycelium Growth inhibition

In all cases, Ag/Ch exhibited higher inhibition of growth. In addition, most fungi showed incremental growth inhibition along with the increment of incubation time, and the extent of inhibition was identical to the test antifungal drug. Ag/Ch shows inhibition 94%, 67% and 78% against Aspergillus sp., Rhizoctonia sp., and Alternaria sp. respectively. Nearly identical inhibitory results were observed in all three strains by both methods tested.

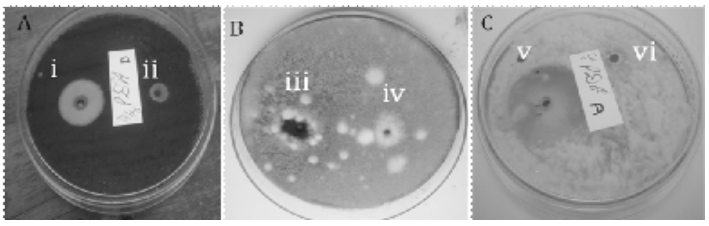

Fig. 2. Zone of inhibition of AgNPs (ii, iv, vi) and Ag/Ch NFs (i, iii, v) against a) A. alterneta, b) R. solani and c) A. flavus.

TABLE 1.
ANTIFUNGAL ACTIVITY OF NANOFORMULATIONS

Sample	Zone of inhibition (mm)		
	A. flavus	A. alterneta	R. solani
Control	0 ± 0	0 ± 0	0 ± 0
Chitosan	10.66 ± 0.76	10 ± 1.73	9.8 ± 1.76
AgNPs	10 ± 1	8.3 ± 0.28	8.16 ±0.76
Ag/Ch	19.66 ± 0.28	16.33 ± 0.29	12.66 ± 0.76
Amphotericine -B	19.5 ± 0.5	19.33 ± 1.52	19.16 ± 1.04

Data given are mean of three replicates ± Standard error

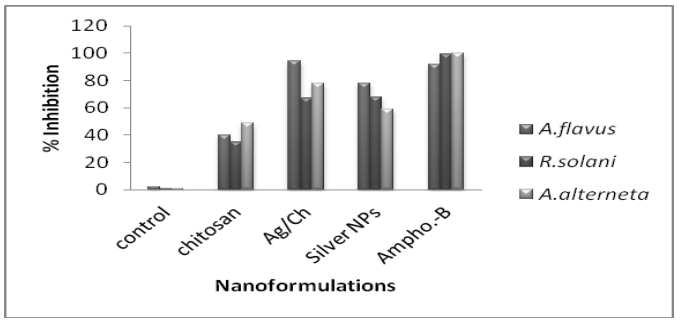

Fig. 3. Percent inhibition of nanoformulations against various fungal strains.

4 CONCLUSION

Management of fungal diseases of food crops is economically important. Recently, a greater effort has been given to development of safe management methods that pose less danger to humans and animals, with a focus on overcoming deficiencies of synthetic fungicides. In the present work it was demonstrated that, Ag/chitosan nanoformulation has significant antifungal activity against the fungi tested, viz. A. flavus, A. alterneta and R. solani, and this was much higher than silver or chitosan nanoparticles used independently. Thus, it can be effectively used against plant phytopathogenic fungi to protect the various crop plants and their products, instead of using the commercially available synthetic fungicides, which show higher toxicity to humans. Moreover, this report opens up for further research, the area of mode of action of silver-chitosan composites on the phytopathogenic fungi.

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