Myco-synthesized silver nanoparticles from *Curvularia affinis* showing inhibitory activity against phyto-pathogenic fungus *Alternaria solani*

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The present study deals with antifungal activity of silver nanoparticles synthesized from phytopathogenic fungus *Curvularia affinis* Boedijn. The fungus is the causal organism of foliar spot disease of several economically important crops. Scanning Electron Microscopy showed that the silver nanoparticles were mostly spherical in shape and less than 10-15nm in size. The effect of these nanoparticles on the first, second and third order branching of *Alternaria solani* (Ell. Martin), causing early blight of tomato was seen. Only 0.5% (v/v) of nanoparticles could alter the normal growth and branching pattern of *Alternaria solani* hyphae as well as having a potent inhibitory effect on their growth. This inhibitory effect was directly proportional to nanoparticle concentration. The inhibitory effect was tested on different media compositions. These nanoparticles were successfully used as nano-fungicide, as it had the potential to inhibit the growth of the test phytopathogenic fungi *A. solani*.

Key words: *Curvularia affinis*, silver nanoparticles, antifungal activity, phytopathogenic fungi, hyphal branching, *Alternaria solani*

INTRODUCTION

Nanotechnology is becoming an active field of research with applications in various fields of science especially in biological science. Now-a-days, nanotechnology is a diverse field involving the fabrication and exploitation of materials at a nanoscale. Biological materials are often preferred due to their eco-friendly, safe, non-toxic and cost effective approach requiring less complicated set-ups. Several biological systems have been used to produce nanoparticles both intracellularly and extracellularly (Boisselier and Astruc, 2009).

Fungi are often preferred for the synthesis of metal nanoparticles over bacteria and other micro-organisms due to their high tolerance capacity towards metals of versatile range of enzymes (Bhargava *et al.* 2016; Jain *et al.* 2011). Nanoparticles are gradually being used in biomedical field in diagnosis and treatment of life threatening diseases like cancer by the virtue of their surface plasmon resonance properties (Yamada *et al*. 2015).

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Biosynthesized gold and silver nanoparticles from *Stenotrophomonas* sp. were successfully used against soil-borne phytopathogenic fungus of chickpea, *Sclerotium rolfsii* (Mishra *et al*. 2017). Gold nanoparticles from fungi EDMnd21 and EDMnd22 showed altered branching pattern of rice sheath blight fungus *Rhizoctonia solani* (Das and Kundu, 2017).

Alternaria solani, a necrotrphic fungi causing early blight of tomato is a dreadful pathogen. Annual yield loss of tomato due to early blight pathogen has been estimated as 79% (Adhikari *et al.* 2017). Visible symptoms including necrotophic cell death occur within 96 hrs of infection (Ray *et al*. 2015). Use of high amount (20%) of chemical fungicides for multiple times before and after infection (2-4 days) is one of the most widely used methods for prevention of spread of early blight in the field and as protection strategy to inhibit the disease progression in already infected plants (Horse field *et al*. 2010). However due to the toxic side effects of fungicidal residues to human and animal health (Axelstad *et al.* 2011), ways to reduce fungicide use is to be considered.

Nanotechnology can offer green and eco-friendly alternatives for plant disease management,

without using hazardous chemicals for controlling pathogens (Aghuthymia *et al.* 2015). Biosynthesized silver nanoparticles (AgNP) against foliar phytopathogenic fungi *Alternaria alternata*, *Curvularia lunata* and *Bipolaris sorokiniana* and soil borne fungal pathogen *Sclerotium rolfsii* started showing its antifungal efficacy only at 2 μg/ ml concentration and complete inhibition occurred at 10 μg/ ml nanoparticles concentration (Mishra *et al*. 2017). 2.5 ppm of biologically synthesized copper nanoparticles from *Streptomyces griseus* were used against *Poria hypolateritia* infested tea plants, and this concentration not only exhibited total control of the pathogen but also improved the soil for better plantation (Ponmurugan *et al*. 2016).

In the present study we wanted to investigate the effect of nanoparticles synthesized from *C. affinis* on the pathogen *A. solani*. The study is aimed at exploring the possibility of utilization of these nanoparticles as nano-fungicide to control early blight pathogen.

MATERIALS AND METHODS

Culture and maintenance of fungus

The hypomycetes plant pathogenic fungus *Curvularia affinis* Boedijn, ITCC (PP /2883) was used in biosynthesis of silver nanoparticles. Fungal mycelia of *Curvularia affinis* was first cultured on potato dextrose agar (PDA) medium since the growth is much faster in PDA than other commonly used fungal growth media (Halder *et al*. 2017). For production of mycelial mats, the mycelium from solid substrate was inoculated in 25 ml of potato dextrose broth (PDB) in 250 ml flasks and grown for seven days in incubators set at 25^{O}C. This mycelial mat was used for biosynthesis of silver nanoparticles according to the published protocol of Chowdhury *et al.* (2014) and Ray *et a*l. (2011). For long term storage, mycelia can be kept in 4^{O}C on PDA and sub-cultured at three months intervals.

Alternaria solani (Ell. Martin), ITCC No. 4632 was used as fungal sample, for treatment with AgNP to study the growth and branching pattern.

Field-Emission Scanning electron microscopy (FE-SEM)

To determine the size and shape of nanoparticles, 5 μl of nanoparticle suspension was spread as thin layer on glass stub (1cm X 1cm) and was vacuum dried then observed under Field emission scanning electron microscope, JEOL JSM-7600F field emission scanning electron microscope with 1kV-15kV electric voltage at 1-1.5nm resolution.

Experimental setup for assaying effect of C. affinis AgNP on growth and branching of Alternaria solani hyphae

Potato dextrose agar (PDA, HiMediaM403), oatmeal agar (OMA, HiMedia RM2565) and malt extract agar (MEA, HiMedia M255) were used for the preparation of experimental setup. 2% agar was added to solidify the media. These media were mixed with 0.5 and 1.0% of silver nanoparticles and were poured in thin layers on grease free slides, and sets were done in triplicate. This experimental setup was kept in 28°C temperature after inoculation with 1 mm mycelial disc of *A. solani*. Slides were observed under compound microscope (Leica DMLS, 20x-100x) for showing the hyphal branching pattern in control and treated slides after 72 hrs of incubation.

RESULTS AND DISCUSSION

Optimization of culture conditions for Curvularia affinis for production of silver nanoparticles: morphology and stages of growth

On PDA media initially the fungal growth was as white mycelia but with maturation it turned grey in colour. The entire plate was covered within 10-12 days. Cottony aerial mycelia were found (Fig. 1A, B).Microscopic study of the fungus showed melanised, septate, cylindrical mycelia (Fig. 1C); sporulation occurred after 96hrs on PDA media. Spores were melanised, 4-celled with the middle cells being larger in size. Average size of spores was 20-30ìm in length and 5-7ìm in width (Fig. 1D). On PDA media the vegetative growthwas more than reproductive growth. Sporulation of *C. affinis*on PDA media started after 96 hrs of inoculation and turned grey in colourwhile on OMA sporulation was faster than MEA and PDA (Halder *et al*. 2017). Mycelial matwas harvested from 15 days old PDB culture.

Field-Emission Scanning electron microscopy (FE-SEM)

FE-SEM image of *C. affinis* AgNp showed the sil-

ver nanoparticles were spherical or polygonal in shape. Particles were well dispersed within the size range of 10-15nm and majority of the particles appeared as oval to circular in shape under scanning electron microscopy (Fig.2).

Small sized (10 nm) nanoparticles are more toxic on microbes than larger ones (60-80nm), Small particles easily enter into cells through the cell wall and inhibits the growth of bacteria and fungi which the large particles are unable to do(Ivask *et al*. 2014).

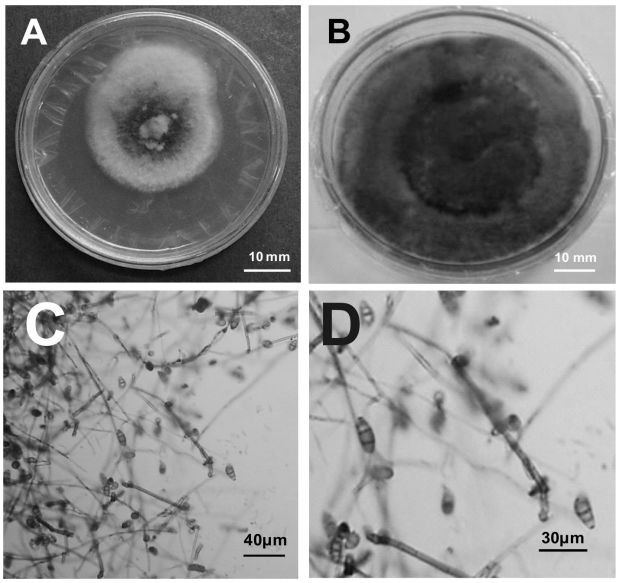

Fig.1: Optimization of culture conditions for *Curvularia affinis* for production of silver nanoparticles (A) Morphoogy of 4 days old plate with white cottony mycelia. (B) Full plate grown 10 days old culture with mature mycelia and spores (C) Microscopic image of *C. affinis* mycelia and spores. (D) Spores of *C. affinis*

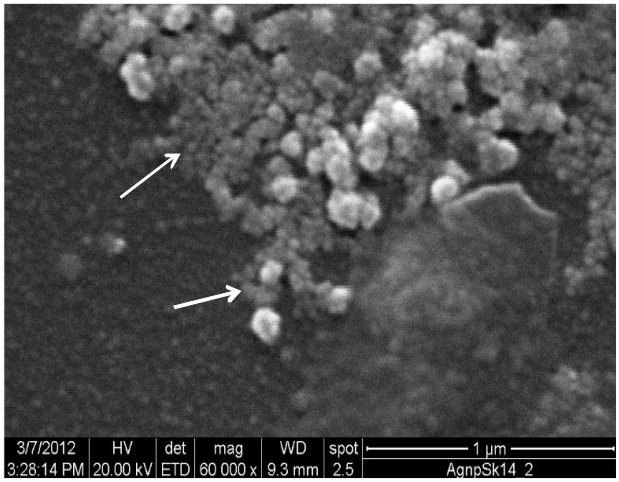

Fig.2: Scanning electron microscopic (SEM) image of silver nanoparticles

Effect of C. affinis AgNP on mycelial branching

The phytopathogenic fungus *Alternaria solani*, the causal organism of early blight disease of tomato, was selected as the test fungus for assaying antifungal activity of the nanoparticles. *A. solani* cultures on different media, were treated with varying concentrations of nanoparticles as described in the methods. In control setup all the three stages of branching i.e. 1st order, 2nd order and 3rd order were found whereas in nanoparticle treated setup the hyphal growth was stunted. In 0.5% nanoparticle concentration the number of all the types of branches were reduced whereas in 1% AgNP concentration 3rd order branches were absent in all media compositions. In case of PDA the branching number was higher than the other two media. (Fig. 3). In an earlier study from this laboratory,

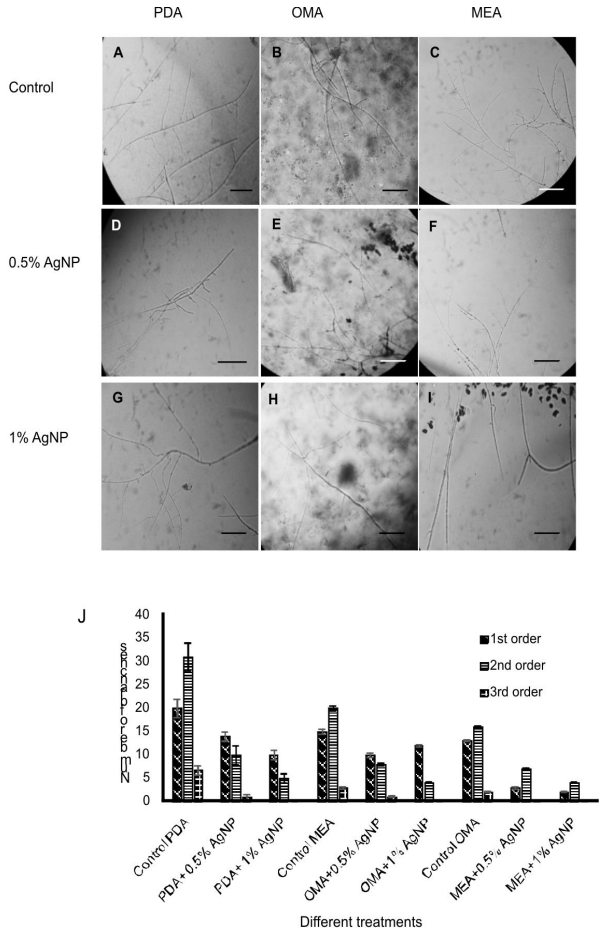

Fig. 3: Branching pattern of *Alternaria solani* hyphae on PDA, OMA and MEA. (A,B and C) Control. (D, E and F) Treated with 0.5% AgNP. (G, H and I) Treated with 1% AgNP (Bar=50ìm). Graphical representation shows number of different types branching on different media at 72 hrs post incubation. (J) Graph showed the Hyphal alteration after treatment with 0.5 and 1% AgNP

inhibition of fungal growth by silver nanoparticles directly correlated with the concentration of nanoparticles was found (Ray *et al*. 2011),The present study confirms to the previous finding. Antifungal activity of nanoparticles depends on the size of nanoparticles, as small sized particles are able to penetrate fungal cell wall they inhibit the growth of pathogenic fungi (Das *et al*. 2017).

Nanoparticles possess antifungal properties and had been used as antifungal agent from past decades against pathogenic fungus (Ray *et al*. 2011). Nanoparticles were reported to decrease the fungal toxicity (Ammar *et al*. 2016). They can inhibit the germination of sclerotia to inhibit the disease progression (Mishra *et al*. 2017). However, the effect of mycosynthesized silver nanoaprticles on branching pattern of fungi has not been reported so far. Silver nanoparticles from mycorrhizal mushroom *Tricholoma crassum* (BERK.) SACC. showed antimicrobial activity against wild and multidrug resistant human bacteria *E. coli* and phytopathogenic bacteria *Agrobacterium tumefaciens.* These nanoparticles were effective against the phytopathogenic fungus *Magnaporthe oryzae* that cause blast disease in rice (Ray *et al*. 2011). Chowdhury *et al*. (2014) reported antimicrobial activity of silver nanoparticle synthesized from phytopathogenic fungus *Macrophomina phaseolina* (Tassi) Goid) showing antibacterial activity against multidrug resistant strain of *E. coli, A. tumefaciens*.The activity of nanoparticles increased with increasing concentration and time point also.

Our mycosynthesized nanoparticles successfully inhibited the growth of phytopathogenic fungus, *A.* solani. Detail studies are required on the mode of action of nanopticles for inhibition of the fungus

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