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Research Article

Green Synthesis of Silver Nanoparticle by *Acalypha indica* and its Antifungal Effect against Phytopathogen *Colletotrichum capsici*

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Abstract

In the present investigation green synthesis of silver nanoparticles was carried out using aqueous leaf extract of *Acalypha indica*. The formation of nanosilver was confirmed by UV-Vis Spectroscopy. The absorbance peak at 450nm indicated the presence of silver nanoparticles in the solution. The antifungal property of silver nanoparticles was evaluated against pathogenic fungus *Colletotrichum capsici* both *in vitro* and in field inoculation experiment. Application of silver nanoparticles produced significant inhibition of growth of fungal mycelia as well as conidial germination *in vitro*. It has been observed that about 54% and 70% reduction in mycelia growth took place at 100 ppm and 200 ppm concentrations of silver nanoparticles respectively. Results of spore germination test revealed about 97% reduction in germination at 10 ppm concentration of silver nanoparticles. In the field trial experiment significant reduction (46%) in anthracnose disease of bean caused by *C. capsici* was observed when the plants were treated with 200 ppm of nanosilver prior to inoculation. Results of the present study highlighted the eco-friendly approach of plant mediated synthesis of nanosilver and its potential application in the field as an alternative to chemical fungicides for disease management.

Keywords: *Acalypha indica*; Silver Nanoparticle; Bean Anthracnose; *Colletotrichum capsici*; Antifungal Property

Introduction

Nanotechnology is one of the most promising area of modern scientific research which deals with synthesis and applications of particles ranging from approximately 1 to 100 nm in size. Novel applications of nano particles and nano materials are growing rapidly in various fronts due to their completely new or enhanced properties based on size, distribution and morphology [1]. Among all the noble metal nano particles, silver nanoparticles have gained interests because of their unique properties such as chemical stability, good conductivity, catalytic and most important antibacterial, antiviral, antifungal and anti-inflammatory activities [2]. The wide applications of silver nano particles has opened up another arena of research interest that is green nanotechnology or biosynthesis of nanoparticles utilizing algae, actinomycetes, bacteria, fungi and plants. Among the various biological synthesis procedures plant mediated synthesis of silver nanoparticles has attracted more attention nowadays. Synthesis of silver nanoparticles has been investigated utilizing many plant extracts like Marigold flowers [3], leaf extract of *Acalypha indica* [4], beet root [5], leaf extract of *Azadirachta indica* [6], plant extract of *Eclipta prostrata* [7], leaf extract of *Abutilon indicum* [8], tissue culture derived callus and leaf extract of *Sesuvium portulacastrum* [9], leaf extract of *Vitex negundo* [10], leaf extract of *Ocimum sanctum* [11], leaf extract of *Centella asiatica* [12], plant extract of *Aloe vera* [13], peel extract of *Citrus sinensis* [14].

Silver is a well-known antimicrobial agent against a wide range of bacteria, fungi and viruses. More recently nanosilver has also been found to be a potent antibacterial and antifungal agent [15-17]. However the full potential of nanosilver is still unexplored for crop protection and presently there has been growing interest to utilize their antimicrobial property for plant disease management. Efficacy of silver nanoparticles was evaluated against powdery mildew under different cultivation conditions *in vitro* and *in vivo*. Silver nanoparticles at various concentrations were applied before and after disease outbreak in plants to determine antifungal activities. In the field tests, the application of 100 ppm silver nanoparticles showed the highest inhibition rate for both before and after the outbreak of disease on cucumbers and pumpkins [18]. The antifungal activity of silver nanoparticle on the phytopathogen *Colletotrichum gloeosporioides* which causes anthracnose in a wide range of fruits was studied. The growth of *Colletotrichum gloeosporioides* was found to be delayed in presence of silver nanoparticles in a dose dependent manner [19].

Acalypha indica Linn belonging to the family Euphorbiaceae is an annual herbaceous plant seen in many parts of Asia including India, Pakistan, Yemen, Sri Lanka and throughout Tropical Africa and South America [20]. The root, stem and leaf of *A. indica* possess herbal activity [21].

In the present study extracellular production of silver nanoparticles was carried out by leaf extract of *A. indica*. The anti-fungal activity of silver nanoparticles against *Colletotrichum capsici* was studied both *in vitro* and in planta inoculation experiment in the field.

Materials and Methods

Synthesis of silver nanoparticles using aqueous extract of leaf of *A. indica*

Preparation of plant extract and synthesis of silver nanoparticles were carried out following the method described earlier [22]. *A. indica* plants were collected from the experimental garden of Scottish Church College, Kolkata. The leaves of *A. indica* were washed thoroughly under running tap water and then with distilled water. The plant material was then air dried and was powdered with the help of mortar and pestle. About 10 gm of powdered sample was then mixed with 100 ml of double sterilized distilled water in Erlenmeyer flask and the mixture was boiled for 5 minutes. The extract was then cooled and filtered through Whatman no 1 filter paper. The resultant filtrate was refrigerated at 4°C for future use (Figure 1). Ten ml of the plant extract was added with 90 ml of 1 mM $AgNO_3$ solution and it was incubated in the dark at 37°C in static condition until a visible colour change was observed. A similar control set up was prepared with 10 ml of double distilled water in 90 ml of $AgNO_3$ (1 mM) solution.

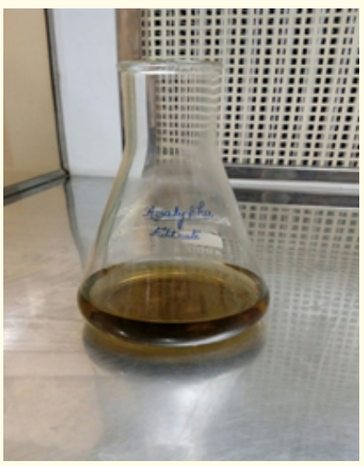

Figure 1: Leaf extract of *A. indica.*

UV-Visible spectroscopy study

The bio synthesis of silver nano particles by the aqueous extract of *A. indica* was monitored by measuring the absorbance at the wavelength 300 - 800 nm using UV-Visible spectrophotometer (ELICO UV-VIS SL 159).

Antifungal Assay

In vitro antifungal assay was performed following the method as described earlier with slight modification [23]. Potato Dextrose Agar medium was prepared. Different concentrations of silver nano particles were prepared like 1 ppm, 5 ppm, 10 ppm, 20 ppm, 50 ppm, 100 ppm and 1 ml of each ppm was added separately in autoclaved Petridishes. Molten hot PDA medium was then poured in the Petridishes and the sets were allowed to solidify. A control set was also prepared containing only PDA medium. The sets were then inoculated with agar discs (4 mm) of *C. capsici* separately and were incubated for 7 days at 30 ± 2°C. After 7days the radial growth of fungal mycelia was noted. The following formula was used for calculation of the inhibition rate (%).

$$\text{Inhibition Rate (\%)} = \frac{R - r}{R}$$

Where R is the radial growth of fungal mycelia on the control plate and r is the radial growth of fungal mycelia on the plate treated with silver nanoparticles.

Spore germination test

On 3 slides 20 µl of different concentrations of silver nanoparticles (0.01 ppm, 0.1 ppm, 1 ppm, 2 ppm, 5 ppm, 10 ppm, 20 ppm) were given separately and a control set was prepared with 20 µl of sterile distilled water. To all the sets including the control 20 µl of spore suspension of *C. capsici* was added separately and four replicas were prepared of each concentration including control. The slides were then incubated for 24 hours. After 24 hours, the slides were stained with cotton blue and mounted with cover slips to observe under low and high power of compound microscope. Percentage of spore germination was calculated and germ tube lengths were noted.

Pathogenicity test

The efficacy silver nanoparticles in reducing anthracnose disease of bean was examined in planta inoculation experiment. The seeds of bean (*Phaseolus vulgaris*) were potted in the earthenware pots containing soil and were allowed to grow. The leaves of 4 week- old- plants were sprayed with 200 ppm concentration of silver nanoparticles. After 48 hours both the treated and untreated control plants were inoculated with the spore suspension of *C. capsici* mixed with tween 20 and then covered with plastic bag. After 2 days the plants were uncovered and after 7 days disease symptoms were noted. Disease symptoms were noted on 7^{th}, 10^{th}, 14^{th} and 21^{th} day after inoculation and disease index was calculated.

Result and Discussion

Formation of silver nanoparticles by the aqueous extract of *A. indica* and its characterization

The reduction of silver and formation of silver nanoparticles using plant extract of *A. indica* were manifested by change in colour of the solution to yellowish to reddish brown within 24 h (Figure 2). Whereas the control set ($AgNO_3$ in double distilled water) showed no colour formation when incubated under the same condition indicating no formation of silver nanoparticles. The for¬mation of reduced silver nanoparticles in reaction mixture was further characterized by UV-Vis spectrophotometry when scanned in the wavelength range of 300 - 800 nm. Generally silver nanoparticles absorb light in the visible re¬gion of the electromagnetic spectrum at 380 - 450 nm. This is due to the surface plasmon resonance (SPR) transition [24]. The maximum absorbance peak was noted at 450 nm which confirmed the presence of silver nanoparticles

in the solution (Figure 3). Synthesis and characterization of silver nanoparticles using leaf extract of *A. indica* was carried out by earlier workers [4,17,24,25].

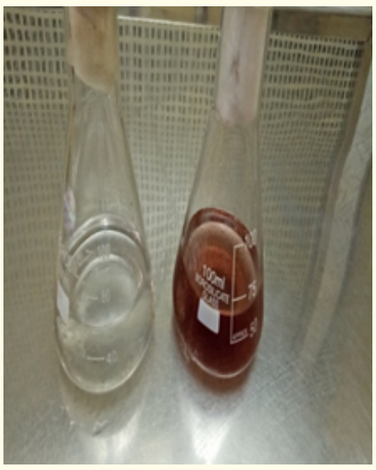

Figure 2: Biosynthesis of silver nanoparticles by extract of *A. indica*

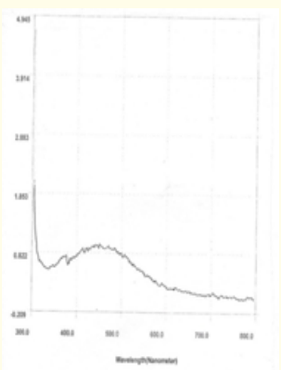

Figure 3: UV –Vis spectroscopy showing peak at 450 nm.

Effect of silver nanoparticles on mycelial growth and spore germination of *C. capsici*

After 7 days of incubation radial mycelia growth of *C. capsici* on PDA medium was noted. It has been observed that about 54% and 70% reduction in mycelia growth took place at 100 ppm and 200 ppm concentrations of silver nanoparticles respectively (Table 1, Figure 4). Results of spore germination test revealed about 97% reduction in germination at 10 ppm concentration of silver nanoparticles (Table 2). However there is no correlation between germ tube length and reduction in spore germination. Similar result was obtained by earlier worker [23] where 18 different plant pathogenic fungi were treated with silver nanoparticles on PDA, Malt Extract Agar and Corn Meal Agar plate. The result indicated silver nanoparticle possesses antifungal properties against pathogens. Most significant inhibition was observed on PDA at 100 ppm concentration of silver nanoparticles. Antifungal activity of silver nano particles synthesized by *A. indica* leaf extract against food borne pathogens *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus flavus* was also reported earlier [24]. Synthesis of silver nanoparticles using turnip leaf extract and its antifungal activity against wood-degrading fungal pathogens such as *Gloeophyllum abietinum, G. trabeum, Chaetomium globosum,* and *Phanerochaete sordida* was studied earlier [25].

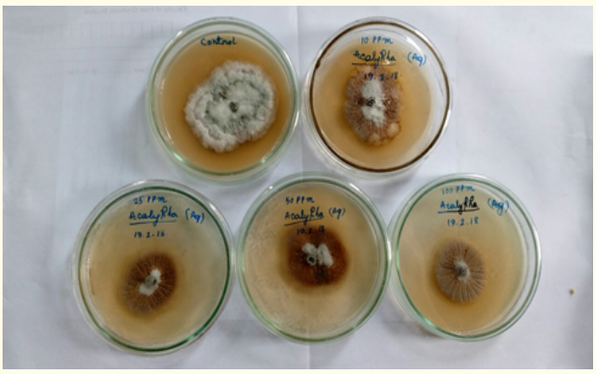

Figure 4: Antifungal effect of silver nano particles synthesised by *A. indica* against *C. capsici.*

*Average of 3 replica

Sl. No.	Treatment	Radial mycelial growth (mm)	% of inhibition
1	Control	7.12 ± 0.03*	0
2	1 ppm*	6.73 ± 0.12	5.47
3	5 ppm	6.20 ± 0.04	12.92
4	10 ppm	6.0 ± 0.02	15.73
5	20 ppm	5.6 ± 0.07	21.34
6	50 ppm	4.9 ± 0.11	31.17
7	100 ppm	3.25 ± 0.14	54.35
8	200 ppm	2.12 ± 0.07	69.8

Table 1

* Concentration of silver nanoparticles; ** Average of 4 replica

Sl. No.	Treatment	Average** of % of spore germination	% of inhibition	Range of germ tube length (µm)
1	Control	85.10 ± 1.24	0	31 - 93
2	0.01 ppm*	75.53 ± 1.49	11.24	57 - 95
3	0.1 ppm	58.88 ± 2.67	30.81	48 - 100
4	1 ppm	48.43 ± 1.91	43.90	35 - 71
5	2 ppm	16.13 ± 0.88	81.04	33 - 78
6	5 ppm	10.27 ± 0.94	87.93	20 - 59
7	10 ppm	2.12 ± 0.29	97.50	25 - 53

Table 2

Effect of silver nano particles against anthracnose of bean in the field.

In the field experiment disease symptoms were noted after 7 days of inoculation. Anthracnose symptoms appeared on leaves as small dark brown spots surrounded by yellow halo. Small lesions coalesce to form irregular lesions and veinal necrosis. Leaves of

treated plants showed less incidence of disease than that of untreated plants. The lesions were graded into three size groups on the basis of visual observation via small, medium and large. Values of 0.5, 1 and 2 were given to the spots respectively to calculate the disease index (Figure 5 and 6). About 15%, 27%, 46% and 41% reduction in disease incidence was noted in treated bean plants in comparison to control after 7, 10, 14 and 21 days of inoculation. The effect of silver nano particles against pepper anthracnose under different culture condition was evaluated earlier. The application of 100ppm conc. of silver nanoparticles produced maximum inhibition of the growth of fungal hyphae as well as conidial germination in comparison to control *in vitro*. In field trials the inhibition of fungi was significantly high when nano silver was applied before disease outbreak on the plants [26]. Various forms of silver ions and nano particles were tested to examine the antifungal activity on two pathogenic fungi *Bipolaris sorokiana* and *Magnaporthe grisea* [27]. Silver nanoparticles synthesized by *Azadirachta indica* were used for the management of banana anthracnose caused by *Colletotrichum musae* [28,29]. The results of the present investigation are in conformity with these earlier reports. In this study it was observed that silver nanoparticles can act as antagonist against *C. capsici* both *in vitro* and in the field experiment. Silver nanoparticles may directly attach to and penetrate the cell membrane to kill the spores, although penetration of silver nanoparticles into microbial cell membranes is not completely understood [15].

(a) **(b)**

Figure 5a and 5b: Untreated inoculated bean plants showing symptoms of anthracnose (a) and treated (200 ppm silver nanoparticles) inoculated plants (after 14 days of inoculation).

Conclusion

In the present investigation green synthesis of silver nanoparticles was carried out by plant extract of *A. indica* and its antifungal effect was noted against plant pathogenic fungus *C. capsici in vitro* and in planta inoculation experiment. Results emphasized that green synthesis of silver nanoparticles by plant extract is not only an eco-friendly approach in terms of production of nanoparticles

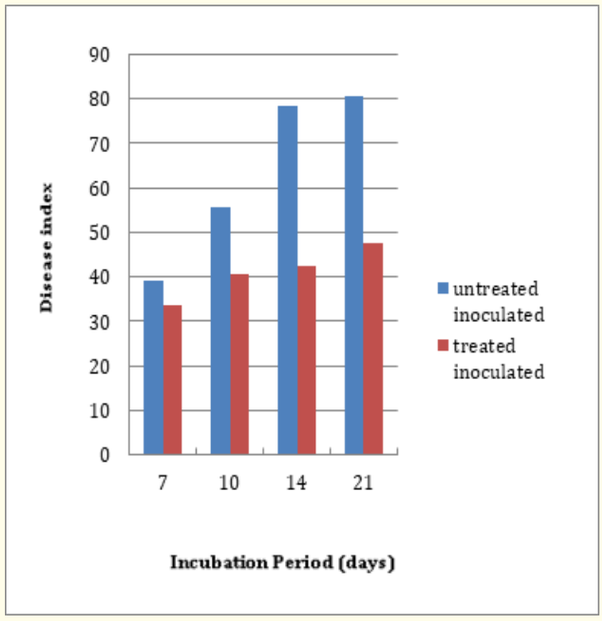

Figure 6: Disease index of untreated inoculated and treated (200 ppm silver nano particles) inoculated bean plants at different incubation period.

but the antagonistic effect of nanoparticles against *C. capsici* in vitro and in planta evaluation indicated its potential application in the field as an alternative to chemical fungicides for management of anthracnose of bean.

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