JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4593-4600

Assessment of Antimicrobial Activity of Biogenic Silver Nanoparticles against Plant Pathogens

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(Received: 16 September 2014; accepted: 30 October 2014)

With the ever increasing demand of the crop production, technology calls for innovative ideas for better disease management leading to higher crop production with available resources. Multiple diseases caused by plant pathogens affect plant crops leads to major losses up to 30% and simultaneously decreasing the quality and safety of agricultural products. The silver nanoparticles used in the present study were prepared through a cost effective and environment friendly green synthesis technique by using leaf extract of *Tagetes patula* L. (french marigold) as reducing as well as capping agent. The prepared biogenic nanoparticles were well characterized using U.V. visible spectroscopy, Transmittance electron microscopy, X-ray diffraction and Fourier-transform infrared spectroscopy which revealed the size of nanoparticles ranging from 15-30nm as well revealed their cubic and hexagonal structure. Further these biologically synthesized nanoparticles were found to be highly toxic against phytopathogenic bacteria and fungi by poison food technique, disc diffusion method and spore germination inhibition test.

Key words: Green synthesis, Silver nanoparticles, Food poisoning method, Disc diffusion method, Spore germination inhibition method.

Nanoscience involves the study of materials on the nanoscale level between approximately 1 to 100 nm. Nanoparticles exhibit completely new or improved properties compare to bulk material and have extensive applications in diverse fields[1]. The field of nanotechnology is one of the most active areas of research in modern material sciences. It opens up a wide array of opportunities in various fields like medicine, pharmaceuticals, electronics and agriculture. In agricultural sciences, this includes insect pest management through the formulations of nanomaterials-based pesticides and insecticides[2, 3]. Nano biotechnological approaches would be the alternative to existing technologies for management of plant diseases in near future[4]. Among various nanomaterials, metal-based nanoparticles are widely used in crop protection[5, 6, 7].

Among the several noble metal nanoparticles, silver metal nanoparticles have attained special focus in view of their distinctive properties, like good electrical conductivity, chemical stability, catalytic and antibacterial activity[8]. More surface area, activation of novel reactive groups and unique physico-chemical properties enable silver nanoparticles more effective against microbes at very low dose[9]. Silver nanoparticles have applications in bimolecular detection, diagnostics, water purification, antimicrobials & therapeutics, catalysis as optical sensor, micro-electronics, photonics, optics, textile

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engineering, DNA sequencing, surface-enhanced Raman spectroscopy, pharmaceuticals, Agriculture and pollution control etc[10-14].

A number of chemical, physical and biological approaches are available for the synthesis of metallic nanoparticles[15, 16]. Nanopartilces synthesized by chemical and physical methods are energy intensive processes and a large amount of capital is involved, moreover the use of hazardous and flammable chemicals makes this method non eco-friendly[17]. Further, chemical synthesis methods lead to presence of some toxic chemicals absorbed on the surface of nanoparticles that may have adverse effect in the medical applications. Hence, there is need for economic, commercially viable and environmentally clean synthesis route.

Biological methods of synthesis have paved way for the "greener synthesis" of nanoparticles and these have proven to be better methods due to slower kinetics, they offer better manipulation and control over crystal growth and their stabilization. A great deal of effort has been put for the search of methods utilizing the biological system. The use of environmentally benign materials like plant leaf extract, plant fruit extract, bacteria, fungi, yeast and enzymes for the synthesis of silver nanoparticles offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and other biomedical applications[18]. Plant cells are rich sources of various secondary metabolites. Plant part extracts contain several water soluble metabolites (alkaloids, phenolic compounds, terpenoids etc.) and coenzymes that can easily reduce metal ion to respective metal or metal oxide nanoparticles[19].

T. patula L. is also called as French marigold, which produces flower throughout the year. In this study, we propose to use leaves of *T. patula* L. for synthesis of silver nanoparticles. In French marigold, once flowers are plucked from the plants, the remaining parts has been discarded hence, we report the beneficial use of these discarded plant leaves towards synthesizing silver nanoparticles and evaluation of their antimicrobial activity. The effect of antimicrobial activity of the silver nanoparticles was observed in plant pathogenic bacteria viz. *Xanthomonas axonopodis pv. vignaeradiatae*, *Xanthomonas axonopodis pv. Punicae*, *Xanthomonas oryzae* and plant pathogenic fungi viz. *Fusarium solani*, *Alternaria* sp. and *Fusarium oxysporum*.

MATERIALS AND METHODS

Plant material and preparation of the Extract

Green leaves of French marigold were used to make the aqueous extract. 5g of leaves were thoroughly washed with water and cut into small pieces and transferred to Erlenmeyer flask (500 ml) containing 100 ml of distilled water and then the mixture was boiled for 5 minutes and filtered through Whatman filter paper number 1 and used for the synthesis of silver nanoparticles.

Synthesis of Silver Nanoparticles

Firstly, 10 ml of leaf extract was added to 90 ml of 2mM aqueous $AgNO_3$ solution for reduction of silver ion (Ag^+) at room temperature for 3 hours. The silver nanoparticles thus obtained after complete reduction was purified by repeated centrifugation at 10,000 rpm for 10-12 minutes followed by redispersion of the pellet in deionized water.

Characterization of silver nanoparticles

Characterization of the synthesized nanoparticles was performed by UV-Vis spectroscopy, XRD, particle size analysis, SEM, EDX, TEM with SEAD. The reduction of pure Ag^+ ions to silver nanoparticles was monitored by measuring the UV-Vis spectrum of the reaction medium from 30 min - 5 hours. UV-Vis spectral analysis was done by using Nanophotometer (Implen, Germany) and absorbance was recorded at a wavelength range of 300-600 nm. It is generally recognized that UV–Vis spectroscopy could be used to examine size and shape controlled nanoparticles in aqueous suspensions[20]. Samples for TEM analysis were prepared on carbon-coated copper TEM grid. The films on the TEM grid were allowed to stand for 2 minutes following which the extra solution was removed using a blotting paper and the grid was allowed to dry prior to measurement.

Antibacterial assays

Luria Bertani (LB) agar plates were prepared, sterilized and solidified. After solidification, bacterial cultures were swabbed on these plates. Antibiotic discs were used as positive control. The sterile disc was dipped in varying concentration of silver nanoparticles solution and placed in the agar plate and kept for incubation at

28° C for 24 hrs. Zone of inhibition was measured and compared with standard antibiotic disc and activity index (A.I.) were also determined using following formula.

$$A.I. = \frac{\text{Diameter of zone of inhibition by sample}}{\text{Diameter of zone of inhibition by standard antibiotic}}$$

In poison food technique, 50 ppm silver nanoparticles were added in plates of LB agar medium. Bacterial culture suspension was spread on the LB agar plates having silver nanoparticles formulation and kept for incubation at 28° C for 24hrs. After incubation bacterial growth (number of colonies) was measured and compared with control (without silver nanoparticles).

Antifungal assay

Poison food technique was used to measure antifungal activity[21, 22]. Four concentrations (100 ppm, 200 ppm, 300 ppm, 500 ppm) of silver nanoparticles were used in antifungal test against three plant pathogenic fungus species viz. *F. solani, Alternaria* sp. and *F. oxysporum* in this technique. Potato dextrose agar medium was prepared and poured in Petri dishes with above mentioned concentrations of silver nanoformulations, separately. Mycelial bits from peripheral end of uniform size (diameter, 5.0 mm) were taken from the 7 days old culture of test pathogens and placed in the centre of test petridishes. All the petridishes were incubated at 28±1 °C for 7 days and the observation of radial mycelial growth was recorded when control petridish cover full growth (90 mm). All the treatments consisted of three replications and experiment was repeated twice. The inoculated plates were compared with control (without nanoparticles) to calculate the % inhibition rate of mycelia of the pathogen the formula[23]:

X 100

$$\text{Inhibition rate (\%)} \quad \frac{M_c - M_t}{M_c} \quad X\ 100$$

Where, M_c = Mycelial growth in control, M_t = Mycelial growth in treatment.

Further, the antifungal effect of silver nanoformulations (100 ppm, 200 ppm, 300 ppm, 500 ppm) on spore germination of *Alternaria.* sp. and *F. solani* pathogens were tested using glass slides. Spore suspensions of *Alternaria.* sp. *and F. solani* were prepared aseptically from 7 day old pure culture; one drop (50µl) of spore suspension and one drop (50µl) of different silver nanoparticle concentrations were taken on glass slide in 10 replicates and compared with control devoid of nanoparticles. All treatments were maintained at an ambient temperature (28 ± 2 °C) for 6-8 hrs. Observations were made under microscope to calculate the % inhibition rate by counting the number of spores germinated compared to control:-

$$\text{Inhibition rate (\%)} \quad \frac{T_c - T_t}{T_c} \quad X\ 100$$

Where, T_c = Germination in control, T_t = Germination in treatment

Fungal spore suspensions with, serially diluted were added into the autoclaved Petri plates. Melted potato dextrose agar (12-15 ml) and silver nanoparticles poured into Petri plates with gentle shaking. Petri plates were placed in incubator at 28 ± 2^0C temperature for 2-3 days. Thereafter, colonies formed were taken out and counted.

Statistical analysis

The observations recorded for antimicrobial experiments were subjected to statistical analysis such as Standard Deviation on Microsoft Excel using standard equations. Furthermore, for determining significant difference among variable treatment on different silver nanoparticles, the analysis has been done using JMP software version 11[24] using Turkey-Kramer HSD test at p=0.05.

RESULTS AND DISCUSSION

Silver nanoparticles exhibit yellow to brown colour in aqueous solution due to surface plasmon resonance (SPR) which arise due to the collective oscillation of free conduction electrons induced by an interacting electromagnetic field[20]. As the *Tagetes patula* L. Leaf extract was mixed in the aqueous solution of the silver ion complex, it started to change the colour from watery to brown due to reduction of silver ion to silver by electron donor metabolites present in the leaf extract, which is the first visible indication of BNP synthesis (Figure 1). Absorption spectra of silver nanoparticles formed in the reaction media revealed

an absorbance peak at 420 nm. Broadening of the peak indicated that the particles are polydispersed (Figure 2). Further, BNP were characterized by XRD, DLS and Transmittance electron microscope (data not shown). The typical XRD pattern and TEM micrograph (Figure 1) revealed that the BNP is in mixed phase (cubic and hexagonal structures) of silver nanoparticles ranged from 15 to 30 nm and majority of the silver nanoparticles were scattered with only a few of them showing aggregation. Further these results were also confirmed with DLS studies (data not shown).

Antibacterial activities of BNP were tested, by both the disc diffusion method and the poison food technique, using Luria-Bertani agar against plant pathogenic bacteria such as XAP, XAV and XO (Figure 3). BNP exhibited antibacterial activity against XAP, XAV & XO as it showed a clear inhibition zone, whereas which standard antibiotics like Tetracycline and Kanamycin were used as positive control, also shown inhibition zones (Figure 4). In poison food technique, 50 ppm BNP was added in the medium that resulted in zero bacterial growth when compared to the control plates. The activity index of silver nanoparticles is represented in Table 1.

To investigate the antifungal effect of BNP against plant pathogenic fungal strains viz. *F. solani, Alternaria* sp. and *F. oxysporum*, both poisoned food technique and spore germination method were performed (Fig. 5, 6). Biogenic Silver nanoparticles show significant antifungal activity against these phytopathogenic fungi summarized in Table 2.

Table 1. Antibacterial activities of Biogeneic silver nanoparticles

Plant Pathogenic Bacteria	Activity Index with refrence to Tetracyclin	Activity Index with refrence to Kanamycin
XAP	0.564	0.709
XAV	0.487	0.740
XO	0.500	0.790

Table 2. Antifungal activities of BPN on *in vitro* mycelial growth and spore germination

Treatment	% inhibition Mycelia growth[a]	% inhibition Spore Germination[a]
F. solani		
Control[d]	0.0D	13.80±1.14 D
100 ppm	30.36±0.39C	65.04±1.70 C
300 ppm	49.97±0.81B	75.57±0.55 B
500 ppm	61.5±0.34A	90.15±1.8 A
Alternaria sp.		
Control[d]	0.0D	13.80±0.95 D
100 ppm	30.21±0.83C	65.04±0.57 C
300 ppm	48.51±1.34B	75.57±1.25 B
500 ppm	58.81±0.92A	90.15±2.37 A
F. oxysporum		
Control[d]	0.0 E	NA
10 ppm	20.07 ± 0.64 D	NA
25 ppm	25.71 ± 0.82C	NA
50 ppm	30.05 ± 0.99B	NA
100 ppm	33.1 ± 1.08B	NA
500 ppm	69.78 ± 0.34A	NA
1000 ppm	73.36 ± 0.80A	NA

[a] Each value is mean of 3 replicates from 2 experiments. Mean ± SE followed by same letter in column of each treatment is not significant difference at p = 0.05 by Tukey–Kramer HSD test, % inhibition rate was calculated compared to the germination of the control (0%). [d]Control without any formulation. (NA: We have not performed this test).

The radial growth of *F. solani*, *Alternaria spp* and *F.oxysporum* were reduced by all concentrations of silver nanoparticles used, as a dose dependent effect. The results clearly showed that the zone of inhibition of nanoparticles strongly depends on the concentration and greatly increases with increasing the concentration of BPN in the medium. The highest growth inhibition in mycelia growth (61.5%) as well as in spore germination in spore suspension test (90.15%) was observed at 500 ppm silver nanoparticles and in pour plate technique no growth or spore germination was observed in a concentrate of spores that were serially diluted. In *Alternaria spp.* the highest inhibition in mycelia growth (58.81%) as well as in spore germination (90.15%) was observed at 500 ppm concentration of silver naoparticles. In *F. oxysporum*, the highest growth inhibition (73.36%) was observed at 500 ppm concentration of silver nanoparticles, followed by 69.78% at a 100 ppm concentration. These concentrations were

significantly more effective in inhibiting the growth of *F.oxysporum* compared to other concentrations. In pour plate technique no spore germination was observed.

Earlier studies suggest BNP are found to have antibacterial[25], antifungal[26, 27] and antiviral[28] properties against phytopathogens. The probable mechanism of antimicrobial of silver nanoparticles is interactions of these particles with phosphorus and sulphur containing compounds, such as DNA and protein which prevent the ability of DNA to replicate since these particles can easily penetrate[29, 30]. The Silver nanoparticles synthesized via green route are highly toxic to phytopathogens hence has a great potential in plant disease management.

Fig. 1. Schematic representation of synthesis of silver nanoparticles using *Tagetes patula* L. Leaf extract

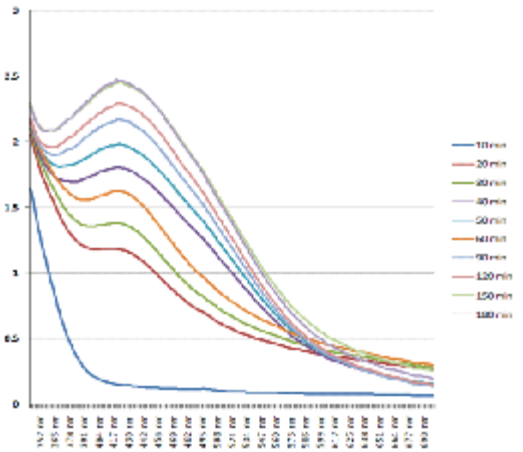

Fig. 2. UV-Vis absorption spectra of BNP synthesized by *Tagetes patula* L. Leaf extract from 30 min to 3 hrs

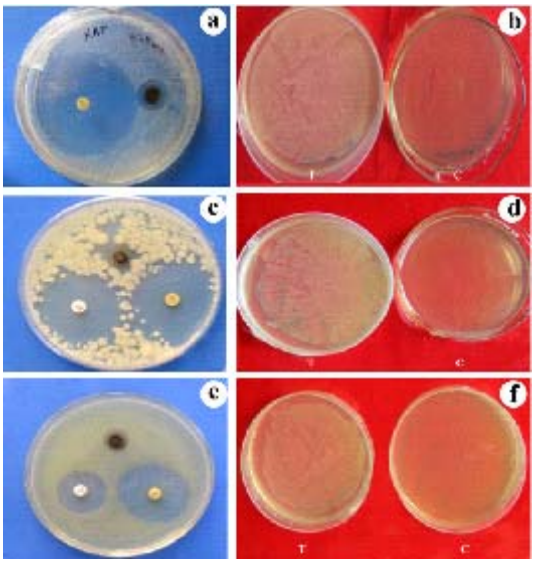

Fig. 3. Antibacterial activities of 50 ppm BNP against (a, b) **XAP**, (c, d) **XAV** and (e, f) **XO**. (a, c & e) Disc diffusion method; (b, d & f) Poisoned food technique (T: treatment, C: control).

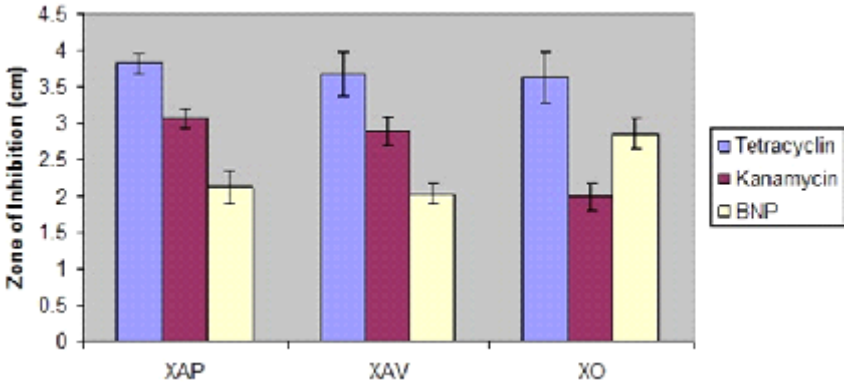

Fig. 4. Statistical representation of antibacterial activities of BNP against XAP, XAV and XO (Error bar show Standard deviation).

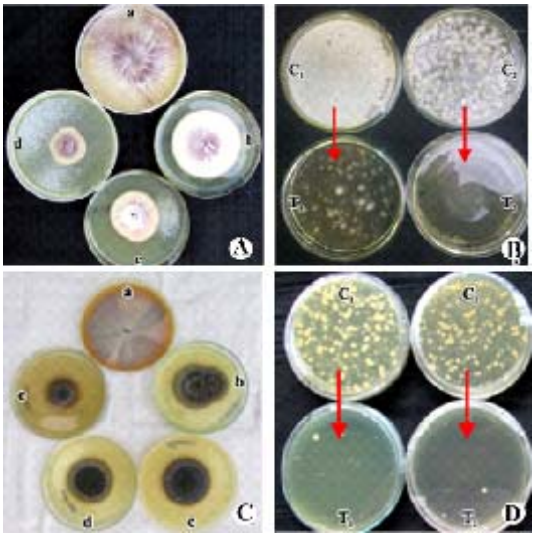

Fig. 5. A representative photograph of antifungal bioassay of BNP against (A) *Fusarium solani* mycelial growth inhibition by poison food technique (a. Control, b. 100 ppm, c. 300 ppm & d. 500 ppm) (B) *Fusarium solani* spore germination inhibition by pour plate technique (C) *Alternaria* sp. mycelial growth by poison food technique (a. Control, b. 100 ppm, c. 200 ppm, d. 300 ppm & e.500 ppm) (D) *Alternaria* sp. spore germination inhibition by pour plate technique. C = Crude spore suspension; C2 = 10^{-1} times diluted spore suspension; T_1 & T_2 = BNP Treatment (500 ppm)

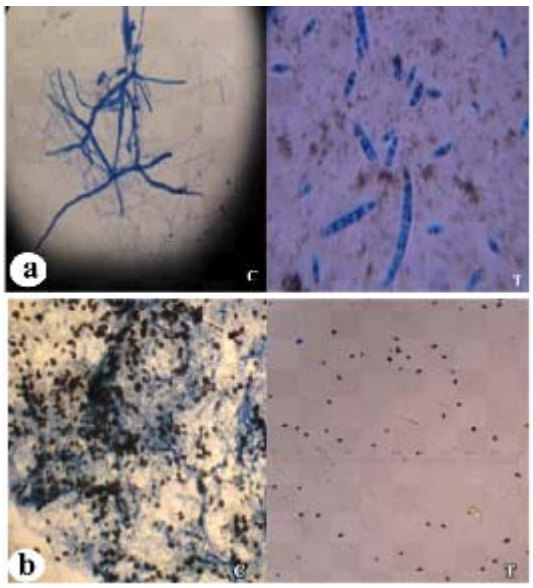

Fig. 6. Microscopic studies of spore germination inhibition of BNP against (a) *Fusarium solani* (b) *Alternaria* sp. C = Control, T =Treatment (500 ppm).

CONCLUSION

The present study involves the synthesis of silver nanoparticles using aqueous leaves extract of *T. patula* L. and evaluation of antimicrobial activity by these bio-synthesized silver nanoparticles against phytopathogens. Biological synthesis provides nanoparticles with good control over size distribution and shape[31] because of slow reaction kinetics. Moreover, the nanoparticles synthesized via green route mostly results in diverse size and shape nanoparticles, which might be the reason of its multitude antimicrobial activities against various microbes. Biosynthesis of metal nanoparticles using plant materials provides advantages such as free from use of harsh, toxic and expensive chemicals and also very cost effective, therefore can be considered as an economic and valuable alternative

for a large scale synthesis of nanoparticles[32]. From this study we have tried to show significant toxic effects exerted by biologically synthesized silver nanoparticles against selected phyto pathogens. Antibacterial characterization, as a function of nanoparticles concentration, was demonstrated against phytopathogenic bacteria i.e. XAP, XAV and XO by Disk diffusion and Poison food technique. Antifungal activity of silver nanoparticles was determined against phytopathogenic fungi i.e. *F. solani, Alternaria sp.* and *F. oxysporum*. The previous studies suggest that the plant cells/ plant parts can be a potential "nanofactory" for the synthesis of metallic nanoparticles. However, extensive research needs to be carried out on the toxicity aspects of silver nanoparticles for the possible use of these silver nanoparticles in effective disease management.

ACKNOWLEDGEMENTS

Thanks are due for the University of Rajasthan for providing TEM, SEM, FTIR, XRD facilities. We are thankful to Dean, RCA for their support and encouragement.

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