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Antifungal effect of biosynthesized silver nanoparticles against *Fusarium* wilt in pea plant

Poonam Rani[1], Lily Trivedi[2], Shailendra Singh Gaurav[3]
[1] Jayoti Vidyapeeth Women's University, Jaipur, Rajasthan, India
[2] Department of Biotechnology, Chaudhary Charan Singh University, Meerut, Uttar Pradesh, India

Abstract
Wilt disease in pea has caused major crop losses worldwide is motivating the researches to focusing on developing innovative disease management methods. In the present time, without application of hazardous chemicals the maintenance of sustainable agriculture is not possible. In present study, we evaluated the effect of plant mediating silver nanoparticles (AgNPs) against *F. pallidoroseum* under *in vitro* and *in vivo* conditions. The AgNPs were synthesized biologically by using plants extract and these AgNPs characterized by using different types of techniques such as UV-Vis Spectroscopy, Atomic force microscope (AFM), Fourier Transform Infra-Red (FTIR), Field Emission Scanning Electron Microscopy (FESEM), and Energy Dispersive X-Ray (EDX).The inhibition percentage (87.5%) of *F.pallidoroseum* caused by AgNPs treatments was established *in vitro*. Field experiment was conducted to compare the efficiency of AgNPs and chemical fungicide at the parameters of pods numbers, pods weight, seed number and seed weight. The data were analysed by SPSS for descriptive statistics and analysis of variance (ANOVA) and results were found to be significant for different parameters at 0.05 significance levels that are average mean of seed weight (53.56 gm), pod weight(2.34 gm), mean of average number of seed (10) and mean of average no of pod (27) is found to be high in AgNPs treatments as compare to chemical fungicides. It is concluded by the experiment that AgNPs may be prove to be potential fungicides in near future and it is an excellent alternative to chemical fungicides.

Keywords: *F. pallidoroseum*, silver nanoparticles, FTIR, SEM, EDX

1. Introduction
Pea *(Pisum sativum*) is a major annual crop of the world and it was originally cultivated in the Mediterranean basin. It is one of the most important multipurpose pulse crops. Pea is the second important food legume crop in the world after pigeon pea.
Pea crop is affected by number of diseases such as wilt, bacterial blight, ascochyta foot rot, downy mildew, rhizoctonia seedling blight, powdery mildew, pythium blight, aphanomyces root and root rot diseases [1]. Wilt disease of pea plant generally caused by *Fusarium oxysporum* which is one of the most common diseases [2]. The Fusarium wilt of pea was first recognized during 1919 [3]. In India, first report of the occurrence of Pea wilt organism during 1949 [4]. Distinct symptoms consist of yellowing of foliage and wilting leading to death of affected plants. The disease appears in scattered areas of the field and eventually may cover bigger areas [5].
Silver ions are very reactive they inhibit microbial respiration and metabolism and they cause physical damage [6, 7]. These biofabricated silvernanoparticles also showed promising antibacterial and cytotoxic effects. Silver has been used to treat medical ailments for over 100 years due to its natural antibacterial and antifungal properties. It is also used in many applications as a pure free metal or as a compound because it possesses antimicrobial activity against pathogens but is nontoxic to humans. Recently, nanotechnology has amplified the effectiveness of silver particles as antimicrobial agents [8]. Silver nanoparticles have extremely large relative surface areas which increases their contact with fungi, vastly improving its fungicidal effectiveness. The larger surface area-to-volume ratio of silver nanoparticles increases their contact with microbes and their ability to permeate cells. When in contact with fungus, they adversely affect cellular metabolism and inhibit cell growth. Silver suppresses respiration, basal metabolism of electron transfer systems, and transport of substrates in the microbial cell membrane.
The extremely small size of nanoparticles (NPs) have emerged as modern agents owing to their large surface to volume ratio which provides a large contact surface with pathogen sources [9]. Among nanoparticles (NPs), silver nanoparticles (SNPs) are more effective they can attack on microorganisms, including the cell membrane structure in largescale biological processes [10, 11]. The antibacterial activity of silver ions has been well established and attributed to the ability of ionised silver nanoparticles (SNPs) to penetrate into the fungal and bacterial cell wall and to modulate cellular signalling [12]. SNPs with fungistatic, bacteriostatic and plasmonic properties are among the eco-friendly inhibitors against plant-pathogens compared with synthetic fungicides [13].

2. Materials and Methods
2.1. Collection of plant sources
Freshly collected leaf of *O. tenuiflorum* (Black Tulsi) washed with double distilled water and shade dried for 10 days. The dried leaf was finely powdered and was filtered through 0.2 mm sieve.

2.2. Isolation of fungus from soil sample
The soil sample was collected from the agriculture field of Meerut (U.P). and cultured on Potato Dextose Agar (PDA) Media (to preserve) and Malt Glucose Yeast Peptone (MGYP) Media for isolation of fungus. After isolation of pure culture, the fungus is characterized by ITCC (Fig.1).

Fig 1: Characterization as *Fusarium pallidoroseum* (ITCC-1936-2016-16092016) from Indian Agriculture Research Institute, New Delhi.

2.3. Biosynthesis of silver nanoparticles by plants resources

The method of biosynthesis of silvernanoparticles through plants extracts has been already used in previous researches. According to this method the aqueous solution of silver nitrate two mili molar (2mM) was prepared for the synthesis of AgNPs [14]. Boiled leaves extract of plants (10 ml) added to the silvernanoparticles (90 ml) solution for reduction on Ag ions kept for the room temperature for overnight in dark conditions. After incubation period, the sample color change from light to dark brown which is indicates the synthesis of silvernanoparticles. The bio-transformation was routinely monitored visually after time intervals (0 hr, 4 hrs, 12 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs).

2.4. Characterization of silvernanoparticles

The biologically synthesized AgNPs were firstly characterized by colour based characterization technique that is UV-Visible Spectrophotometer which is facilitated in Department of Genetics and Plant Breeding, CCSU, Meerut), FESEM (facilitated from Indian Institute of Technology, Kanpur), EDX (facilitated from Indian Institute of Technology, Kanpur), FTIR (facilitated from SAIF, Indian Institute of Technology, Bombay), AFM (facilitated from Indian Institute of Technology, Kanpur) and ICPMS (facilitated from Indian Institute of Technology, New Delhi).

2.5. Antifungal effect of silver nanoparticles on *F. pallidoroseumin* under *in vitro* condition

In the present research, antifungal effect of silver nanoparticles was done against *F. pallidoroseum* under two conditions that are control (*F. pallidoroseum* alone), SNPs with *F. pallidoroseum*). Potato dextrose agar media was used to cultivate the test fungal species.1 ml of silver nanoparticles solution was poured with PDA medium into each Petri plate and after solidification of the medium, inoculated the *F. pallidoroseum* on it for seven days at at 28±2°C. Previous researchers also examined the antifungal activity of silvernanoparticle on the basis of colony formation technique in *in vitro* condition on Petri dish [15].

The diameters of the colonies were recorded after 72 hrs and the percentage inhibition was calculated using the formula:

$$I =$$

Where, I= Inhibition percentage; C = Radial growth to the fungus in control plates; T= Radial growth of the fungus in the petridish with medium containing the silver nanoparticles.

2.6. *Invivo* examination of SNPs on wilt disease caused by *F. pallidoroseum* under field conditions (Spores/ml = 10^8 c.f.u: colony forming unit)

In vivo examination were started during winter seasons of year 2018 (sowing date 11 Nov, 2018), at Chaudhary Charan Singh University, Meerut (Uttar Pradesh, India) to evaluate the efficacy of silver nanoparticles application on severity of fusarium wilt disease of pea plants under natural field conditions. The 'Ks 10' variety of pea was sown, which is generally cultivated in western Uttar Pradesh (India). The AgNPs solution were compared with recommended dose of commercially available fungicide Abic® syngenta® and Saaf ® (containing Mancozeb and Arbendazim), with (1000 ppm) and a control (pea plants without any treatment). Each treatment was applied in three replication rows, each row containing 15 plants. The spore suspension of *F. pallidoroseum* was sprayed on the plants after 20 days of sowing.

All treatments were applied as foliar spray three times with 10 days interval. At harvest time, 90 days after planting, the average harvested yield was calculated for all applied treatments. Randomly, five plants were taken from each replication of treatments and average yield was calculated as average number of pods /plant and weight of pods/ plant, number of green seeds /pods and weight of green seeds/ pods. All the statistical analysis including determination of least significant difference (LSD) was done with SPSS (Statistical Package for Social Sciences) software.

3. Result and Discussion

3.1. Biosynthesis of silvernanoparticles

The plant extract of *O. tenuiflorum* was mixed with aqueous solution of $AgNO_3$, it started to appear mustard dark brown after 72 hrs in the present case (Fig.2), which indicated the formation of AgNPs. Previous researchers also reported the synthesis of extracellular AgNPs with the help of plants extract. Plant extract have been used for AgNPs synthesis in previous researches also [16, 17, 18, 19, 20, 21]. Previous researchers used the number of plants like *Azadirachta indica* [22], *Bergenia ciliata, Clitoria ternatea, Cochlospermum religiosum, Dianthus raryophyllus, Garcinia mangostana, Hyacinthus orientalis, Pinus eldarica, Rumex hymenosepalus and Saraca indica* [23] for the biosynthesis of extracellular silver nanoparticles.

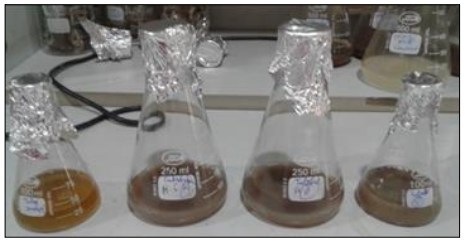

Fig 2: Synthesis of silvernanoparticles from *O. tenuiflorum* in three flask after 96 hr.and compression with control flask (left).

3.2. Analysis of AgNPs through UV spectrophotometer

The AgNPs were characterized by UV-Vis double beam spectrophotometer (Lasany LI-295). All spectra were measured at room-temperature, in a quartz cell with 1 cm optical path, to know the kinetic behavior of AgNPs. The

scanning range of the UV spectrophotometer for the samples was 200-800 nm. The spectrophotometer was equipped with "UV prov software" to record and analyze the data. Base line correction of the spectrophotometer was carried out by using a blank distil water as a reference. The samples were analyzed at 0, 4, 12, 24, 48, 72, 96 and 120 hrs. AgNPs generally show a broad peak in the UV-Visible spectrum in the range of 400-460 nm. In the present study the reaction stabilized after 96 hrs and optical changes have been noted at 420, 520 and 540 nm (Fig.3). The characteristic fluorescence peak of AgNPs in the water phase at 465 nm was already reported [24].

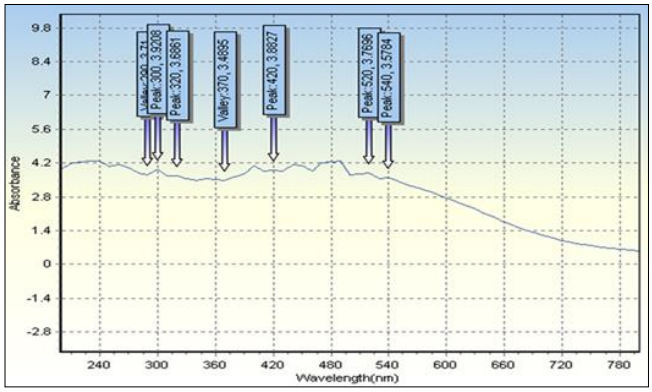

Fig 3: UV- spectrophotometer groph of AgNPs after 96 hrs.

3.3. Analysis of AgNPs by FESEM

SEM is a surface imaging method, fully capable of resolving different particle sizes, size distributions, nanomaterial shapes and the surface morphology of the synthesized particles at the micro and nanoscales [25]. The AgNPs dried samples were prepared by placing two drops (200µl) of AgNPs solution on aluminum foil and let air dry followed with placing it in hot air oven at 50ºC for 24 hrs. The FESEM facility was availed from Advance Imaging Centre, Indian Institute of Technology (IIT), Kanpur (UP, India). The image taken indicated that nanoparticles are well distributed with the lowest agglomeration of nanoparticles (Fig.4). The particles were discreet, spherical in nature and mostly polydispersed. Studies of FESEM micrograph also revealed nanoparticles with a few monoclinic non-spherical structures. In previous rresearches similar results were reported for phyto-synthesised silver nanoparticles [26, 27, 28, 29]. Also in previous researches biosynthesis of silvernanoparticles through *T. involucrata, C.citronella, S. verbascifolium* and *T. ovata* was analysed [30].

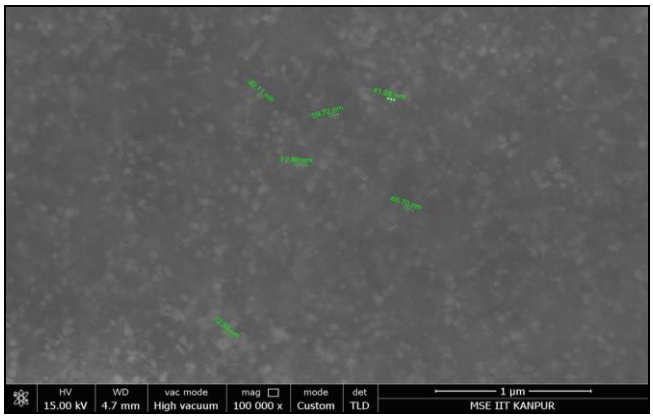

Fig 4: Image obtained from FESEM analysis.

3.4. Analysis of AgNPs by EDX

This facility was also availed from Advance Imaging Centre, IIT, Kanpur (UP, India). The EDX report shows the EDX spectrum of AgNPs (Fig.5). EDX spectrum showed peaks of silver (Ag) and aluminum (Al). EDX analysis showed the optical absorption peak at 3 *keV*. The peak corresponding to aluminum is obvious as the sample smear was prepared on the aluminum foil base. Weight percentage of Ag was found to be 100 %. (Fig.4). Mostly metallic silver nanocrystals show typical optical absorption peak approximately at 3 keV due to surface plasmon resonance [31].

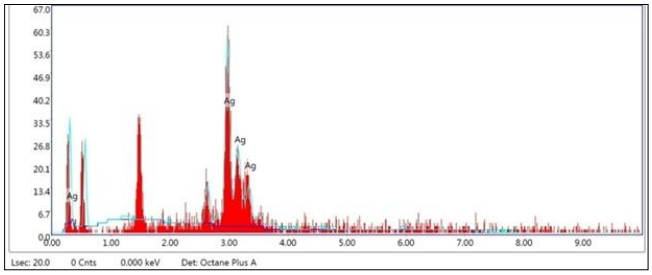

Fig 5: EDX analysis graph, where X-axis is showing the energy in keV and Y-axis is signifying intensity count.

3.5. Analysis of silvernanoparticles through AFM

The AgNPs dried samples were prepared by placing two drops of AgNP solution on aluminum foil and placing it in hot air oven at 80ºC for 12 hrs. The AFM facility was availed from Advance Imaging Centre, Indian Institute of Technology (IIT), Kanpur (UP, India). The three-dimensional (3D) surface morphology and size analysis of silver nanoparticles were obtained from AFM. The image taken indicated that silver nanoparticles are well distributed with the lowest agglomeration of nanoparticles. Particles with 25 nm size were found to be present in maximum quantity and the shapes of the particles are spherical (Fig.6). Previous researches strongly supported this study with the particle size of the silver nanoparticles was found to be 28 nm, 26.5 nm, 65 nm, 22.3 nm and 28.4 nm corresponding to *O. tenuiflorum, S. cumini, C. sinensis, S. tricobatum and C. asiatica* respectively [32].

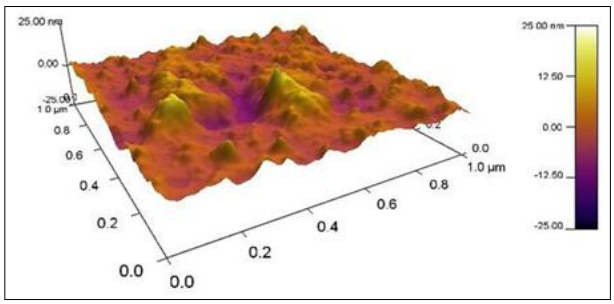

Fig 6: Image obtained from atomic force microscope

3.6. Analysis of AgNPs by FTIR

The FTIR facility was availed from Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology (IIT), Bombay to recognize the organic, inorganic, biomolecule residues along with nanoparticle formation, which may come along via reducing agent on to the surface of AgNPs (Fig.7). Absorption bands for AgNPs were found to be at 495.38 cm^{-1}, 602.42 cm^{-1}, 696.32 cm^{-1}, 759.75 cm^{-1}, 1025.70 cm^{-1}, 1152.43 cm^{-1}, 1212.25 cm^{-1}, 1382.25

cm^{-1}, 1457.87 cm^{-1}, 1519.86 cm^{-1}, 1649.72 cm^{-1}, 1711.38 cm^{-1}, 3615.67 cm^{-1}, 3746.03 cm^{-1} and 3861.12 cm^{-1}. The peaks at 1649.72 cm^{-1} and 1711.38 cm^{-1} are corresponding to C=C stretch in the aromatic ring. The band at 1631 cm^{-1} in the spectra corresponds to C–N and C–C stretching indicating the presence of proteins [33]. 1527.47 cm^{-1}, 1545.93 cm^{-1}, 1567.39 cm^{-1}, alkanes and C-O and C-H bending. 1024.34 cm^{-1}, 1108.70 cm^{-1}, 1155.88 cm^{-1}, 1218.35 cm^{-1}, 1258.78 cm^{-1}, 1383.57 cm^{-1}, C-O bonding, 415.38 cm^{-1}, 497.90 cm^{-1},606.58 cm^{-1}, alkyl halides [34].

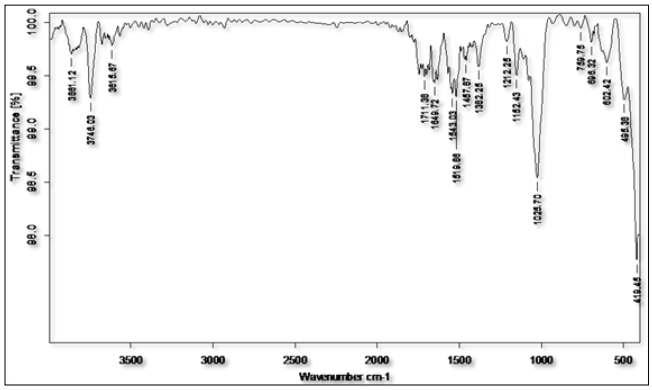

Fig 7: Image obtained from Fourier Transform Infrared Spectra

3.7. Analysis of antifungal effect of silver nanoparticles under *in vitro*

In this research the inhibition effect of AgNPs was estimated against *F.pallidoroseum*. Size of the inhibition zone for AgNPs synthesized by *O. tenuiflorum* against the *F. pallidoroseum* was determined (Table.1). In petri plate (B) the inhibition rate of *F. pallidoroseum* is very high as compared with control, so it was analysed by this research that AgNPs have the antifungal effect on *F.pallidoroseum* (Fig.8). Previous researches also analysed the antifungal effect of silver nanoparticles against fusarium wilt in *Fusarium solani Fusarium solani* and *Macrophomina phaseolina* [35, 36].

Table 1: Size of the inhibition zone for AgNPs synthesized by *O. tenuiflorum* against the F. *pallidoroseum*

S. No.	Treatment	Zone of Inhibition(cm.)	Inhibition %
1	Control	4.0 cm.	No Inhibition
2	Ag-NPs, treatment	0.5cm.	87.5 %

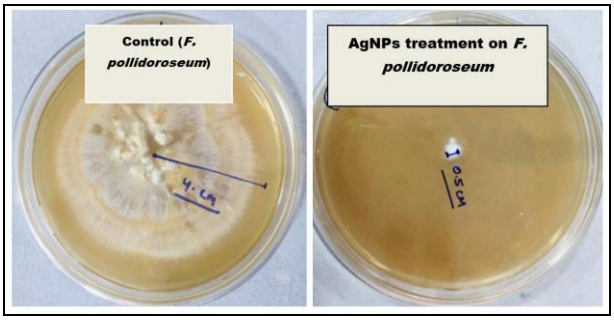

Fig 8: Antifungal activities of (A) Control, (B) Ag-NPs, treatment against *F. pallidoroseum* after 5 days of incubation periods.

3.8. Results of SNPs on wilt disease *F. pallidoroseum* caused by under field conditions.

The results of average weight and number of pods and number of seeds per treatment are given in table 2. The highest weights of pods and seeds were recorded in plants treated with AgNPs and chemical fungicide and their difference was found to be significant as compared to control at 0.05 significance levels. The decimal values obtained for pods numbers and seeds numbers were rounded off to whole number as pod numbers and seeds number can't be a decimal value. The ANOVA was found to be significant at 0.05 levels for the difference in pod numbers and seeds number among control, AgNPs treatment and chemical fungicide treatment.

Table 1: Average number of pods, Mean weight of pods, number of seeds and weight of seeds in per treatment.

S. No.	Treatments	Average Number of Pods (Mean)	Mean weight of Pods (Mean) & Standard Deviation	Average Number of Seeds (Mean)	weight of Seeds (Mean) & Standard Deviation
1	Control	21	1.94 (±2.71)	6	35.07 (±2.84)
2	AgNPs (2Mm)1	27	2.34 (±11.51)	10	53.56 (±1.76)
3	AgNPs (1Mm)2	26	2.32 (±4.62)	9	52.86 (±2.59)
4	Chemical fungicide 1	24	2.12 (±8.71)	8	49.29 (±0.93)
5	Chemical fungicide 2	24	2.07 (±5.40)	8	49.47 (±0.55)
6	Chemical fungicide 3	25	2.06 (±6.17)	6	47.23 (±1.29)

*The decimal values were rounded off.

4. Conclusion

The present study analysed the antifungal effects of AgNPs on *F. pallidoroseum which* was tested *in vitro*, based on zone of inhibition, it was concluded that AgNPs have considerable antifungal activity against *F. pallidoroseum*. AgNPs were also found effective as potential control agent against *fusarium wilt* of pea crop as against the extensively used chemical fungicides, which are known to cause various health and environmental hazards. Further study is required to set the minimal inhibitory concentration of AgNPs against this fungus, so that nano formulations may be prepared accordingly.

5. Acknowledgement

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