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EVALUATION OF MYCOSILVER NANOFUNGICIDES AS POTENTIAL CONTROL AGENT AGAINST *Phytophthora infestans*

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ABSTRACT

Potato (*Solanum tuberosum* L.) is one of the most important vegetable crops across world and India is second largest producer of this crop across the world. Late blight disease has been the most serious threat to world's potato production, resulting in 80-100 % yield loss. The causal agent of late blight is fungi, *Phytophthora infestans.* The present investigation was carried out to evaluate the efficacy of mycogenic silver nanoparticles as an antifungal agent against *P. infestans*. The silver nanoparticles were synthesized biologically by using *Aspergillus niger* biomass and characterization of silver nanoparticles (AgNPs) was done by UV-Vis Spectroscopy, Field Emission Scanning Electron Microscopy (FESEM), Energy Dispersive X-Ray (EDX), Dynamic Light Scattering (DLS) and Fourier Transform Infra Red (FTIR). The inhibition percentage of *P. infestans* caused by silver nanoparticles treatment was established *in vitro*. Field experiment was conducted to compare the efficiency of silver nanoparticles and chemical fungicide at the parameters of disease severity, tuber number and tuber weight. The data were analysed by SPSS software for descriptive statistics and analysis of variance (ANOVA) including least significance difference (LSD). The development of mustard yellow colour in the reaction mixture preliminary confirmed the synthesis of AgNPs. The UV-Vis Spectral report showed peaks corresponding to AgNPs at 420, 430, 440 and 460 nm. The FESEM images confirmed synthesis of roughly spherical nano-sized particles and the elemental composition of the same was confirmed to be silver by EDX analysis report. DLS analysis depicted the average size of nanoparticles as 37.2 nm. The FTIR spectral report provided information about the molecular interactions between AgNPs and surrounding chemical functional groups. The AgNPs showed 75% inhibition percentage during *in vitro* analysis. The average disease severity was found to be significantly higher in control plants as compared to AgNPs and chemical fungicide treated plants. Also, AgNPs and chemical fungicide treated plants showed significantly higher average

tuber production as compared to control as tested at 0.05 significance levels. There was no significant difference between AgNPs and chemical fungicide treated plants at all the parameters taken under consideration. It may be concluded by the study that AgNPs may be proved to be potential fungicides in near future and it is an excellent alternative to chemical fungicides.

Keywords: *Solanum tuberosum; Phytophthora infestans; Aspergillus niger;* nanoparticles; nano-fungicides; SEM; EDX.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important and edible vegetable crops in the world. In India, 90% of the potato crops are grown in the plains and 20% are grown in hill region and the country holds third position in worldwide potato cultivation area and second position in total potato production i.e. contributes 12.32% of total potato production in world [1]. Nearly 45% of global potato production occurs in three largest potato producing countries that are China, India and Russia [2]. In India, potato is majorly cultivated in planes and the three largest potato producing states are Uttar Pradesh, West Bengal and Bihar (contribute 32.38%, 26.94% and 14.56% of total production respectively), collectively contribute about 75% of the national production [3]. Potatoes are exposed to various diseases caused by plant pathogens that present in soil and airborne which are causing significant yield loss worldwide. Potato is attacked by number of diseases like Late blight, Early Bligh, Potato Leaf roll virus, Black leg, Scab, Black scurf, and Wilt, etc. Among these diseases, late blight is the most important one affecting potatoes [4]. Late blight has been the most serious threat to world's potato and has the tendency to destroy the crop completely, resulting in 80-100% yield loss [5]. The causal agent of late blight is an oomycete, *Phytophthora infestans* (Mont.) De Bary [6]. Complete field destruction due to late blight is common worldwide leading to 3-5 billions economic loss each year [7-10]. *P. infestans* produces various factors including effector molecules coded by avirulence genes that allow rapid infection and host tissue colonization [11]. Blight disease has been a problem since 150 years, and many approaches have been undertaken to control it [12]. In order to prevent and protect the crop plants diseases against pathogens, different strategy has been used i.e. chemical control, fungicides and bio-fungicides are in practice. Used of fungicides and antibiotics on the plant surfaces for disease management lose their activity against plant pathogens [13]. Due to the extensive use of fungicides and pesticides there is rapid increase in ecotoxicity and resistance development in plant pathogenic microbes [14-16]. Biological control methods for the disease management of phyto-pathogens have been useful [17]. In contrast to conventional application of fungicides antibiotics, nanoparticles are most important strategy to manage plant diseases [18]. The use of silver nanoparticles as an alternative to chemical pesticides could make crop production more economical [19]. Nano-biotechnology has emerged as one of the fastest growing modern areas of research in materials science and technology [20]. Nanoparticles are synthesized by physical, chemical and biological methods. Physical and chemical methods are energy intensive and cause toxic chemicals whereas, biogenic technique is eco-friendly, non-toxic and economically viable [21]. Silver nanoparticles (AgNPs) have long been used as antimicrobial agents [22]. The present research was carried out to investigate the antimicrobial activity of silver nanoparticles against *P. infestans.*

MATERIALS AND METHODS

Culturing of *Aspergillus niger*

The pure culture of *Aspergillus niger* was obtained from Department of Biotechnology, Chaudhary Charan Singh University, Meerut (UP, India) and sub-cultured on Potato Dextrose Agar (PDA) Media (to preserve) and Malt Glucose Yeast Peptone (MGYP) Media (to generate biomass for nanoparticle synthesis).

Biosynthesis of Silver Nanoparticles by *A. niger* Biomass

This was done as per the method given by Sagar and Ashok (2012) [23]. Two mili molar (2mM) aqueous solution of silver nitrate ($AgNO_3$) was prepared for the synthesis of AgNPs. About 10

gram of wet fungal biomass was taken and suspended in 1000 ml of the 2 mM aqueous $AgNO_3$ solution for reduction of $AgNO_3$ into AgNPs. Fungal biomass with water was kept as control. The control and test flasks were placed in an incubator shaker at 30°C (at 150 RPM) and the reaction was carried out for a period of 120 hours. 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). The biomass was separated by filtration using Whatman No. 1 filter paper followed by syringe filter (pore size 0.45 µm).

Characterization of AgNPs

The synthesized AgNPs were characterized by the techniques UV (ultra voilet) - Visible Spectroscopy, Field Emission Scanning Electron Microscopy (FESEM), Energy Dispersive X-Ray (EDX), Dynamic Light Scattering (DLS) and Fourier-transform infrared spectroscopy (FTIR).

In vitro* Testing of AgNPs against *P. infestans

The antimicrobial assay was done against *P. infestans* by method as given by Banik and Luque (2017) [24]. Potato dextrose agar media was used to cultivate the test fungal species. One ml of silver nanoparticles solution was poured with PDA medium into each Petri plate and after solidification of the medium, each Petri plate was inoculated centrally with 5 mm diameter discs from the growing margins of the seven days old culture of the test fungi. Petri plates were incubated for 5 days at 25±1°C. The diameters of the colonies were recorded after 72 hrs and the percentage inhibition was calculated using the formula:

$$I = \frac{C - T}{C} \times 100$$

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

Field Experiments and Statistical Analysis

Field experiments were carried out at Chaudhary Charan Singh University, Meerut (Uttar Pradesh, India) during winter seasons of year 2017 (sowing date 29 Oct, 2017), to evaluate the efficacy of silver nanoparticles application on severity of early blight disease of potato plants under natural field conditions. The 'Kufri - Pukhraj' variety of potato (developed by Central Potato Research Institute, India) was sown. This is the generally cultivated potato variety in western Uttar Pradesh (India). The AgNPs solution were compared with recommended dose of commercially available chemical fungicide (CF) Abic® syngenta® (containing Mancozeb), Tween 80 (T-80) in 1000 ppm and 2000 ppm concentrations and a control (potato plants without any treatment). Each treatment was applied in three replication rows, each row containing 55 plants. The spore suspension ($1X10^5$ spores per ml) of *P. infestans* was sprayed on the plants after 45 days of sowing. All treatments were applied as foliar spray three times with 10 days interval, starting from the day of visible disease symptoms on the crop. Disease severity (DS) was calculated as per the method followed by El-Batal et al. (2016) [25]. At harvest time, 90 days after planting, the average harvested yield was calculated for all applied treatments. Randomly, ten plants were taken from each replication of treatments and average yield was calculated as average number of tubers/plant and weight of tubers/ plant. All the statistical analysis including ANOVA (Analysis of Variance) and determination of least significant difference (LSD) was done with SPSS (Statistical Package for Social Sciences) software version 16.0.

RESULTS AND DISCUSSION

Biosynthesis of AgNPs using *Aspergillus niger*

As the fungal biomass was mixed with aqueous solution of $AgNO_3$, it started to appear mustard yellow after 24 hrs in the present case, which indicated the formation of AgNPs. A number of the workers in the past have reported the synthesis of extracellular AgNPs with the help of fungal biomass. *Aspergillus niger* have been used for AgNPs synthesis in various researches in past [23,26-30]. Various other fungal species have been reported to facilitate synthesis of AgNPs viz *Penicillium oxalicum, Trichoderma longibrachiatum, Arthroderma fulvum, Aspergillus fumigatus*, *Aspergillus flavus*,

Candida albicans, *Penicillium italicum*, *Syncephalastrum racemosum*, *Fusarium oxysporum*, *Alternaria solani*, *Aspergillus ochraceus* etc. [31-34].

UV-Vis Analysis of AgNPs

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 spectrophotometer was equipped with "UV prov software" to record and analyze the data. 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 400-450 nm [35]. In the present study the optical transitions have been observed at 420, 430, 440 and 460 nm (Fig. 1). The reaction stabilized after 96 hrs.

Analysis of AgNPs by FESEM

Among various electron microscopy techniques, FESEM 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 [36]. 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 50°C for 24 hrs. The FESEM facility was availed from Advance Imaging Centre, Indian Institute of Technology (IIT), Kanpur (UP, India). The Nova NanoSEM 450 (FESEM) instrument was used. The image taken indicated that nanoparticles are well distributed with the lowest agglomeration of nanoparticles (Fig. 2). The particles were discreet, spherical in nature and mostly polydispersed. Studies of FESEM micrograph also revealed nanoparticles with a few monoclinic non-spherical structures.

Analysis of AgNPs by EDX

This facility was also availed from Advance Imaging Centre, Indian Institute of Technology, Kanpur (UP, India). The EDX analysis was collaboratively available with the Nova NanoSEM 450 (FESEM). The EDX report shows the EDX spectrum of AgNPs (Fig. 3). 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 and Al were found to be 65.19% and 34.81% respectively.

Analysis of AgNPs by DLS

Dynamic light scattering (DLS) which is based on the laser diffraction method with multiple scattering techniques was employed to study the average particle size of AgNPs. Among the techniques of nanoparticles characterization DLS is the most commonly used [37-39]. The samples were sent to Centre for interdisciplinary Research, Motilal Nehru National Institute of Technology, Allahabad, Uttar Pradesh, India. Microtrac Particle Size Analyser was used for the purpose. The aqueous sample was ultrasonicated before processing under DLS. The DLS size distribution image of biosynthesized AgNPs is shown in Fig. 4. It showed that the size of AgNPs is ranging from 32.5 nm to 42.1 nm with average size peak at 37.2 nm.

Analysis of AgNPs by FTIR

The FTIR (3000 Hyperion Microscope with Vertex 80 FTIR System) facility was availed from Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Bombay to analyse the organic, inorganic biomolecule residues associated with nanoparticles, which may come along via reducing agent on to the surface of AgNPs (Fig. 5). Absorption bands for AgNPs were found to be at 639.43 cm^{-1}, 1383.92 cm^{-1}, 1631.43 cm^{-1}, 3456.26 cm^{-1}. The intense band at 3456.26 corresponds to O–H stretching [40]. The peak at 1631.43 cm^{-1} is corresponding to C=C stretch in the aromatic ring [41]. Peak at 1383.92 cm^{-1} indicates the significance presence of C–N bending vibration [42]. Peak at 639.43 cm^{-1} shows presence of alkyl halides [43].

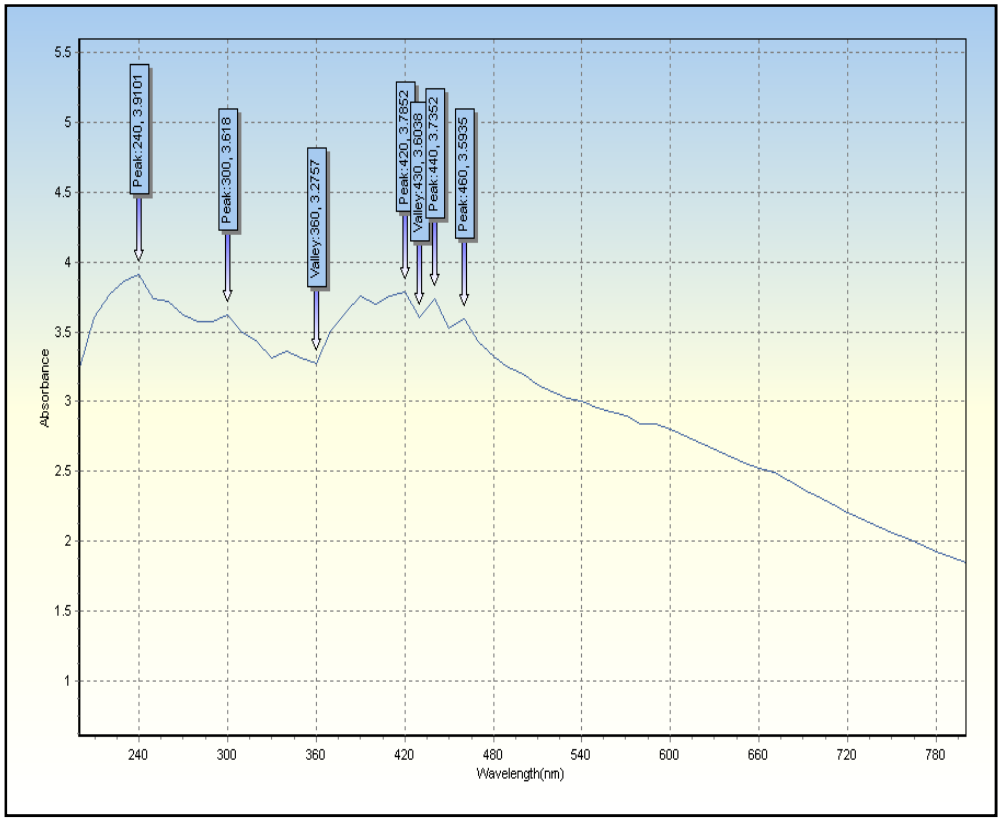

Fig. 1. UV-Vis graph of 96 hrs sample of AgNPs

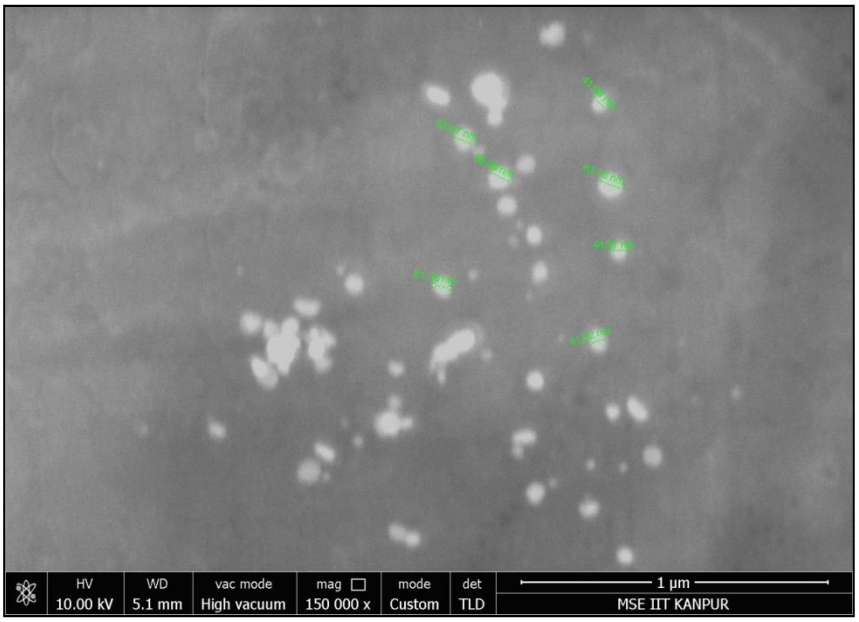

Fig. 2. AgNPs image obtained from FESEM analysis

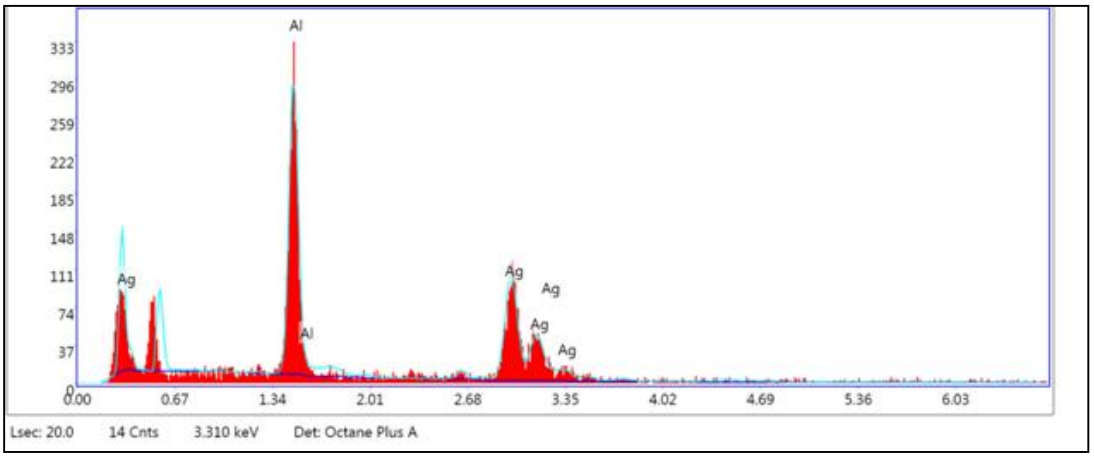

Fig. 3. EDX analysis graph, where X-axis is showing the energy in keV and Y-axis is signifying intensity count

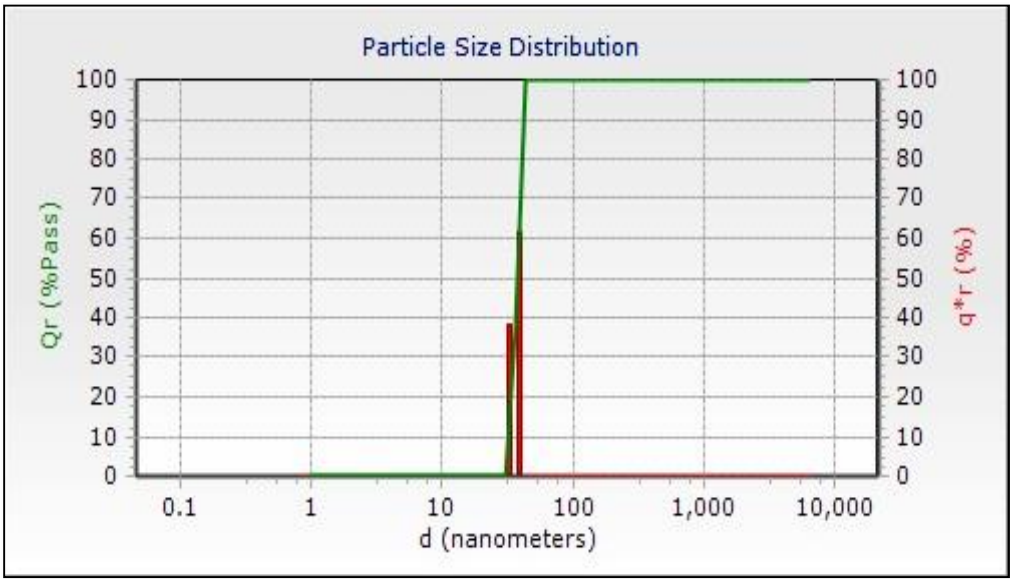

Fig. 4. DLS-data of AgNPs

Inhibition Percentage in vitro

The silver nanoparticle plate showed inhibition percentage (IP) as 75% after 72 hrs of fungal inoculation in media (Fig. 6). Gunalan et al. illustrated about the antifungal activity of zinc oxide nanoparticles [44]. Jayaseelan et al. (2012) evaluated antimicrobial activity of zinc oxide nanoparticles against *Pseudomonas aeruginosa* and *Aspergillus flavus* and reported that the maximum zone of inhibition was observed at 25 µg/mL against *Pseudomonas aeruginosa* (22 ± 1.8 mm) and *Aspergillus flavus* (19 ± 1.0 mm) [45]. Shinde (2015) evaluated the antimicrobial activity and formulated minimal inhibitory concentration (MIC) of zinc oxide nanoparticles against various pathogenic bacteria and fungi [46]. Jasim (2015) determined the antifungal activity of zinc oxide nanoparticles on *Aspergillus fumigates* fungus & *Candida albicans* yeast and formulated MIC [47].

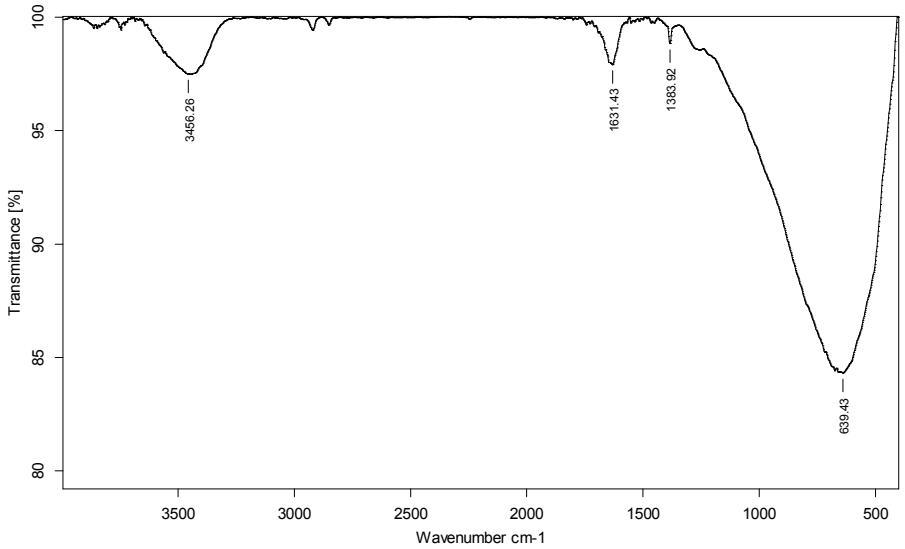

Fig. 5. FTIR-spectrum of AgNPs

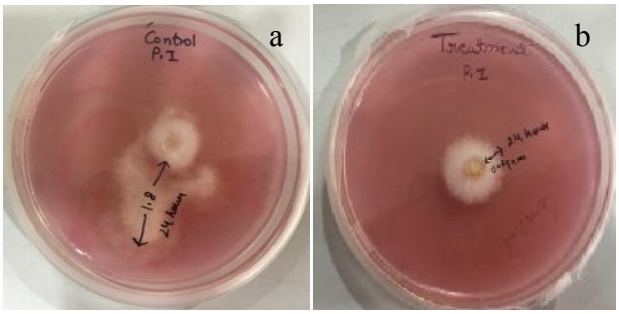

Fig. 6. Growth of *P. infestans* in control (a) and treatment (b) plates after 24 hrs time

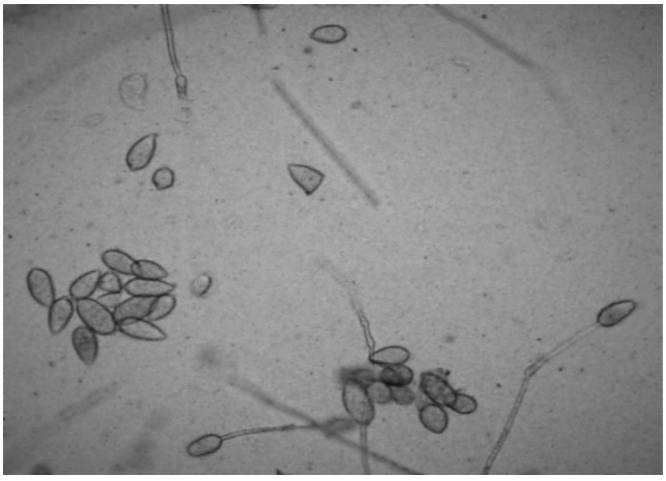

Fig. 7. Microscope image showing spores of *P. infestans*

Results of Field Experiments

The onset of late blight was observed in the late in December. The causative agent (*P. infestans*) was confirmed by testing the fungi. The infected leaves were surface sterilized under aseptic conditions and dried black spot of the leaf was pressed onto the SDA media. The inoculated media was kept at 25°C for 72 hrs. The grown fungus was stained with lactophenol blue on glass slide and was observed under Nikon Eclipse T2 Inverted Phase Contrast Microscope (Fig. 7).

The highest and lowest DS% was recorded in control and CF treatment respectively. DS% was found to be significantly higher in control as compared to all the treatments. The DS% in T-80 treated plants was significantly higher as compared to AgNPs and CF treated plants. However, there was no significant difference between AgNPs treated and CF treated plants at 0.05 significance levels in terms of DS% (Table 1).

The results of average number and weight of tubers in control and treated plants along with significance values are given in Tables 2 and 3 respectively. The highest average number and weight of tubers were recorded in plants treated with AgNPs followed by CF and it was found to be significantly higher as compared to control, T-80 (1000 ppm) and T-80 (2000 ppm) at 0.05 significance levels. There was no significant difference in average tuber number and weight of AgNPs and CF treated plants. The difference between average tuber number in control plants and T-80 treated plants was found to be statistically insignificant. However, the average tuber weight of T-80 (2000 ppm) treated plants was found to be significantly higher than control plants. The T-80 (1000 ppm) treated plants showed no significant difference with T-80 (2000 ppm) treated and control plants in terms of average tuber weight at 0.05 significance levels. Giannousi et al. (2013) studied effect of Cu-based NPs on tomato and concluded that it can be applied in a lower formulated product and are more effective than the trade agrochemicals [48]. Ali et al. (2015) reported inhibition of *Phytophthora parasitica* and *P. capsici* by silver nanoparticles [49]. Zhan et al. (2018) prepared a gold nanoparticle-based lateral flow biosensor for detection of potato late blight [50]. Fukamachi et al. (2019) reported control of *P. infestans* by using cyazofamid encapsulated in biodegradable poly lactic-co-glycolic acid (PLGA) nanoparticles [51].

Table 1. Mean DS caused by *P. infestans* for control and all treatments on potato crop

Study groups	DS (%)	SD(±)	SE(±)	ANOVA with LSD analysis results	
				MCG	SV
Control	85.0	0.0	0.0	T-80 (1000 ppm)	0.022*
				T-80 (2000 ppm)	0.000*
				AgNPs	0.000*
				CF	0.000*
T-80 (1000 ppm)	74.1	3.8	2.2	Control	0.022*
				T-80 (2000 ppm)	0.072
				AgNPs	0.000*
				CF	0.000*
T-80 (2000 ppm)	65.8	3.8	2.2	Control	0.000*
				T-80 (1000 ppm)	0.072
				AgNPs	0.000*
				CF	0.000*
AgNPs	38.9	1.9	1.1	Control	0.000*
				T-80 (1000 ppm)	0.000*
				T-80 (2000 ppm)	0.000*
				CF	0.000*
CF	21.3	3.3	1.9	Control	0.000*
				T-80 (1000 ppm)	0.000*
				T-80 (2000 ppm)	0.000*
				AgNPs	0.000*

SD = Standard deviation, SE = Standard error, MCG = Multiple comparison groups, SV = Significance value
*Values are significant at 0.05 significance level

Table 2. Average number of tubers control and all treatments on potato crop

Study groups	Mean number of tubers	SD(±)	SE(±)	ANOVA with LSD analysis results	
				MCG	SV
Control	4.7	0.1	0.0	T-80 (1000 ppm)	0.169
				T-80 (2000 ppm)	0.077
				AgNPs	0.000*
				CF	0.000*
T-80 (1000 ppm)	5.0	0.1	0.0	Control	0.169
				T-80 (2000 ppm)	0.675
				AgNPs	0.000*
				CF	0.000*
T-80 (2000 ppm)	5.1	0.2	0.1	Control	0.077
				T-80 (1000 ppm)	0.675
				AgNPs	0.000*
				CF	0.000*
AgNPs	7.3	0.7	0.4	Control	0.000*
				T-80 (1000 ppm)	0.000*
				T-80 (2000 ppm)	0.000*
				CF	0.332
CF	7.0	0.0	0.0	Control	0.000*
				T-80 (1000 ppm)	0.000*
				T-80 (2000 ppm)	0.000*
				AgNPs	0.332

*Values are significant at 0.05 significance level

Table 3. Mean weight of tubers for control and all treatments on potato crop

Study groups	Mean weight of tubers (g)	SD(±)	SE(±)	ANOVA with LSD analysis results	
				MCG	SV
Control	221.27	5.68	3.28	T-80 (1000 ppm)	0.108
				T-80 (2000 ppm)	0.020*
				AgNPs	0.000*
				CF	0.000*
T-80 (1000 ppm)	267.17	10.15	5.86	Control	0.108
				T-80 (2000 ppm)	0.430
				AgNPs	0.000*
				CF	0.000*
T-80 (2000 ppm)	289.26	8.79	5.07	Control	0.020*
				T-80 (1000 ppm)	0.430
				AgNPs	0.000*
				CF	0.000*
AgNPs	595.93	14.47	8.35	Control	0.000*
				T-80 (1000 ppm)	0.000*
				T-80 (2000 ppm)	0.000*
				CF	0.163
CF	556.33	56.43	32.58	Control	0.000*
				T-80 (1000 ppm)	0.000*
				T-80 (2000 ppm)	0.000*
				AgNPs	0.163

*Values are significant at 0.05 significance level

CONCLUSION

It may be concluded by the above study that AgNPs can be a potential control agent against late blight of potato as extensively used chemical fungicides 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. Further studies are needed in future to investigate whether the application of AgNPs on plants may

cause unwanted damage to useful flora and fauna. Also, lethal dosage against pathogen and formulation of effective dosage need to be done, so as to minimize the chances of harm to humans and animals due to bio-accumulation of AgNPs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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