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EVALUATION OF MYCOGENIC SILVER AND ZINC OXIDE NANOPARTICLES AS POTENTIAL CONTROL AGENT AGAINST EARLY BLIGHT (*ALTERNARIA SOLANI*) OF POTATO (*SOLANUM TUBEROSUM* L.)

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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. Early blight disease has been the most serious threat to world's potato production, resulting in 80-100 % yield loss. The causal agent of early blight is fungi, *Alternaria solani*. The present investigation was carried out to evaluate the efficacy of mycogenic silver nanoparticles (AgNPs) and zinc oxide nanoparticles (ZNOPs) as antifungal agents against *A. solani*. The nanoparticles were synthesized biologically by using *Aspergillus flavus* biomass and characterization was done by UV-Vis Spectroscopy, Field Emission Scanning Electron Microscopy (FESEM), Energy Dispersive X-Ray (EDX), Dynamic Light Scattering (DLS), Fourier Transform Infra Red (FTIR) and inductively coupled plasma mass spectrometry (ICPMS). The inhibition percentage of *A. solani* caused by nanoparticle treatments was established *in vitro*. Field experiment was conducted to compare the efficiency of nanoparticles and chemical fungicide at the parameters of disease severity, tuber number and tuber weight. The data were analyzed by SPSS for descriptive statistics and analysis of variance (ANOVA) including least significance difference (LSD) and results were found to be significant for different parameters at 0.05 significance levels. It may be concluded by the experiment that AgNPs and ZONPs may be proved to be potential fungicides in near future and it is an excellent alternative to chemical fungicides.

Keywords: *Solanum tuberosum, Alternaria solani, Aspergillus flavus,* Silver nanoparticles, Zinc oxide nanoparticles, Nono-fungicides, DLS, FTIR, SEM, EDX.

1. INTRODUCTION

Potato does not need exceptional growth conditions; it has been for a long time a most important field crop in temperate regions, and progressively in warmer region [1]. This is one of the most productive and widely grown food crops in the world and produces approximately twice as many calories per hectare as rice and wheat [2]. There are several factors responsible for low potato production in India. 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 diseases early blight is the most important one affecting potatoes [3]. Early 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 [4]. Early blight is a challenging plant disease to the major economic host crops potato (*Solanum tuberosum* L.) and tomato (*S. lycopersicum* L.). The causal agent of early blight is *Alternaria solani*, which are well host plants susceptible to infection [5]. Complete field destruction due to late blight epidemics are relatively common in worldwide leading to 3-5 billions of economic loss 6].

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. Repetitive use of fungicides and antibiotics on the plant surfaces for disease management leads to development of resistance in the plant pathogens [7]. Due to the extensive use of fungicides and pesticides there is rapid increase in ecotoxicity and resistance development in plant pathogenic microbes [8-10]. Biological control methods for the disease management of phyto-pathogens

have been useful [11]. In contrast to conventional application of fungicides and antibiotics; nanoparticles can be proved to be excellent strategy to manage plant diseases [12]. Nano-biotechnology has emerged as one of the fastest growing modern areas of research in material sciences and technology [13]. Nanoparticles are synthesized by physical, chemical and biological methods. Physical and chemical methods are energy intensive and cause toxicity to environment whereas, biogenic technique is eco-friendly, non-toxic and economically viable [14]. In the present research the antimicrobial activity of biologically synthesized silver nanoparticles (AgNPs) and zinc oxide nanoparticles (ZONPs) has been evaluated against *A. solani*.

2. MATERIAL AND METHODS

2.1. Culturing of *Aspergillus flavus*

The pure culture of *Aspergillus flavus* was obtained from Department of Biotechnology, C.C.S. University, Meerut and sub-cultured on Potato Dextose Agar (PDA) Media (to preserve) and Malt Glucose Yeast Peptone (MGYP) Media (to generate biomass for nanoparticle synthesis).

2.2. Biosynthesis of AgNPs and ZONPs by A. flavus biomass

This was done as per the method given by Majeed et al [15]. Fungal biomass was separated from media by filtration and washed to remove any particle of media. Then 10 g of biomass was introduced in 1000 ml of distilled water and flask was kept at 160 rpm for 72 hrs. The biomass was filtered and 0.17 g of silver nitrate ($AgNO_3$) was mixed with the 500 ml filtrate to prepare 2mM $AgNO_3$. Rest of the 500 ml filtrate was kept as control. Same protocol was followed for synthesis of zinc nanoparticles using zinc nitrate hexa hydrate. 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).

2.3. Characterization of AgNPs and ZONPs

The synthesized AgNPs and ZONPs were characterized by UV-Visible Spectrophotometer (in Department of Genetics and Plant Breeding, CCSU, Meerut), Field Emission Scanning Electron Microscopy (FESEM) facilitated from Indian Institute of Technology, Kanpur, Energy Dispersive X-Ray (EDX) facilitated from Indian Institute of Technology, Kanpur, Fourier Transform Infra Red (FTIR) facilitated from SAIF, Indian Institute of Technology, Bombay and Dynamic Light Scattering (DLS) facilitated from Motilal Nehru National Institute of Technology, Allahabad.

2.4. *In vitro* testing of AgNPs and ZONPs against *A. solani*

The antimicrobial assay was done against *A. solani* by method as given by Banik and Luque [16]. Potato dextrose agar media was used to cultivate the test fungal species. One ml of AgNPs and ZONPs solutions were poured with PDA medium into different petri plates and media without nanoparticles was used as control. After solidification of the medium, each Petri plate was inoculated centrally from the growing margins of the seven days old culture of the test fungi using inoculating loop. Petri plates were incubated for 5 days at 25 ± 1^0C. The diameters of the colonies were recorded after 72 hrs and the percentage inhibition was calculated using the formula:

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

Where, I= Inhibition percentage; C = Radial growth to the fungus in control plates; T= Radial growth of the fungus in the petri dish with medium containing the AgNPs and ZONPs.

2.5. Field experiments and statistical analysis

Field experiments were carried out at Chaudhary Charan Singh University, Meerut (Uttar Pradesh, India) during winter seasons of year 2018 (sowing date 29 Oct, 2018), to evaluate the efficacy of AgNPs and ZONPs application on severity of early blight disease of potato plants under natural field conditions. The 'Sadabahar 3797' variety of potato was sown, which is generally cultivated in western Uttar Pradesh (India). The nanoparticles were compared with recommended dose of commercially available fungicide Abic® syngenta® (containing Mancozeb), Tween 80 (1000 ppm and 2000 ppm) and a control (potato plants without any treatment). Each treatment was applied in three replication rows, each row containing 55 plants. The spore suspension of *A. solani* was sprayed on the plants after 20 days of sowing. All treatments were applied as foliar spray three times with 10 days interval. Disease severity (DS) was calculated as per the method followed by El-Batal et al [17]. 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 determination of least significant difference (LSD) was done with SPSS (Statistical Package for Social Sciences) software.

3. RESULTS AND DISCUSSION

3.1. Biosynthesis of AgNPs and ZONPs using *Aspergillus flavus*

The solutions started to appear mustard yellow and pale yellow after 24 hrs in the reaction mixtures for AgNPs and ZONPs respectively, indicated the formation of nanoparticles. A number of the workers in the past have reported the synthesis of extracellular AgNPs and ZONPs with the help of fungal biomass. *Aspergillus sp* has been used for nanoparticles synthesis in various researches in past [18-23]. Various other fungal species have been reported to facilitate synthesis of AgNPs and ZONPs viz *Penicillium oxalicum, Trichoderma longibrachiatum, Arthroderma fulvum, Aspergillus fumigatus* , *Aspergillus niger*, *Candida albicans*, *Penicillium italicum*, *Syncephalastrum racemosum*, *Fusarium oxysporum*, *Alternaria solani*, *Aspergillus ochraceus* etc [24-29].

3.2. UV-Vis analysis of AgNPs and ZONPs

The nanoparticles 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 and ZONPs. The scanning range 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 reference. The samples were analyzed at 0, 4, 12, 24, 48, 72, 96 and 120 hrs. The band gap increases with decreasing particle size. AgNPs generally show a broad peak in the UV-Visible spectrum in the range of 400-450 nm [30]. In the present study the optical transitions for AgNPs have been observed at 430 nm (Fig 1). ZONPs gave peak at 290 nm, 330 nm and 350 nm (Fig 2). The result was matched with Fakhari et al. [31]. According to Jamdagni et al., the range of UV spectrum for ZnO NPs is 320-390 nm [32]. The reaction stabilized after 96 hrs.

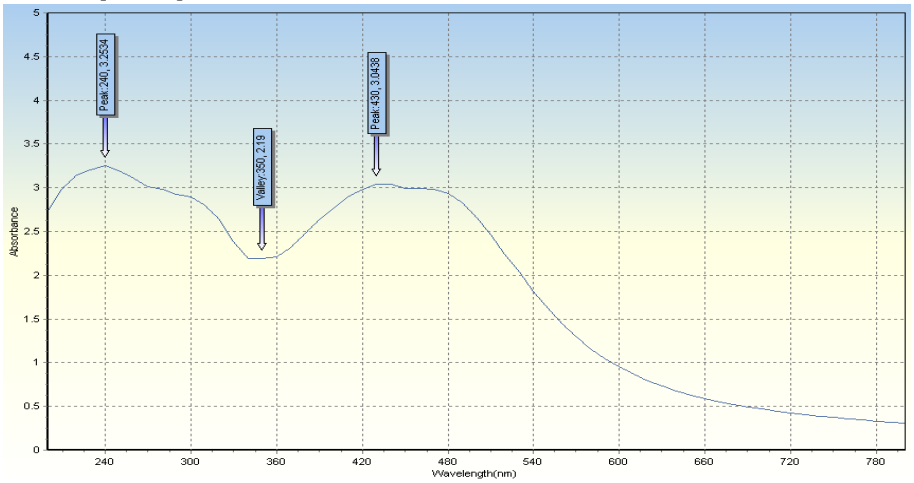

Fig 1: UV-Vis graph of 96 hrs sample of AgNPs

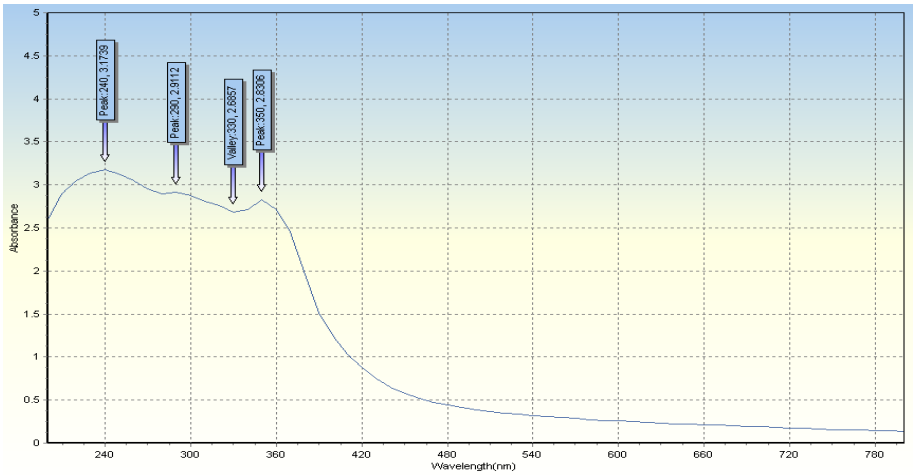

Fig 2: UV-Vis graph of 96 hrs sample of ZONPs

3.3. Analysis of AgNPs and ZONPs by FESEM

Among various electron microscopy techniques, 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 [33]. The nanoparticles dried samples were prepared by placing two drops (200µl) of AgNPs and ZONPs mixture 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 image taken indicated that nanoparticles are well distributed with the lowest agglomeration of nanoparticles (Fig 3 and 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.

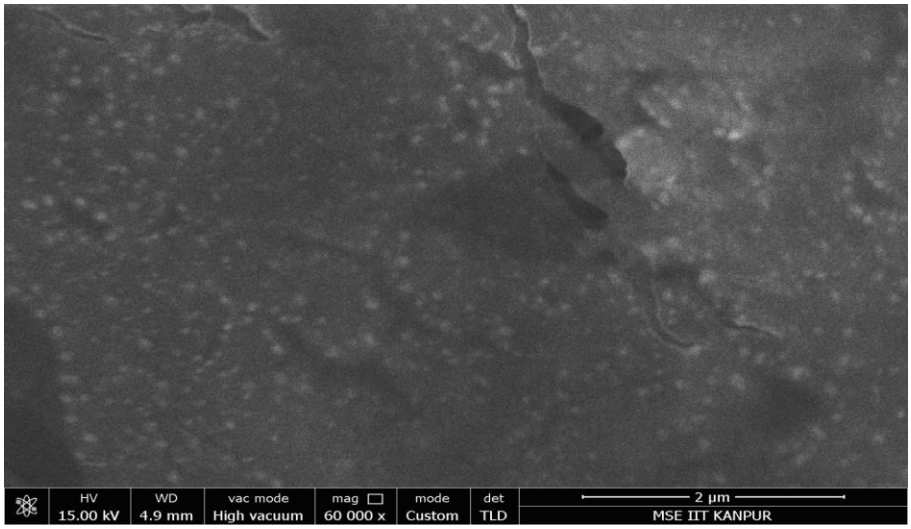

Fig 3: FESEM image of AgNPs

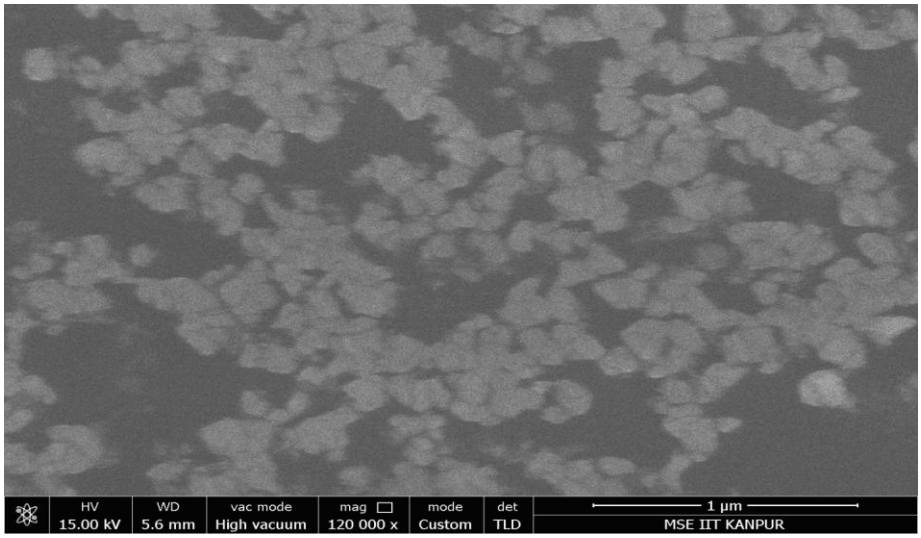

Fig 4: FESEM image of ZONPs

3.4. Analysis of AgNPs and ZONPs 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) and ZONPs (Fig 6). 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, O and Al were found to be 23.33%, 26.56% and 50.11% respectively. Peaks corresponding to Zn, O, P (phosphorous), K (potassium) and Al were detected in ZONPs sample.

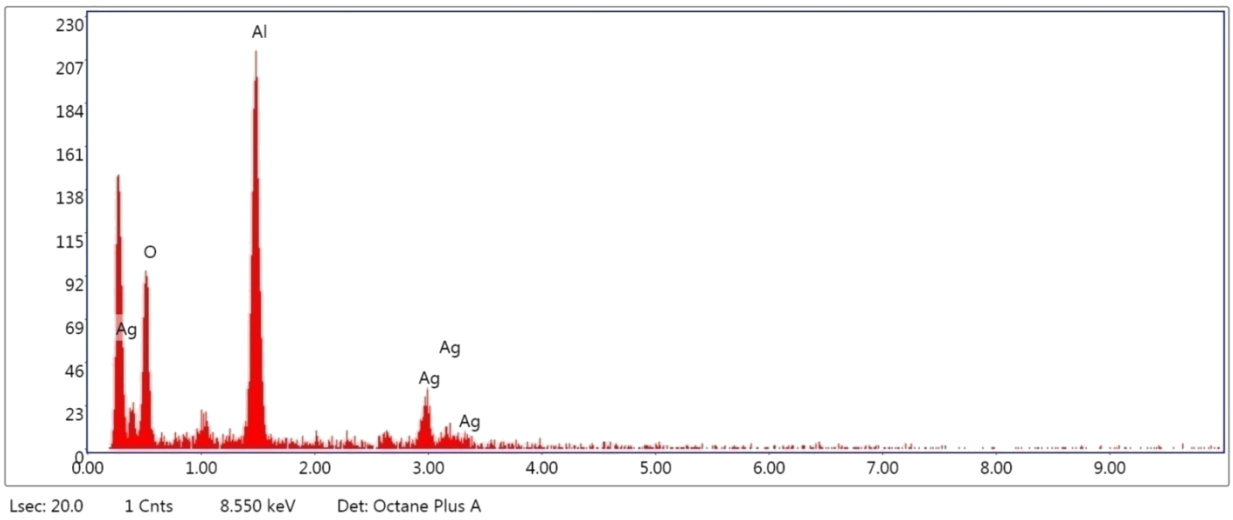

Fig 5: EDX analysis graph of AgNPs, where X-axis is showing the energy in keV and Y-axis is signifying intensity count

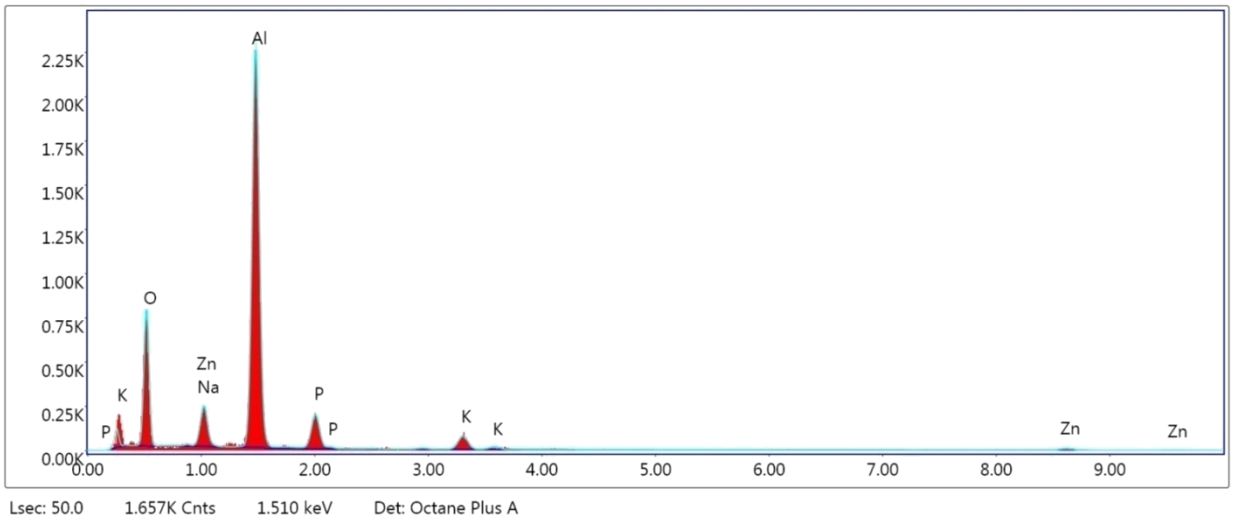

Fig 6: EDX analysis graph of ZONPs

3.5. Analysis of AgNPs and ZONPs 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 and ZONPs. Among the techniques of nanoparticles characterization DLS is the most commonly used [34-36]. The samples were sent to Centre for interdisciplinary Research (CIR), Motilal Nehru National Institute of Technology (MNNIT), Allahabad (U.P.), India. The aqueous samples were ultrasonicated before processing under DLS. The DLS size distribution images of biosynthesized AgNPs and ZONPs are shown in Fig 7 and 8. It showed that the average sizes of AgNPs and ZONPs are 85.21 nm and 90.05 nm respectively.

3.6. Analysis of AgNPs and ZONPs 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 nanoparticles (Fig 9 and 10). Absorbance peaks of AgNPs & ZONPs and their corresponding functional groups are given in table 1.

3.7. ICPMS analysis

ICPMS technique availed from Indian Institute of Technology (IIT), New Delhi to determine the parts per million (ppm) concentrations of synthesized AgNPs and ZONPs which was found to be 344.411 ppm 57.654 ppm respectively.

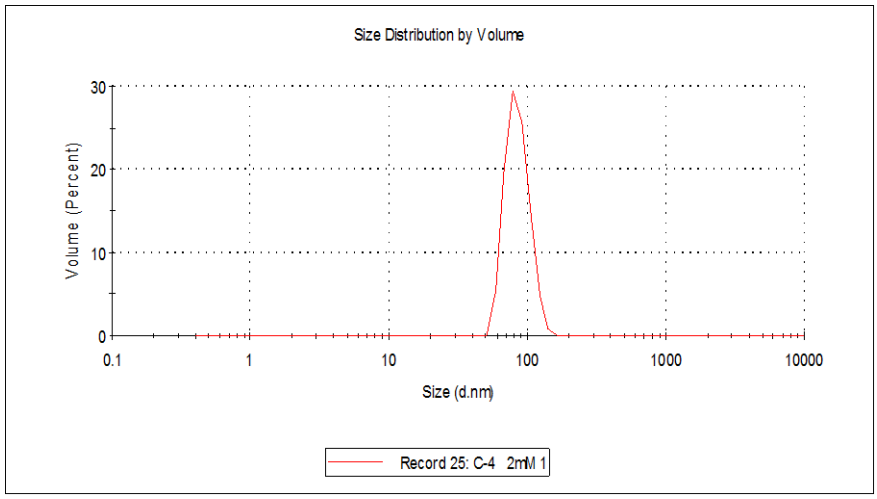

Fig 7: DLS distribution of AgNPs

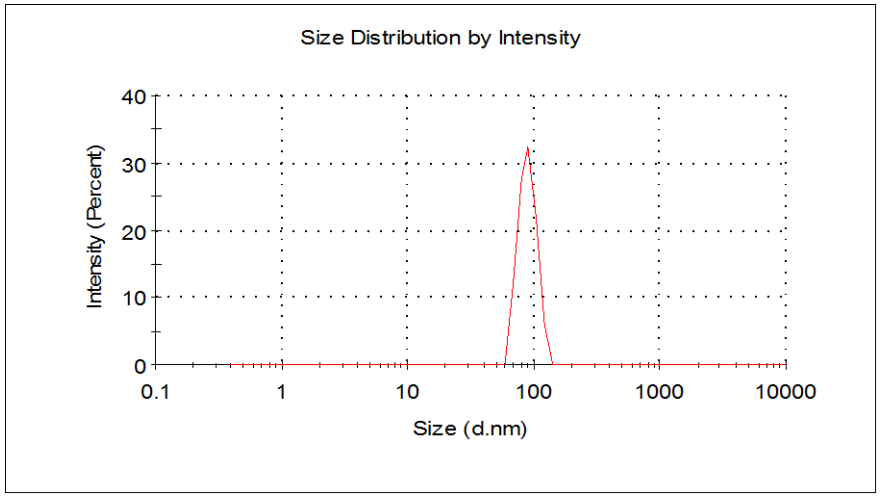

Fig 8: DLS-data of ZONPs

Table 1: FTIR Peak values and corresponding functional groups or compounds

Wave Numbers cm^{-1}		Functional Groups/ Compound	Reference
AgNPs	ZONPs		
3862.12 3745.06 3454.83	3848.59 3745.80 3562.72	O – H	[37]
2919.76 2851.21	–	C – H	[37]
1639.75	1646.68	C=C	[38]
1514.17	1551.11	N – O	[38]
1464.44	1451.20	Alkane, aromatic compound	
1383.65 1237.39	1276.46	Alhiphetic chain, sulphonic acid	
–	1153.75		[39]
1026.30	996.53	Si –O – C	[39]
609.14	618.49	Alkyl halides	[40]
498.75 422.68	498.80 420.93	S – S	[40]

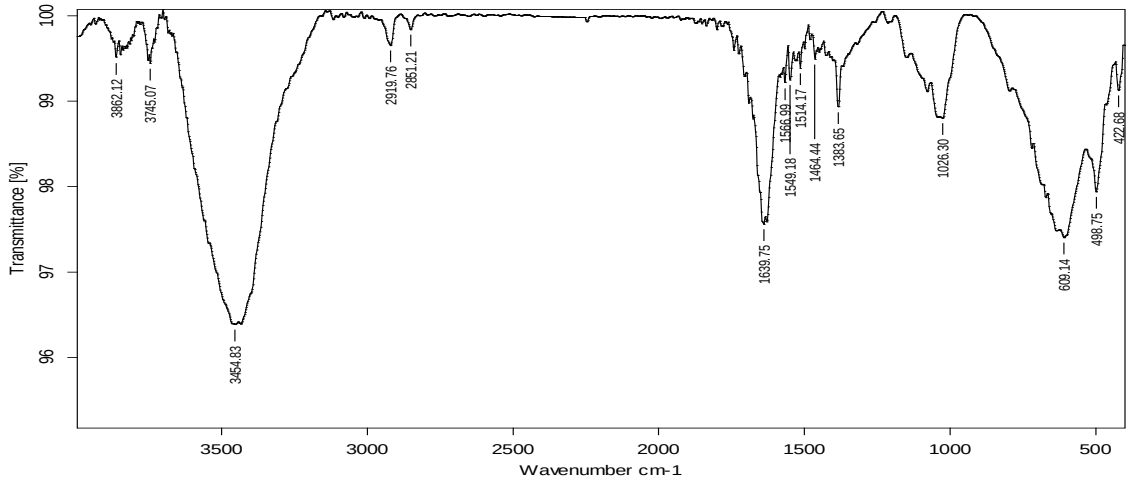

Fig 9: FTIR-spectrum of AgNPs

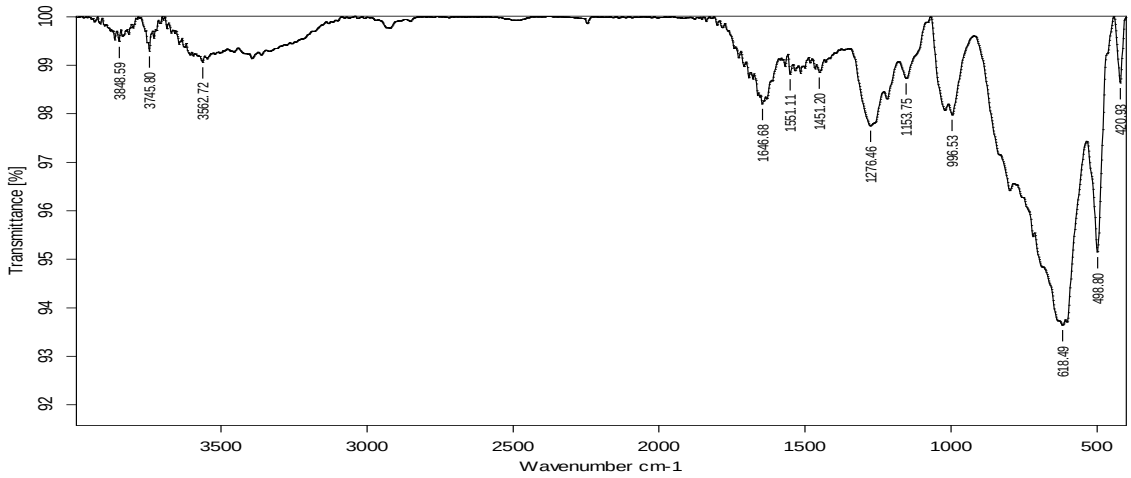

Fig 10: FTIR-spectrum of ZONPs

3.8. Inhibition Percentage *in vitro*

The AgNPs, ZONPs and chemical fertilizer treatments showed inhibition percentage (IP) as 85.29%, 47.05%, 41.11% respectively after 72 hrs of fungal inoculation in media (Fig. 11).

3.9. Results of Field experiments

The highest and lowest DS was recorded in control and AgNPs treatment respectively (table 2). DS was found to be significantly different in control and nanoparticle treatmets at 0.05 significance levels. The results of average weight and number of tubers per treatment are given in table 3 and 4 respectively. The highest weights of tubers 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. For number of tubers, ANOVA was found to be significant at 0.05 levels for the difference in tuber numbers among control, AgNPs, ZONPs and chemical fungicide treatment.

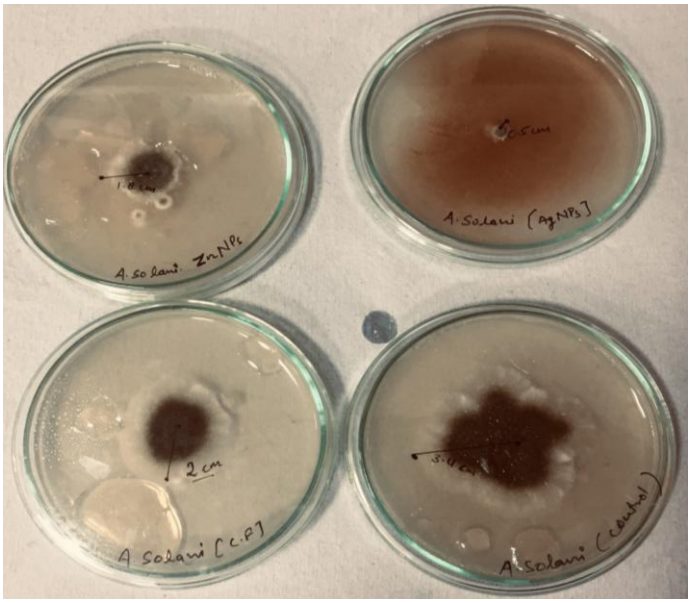

Fig.11: Growth of *A. solani* under (a) ZONPs treatment, (b) AgNPs treatment; media is appearing brown due to color of AgNPs, (c) Chemical fertilizer treatment and (d) Control plate.

Table 2: Mean DS caused by *A. solani* for all treatments on potato crop

Treatments	Mean DS (%)	Std.Deviation (SD)
Control	76.2	±3.67
AgNPs	17.3	±0.70
ZONPs	19.9	±1.21
Chemical fungicide	23.3	±2.58

Table 3: Mean weight of tubers per treatment

Treatments	Mean weight of tubers (g) (%)	(SD)
Control	74.1	±2.75
AgNPs	118.5	±4.52
ZONPs	109.5	±3.26
Chemical fungicide	113.9	±1.94

Table 4: Average number of tubers per treatment

Treatments	Average number of tubers per treatment	(SD)
Control	6.2	±0.20
AgNPs	8.3	±0.30
ZONPs	8.0	±0.26
Chemical fungicide	6.9	±0.30

4. CONCLUSION

It may be concluded by the above study that nanoparticles can be a potential control agent against early 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 and ZONPs against this fungus, so that nano formulations may be prepared accordingly.

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