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Effect of Zinc Oxide, Sulphur and Silver Nanoparticles against Soybean Rust *Phakopsora pachyrhizi* Syd.

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ABSTRACT

Background: Soybean rust caused by *Phakopsora pachyrhizi* Syd. is one of the major constraints in the production of soybean. Nanotechnology is a new scientific approach that includes use of materials and equipment capable of using physical and chemical properties of a substance at molecular level to explore the biological and material worlds in nanometer-scale and use it in various carriers from medicine to agriculture. Nanotechnology has emerged as one of the most innovative scientific field in agriculture. Among various synthesis methods, green synthesis of nanoparticles is widely used by researchers because it is simple, convenient, eco-friendly and requires less reaction time.

Methods: The chitosan based zinc oxide nanoparticles (Ch-ZnONPs), pomegranate aril based sulphur nanoparticles (PA-SNPs) and pomegranate aril based silver nanoparticles (PA-AgNPs) were synthesised, characterised and evaluated under *in vitro* conditions against soybean rust pustules.

Result: The maximum per cent spore inhibition of 70.25, 74.70 and 86.68 per cent over control was recorded in Ch-ZnONPs (1250 ppm), PA-SNPs (2000 ppm) and PA-AgNPs (500 ppm) respectively.

Key words: Chitosan, Nanoparticle, Pomegranate, Silver, Soybean rustzinc.

INTRODUCTION

Soybean (*Glycine max* L.), an oilseed crop of leguminaceae, which is also known as soja bean or soya bean, is the most important source of vegetable protein with all the essential amino acids to the human kind. In the World, soybean crop covers an area of 122.65 million hectares (mha) with a production and productivity of 339.42 million metric tons (mMT) and 2.77 metric tons per hectare (MT/ha) cultivated across United States of America, East Asia. European countries, Middle East, African countries and Soviet Union. In India, the crop coverage accounts to 12.19 m ha with 9.30 mMT and 0.76 MT/ha as production and productivity respectively. Rajasthan, Madhya Pradesh, Maharashtra andhra Pradesh, Chattisgarh, Gujarat and Karnataka are the states of India, where this particular crop is grown.

The production of soybean is vulnerable to many diseases, among which 35 are the economically important diseases. The major fungal diseases are rust (*Phakopsora pachyrhizi*), collar rot (*Sclerotium rolfsii*), charcoal rot (*Macrophomina phaseolina*), purple seed satin (*Cercospora kikichii*), fungal foliar spots like Alternaria leaf spot (*Alternaria* sp.), frog eye leaf spot (*Cercospora sojina*), anthracnose (*Colletotrichum truncatum*), target leaf spot (*Corynespora cassicola*), Myrothecium leaf spot (*Myrothecium roridum*) and brown spot (*Septoria glycine*).

Soybean rust is one of the major foliar fungal diseases caused by *Phakopsora pachyrhizi* which was first reported in Japan during 1903 (Sinclair and Shurtleff, 1975). In India it was first reported from Pantnagar during 1970 (Sarbhoy *et al*., 1972) and later the incidence was reported from other parts of the country. The disease can cause a yield loss of upto 80 per cent (Patil and Basavaraj, 1997). The symptoms are characterized by the appearance of yellow lesions with a brown speck at its center, found on the lower surface of the leaf. The lesions continue to develop into light to dark brown pustules, which later advances in plant symptomatically recorded as yellowing, pre-mature drying and defoliation (Singh and Thapliyal, 1977).

Chemical management strategies that include the plant protection pesticides have been proved to impose risk to the environment beside the development of resistance to plant protection chemicals in plant pathogens. This has led to an era to explore new management strategies of which nanotechnology is gaining scope in management of disease as well as in detection of the pathogen. Nanotechnology is an approach that deals with the reduction of bulk material into Nano size that ranges between 0.1 to 100 nm. Richard Philips Feynman introduced the concept of nanotechnology in 1959 and Professor Norio Taniguchi coined the term.

Exploration of new molecules or materials for the control of diseases is taking a scope due to the development of

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resistance in plant pathogens by excessive of antimicrobial compounds that are used in the field production process and nanotechnology has become a novel technique in the field of plant pathology. Hence, this study was taken up to synthesize and evaluate the effect of nano particles against soybean rust disease. The present study focuses on the synthesis of chitosan-based zinc oxide nanoparticles and pomegranate aril extract based Sulphur and silver nanoparticles.

MATERIALS AND METHODS

In this study, synthesis of chitosan-based Zinc oxide (ZnO) nano particles, Pomegranate aril extract-based Sulphur nanoparticles and pomegranate aril extract-based silver nano particles were synthesized.

Chitosan-based ZnO nanoparticle synthesis (CZNP)

The bulk ZnO was used to prepare zinc nanoparticles by dissolving 125 mg of ZnO in hot distilled water using hot distilled water and subsequently added 10 ml of 1 per cent water soluble chitosan followed by 1 ml of glycerol. The mixture was continuously stirred for 24 hours, later irradiated for 30 seconds and sonicated for 30 minutes.

Green synthesis of sulphur and silver nanoparticles

Locally available pomegranate fruits were used to obtain the peel extract. This extract was used as reducing agent in the synthesis of Sulphur (S) and Silver (Ag) nanoparticles.

The pomegranate aril based sulphur nanoparticle (PSNP) synthesis involves, preparing mixture of 100 ml of 18 per cent pomegranate aril extract in which 3 g of sodium thiosulphate was dissolved, this solution was stirred for 5 minutes. To this mixture, 2 ml of 20 per cent citric acid was added and the components were continuously stirred for 6 hours using magnetic stirrer. After 6 hours, the components were cooled and subjected to ultrasonication for 30 minutes.

Pomegranate aril-based silver nanoparticles (PAgNP) was prepared by adding 3 ml of 20% pomegranate aril extract into 50 ml of silver nitrate ($AgNO_3$) and stirring using magnetic stirrer continuously for 60 minutes. The mixture was cooled and sonicated for 30 minutes.

Characterization of the nanoparticles

The synthesized nanoparticles *i.e.*, CZNP, PSNP and PAgNP were subjected to particle size analyzer and scanning electron microscope (SEM) to determine the shape and size. Later the presence of nanoparticles was detected using energy dispersive X-Ray analysis (EDAX).

In vitro assay of nanoparticles on uredospore germination

Soybean leaf samples showing severe infection with maximum rust pustules were collected from highly susceptible variety JS 335. Rust spores were harvested immediately from the infected leaves by scrapping the spores from the infected leaf tissue of soybean with help of a sterile scalpel. Uredospores were collected in sterilised two ml centrifuge tubes, labelled and stored at -20°C in deep freezer for further studies (Sachin, 2012).

The CZNP was evaluated at 100, 500, 1000 and 1250 ppm concentration along with control namely bulk zinc oxide (ZnO) at 1250 ppm, Water soluble chitosan (WSC) at 10000 ppm, hexaconazole at 1000 ppm concentration as treatments and water as control (Table 1). PSNP was used at 100,500,1000 and 2000 ppm concentration, with sodium thiosulphate (2000 ppm), pomegranate aril extract (10%), hexaconazole (1000 ppm) and water as control (Table 2). Apart from these, PAgNP was used at 50, 100, 250 and 500 ppm concentration along with bulk $AgNO_3$ (500 ppm), pomegranate aril extract (10%), hexaconazole (1000 ppm) and water as control (Table 3).

Three nanoparticles at four different concentrations along with corresponding bulk materials and control were tested *in vitro* on the germination of uredospores of *P. pachyrhizi* by following the cavity slide method. Each treatment was replicated three times. Uredospores were observed for germination at 24 h. after incubation in moist chamber (Sachin, 2012). A control treatment was maintained with distilled water. Per cent inhibition over control was calculated by using the formula given by Vincent (1947). The uredospores were observed for germination at 24 hours after incubation in moist chamber. The per cent inhibition over control was calculated by using the formula given below by Vincent (1947).

Per cent spore germination =

$$\frac{\text{Spore germination in control-} \\ \text{Spore germination in treatment}}{\text{Spore germination in control}} \times 100$$

RESULTS AND DISCUSSION

Three nanoparticles *viz.,* chitosan based zinc oxide (Ch-ZnONPs) nanoparticles, pomegranate aril based sulphur nanoparticles (PA-SNPs) and pomegranate aril based silver nanoparticles (PA-AgNPs) were synthesized by using the standardised protocol as mentioned in materials and methods. The characterisation of the above synthesised nanoparticles was done by Particle size analyser (PSA) and Scanning Electron Microscope (SEM) and Energy Dispersive X-Ray Analysis (EDAX). In PSA, the mean diameter of Ch-ZnONPs, PA-SNPs and PA-AgNPs was recorded as36.5 nm, 79.8 nm and 78.7 nm respectively (Plate 1). The SEM images show the size and shape of the nanoparticles. In Ch-ZnONPs, the particle is rod shaped and in PA-SNPs and PA-AgNPs, the shape is spherical to irregular (Plate 2). The EDAX image confirms the presence of element in synthesised nanoparticle (Plate 3).

Chitosan (Ch) is used in the synthesis of zinc oxide nanoparticles, which is one of the most abundant natural polymers, powerful chelating agent, non-toxic, biodegradable and biocompatible, which easily form complexes with transition metals and heavy metals and shows antimicrobial and antibacterial activity (Liu and Huang, 2008). The antimicrobial activity of chitosan was observed against microorganisms *viz.,* fungi, algae and some bacteria (Eun *et al.* 2010; Allan and Hadwiger, 1979). The higher antimicrobial activity is because of the stronger positive charge after complexation. Du *et al.* (2009) found that antimicrobial properties of chitosan were enhanced by loading chitosan with various metals, especially for Cu and Zn, as compared to nanoparticles of chitosan or metal nanoparticles alone. The disruption of membrane structure, the release of reactive oxygen species and hydrogen peroxide are mainly involved in the antimicrobial potential of ZnO NPs (Lipovksy *et al.* 2011; Sawai *et al.* 1998 and Sinha *et al.* 2011).

The cost effective and eco-friendly technique of green synthesis of silver and sulphur nanoparticles is from the pomegranate aril extract, which is used as a reducing agent and it has good antioxidant and antimicrobial properties. In the synthesis of silver nanoparticles, the change in colour was noticed when pomegranate aril extract was incubated with aqueous solution of $AgNO_3$. It started to change colour from watery to yellowish brown due to the reduction of silver ions, this exhibit the formation of silver nanoparticles. The change in colour increased with increase in time initially, later after dark brown colour formation further there is no

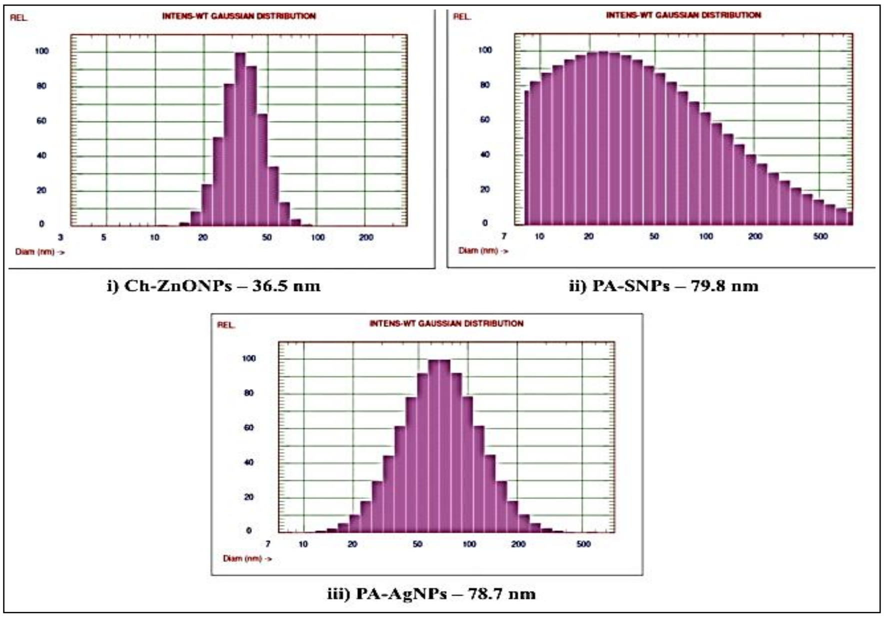

Plate 1: Characterization of synthesized nanoparticles using particle size analyzer (PSA).

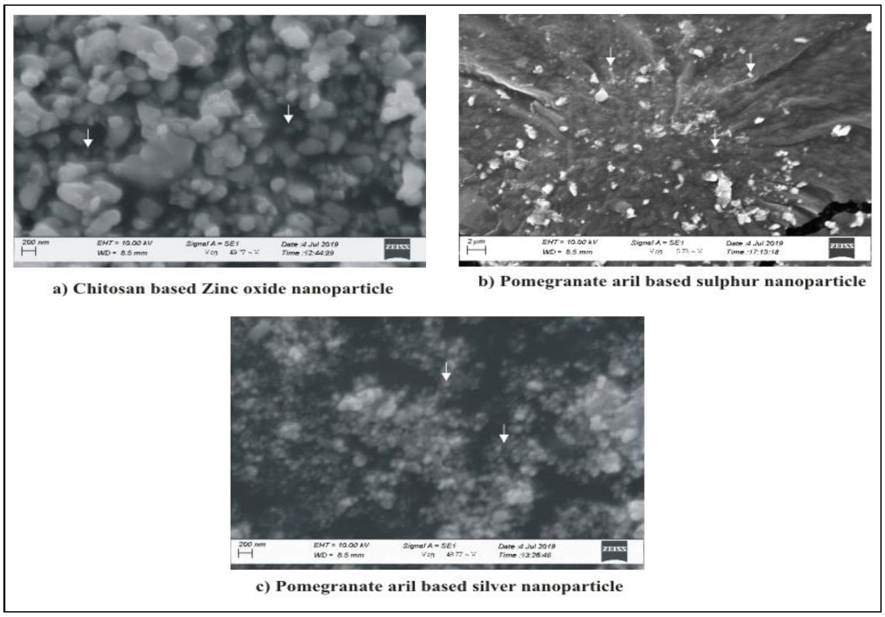

Plate 2: Characterization of synthesized nanoparticles using scanning electron microscope (SEM).

colour change. The pomegranate aril extract without silver nitrate solution did not show any change in colour. The similar observations were made by earlier workers (Chauhan *et al.* 2011 and Shanmugavadivu *et al.* 2014) and demonstrated that pomegranate seed extract is capable of producing Ag from the aqueous solution of Ag^{+}. Khalil *et al.* (2013) reported change in colour (brown) of aqueous solution of $AgNO_3$ resulting in silver nanoparticle formation when olive leaf extract was used as reducing agent.

In PSA, the average diameter of particles in Ch-ZnONPS, PA-SNPs and PA-AgNPs were 36.5 nm, 79.8 nm and 78.7 nm respectively which indicates that the size of particle is at nanoscale. The chitosan, pomegranate aril, olive leaf extract and garlic leaf extract and so on used as reducing agents, results in reduction in mean diameter of zinc, sulphur and silver nanoparticles (Jobitha *et al*., 2013; Choudhary *et al*., 2019; Khalil *et al*., 2013).The average particle size of nanoparticle is highly influenced by concentration of the reducing agent. Khalil *et al.* (2013) reported that increase in olive leaf extract concentration in the synthesis of silver nanoparticles resulted in decrease in particle size.

The SEM images of Ch-ZnONPS appeared as rod shape, whereas, PA-SNPs and PA-AgNPs were found to be spherical to irregular, represents that nanoparticles are poly dispersed and less than 15 nm in size, imparting long term stability and redispersibility to the nanocomposites without affecting their physical and chemical characteristics (Liu and Huang, 2008). The SEM image of ZnONPs is rod shaped which is in accordance with earlier worker Dhillon *et al.* (2014), that the shape of zinc oxide nanoparticles may be circular, elliptical and rod shaped, whereas Kaur *et al.* (2014) and Choudary *et al.* (2019) reported spherical shape of zinc nanoparticles. In SEM-EDX image of Ch-ZnONPs, showed the presence of oxygen and zinc in the peak which indicates the presence of chitosan and formation of ZnO nanoparticles in chitosan matrix as reported by Yin *et al.* (2004) and Vasile *et al.* (2011).

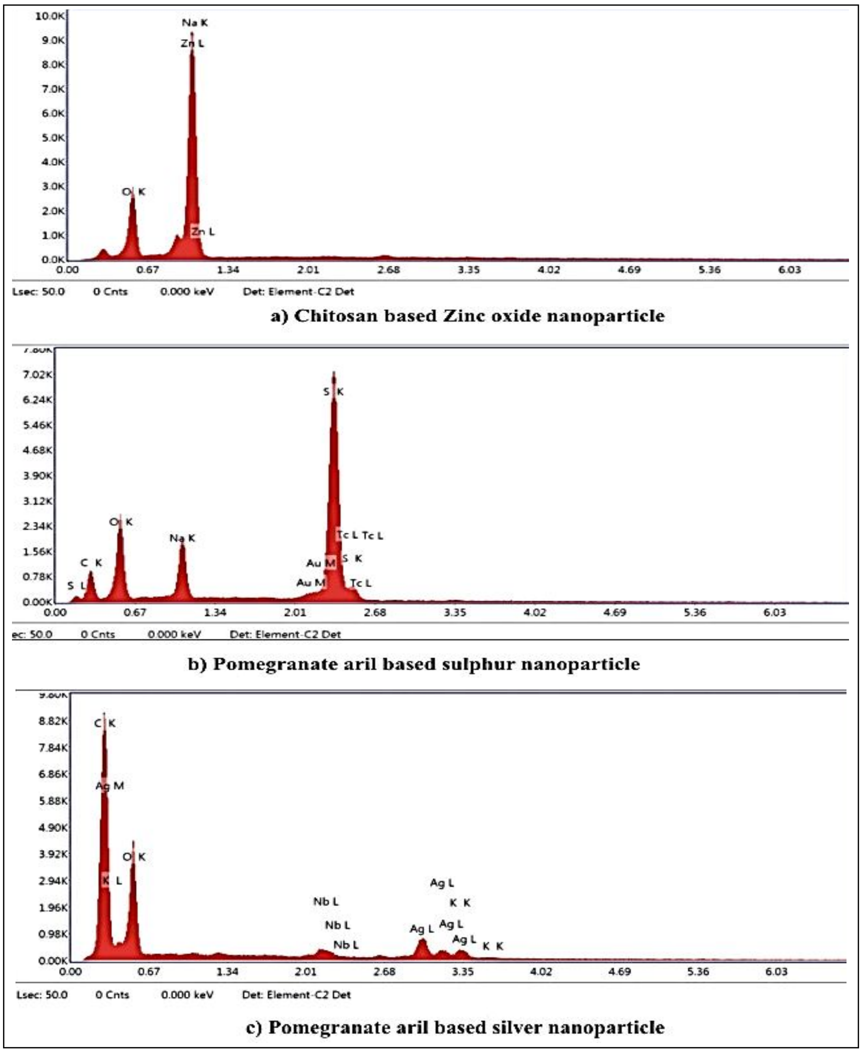

Plate 3: EDAX images of the respective synthesized nanoparticles.

The shape of PA-SNPs was spherical to irregular. Similar shape (smooth spherical shape) was reported by Khairan *et al.* 2019 and Salem *et al.* (2016) when synthesised sulphur nanoparticles using aqueous garlic extract and pomegranate peel extract respectively by reducing sulphur atoms to sulphur nanoparticles through nucleation process. The SEM-EDX images represented the presence of S, C, O and K.

The SEM image of PA-AgNPs was spherical to irregular. The findings were similar to earlier workers. Chauhan *et al.* (2011) reported silver nanoparticles as spherical in shape could be controlled by simple variation in the amount of extract with aqueous solution. The reduction of metal ions through fruit extracts leads to formation of spherical silver nanoparticles. Khalil *et al.* (2013) stated that at low quantities of olive leaf extract can reduce silver ions, but do not protect from aggregating because of the deficiency of biomolecules to act as a protecting agent. But at higher extract concentration, the biomolecules act as reducing agents and cap the nanoparticle surfaces protecting from aggregation. The EDAX images of silver nanoparticles confirm the presence of Ag along with O, C. The results were in accordance with Jobitha *et al.* (2013) and Ibrahim *et al.* (2016) that the silver nanoparticles were spherical in shape and the presence of some large particles may be due to the high surface activity and aggregation of smaller particles. EDS analysis of AgNPs revealed highest percentage of Ag followed by O, C, Ca, Cl and Al. The optical absorption peak appeared at 3 keV was due to the surface plasmon resonance of AgNPs.

In vitro evaluation

The green synthesised and characterised nanoparticles were evaluated under *in vitro* conditions against *Phakopsora pachyrhizi* at different concentrations by cavity slide method.

The chitosan based zinc oxide nanoparticles (Ch-ZnONPs) were evaluated against *Phakopsora pachyrhizi*. Among different concentrations of nanoparticles tested, the maximum per cent spore inhibition over control was recorded at 1250 ppm (70.25%) which was on par with 1000 ppm (70.00%) followed by 500 ppm (65.00%), 250 ppm (43.31%) and 100 ppm (28.88%). The bulk ZnO (1250 ppm) and water soluble chitosan (1%) resulted in 60 per cent and 31.64 per cent spore inhibition over control respectively, whereas, 100 per cent inhibition was observed in hexaconazole (Table 1).

In pomegranate aril based sulphur nanoparticles (PA-SNPs), the highest per cent spore inhibition was recorded at 2000 ppm (74.70%) which was statistically on par with 1000 ppm (74.08%) followed by 500 ppm (61.09%) and 100

Table 1: *In vitro* evaluation of Chitosan based zinc oxide nanoparticles (Ch-ZnONPs) on *Phakopsora pachyrhizi*.

Treatment	Concentration (ppm/%)	Spore germination inhibition (%)
Ch-ZnONPs	100 ppm	28.88 (32.49)*
	250 ppm	43.31 (41.14)
	500 ppm	65.00 (53.71)
	1000 ppm	70.00 (56.76)
	1250 ppm	70.25 (56.92)
Bulk ZnO	1250 ppm	60.00 (50.75)
Water soluble chitosan(WSC)	10000 ppm	31.64 (34.20)
Hexaconazole	1000 ppm	100.00 (89.96)
Control	-	-
S.Em. ±		0.55
C.D. @ 1%		2.30

*Arcsine values.

Table 2: *In vitro* evaluation of Pomegranate aril based sulphur nanoparticles (PA-SNPs) on *Phakopsora pachyrhizi*.

Treatment	Concentration (ppm)	Spore germination inhibition (%)
PA-SNPs	100 ppm	37.01 (37.45)*
	500 ppm	61.09 (51.39)
	1000 ppm	74.08 (59.38)
	2000 ppm	74.70 (59.78)
Sodium thiosulphate	2000 ppm	69.11 (56.21)
Pomegranate aril extract (PAE)	10%	30.88 (33.74)
Hexaconazole	1000 ppm	100.00 (89.96)
Control	-	-
S.Em. ±		0.56
C.D. @ 1%		2.38

*Arcsine values.

Table 3: *In vitro* evaluation of Pomegranate aril based silver nanoparticles (PA-AgNPs) on *Phakopsora pachyrhizi*.

Treatment	Concentration (ppm)	Spore germination inhibition (%)
PA-AgNPs	50 ppm	63.55 (52.85)*
	100 ppm	74.00 (59.32)
	250 ppm	86.15 (68.14)
	500 ppm	86.68 (68.58)
Bulk $AgNO_3$	500 ppm	75.73 (60.47)
Pomegranate aril extract (PAE)	10%	47.98 (43.83)
Hexaconazole	1000 ppm	100.00 (89.96)
Control	-	-
S.Em. ±		0.56
C.D. @ 1%		2.34

*Arcsine values.

ppm (37.01%). The sodium thiosulphate and pomegranate aril extract resulted in 69.11 and 30.88 per cent spore inhibition respectively over control (Table 2).

In pomegranate aril based silver nanoparticles (PA-AgNPs), the maximum per cent spore inhibition was recorded at 500 ppm (86.68%) which was on par with 250 ppm (86.15%) followed by 100 ppm (74.00%) and 50 ppm (63.55%). The bulk silver nitrate and pomegranate aril extract resulted in 75.73% and 47.98% spore inhibition respectively over control, whereas, 100 per cent inhibition was recorded in hexaconazole (Table 3).

In PSA, the average diameter of particles in Ch-ZnONPS, PA-SNPs and PA-AgNPs were 36.5 nm, 79.8 nm and 78.7 nm respectively which indicates that the size of particle is at nanoscale. The chitosan, pomegranate aril, olive leaf extract and garlic leaf extract and so on used as reducing agents, results in reduction in mean diameter of zinc, sulphur and silver nanoparticles (Jobitha *et al*., 2013; Choudhary *et al*., 2019; Khalil *et al*., 2013). The average particle size of nanoparticle is highly influenced by concentration of the reducing agent. Khalil *et al.* (2013) reported that increase in olive leaf extract concentration in the synthesis of silver nanoparticles resulted in decrease in particle size.

The Ch-ZnONPs showed strong *in vitro* antifungal activity against *Phakopsora pachyrhizi* at 1250 ppm (70.25%) which was on par with 1000 ppm (70%) compared to bulk ZnO (60%). The observations were found to be similar with He *et al.* (2011) in *Botrytis cinerea* and *Penicillium expansum*, Yehia and Ahmed (2013) in *Fusarium oxysporum* and *Penicillium expansum* and Al-Dhabaan *et al.* (2017) in *Ralstonia solani*.

In SNPs, the maximum per cent spore inhibition was recorded at 2000 ppm (74.70%) followed by 1000 ppm (74.08%) compared to sodium thiosulphate (69.11%). The AgNPs resulted in maximum spore inhibition at 500 ppm (86.68%) followed by 250 ppm (86.15%) compared to bulk silver nitrate (75.73%). Similar inhibitory effect of silver nanoparticles was made by Chauhan *et al.* (2011); Mishra *et al.* (2014); Ouda (2014), Alananbeh *et al.* (2017) in *Aspergillus flavus*, *Serratia* sps., *Alternaria alternate* and *Botrytis cinerea* and *A. niger* and *A. terreus* respectively. Gado *et al.* (2018) reported that the germination of uredospores was found to be strongly inhibited at all the concentration (0.25, 0.5 and 1 mM) tested against rose rust.

CONCLUSION

ZnONPs, SNPs and AgNPs were synthesised from water soluble chitosan and pomegranate aril extract as reducing agents by using the standardised protocols and characterization was done by Particle Size Analyser (PSA) and Scanning Electron Microscope (SEM). In PSA, the average diameter of particles in Ch-ZnONPS, PA-SNPs and PA-AgNPs were 36.5 nm, 79.8 nm and 78.7 nm respectively. In SEM, the Ch-ZnONPS appeared as rod shape. Whereas, PA-SNPs and PA-AgNPs were found to be spherical to irregular. Under *in vitro conditions*, Ch-ZnONPs resulted in maximum per cent spore inhibition (70.25%) at 1250 ppm and was on par with 1000 ppm (70.00%) over bulk ZnO (60.00%) and control. The PA-SNPs resulted maximum per cent spore inhibition at 2000 ppm (74.70%) over control. About 69.11, 30.88 and 100 per cent spore inhibition were observed in sodium thiosulphate, pomegranate aril extract and hexaconazole respectively. The maximum per cent spore inhibition (86.68%) was observed at 500 ppm in PA-AgNPs over bulk silver nitrate (75.73%), pomegranate aril extract (47.98%) and hexaconazole (100%).

Nanotechnology has been used in many fields like physics, chemistry, pharmaceutical science, material science, medicine and agriculture. Nanotechnology has emerged as one of the most innovative scientific field in agriculture. Now days, nanotechnology is extensively used in modern agriculture to make true, the concept of precision agriculture. Due to small size, high surface to volume ratio and unique optical properties, it is used in plant protection, nutrition and management of farm practices.

Conflict of interest: None.

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