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First Report on Synthesis of Green Nanoparticles and Their Bio-Efficacy against *Colletotrichum truncatum* Causing Pod Blight Disease in Soybean

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ABSTRACT

Background: Nanotechnology is emerging approach, which involves the process of reduction of bulk material into nano size particles. Metal based and green based particles can be used in crop management against disease causing pathogens. This study focuses on synthesis of nanoparticles to evaluate its effect on *Colletotrichum truncatum* that causes pod blight disease in soybean crop.

Methods: In this study, green nanoformulation namely, Chitosan based zinc oxide nanoformulation (ChZnNF), *P. fluorescens* based zinc nanoformulation (PfZnNF), Pomegranate aril-based Sulphur (PASNF) and Pomegranate aril-based silver nano formulations (PAAgNF) were synthesised through irradiation method. These nanoformulations were evaluated against *C. truncatum* through *in vitro* technique using poison food technique. The effective concentration of each formulation was also evaluated against *C. Truncatum* challenge inoculated soybean plants (two varieties). Per cent disease index in the glasshouse condition was recorded.

Result: In the *in vitro* analysis, it was found that among the four nanoformulations, PAAgNF was found to be the effective one to inhibit the mycelial growth of *C. truncatum* with per cent inhibition of 84.71. In the glasshouse study, PAAgNF did not record any disease symptoms besides no record of phytotoxicity symptoms. Thus the outcome indicated that PAAgNF is the effective green nanoformulation against *C. truncatum*.

Key words: Bioefficacy, Green nanoformulation, *In vitro*, Soybean, Synthesis.

INTRODUCTION

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. Nano agriculture has become a trend in researchers to promote application of non-toxic metal based and green nanoparticles in crop production and plant protection and thus, nanotechnology has scope and application in agriculture production (Khan and Rizvi, 2014). Risks involved in chemical management strategies 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. Nanoparticles can be synthesised through physical and chemical methods (Iravani *et al*., 2014), wherein microbial and plant extracts can beused (Mittal *et al*., 2013).

In disease management, nanotechnology exploits metalloids, metallic oxides, non-metals and plant and microbial based sources (Hou *et al*., 2016; Souza *et al*., 2020) and also involves to combine plant or microbial extracts.

Soybean crop production faces major challenges due to attack of plant pathogens of which anthracnose caused by *Colletotrichum truncatum* is economically important.

Nanotechnology has become a novel technique in the field of plant pathology and there is limited research in the analysis of efficacy of different nanoparticles against *C. truncatum*. Hence, this study was taken up to assess the efficacy under *in vitro* conditions.

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MATERIALS AND METHODS

ChZnNF

Bulk Zinc oxide (5 g) was dissolved in hot distilled water and stirred using magnetic stirred until completely dissolved. Water soluble chitosan (1 per cent) was filtered and added to the zinc oxide solution and stirred continuously for 48 hours and subjected to ultrasonication to obtain the nanoformulation (Vinay *et al*., 2016).

PfZnNF

Fresh broth culture of *P. fluorescens* was centrifuged to obtain the microbial extract. The bulk zinc oxide (5 g) was added to heated distilled water (850 ml) and stirred well. To this zinc oxide mixture and the microbial extract (150 ml)

was added and stirred for 24 hours. The final solution was subjected to ultrasonication to reduce the particles to nano size (Vinay *et al*., 2016).

PASNF and PAAgNF

Pomegranate aril obtained from fruits was sun dried and crushed in sterile pestle and mortar. The extract was filtered in Whattman 5 filter paper to remove impurities. This filtrate was used for the synthesis of sulphur and silver nanoformulation (Srikanth, 2018).

Sodium thiosulphate (3 g) was used as precursor for sulphur, which was added to 100 ml of 18 per cent of pomegranate aril filtrate and stirred well using magnetic stirrer. Further, ml of 20 per cent citric acid was added to the above mixture and stirred for 24 hours. The final solution was then subjected to ultrasonication to produce nanoparticles (PASNF).

For PAAgNF, silver nitrate (3 g), was added to 490 ml of distilled water and stirred well before boiling in microwave oven. Later, was stirred for 24 hours and subjected to ultrasonication for 30 minutes.

Characterization of nanoformulation

ChZnNF, PfZnNF, PASNF and PAAgNF were subjected to Particle Size Analyser (PSA) to determine the size of the nanoparticle in each of the formulation synthesised. Scanning Electron Microscope (SEM) (Carl Zeiss-EVO-18-UK) with Energy Dispersive X-Ray Spectroscopy (EDS was used to analyze the surface topology and particle morphology images of the nanoparticle in the nanoformulation. Besides,the element present in the nanoformulation was discerned in Energy Dispersive X-Ray Analysis (EDAX).

Evaluation of nanoformulation against *C. truncatum* by *in vitro* assay

In order to evaluate the affectivity of the nanoformulation, *in vitro* assay was carried out using the standard poison food technique (Shravelle, 1961) against *C. truncatum* using sterilized Potato Carrot Agar medium. The concentrations of each formulation are as discussed in Table 1-5.

Control was maintained by using PCA medium without incorporation of nanoformulation. Radial mycelial growth of the test fungus in treated and control treatments was recorded. Further, percent inhibition was calculated by using the formula given by Vincent (1947).

$$\text{Per cent inhibition} = \frac{(C-T)}{C} \times 100$$

Where,

C= Radial growth of mycelium in unamended medium (Control).

T= Radial growth of mycelium in amended medium.

Evaluation of nano formulation against *C. truncatum* under glasshouse condition

The effective concentration from each of the nano formulation was subjected to evaluation against *C. truncatum* under glass house pot experiment. Seeds of soybean (JS-355 and DSb21 varieties) were treated accordingly as explained in Table 5 and sown in pots filled with sterile pot mixture. Spraying of respective treatment was carried out at 30 and 40 days after sowing. The seedlings were challenged with the test fungus by artificial inoculation technique. Observations were recorded from the first day symptom appearance; Per cent disease index was calculated at three days interval and FESEM analysis.

Table 1: *In vitro* evaluation of Chitosan based zinc nanoformulation (ChZnNF) against *Colletotrichum truncatum*.

Treatments	Concentration	Per cent mycelial inhibition
Chitosan based zinc nanoformulation (ChZnNF)	500 ppm	8.20 (16.64)*^{i}
	1000 ppm	24.40 (29.52)^{h}
	1250 ppm	35.40 (36.51)^{g}
	1500 ppm	49.00 (44.44)^{ef}
Commercial zinc nanoparticle	500 ppm	44.30 (41.73)^{f}
	1000 ppm	50.10 (45.04)^{e}
	1250 ppm	57.30 (49.17)^{d}
	1500 ppm	63.70 (52.93)^{c}
Water soluble chitosan (WSC)	1%	61.20 (51.47)^{cd}
Bulk zinc oxide (ZnO)	1250 ppm	28.10 (31.96)^{h}
Carbendazim 50 WP	2000 ppm	73.50 (59.00)^{b}
Carboxin 37.5%+Thiram 37.5% DS	2000 ppm	100.00 (89.54)^{a}
Control	-	0.00 (0.46)^{i}
S.Em. ±		1.00
C.D. (0.01)		3.92
C.V. (%)		4.09

*Values are mean of three replication.

Figures in parenthesis are arc sine transformed value.

Phytotoxic effect of nano formulation on soybean plants

Phytotoxicity of nano formulation was assessed by spraying plants with various concentrationsat 1, 3, 5, 7, 9, 11, 13 and 15 days after the spray.

RESULTS AND DISCUSSION

Characterization of synthesized green nano formulation

The color of the end product of ChZnNF and PASNF was off white precipitate, PfZnNF was white to yellow precipitate and PAAgNF was chocolate brown to black respectively (Fig 1). The mean diameter recorded for ChZnNF, PfZnNF, PASNF and PAAgNF using Particle Size Analyser was 73.6 nm, 9.9 nm, 79.8 nm and 83.5 nm respectively as indicated in Fig 2-5. The SEM analysis with EDS revealed that zinc element was present in ChZnNF and PfZnNF, which was rod shaped (Fig 2) and rod to irregular shaped (Fig 3) respectively. Sulphur was detected in PASNF and was spherical to irregular in shape (Fig 4), while silver nanoparticle was detected in PAAgNF, which was spherical in shape (Fig 5).

Similar results were recorded by Vinay *et al*. (2016) who synthesized green nanoformulations *i.e. Pseudomonas fluorescens* and chitosan based zinc nanoformulation. The findings are in similarity with the results of various researchers who used chitosan in synthesis of zinc oxide nanoparticles (Jae-Wook *et al*., 2019).

Table 2: *In vitro* evaluation of *Pseudomonas fluorescens* based zinc nanoformulation (PfZnNF) against *Colletotrichum truncatum*.

Treatments	Concentration	Per cent mycelial inhibition
Pseudomonas fluorescens based zinc	500 ppm	36.60 (37.22)*^{i}
nanoformulation (PfZnNF)	1000 ppm	50.72 (45.41)^{fg}
	1250 ppm	54.12 (47.36)^{ef}
	1500 ppm	58.17 (49.70)^{d}
Commercial zinc nanoparticle	500 ppm	44.31 (41.73)^{h}
	1000 ppm	50.07 (45.04)^{g}
	1250 ppm	57.25 (49.17)^{de}
	1500 ppm	63.66 (52.93)^{c}
Pseuodomonas fluorescens extract	20%	14.90 (22.64)^{k}
Bulk zinc oxide (ZnO)	1250 ppm	28.10 (31.99)^{j}
Carbendazim	2000 ppm	73.46 (59.00)^{b}
Carboxin 37.5%+Thiram 37.5% DS	2000 ppm	100.00 (89.54)^{a}
Control	-	0.00 (0.46)^{l}
S.Em.±		0.74
C.D. (0.01)		2.90
C.V. (%)		2.90

*Values are mean of three replication.

Figures in parenthesis are arc sine transformed value.

Table 3: *In vitro* evaluation of pomegranate aril based sulphur nano formulation (PASNF) against *Colletotrichum truncatum*.

Treatments	Concentration	Per cent mycelial inhibition
Pomegranate aril based sulphur	500 ppm	14.77 (22.59)*^{h}
nanoformulation (PASNF)	1000 ppm	20.39 (26.84)^{f}
	1500 ppm	24.05 (29.37)^{e}
	2000 ppm	27.06 (31.34)^{d}
Commercial sulphur nanoparticle	500 ppm	16.34 (23.84)^{g}
	1000 ppm	17.25 (24.54)^{g}
	1500 ppm	19.74 (26.36)^{f}
	2000 ppm	23.92 (29.28)^{e}
Pomegranate arilextract	10%	21.18 (27.39)^{f}
Sodium thiosulphate($Na_2S_2O_3$)	2000 ppm	39.87 (39.15)^{c}
Carbendazim	2000 ppm	73.46 (59.00)^{b}
Carboxin 37.5%+Thiram 37.5% DS	2000 ppm	100.00 (89.54)^{a}
Control	-	0.00 (0.46)^{i}
S.Em. ±		0.42
C.D. (0.01)		1.66
C.V. (%)		2.22

*Values are mean of three replication.

Figures in parenthesis are arc sine transformed value.

Various researchers Kouzegaran and Farhadi (2017), Tripathi *et al.* (2018), Ragab and Saad-Allah (2020) synthesized sulphur nanoparticles through green approaches by using plant extracts. Characterization of these nanoparticles revealed that the size was ranging from 20 to 120 nm and shapes were ranging from spherical to random shape. Pomegranate peel as reducing agent was used in nanoparticle synthesis by Phongtongpasuk and Poadang (2015) and Upadhyay (2018).

Table 4: In vitro evaluation of pomegranate aril based silver nanoformulation (PAAgNF) against Colletotrichum truncatum.

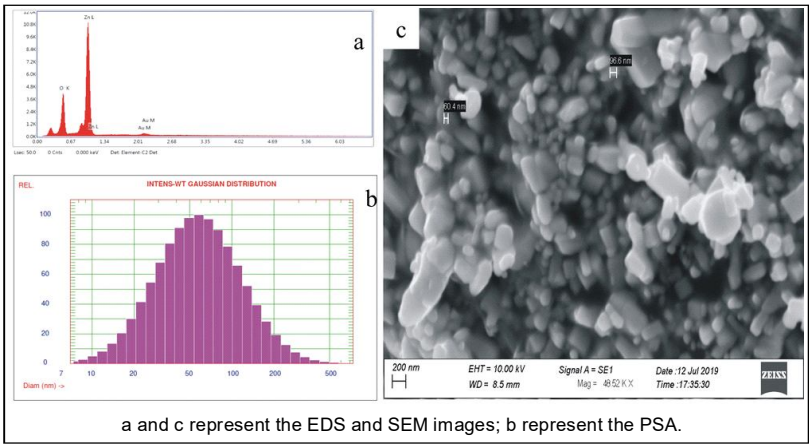
Treatments	Concentration	Per cent mycelial inhibition
	50 ppm	11.90 (20.17)*[i]
Pomegranate aril based silver	100 ppm	20.78 (27.04)[fg]
nanoformulation (PAAgNF)	250 ppm	37.91 (38.00)[d]
	500 ppm	84.71 (66.99)[b]
	50 ppm	15.69 (23.33)[h]
	100 ppm	18.82 (25.67)[g]
Commercial silver nanoparticle	250 ppm	23.14 (28.73)[f]
	500 ppm	31.90 (34.38)[e]
Pomegranate aril extract	10%	21.18 (27.39)[fg]
Silver nitrate ($AgNO_3$)	500 ppm	82.75 (65.47)[b]
Carbendazim	2000 ppm	73.46 (58.99)[c]
Carboxin 37.5%+Thiram 37.5% DS	2000 ppm	100.00 (89.54)[a]
Control		0.00 (0.46)[j]
S.Em.±		0.78
C.D. (0.01)		3.06
C.V. (%)		3.47

*Values are mean of three replication.

Figures in parenthesis are arc sine transformed value.



Fig 1: Green nanoparticles synthesised in the study namely Chitosan based zinc nanoformulation (ChZnNF), *Pseudomonas fluorescens* based zinc nanoformulation (PfZnNF), pomegranate aril based sulphur nano formulation (PASNF) and pomegranate aril based silver nanoformulation (PAAgNF).

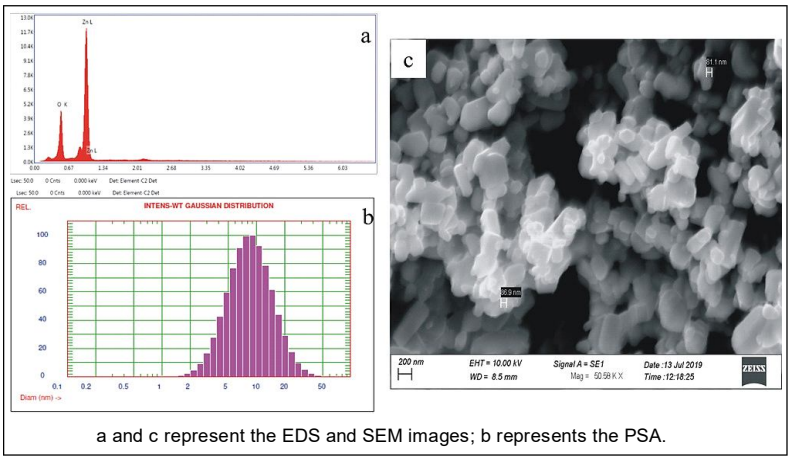


a and c represent the EDS and SEM images; b represent the PSA.

Fig 2: Characterization of chitosan based zinc nanoformulation (ChZnNF).

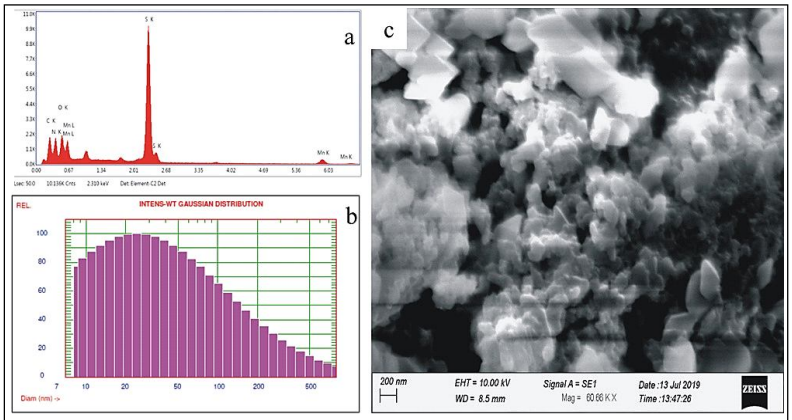
In vitro assay of nanoformulation against *C. truncatum*

The observation recorded on percent mycelial inhibition for respective treatments are represented in Table 1-4. ChZnNF at its highest concentration (@1500 ppm) showed 49 per cent inhibition, while PfZnNF (@ 1500 ppm) showed inhibition of 58.17 per cent which was comparatively higher than ChZnNF as indicated in Table 1 and 2 and depicted in Fig 6 and 7. In case of PASNF, highest inhibition of 27.06 per cent at 2000 ppm concentration (Table 3) was noted. Whereas, PAAgNF showed an inhibition of 84.71 per cent



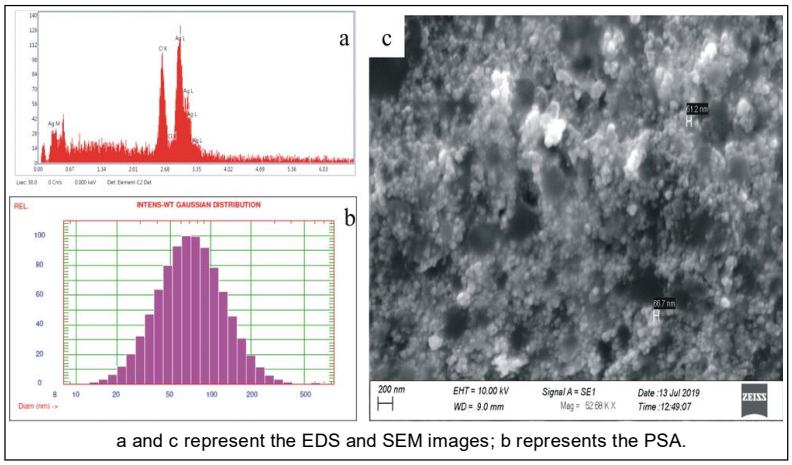
a and c represent the EDS and SEM images; b represents the PSA.

Fig 3: Characterization of *Pseudomonas fluorescens* based zinc nanoformulation (PfZnNF).



a and c represent the EDS and SEM images; b represents the PSA.

Fig 4: Characterization of pomegranate aril based sulphur nanoformulation (PASNF).



a and c represent the EDS and SEM images; b represents the PSA.

Fig 5: Characterization of pomegranate aril based silver nanoformulation (PAAgNF).

at 500 ppm (Table 4). Thus, among all the green nanoformulations, PAAgNF was found to be effective in inhibiting the mycelial growth of *C. truncatum* (Fig 8 and 9).

The results were in conformity with the results of Lamsal *et al*. (2011), Rosa-Garcia *et al.* (2018) and Srikanth (2018) against *Colletotrichum* spp.

Evaluation of nanoformulations on soybean challenge inoculated with *Colletotrichum truncatum* under glasshouse condition

Among 16 treatments (Table 5), silver nitrate at 500 ppm concentration did not record any disease symptoms after second spray in the two varieties. Pomegranate aril based

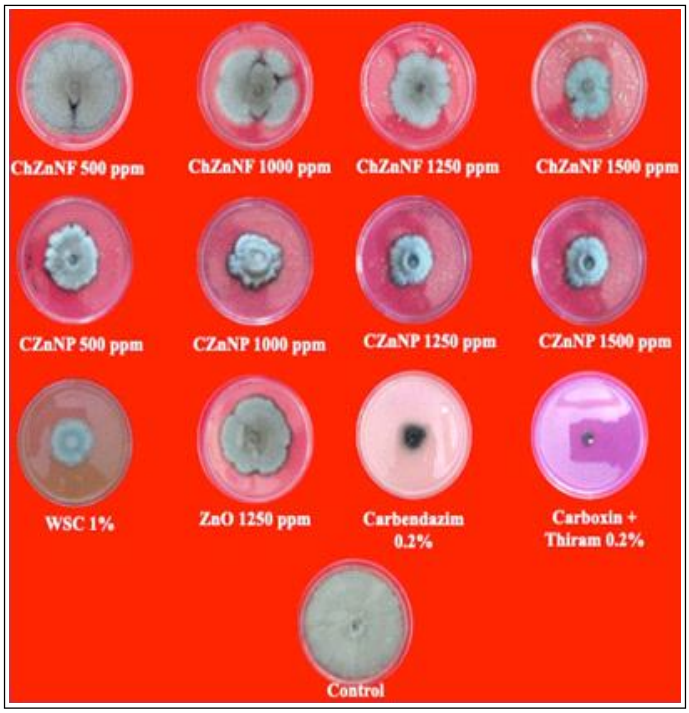


Fig 6: *In vitro* evaluation of Chitosan based zinc nanoformulation against *C. truncatum*.



Fig 7: *In vitro* evaluation of *Pseudomonas fluorescens* based zinc nanoformulation against *C. truncatum*.

silver nanoformulations (PAAgNF) did not record any anthracnose symptoms on JS 335 variety 15 days after second spray whereas DSb 21 recorded 0.62 disease severity. Carboxin 37.5%+Thiram 37.5% DS was on par with PAAgNF at 500 ppm concentration in case of DSb 21 variety with 1.23 PDI. In case of JS 335 variety it recorded 2.47 percent severity. JS 335 and DSb 21 sprayed with PAAgNF at 500 ppm concentration reduced 100 and 96.97 per cent anthracnose disease over control whereas Carboxin 37.5% +Thiram 37.5% DS recorded 95.74 and 96.97 per cent

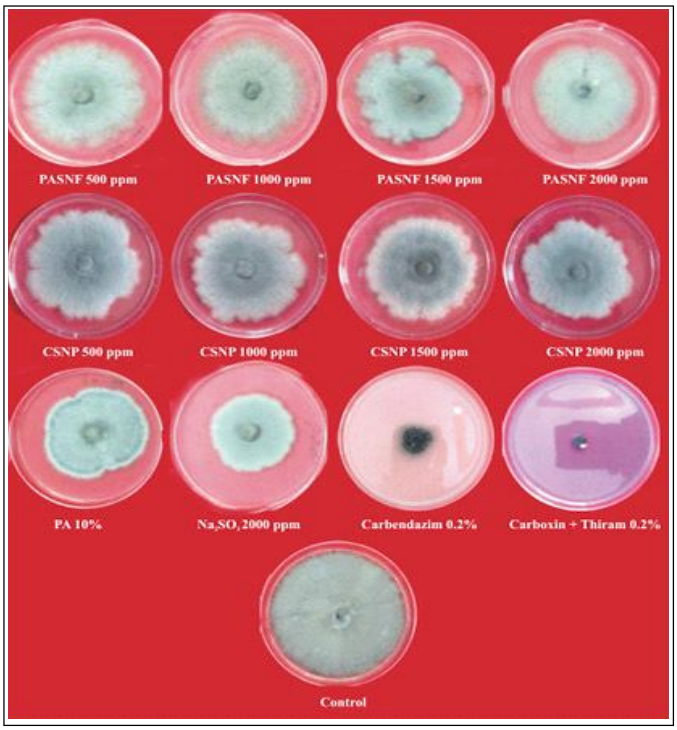


Fig 8: *In vitro* evaluation of pomegranate aril based sulphur nanoformulation against *C. truncatum*.

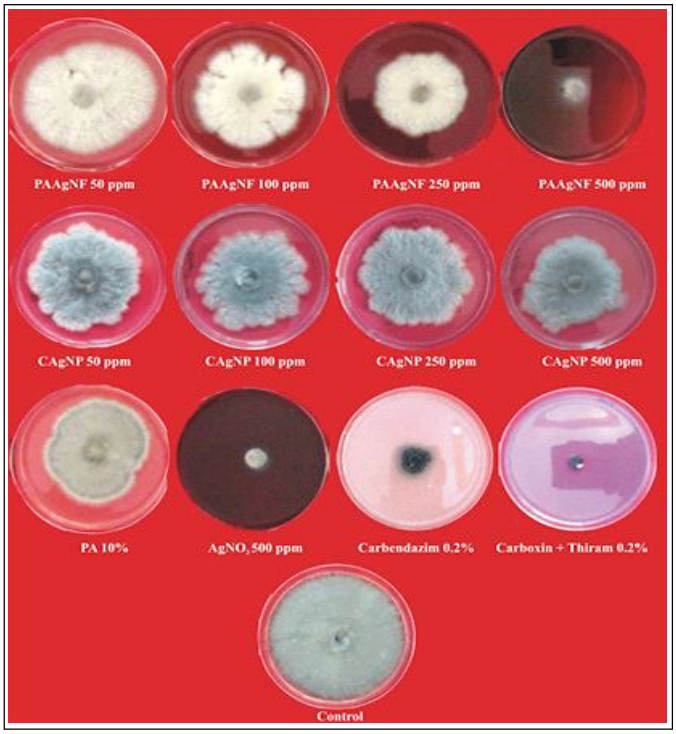


Fig 9: *In vitro* evaluation of pomegranate aril based silver nanoformulation against *C. truncatum*.

Table 5: Evaluation of nanoformulation on soybean plants challenge inoculated with *Colletotrichum truncatum* under glasshouse condition

Treatment no.	Treatments	Concentration (ppm/%)	PDI					
			15 1A			15 2A		
			JS 335	DSb 21	Mean	JS 335	DSb 21	Mean
T_1	Chitosan based zinc nanoformulation (ChZnNF)	1500 ppm	4.94 (12.66)^{de}	3.70 (9.10)^{bc}	4.32	12.35 (20.53)*^{cd}	7.41 (15.45)^{bc}	9.88
T_2	*Pseudomonas fluorescens* based zinc nanoformulation (PfZnNF)	1500 ppm	7.41 (15.79)^{e}	7.41 (15.45)^{de}	7.41	14.81 (22.53)^{cde}	9.88 (18.25)^{bcd}	12.35
T_3	Pomegranate aril based sulphur nanoformulation (PASNF)	2000 ppm	14.81 (21.96)^{g}	16.05 (23.18)^{g}	15.43	28.40 (31.92)^{f}	33.33 (35.18)^{fg}	30.86
T_4	Pomegranate aril based silver nanoformulation (PAAgNF)	500 ppm	0.00 (0.41)^{a}	0.00 (0.41)^{a}	0.00	0.00 (0.41)^{a}	1.23 (3.97)^{a}	0.62
T_5	Commercial zinc nanoparticle	1500 ppm	3.70 (11.10)^{cd}	4.94 (12.66)^{cd}	4.32	8.64 (17.02)^{c}	8.64 (17.02)^{bcd}	8.64
T_6	Commercial sulphur nanoparticle	2000 ppm	13.58 (21.31)^{g}	11.11 (19.47)^{f}	12.35	22.22 (28.07)^{ef}	16.05 (23.41)^{de}	19.14
T_7	Commercial silver nanoparticle	500 ppm	8.64 (17.02)^{f}	11.11 (19.47)^{f}	9.88	19.75 (26.37)^{def}	23.46 (28.90)^{ef}	21.60
T_8	Water soluble chitosan	1%	8.64 (17.02)^{f}	7.41 (15.45)^{de}	8.02	14.81 (22.53)^{cde}	11.11 (18.69)^{bcd}	12.96
T_9	*Pseudomonas fluorescens* extract	20%	9.88 (18.25)^{fg}	7.41 (15.45)^{de}	8.64	17.28 (24.54)^{de}	14.81 (22.53)^{cde}	16.05
T_{10}	Pomegranate aril extract	10%	13.58 (21.31)^{g}	8.64 (17.02)^{f}	11.11	22.22 (27.95)^{ef}	14.81 (22.53)^{cde}	18.52
T_{11}	Bulk zinc oxide (ZnO)	1250 ppm	2.47 (7.54)^{bc}	6.17 (14.23)^{de}	4.32	3.70 (9.10)^{b}	8.64 (17.02)^{bcd}	6.17
T_{12}	Sodium thiosulphate ($Na_2S_2O_3$)	2000 ppm	2.47 (7.54)^{bc}	2.47 (7.54)^{b}	2.47	7.41 (15.79)^{c}	4.94 (12.66)^{b}	6.17
T_{13}	Silver nitrate ($AgNO_3$)	500 ppm	0.00 (0.41)^{a}	0.00 (0.41)^{a}	0.00	0.00 (0.41)^{a}	0.00 (0.41)^{a}	0.00
T_{14}	Carbendazim	2000 ppm	4.94 (12.66)^{de}	7.41 (15.45)^{de}	6.17	11.11 (19.30)^{cd}	12.35 (20.53)^{cd}	11.73
T_{15}	Carboxin 37.5% + Thiram 37.5% DS	2000 ppm	1.23 (3.97)^{a}	0.00 (0.41)^{a}	0.62	2.47 (7.54)^{b}	1.23 (3.97)^{a}	1.85
T_{16}	Control		33.33 (35.18)^{h}	20.99 (27.20)^{h}	27.16	58.02 (49.63)^{g}	40.74(39.62)^{g}	49.38
	S.Em. ±		1.30	1.25		2.21	2.33	
	C.D. (0.01)		5.04	4.85		8.57	9.04	
	C.V. (%)		16.09	16.33		18.67	21.30	

*Values are mean of three replication.

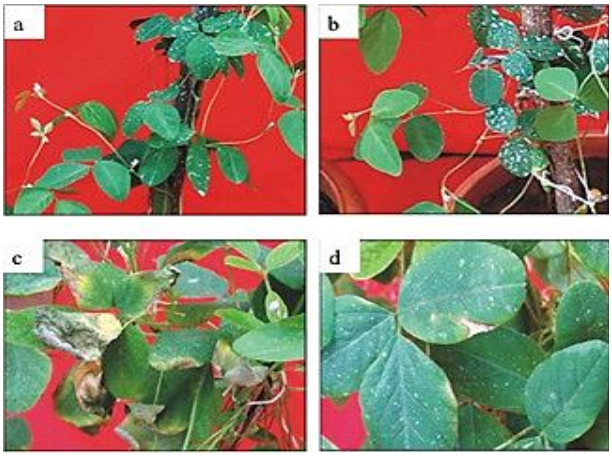
Figures in parenthesis are arc sine transformed value.

Note: 15 1A- 15 days after first application; 15 2A- 15 days after second application.

reduction of disease severity over control. This was followed by zinc oxide at 1500 ppm concentration with 3.70 and 8.64 PDI in case of JS 335 and DSb 21 variety and sodium thiosulphate at 2000 ppm concentration with 7.41 and 4.94 PDI.The results are in conformity Srikanth (2018) and Supriya (2019) in pulse crop against fungal pathogen. *Bacillus subtilis* based zinc nanoparticle and chitosan based zinc nanoparticle at 1250 ppm concentration effective in managing citrus canker and bacterial wilt of tomato respectively (Vinay *et al*., 2016).

Phytotoxicity of green synthesized nanoformulation

It was observed under the study that ChZnNF and PfZnNF along with precursor did not show any phytotoxicity upto 2000 ppm. PASNF, sodium thiosulphate, showed toxicity with symptoms such as scorching, curling and drying of leaves. PAAgNF did not show any toxicity up to 500 ppm concentration. Toxicity to the extent of 20 per cent was observed at 15 days after spray of 1000 ppm of PAAgNF. Precursor of PAAgNF, silver nitrate showed toxicity up



a) ChZnNF at 2000 ppm, b) PfZnNF at 2000 ppm, c) PASNF at 3000 ppm and d) PAAgNF at 1000 ppm.

Fig 10: Phytotoxic effect of green nanoformulation on soybean plants.

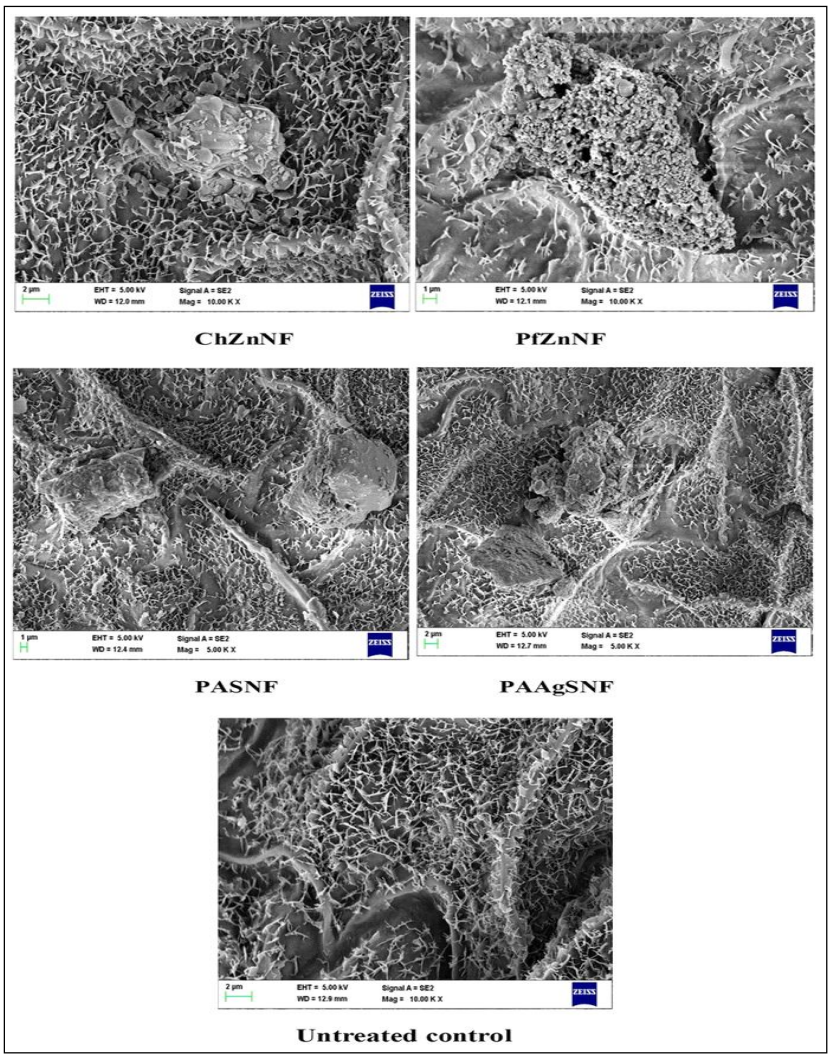


Fig 11: SEM images of leaf samples sprayed with green nanoformulation along with untreated control.

to 20 per cent from 10 days after spray. Toxicity symptoms observed were tip scorching and veinal chlorosis (Fig 10).

Phytotoxicity of precursor and non-phytotoxicity of the nanoparticles in case of sulphur and silver was also recorded by Supriya (2019). Similarly, non-phytotoxicity of silver nanoparticles was reported by Vanti *et al.* (2018).

Confirmation of nanoparticles on sprayed leaves

FESEM performed by selecting the treated leaves, showed the presence of the respective nanoparticle (Fig 11). Whereas, in the control there was no record of nanoparticle present.

CONCLUSION

Nanoscience in phytopathology is worthiest option as it reduces the environment pollution. There are few nano-capsules with controlled and effective release, which increases the efficiency of fungicide. Synthesis of nanoparticles through green approaches by using plant extract or beneficial microorganisms has advantages over chemical or physical method. Green synthesis of nanoparticles is simple, more convenient, requires less reaction time and is eco-friendly. In this study we evaluated four green nanoparticles, among which PAAgNF was found to be effective against *C. truncatum*.

Conflict of interest

There is no conflict of interest in publication of paper on behalf of all the authors.

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