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RESEARCH ARTICLE

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Cu-Chitosan Nanoparticle Induced Plant Growth and Disease Resistance Efficiency of Soybean [*Glycine max* (L.)]

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ABSTRACT

Background: Inefficient and excessive use of inorganic fertilizers and pesticides causes environmental risks. Concurrent with the recent increase in agricultural productivity, agricultural systems are now also recognized to be a significant source of environmental damage. Chitosan is a biocompatible, biodegradable and nontoxic polymer with various applications.

Methods: In the present investigation, the efficacy of Cu-chitosan nanoparticles (NPs) to plant growth promoting activity and antibacterial activity against bacterial pustule disease of soybean were evaluated. NPs treatments exhibited growth promoting effect in terms of plant height, root length, root weight, nodule number, weight of nodule and number of pods per plant in pot experiments. In field experiment, plant height, root length, root weight, nodule number, weight of nodule and number of pods per plant weight were enhanced in NPs treatments.

Result: In control plants (water treated+inoculation) showed average disease severity 66.0%. All plants treated with 0.02 to 0.12% Cu-chitosan NPs showed significant antibacterial activity, express lower disease severity 50.0% to 33.3% and 55.3% to 34.0% in pot and in field condition respectively.

Key words: Bacterial pustule, Cu-chitosan, Disease resistance, Nanoparticles, Plant growth, *Xanthomonas axonopodis* pv. *glycine*.

INTRODUCTION

Sustainable food production for a rapidly growing human population is one of the major challenges faced by the agriculture sector globally (McClung 2014). Therefore, the increased uses of pesticides have become essential to maximize the agricultural productivity. Despite their beneficial role in agriculture, pesticides can be hazardous to humans and other non-targeted organisms (Kohler and Triebskorn, 2013). An estimated 3.2 million tons of pesticides are used on crops each year (FAO, 2017).

The efficiency of pesticides could be improving by using the new formulations and systems of nanobiotechnology (Raliya *et al.* 2017). Chitosan has emerged as one of the most promising polymers for the efficient delivery of agrochemicals and micronutrients in nanoparticles Choudhary *et al.* (2017). In the plant system, chitosan has been reported to induce multifaceted disease resistance M.A. Rezaie *et al*. (2020). Furthermore, the benefits of nanotechnology innovations have been initiated to discover the synthesis of various chitosan-based nanoparticles Chandra *et al.* (2015)

Chitosan based nanoparticles blend with various active components have been synthesized Kashyap *et al.* (2015). Among the active components, metals showed more affinity towards chitosan (Rhazi *et al*., 2002; Guibal, 2004). Among, the metals copper (Cu) played an important role in crop protection and act as micronutrient. By an ionic gelation method, we have already reported a reproducible method for the synthesis of stable and monodisperse Cu chitosan nanoparticles (NPs) (mean size= 374±8.2 nm). Our studies have resulted 80% Cu-encapsulation efficacy of chitosan nanomaterial in a highly porous network Saharan *et al.* (2015). Subsequently, we systematically studied the growth-promoting effect of Cu chitosan NPs on tomato and maize seedlings by measuring germination, shoot/root length, and seed vigor index. Amine *et al*. (2020). Recently, chitosan-based NPs have been evaluated as potent inducer of antioxidant and defense enzymes. Increased level of defense responses was noticed in chitosan NPs treated plants due to high expression of defense related genes.

Soybean [*Glycine max* (L.)] is one of the most important crop worldwide that delivers two third of calories derived from agriculture (Ray *et al.* 2013) and accounts for half of the global demand for oil and vegetable protein (Abhishek *et al*. 2022). Bacterial pustule caused by *Xanthomonas axonopodis* pv. *glycine* is significant bacterial disease that limit soybean production worldwide 20-40% (Kaewnum *et al.* 2005).

In the present investigation, we report for the first time the efficacy of Cu-chitosan NPs to induce plant growth

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promotry activity and the defense responses against bacterial pustule disease in soybean under net house and field conditions. Our results convincingly establish Cu-chitosan NPs as a potent inducer of growth promotry activity and systemic acquired resistance for effective control of bacterial pustule disease of soybean.

MATERIALS AND METHODS

Chitosan (Mol. Wt. 50,000–190,000 and 80% N-deacetylation) and sodium tri-polyphosphate (TPP) were procured from Sigma-Aldrich, St. Louis, MO, USA. Chemicals for enzyme assay and other experiments were procured from HiMedia and SRL, Mumbai, India. The seeds of cultivar soybean cultivar JS-335 susceptible towards bacterial pustule disease was selected. Highly infectious *Xanthomonas axonopodis pv. glycine* causing bacterial pustule disease of soybean, were procured from Department of Plant Pathology, RCA, MPUAT, Udaipur in 2019.

Preparation and characterization of Cu-chitosan nanoparticles

Cu-chitosan NPs were prepared by following methods developed in our laboratory based on the ionic gelation of 0.1 gm of chitosan (low molecular weight and 80% N-deacetylation) with 1.0 gm of TPP (Sodium tripolyphosphate anhydrous, Loba Chemie) anions Saharan *et al.* (2015). Synthesized NPs were characterized for physicochemical analyses using dynamic light scattering (DLS), Fourier transform infrared (FTIR), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS). The characteristic details of synthesized NPs were the same as we reported in our earlier paper (Saharan *et al.* 2015).

Pot experiment for plant growth and disease assessment

After 4 h treatment of the seeds of variety JS-335 were sown in earthen pots filled with standard potting soil obtained from field and kept in net house in natural environment. Foliar spray of Cu-chitosan NPs (until run-off) was applied after emergence of first trifoliate stage. Artificial inoculation of *X. axonopodis* pv. *glycine* was carried out after 35 days of sowing as describe earlier (Kim *et al.,* 2011). Second spray of Cu-chitosan NPs (0.02, 0.04, 0.06, 0.08, 0.10 and 0.12% w/v) along with controls [untreated, bulk chitosan (0.01%) and $CuSO_4$ (0.01%)] in aqueous suspension was applied after disease occurrence. After 48h of second foliar spray various enzymes assay were conducted in inoculated plants as well as in control (Jung *et al.* 2011). Various growth characters like plant height, root length, root weight, nodule number, weight of nodule, number of pods per plant and 100 seed weight were recorded at the end of physiological maturity.

Field experiment for crop yield and disease assessment

Most effective three treatments of Cu-chitosan NPs selected from pot experiments were used for seed treatment as well as foliar application. The treated seeds of cultivar JS-335 were sown in field in randomized block design (RBD). The test field were maintained as per standard agronomic and plant protection management. First foliar application was applied after emergence of first trifoliate stage. Artificial inoculation of *X. axonopodis* pv. *glycine* was carried out in field after 35 days of sowing. Second spray was applied after disease occurrence. Disease incidence and disease severity was assessed by using Bull and Koike scale as described earlier Odubanwo *et al.* (2013). Various growth characters like plant height, root length, root weight, nodule number, weight of nodule, number of pods per plant and 100 seed weight were recorded at the end of physiological maturity.

Disease assessment

Disease incidence and disease severity was assessed in pot and field by using Bull and Koike scale as describe earlier. Disease incidence was determined by calculating the proportion of diseased plants in each treatment. Disease severity (DS) was evaluated by rating the most severely damaged area on the plant on a scale of 0 to 5 (Table 1). Further the disease severity and percentage efficacy of disease control (PEDC) was calculated by using formula given by Chester (1959) and Wheeler (1969).

Disease severity =

$$\frac{\text{Sum of all individual disease rating}}{\text{Total number of leaf assessed} \times \text{maximum rating}} \times 100$$

PEDC =

$$\frac{\text{Disease severtiy in control-disease severity in treatment}}{\text{Infection index in control}} \times 100$$

Statistical analysis

Statistical analysis of the data was performed with JMP software version 12. The significant differences among treatment groups were determined using the Turkey Kramer HSD at p = 0.05. All experiments were performed in three replications (triplicates) and each replication consisted of minimum three (for pot experiments) and ten samples (for field experiments) from randomly selected plants.

RESULTS AND DISCUSSION

Cu-chitosan NPs

In this study we have synthesized stable and monodisperse Cu-chitosan NPs. DLS study revealed results of mean hydrodynamic diameter (314±2.5), PDI value (0.48)

Table 1: Rating scale for Bacterial pustule.

Description	Disease rating
Plants with no visible symptoms	0
A few individual lesions	1
Many individual lesions	2
Small patches of coalesced lesions	3
Medium sized patches of coalesced lesions	4
Large patches of coalesced lesions	5

indicated monodisperse nature of Cu-chitosan nanoparticales and zeta potential (+ 19.5 mV) of Cu-chitosan NPs showed overall positive charge, which is important parameter for the stability and higher affinity towards biological membranes in aqueous. These are almost same characteristics as reported in our previous studies (Saharan *et al.,* 2015). FTIR analysis further confirmed the functional groups of bulk chitosan and Cu-chitosan NPs. TEM study expressed the actual behaviour of nanoparticles in aqueous suspension. Sphere-shaped NPs verified by TEM. Further nano-organization of Cu-chitosan NPs was confirmed by SEM micrograph. Cu-chitosan NPs possess highly porous structure (like a barred enclosure) was displayed at higher magnification (20,000X). Porous surface was observed as per SEM micrograph. XPS study was demonstrated the presence of C, O, N and P elements. The most abundant elements detected in NPs were carbon and oxygen, while nitrogen and phosphorus were detected at lower concentrations.

Effect of Cu chitosan NPs on the plant growth and disease assessment in Pot experiment

In batch experiments conducted in pots, bacterial pustule disease symptoms were observed after 10 days of artificial inoculation of *X. axonopodis* pv. *glycine*. Thereafter, foliar spray of water (control), bulk chitosan, $CuSO_4$ and different concentrations of nanoemulsion was applied. After 10 days of application, data for disease severity (DS) and percent efficacy of disease control (PEDC) were recorded. Small, pale-green spot with elevated pustule were critically analyzed on the experimental plants. In control plants, lesions expanded and merged leading DS to the extent of 66.3% (Fig 1A) while in Cu-chitosan NPs treated plants, small yellow to brown lesions were observed (Fig 1B). All plants treated with 0.02 to 0.12% Cu-chitosan NPs showed significant antibacterial activity, express lower disease severity 50.0% to 33.3%. Bulk chitosan and $CuSO_4$ were showed 40.0% and 26.3% disease severity respectively. PEDC was found maximum (49.7%) at 0.06% of Cu-chitosan NPs. At statistical level significantly higher PEDC value was observed in 0.06% of Cu-chitosan NPs from all other treatments (Table 2).

Another aim of pot experiment was to find out the effect of Cu chitosan NPs on growth characteristics of soybean plant. Plant height, root length and pod number were recorded maximum at 0.06% of Cu-chitosan NPs and minimum at $CuSO_4$ treated plants. Root weight was recorded maximum at 0.02% of Cu-chitosan NPs and minimum at $CuSO_4$. Nodule number was reported maximum at 0.02% of Cu-chitosan NPs and minimum was at bulk chitosan treated plants. Nodule weight was found maximum in 0.02% of Cu-chitosan NPs and minimum in $CuSO_4$ treatment. 100 seed weight was reported maximum at control I and minimum at $CuSO_4$ treated plants (Fig 2). Although, at higher concentrations of NPs, a slight decrease in various growth parameters were observed as compared to lower concentrations of NPs treatments. Growth promotory effect of bulk chitosan has been recorded significantly lower as compared to chitosan NPs. Similarly, as compared to control and $CuSO_4$, bulk chitosan has been reported to have higher value for all parameters except for percent germination and SVI. In another study, Cu–chitosan NPs, at 0.08, 0.10, and 0.12% treatments, showed significantly growth promotory effect on seed germination, seedling length, and fresh and dry weight in tomato plants.

Effect of Cu chitosan NPs on the crop yield and disease assessment in field experiment

With 4 hr of seed treatment and two foliar sprays, control plants (water treated + inoculation) showed average disease severity 66.0%. All plants treated with 0.02 to 0.10% Cu-chitosan NPs showed significant antibacterial activity, express lower disease severity 55.3% to 34.0%. Bulk chitosan and $CuSO_4$ were showed 40.0% and 29.3% disease severity respectively. PEDC was found maximum (51.3%) at 0.06% of Cu-chitosan NPs. At statistical level significantly higher PEDC value was observed in 0.06% of Cu-chitosan NPs from all other treatments (Table 3).

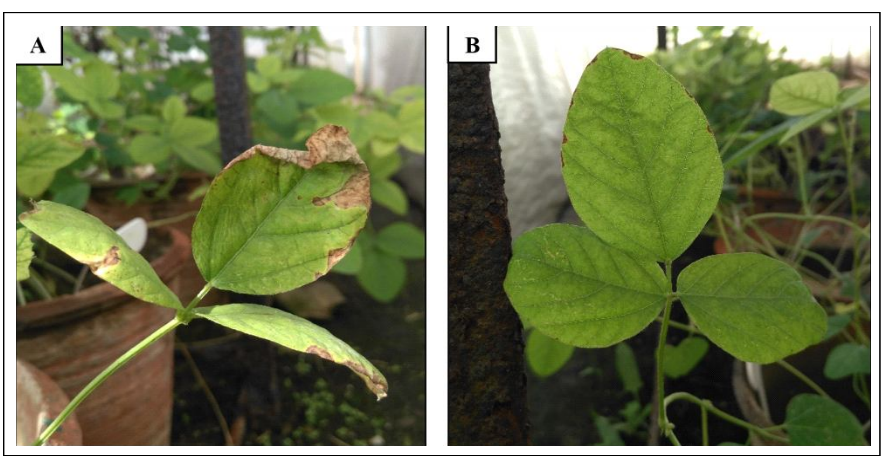

Fig 1: Symptoms of bacterial pustule disease on soybean plants in pot experiments (A) lesions expanded and merged in control (B) small yellow to brown lesions in soybean leaf at 0.06%,v/v Cu-chitosan NPs.

Table 2: Effect of Cu-chitosan NPs on bacterial pustule disease in pots (under net house condition).

Treatment (%)	Disease severity (%)^A	PEDC (%)^A
Control I	66.3±0.88^a	0.00±0.00^f
Control II	64.3±1.33^a	3.67±1.33^f
Bulk chitosan (0.01)	40.0±1.15^c	39.2±1.94^d
$CuSO_4$ (0.01)	26.3±0.88^e	60.1±1.25^a
Cu-chitosan NPs		
0.02	49.0±1.15^b	26.1±1.74^e
0.04	50.0±0.00^b	24.6±0.00^e
0.06	33.3±1.20^d	49.7±1.81^b
0.08	34.3±0.66^d	48.2±1.00^{bc}
0.10	37.6±0.88^{cd}	43.2±1.32^{cd}
0.12	39.3±0.33^c	40.6±0.50^d

Table 3: Effect of Cu-chitosan NPs on bacterial pustule disease in field condition.

Treatment (%)	Disease severity (%)^A	PEDC (%)^A
Control I	70.6±1.15^a	0.00±0.00^e
Control II	66.0±2.30^a	6.48±3.25^e
Bulk chitosan (0.01)	40.0±1.15^{cd}	43.3±1.63^{bc}
$CuSO_4$ (0.01)	29.3±1.33^e	58.2±2.10^a
Cu-chitosan NPs		
0.02	55.3±1.33^b	21.6±1.89^d
0.06	34.0±1.15^{de}	51.3±1.45^{ab}
0.10	43.3±0.66^c	38.5±0.96^c

Disease data were recorded after visible appearance of symptoms following 15 days of inoculation using 0 to 5 standard disease rating scale. ^{A}Each value is mean of triplicates and each replicate consisted of 10 plants samples. Mean±SE followed by same letter is not significantly different at p= 0.05 as determined by Tukey-Kramer HSD. Control I (without water). Control II (water treated+inoculation). Chitosan dissolved in 0.1% acetic acid. PEDC = Percentage efficacy of disease control was calculated compare to control.

The antibacterial activity of copper nanoparticles against a number of bacterial diseases has been reported previously (Badawy *et al.,* 2016; Syame *et al.,* 2017; Swati *et al*., 2017 and 2018; Shailesh *et al*., 2018). The antimicrobial activity of chitosan is well known against a variety of bacteria and fungi, several researchers have presented their practical point of view. Goy *et al*. (2009) suggested three antibacterial mechanisms of chitosan; firstly, ionic surface interaction resulting in cell wall leakage; secondly, permeation of chitosan into microorganism nuclei inhibits their protein and mRNA synthesis, and thirdly, formation of an external film over the plant surface, limiting the nutrient availability for microorganisms (Liang *et al*., 2014). stated that chitosan is responsible for the destruction of the bacterial cell membrane which causes death due to the leakage of intracellular substances. However, in recent times, it has been reported that chitosan is responsible for the hydrolysis of peptidoglycans, increasing electrolyte leakage and potentially causing the death of the pathogen.

Living organisms requires copper at low concentrations as cofactors for metalloproteins and enzymes. However, at high concentrations, Cu induces an inhibition of growth in bacteria. This effect may involve substitution of essential ions and blocking protein's functional groups, inactivation of enzymes, production of hydroperoxide free radicals by membrane bound copper and alterations of membrane integrity Faundez *et al*. (2004). When chitosan chelated with Cu ions, the positive charge on the amino groups of chitosan was strengthened. As a result, the complex was easier to interact efficiently with anionic components of cell surface following the same mechanism as chitosan but with enhancement of adsorption ability, exhibiting thus higher inhibitory activities.

After 4 hr of seed treatment, plant height was recorded maximum at control- I and minimum at $CuSO_4$ treated plants. Root length and Root weight was recorded maximum at 0.06% of Cu-chitosan NPs and minimum at bulk chitosan treated plants. Nodule number was reported maximum at bulk chitosan and minimum was at $CuSO_4$ treated plants.

Table 4: Effect of Cu-chitosan NPs on growth parameter of soybean in field condition. Various growth parameters were recorded at physiological maturity of crop. Each value is mean of triplicates and each replicate consisted of 10 plants samples.

Treatment (%)	Plant growth^A						
	Plant height (cm)	Root length (cm)	Root weight (gm)	Nodule number	Nodule weight (gm)	Pod number	100 seed weight (gm)
Control I	76.0±1.4^a	22.5±0.1^d	3.80±0.4^b	5.2±0.5^a	226.0±31.6^b	56.6±0.3^b	13.3±0.8^a
Control II	53.5±7.4^{bc}	24.0±0.1^{cd}	4.31±0.0^b	6.1±0.1^a	254.7±33.8^a	54.1±1.8^b	11.8±0.1^{ab}
Bulk chitosan (0.01)	44.7±1.4^{cd}	15.8±0.4^f	2.93±0.0^c	6.1±0.2^a	265.6±35.1^a	27.6±0.5^c	11.0±0.8^{ab}
$CuSO_4$	36.0±2.1^d	19.0±0.3^e	3.80±0.1^b	4.1±0.1^b	212.3±30.7^{bc}	32.5±1.5c	10.1±0.1^b
Cu-chitosan NPs							
0.02	54.7±2.2^{bc}	26.5±0.6^b	4.31±0.0^b	5.1±0.7^a	198.8±26.9^c	57.1±1.0^b	12.1±0.1^{ab}
0.06	60.8±2.3^{ab}	36.2±0.5^a	5.58±0.0^a	4.4±0.2^b	209.7±28.6^{bc}	73.4±0.9^a	12.6±0.8^{ab}
0.10	55.7±0.5^{bc}	26.3±0.8^{bc}	4.12±0.0^b	5.1±0.9^a	190.8±21.7^c	51.8±1.8^b	11.8±0.1^{ab}

Mean±SE followed by same letter is not significantly different at p=0.05 as determined by Tukey–Kramer HSD. Control I (without water). Control II (water treated+inoculation). Chitosan dissolved in 0.1% acetic acid.

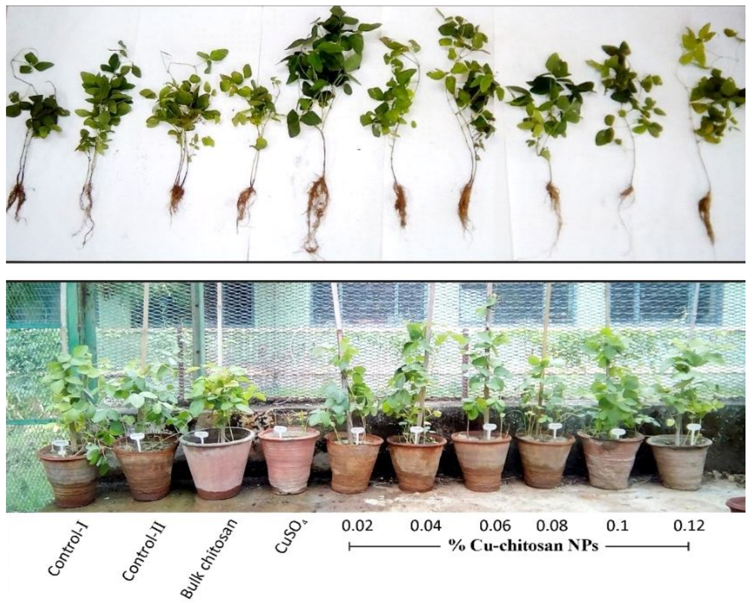

Fig 2: Effect of Cu-chitosan NPs on plant growth of soybean. Concentrations of Cu-chitosan ranging from 0.02 to 0.12% v/v, exhibited visual differences in plant growth.

Nodule weight was found maximum in bulk chitosan and minimum in 0.12% of Cu-chitosan NPs. Pod number was recorded maximum at 0.02% of Cu-chitosan NPs and minimum at bulk chitosan treated plants. 100 seed weight was reported maximum at control Ð and minimum at $CuSO_4$ treated plants (Table 4).

CONCLUSION

Overall, in present study, Cu-chitosan NPs has established as very effective antibacterial agent against bacterial pustule of soybean in pot condition as well as at field condition. Cu-chitosan NPs developed in this study showed growth promontory effect in soybean and also effective in controlling disease. The potential of Cu-chitosan NPs is this study anticipated that developed NPs could be further exploited in large scale experiments.

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Conflict of interest: None.

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