RESEARCH ARTICLE

INDUCTION OF DEFENCE RESPONSES IN SOYBEAN AGAINST *XANTHOMONAS AXONOPODIS* BY COPPER-CHITOSAN NANOPARTICLES

Swati* and Arunabh Joshi[1]

[1]*Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, Rajasthan, India*
Email: swatijhajhria90@gmail.com

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Abstract: Inefficient and excessive use of inorganic fertilizers and pesticides causes environmental risks. Concurrent with the recent increase in agriculturalproductivity, agricultural systems are now also recognized to be a significant source of environmentaldamage. Chitosan is a biocompatible, biodegradable and nontoxic polymer with various applications. In the present investigation, the efficacy of Cu-chitosan nanoparticles (NPs) to boost defense responses against *Xanthomonas axonopodis*pv. *glycines*were evaluated. *X. axonopodis*causes Bacterial Pustule disease in soybean. Cu-chitosan NPs treated plants showed significant defense response through higher activities of antioxidant (SOD and POD) and defense enzymes (PPO and PAL). Significant control of Bacterial pustule disease of soybean was recorded at 0.06%concentration of Cu-chitosan NPs treatments in pot and field condition. The potential of Cu-chitosan NPs is this studyanticipated that developed NPs could be further exploited in large scale experiments.

Keywords: Chitosan, Cu-chitosan, Nanoparticles, Bacterial pustule, Defence enzymes

INTRODUCTION

Sustainable food production for a rapidly growing human populationis one of the major challenges faced by the agriculture sector globally (Godfray and Garnett, 2014; 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 nano biotechnology (Raliya *et al*., 2017). Chitosan has emerged as one of the most promising polymersfor 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(Chandra *et al*., 2015). Furthermore, the benefits of nanotechnology innovations have been initiated to discover the synthesis of various chitosan-based nanoparticles (Chandra *et al*.,2015; Saharan *et al*., 2016).

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 (Swati *et al*., 2020; Saharan *et al*., 2015, 2016) and act as micronutrient (Choudhary *et al*., 2017; Swati *et al*., 2020).

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. Bacterial pustule caused by *Xanthomonasaxonopodis*pv. *glycine* is significant bacterial disease that limits 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 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 systemic acquired resistance for effective control of bacterial pustule disease of soybean.

MATERIALS AND METHODS

Preparation & characterization of Cu-chitosan nanoparticles

Cu-chitosan NPs were prepared by following the methods developed in our laboratory based on the ionic gelation of 0.1 gm of chitosan(low molecular weight and 80% N-deacetylation, Sigma-Aldrich, St.Louis, USA) 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), X-ray photoelectron spectroscopy (XPS) and double-beam atomic absorption spectrophotometry (AAS). The characteristic details of synthesized NPs were the same as we reported in our earlier paper (Saharan *et al*., 2015).

*Corresponding Author

***In vitro* antibacterial activity of Cu-chitosan NPs:**
Bacterial strain of *Xanthomonasaxonopodis*pv. *glycine*was obtained from Department of Pathology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology.For the preparation of inoculums, a loopful of the stock culture were transfer in 50 ml of Luria Bertani broth and incubated at 37° C on a shaker (120 rpm). OD were measured at 660 nm at various time duration (0, 12, 24, 36 and 48h). Then 100µl bacterial suspension were collected from appropriate growth innoculum and added to sterile test tubes containing LB Broth supplemented with different concentration of Cu-chitosan NPs *viz*. 0.02, 0.04, 0.06, 0.08, 0.10 and 0.12% w/v along with control (without treatment) Bulk controls (CuSO4 and chitosan). Bacterial growth was assessed by measuring OD at 660nm at various time duration (0, 12, 24, 36 and 48 h). At appropriate growth of test sample, test sample were collected and serial diluted to achieve countable colony number. Further, 50 µl of diluted test sample were spread on 90 mm Petri-plates containing King's medium B Base (King *et al*., 1954). Colony numbers were counted after incubation of the plates for 24 h in an incubator at 29±1ºC. CFU/ ml were calculated using the equation given below:

$$\text{CFU} = \frac{\text{Colony no.}}{\text{Plated sample (ml)}} \times \text{dilution factor}$$

Activities of antioxidant and defense enzymes in Pot experiment:
Activity of defense [polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL)] and antioxidant enzymes [peroxidase (POD) and superoxide dismutase (SOD)] were estimated in 3rd leaf after 24 h of foliar spray of various treatments. SOD activity was determined at 560nm by (Rao *et al*., 1996) method. POD activity was measured spectrophotometrically (at 470nm) as described by (Chance and Maehly, 1955). PPO was assayed according to (Taneja and Sachar, 1974) and activity was expressed as change in absorbance at 490nm. PAL was estimated as described by (Moerschbacher*et al*., 1988) where activity measured at 290nm. Activities of all the enzymes were expressed in µmol/min/g tissue.

Pot experiment for plant growth and bacterial pustule disease assessment:
After 4h 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 (Khan *et al*., 2003; 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 assessedby using Bull and Koike scale as described earlier (Odubanwo *et al*., 2013). 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. Further, the disease severity and percentage efficacy of disease control (PEDC) was calculated by using formula given by Chester (1959) and Wheeler (1969). 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.

$$\text{Disease severity} = \frac{\text{Sum of all individual disease rating}}{\text{Total number of leaf assessed} \times \text{Maximum rating}} \times 100$$

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

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) indicated monodisperse nature of Cu-chitosan nanoparticles 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.

Cu encapsulation profile: Release of Cu from Cu-chitosan NPs was studied in the pH range 1 to 6 (Fig. 1). With decrease in pH from 4 to 1, Cu was encapsulated in increasing order (21, 51.2, 73.8 and 93.9%) from pH 1-4. Further Cu encapsulation was decreased at pH 5 and 6 compared to pH 4.

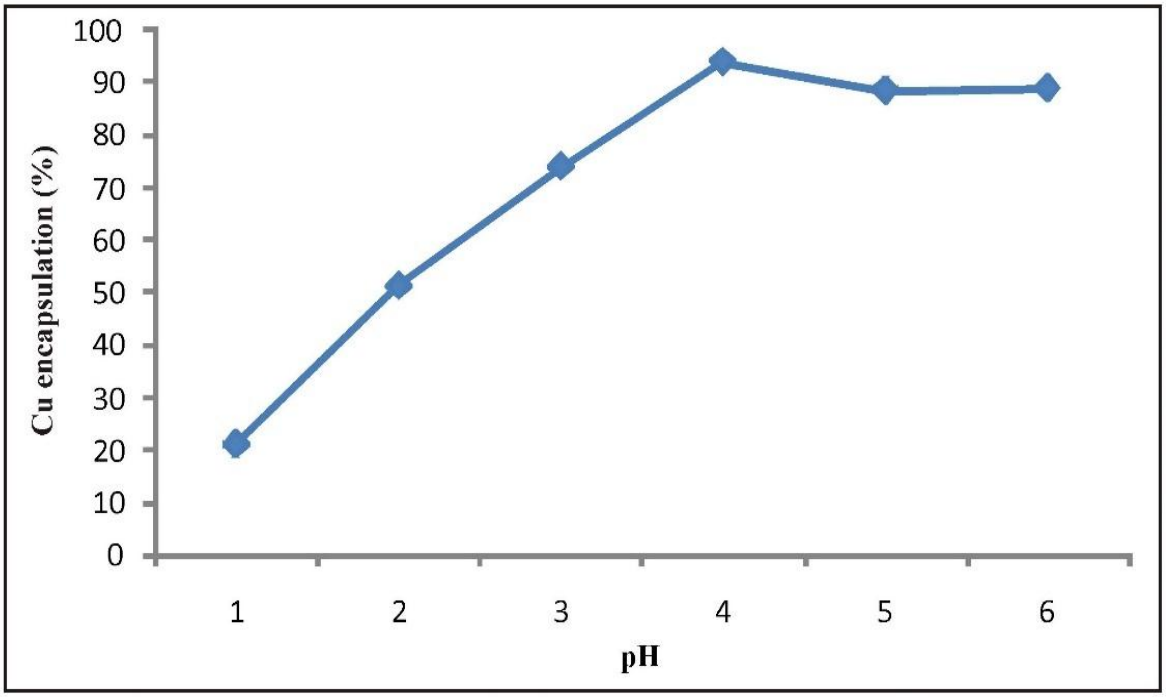

Fig 1: Cu-encapsulation in Cu-chitosan NPs under varying pH

***In vitro* antibacterial activity of Cu-chitosan NPs:** All concentrations of Cu-chitosan NPs showed higher antibacterial activity as compare to control and bulk chitosan treatments. Complete (100%) inhibition was observed in 0.04 to 0.12% concentrations of Cu-chitosan NPs and CuSO4(Fig. 2). Maximum log (CFU/ml) was found in control, followed by bulk chitosan and 0.02 concentrations of Cu-chitosan NPs treatments (Table. 1). Nanochitosan acquired remarkable advantage over bulk chitosan due to large surface area and small size. Being nanosize of chitosan, it can easily interact withbacterial system that will lead to disturbs the normal physiological activities of the bacteria and kills them (Mekahlia and Bouzid; 2009). Cu-chitosan NPshave previously been reported very effective against *E. coli*, *Salmonella choleraesuis*, *Salmonella typhimurium*, *Staphylococcus aureus* (Qi *et al*; 2004), *Salmonella Enteritidis* (Mekahlia and Bouzid; 2009), *Pseudomonas syringae* (Swati *et al.*, 2017) and *Streptococcus mutans* (Covarrubias *et al;* 2018).

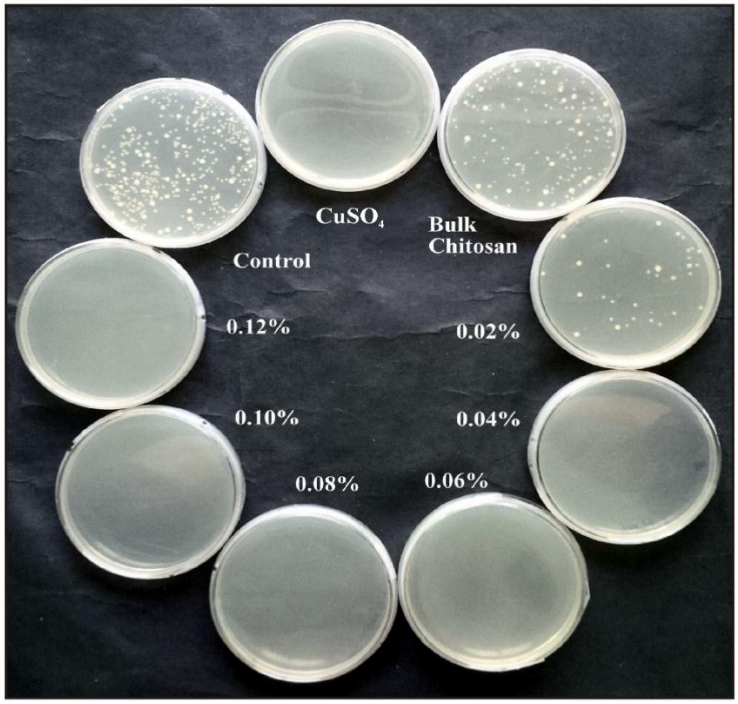

Fig 2: Antibacterial activity of different concentration of Cu-chitosan NPs (0.02, 0.04, 0.06, 0.08, 0.10 and 0.12%) with control, $CuSO_4$ and bulk chitosan in clockwise direction against colony number of *Xanthomonas axonopodis* pv. *glycine in vitro*.

Table 1. Effect of Cu-chitosan NPs on bacterial growth as log(cfu/ml)

Treatment (%)	Log(cfu/ml)
Control (water)	9.80 ± 0.50^{a}
Bulk chitosan (0.01)	9.49 ± 0.05^{c}
CuSO4 (0.01)	0.00 ± 0.00^{d}
Cu-chitosan NPs	
0.02	8.86 ± 0.50^{c}
0.04	0.00 ± 0.00^{d}
0.06	0.00 ± 0.00^{d}
0.08	0.00 ± 0.00^{d}
0.10	0.00 ± 0.00^{d}
0.12	0.00 ± 0.00^{d}

Data were recorded after incubation of the plates for 24 hr at 29±1ºC. Mean ± SE followed by same letter is not significantly different at $p = 0.05$ as determined by Tukey–Kramer HSD. Chitosan dissolved in 0.1% acetic acid.

Effect of Cu chitosan NPs on the activities of antioxidant and defense enzymes in Pot experiment:

Application of NPs substantially induced the enzyme activities in leaves. SOD activity was significantly higher in 0.02, 0.06 and 0.10% of NPs (Fig. 3A). Similarly, 1.5–2 folds higher POD activity was recorded in 0.02 to 0.06% NPs treated plant leaves as compared to control and bulk chitosan treated plants (Fig. 3B). The higher activity of SOD effectively converts highly toxic superoxide radicals into less toxic H_2O_2 species. A significantly higher activity of POD, a key enzyme to scavenge H_2O_2 into H_2O and O_2. So that the elevated activities of SOD and POD in NPs treated plants protect the plant from oxidative stress during pathogen invasion. The activity of PAL was also enhanced by NPs treatments (0.06 - 0.10%) as compared to control, bulk chitosan and $CuSO_4$ treatments (Fig. 3C). Likewise, Cu-chitosan NPs treated plants leaves showed 1–2 folds increased PPO activity as compared to control and bulk chitosan treatment (Fig. 3D). These enzymes are responsible for scavenging of reactive oxygen species (ROS) by the oxidation of phenolic compounds for resistance to pathogens in the host (Gao *et al.*, 2008; Chandra *et al.*, 2015). In a recent study in maize, chitosan NPs were found to induce activation of defense-related enzymes and antioxidant enzymes, namely, peroxidase (POX), polyphenol oxidase (PPO), phenylalanine ammonia-

lyase (PAL), superoxide dismutase (SOD), and catalase (CAT), respectively (Choudhary *et al*., 2017). In many plant species, metal and metal-based NPs have previously been reported as immune modulator through inductionof antioxidant/defense enzymes activity (Dimkpa *et al*. 2012; Shaw and Hossain 2013; Cui *et al*.,2014; Ma, *et al*., 2015; Zhang *et al*., 2015 & 2017). Copper is an essential micronutrient for most organismsbecause it acts as a catalyst of redox reactions as well as reactions involving molecular oxygen (such as reactive oxygen species). As a result, too much copper is toxic, so the incorporation of copper into chitosan by chelation mechanism, exhibit tightly regulated and highly selective transport of copper.

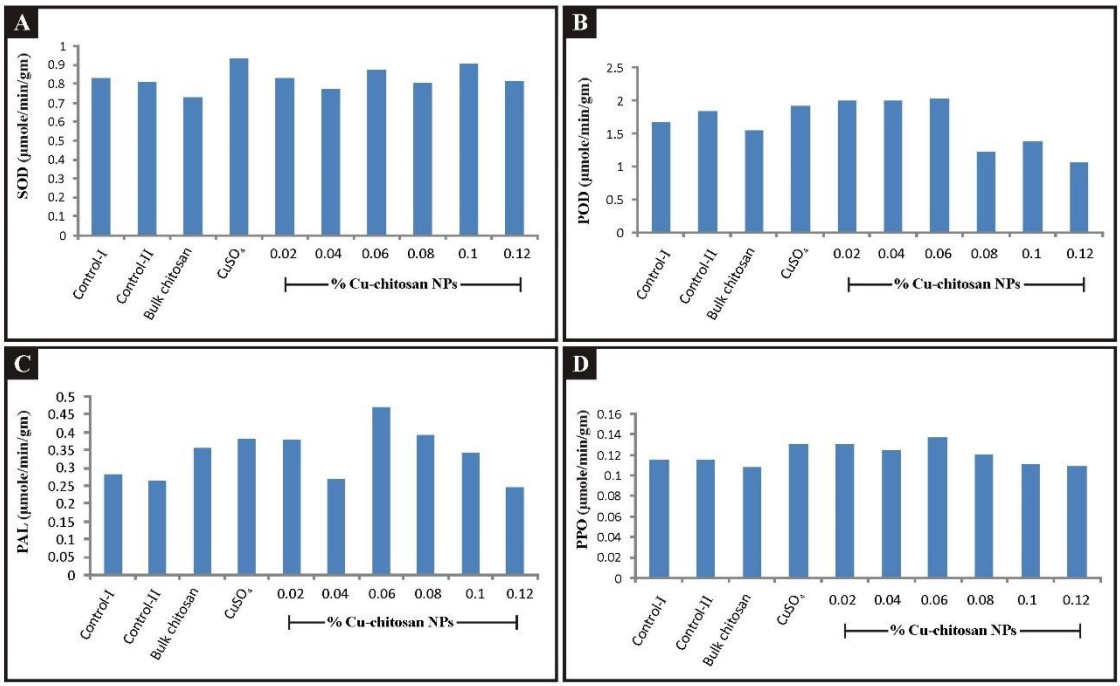

Fig 3: Effect of Cu-chitosan NPs on (A)SOD (B)POD (C)PAL (D)PPO enzyme activity in soybean plant leaves After 48 hr of foliar spray in pots (under Net house condition). Control I (without water). Control Π (water treated + inoculation). Bulk chitosan, (0.01%) dissolved in 0.1% acetic acid. $CuSO_4$ (0.01%)

Effect of Cu chitosan NPs on Bacterial pustule disease in pot experiments:

With 4h of seed treatment and two foliar sprays, control plants (water treated + inoculation) showed average disease severity 64.30%. 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; Fig 4).

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 Π	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}

Disease data were recorded after visible appearance of symptoms following 10 days of inoculation using 0 to 5 standard disease rating scale. [A]Each value is mean of triplicates and each replicate consisted of 5 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.

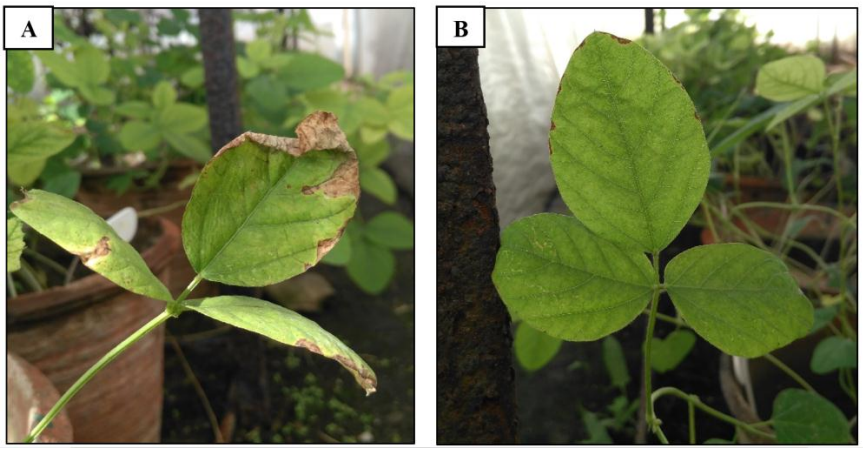

Fig 4: Symptoms of bacterial pustule disease on soybean plants in pot experiments (A) lesions expanded and merged in control (B) small yellow to brown lesions in soybean leaf at 0.06%,v/v Cu-chitosan NPs.

Effect of Cu chitosan NPs on Bacterial pustule disease in field experiments:

All plants treated with 0.02 to 0.10% Cu-chitosan NPs showed lower disease severity 55.3% to 34.0%. At statistical level significantly higher PEDC value was observed in 0.06% of Cu-chitosan NPs from all other treatments (Table.3). The antibacterial activity of copper nanoparticles against a number of bacterial diseases has been reported previously (Badawy *et al*., 2016; Syame *et al*., 2017; 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. For example, Goy et al. 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 (Goy *et al*., 2009). Liang et al. stated that chitosan is responsible for the destruction of the bacterial cell membrane which causes death due to the leakage of intracellular substances (Liang *et al*., 2014). 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 (Goy *et al*., 2016).

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 aresult, the complex was easier to interact efficiently with anionic components of cell surface following the samemechanism as chitosan but with enhancement of adsorption ability, exhibiting thus higher inhibitory activities.

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.

In summary, the present study Cu-chitosan NPs act as antibacterial agent and proven that it can significantly enhance the activities of antioxidant and defense enzymes in soybean plants. These biodegradable nanoparticles could be essential towards sustainable agriculture without damaging ecosystem. The synthesized NPs have enormous potential to be commercially explored for agriculture use.

DECLARATION

Conflict of interest: The authors declare that they have noconflict of interest.

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