Original Research Article

Cu-Chitosan Nanoparticles Stimulated Defence responses against Bacterial Pustule disease in Soybean crop

ABSTRACT

Aim: To assess the effect of Cu-chitosan nanoparticles on controlling Bacterial disease by enhancing the activity of different antioxidant and defence enzymes in soybean.

Study design: Randomized block Method

Place and Duration of Study: Department of Molecular biology and Biotechnology, RCA, MPUAT, Udaipur, Sub humid southern plain region of India between 2017-2019.

Methodology: We synthesized, characterized and tested the efficacy of Cu-chitosan Nanoparticles against Bacterial pustule disease in Soybean. Activity of antioxidant [superoxide dismutase (SOD) and peroxidase (POD)] and defence enzymes [phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO)] were estimated after treating the plants with different concentrations of Cu-chitosan nanoparticles.

Results: Three treatments of Cu-chitosan treatments (0.02, 0.06 and 0.10%) were found most effective in disease control in both laboratory and net house condition) experiments. Cu-chitosan Nanoparticles application to soybean plants significantly enhanced the activity of antioxidant enzymes (SOD and POD) and defence enzymes (PAL and PPO) from 1.1 to 2.29 folds.

Conclusion: Chitosan Nanoparticles have been investigated as a carrier for active ingredient delivery for various applications due to their biocompatibility, biodegradability, high permeability, cost-effectiveness, non-toxicity, excellent film forming ability and antimicrobial activities. Cu-chitosan NPs developed in this study were effective in controlling disease by enhancing the activity of different antioxidant [superoxide dismutase (SOD) and peroxidase (POD)] and defence enzymes [phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO)]. This is an important development in agriculture nanomaterial research where biodegradable Cu-chitosan nanoparticles are better compatible with biological control as NPs "mimic" the natural elicitation of the plant defence and antioxidant system for disease protection and sustainable growth.

The potential of Cu-chitosan NPs is this study anticipated that developed NPs could be further exploited in large scale experiments. In the present investigation, the efficacy of Cu-chitosan nanoparticles to boost defence responses against Bacterial disease of soybean were evaluated.

Keywords: *Chitosan, Antioxidants, Defence enzymes and Copper Chitosan Nanoparticles.*

1. INTRODUCTION

Environmental contamination has become a challenging issue because of uncontrolled and extensive use of synthetic agrochemicals for plant growth and protection [30]. The continuous use of agrochemicals causes several adverse effects including, increased resistance in plant pathogenic microbes, negative impact on non-target organisms and deterioration of soil health [9] and [35]. Globally, crops are severely affected by diseases which lead to qualitative and quantitative losses in agriculture. Consequently, potential emphasis needs to be concentrated on development of biomaterial based biodegradable agrochemicals for effective and safe application in crops. To address the increasing challenges of sustainable production and food security, significant technological advancements and innovations have been made in recent years in the field of agriculture [5, 14 and 33]. Such continuous agricultural innovations are crucial to meet the increasing food demand of exploding global population through the uses of natural and synthetic resources. In particular, nanotechnology has potential to provide effective solutions to the multiple agriculture-related problems. To bridge the gap between bulk materials and atomic or molecular structures, nanoparticles provide a great scientific interest. Over the last two decades, a significant amount of research has been carried out on nanotechnology emphasizing its numerous applications in agriculture sectors [3, 15and 19].

Chitosan, a versatile biomaterial that is of a non-toxic, biocompatible and biodegradable nature, is being exploited in agriculture [10 and 34]. It is well recognized as an antimicrobial [6 and 13] immuno modulatory [1, 18, 23 and 24] and plant growth promotory agent in concentration of 0.04 to 0.16% [8]. Higher physiological and biochemical responses of chitosan based NPs as compared to bulk chitosan is due to its high surface to volume ratio and surface charge [20, 22 and 31]. Hence, chitosan based NPs have been used for various applications in agriculture including plant growth [2, 21, 27 and 28). Recently, chitosan based NPs have been evaluated as potent inducer of antioxidant [superoxide dismutase (SOD) and peroxidase (POD)] and defence enzymes [phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO)]. Transcript analysis of chitosan NPs treated plants showed that increased level of defence responses was due to high expression of defence related genes. These finding supported the enhanced innate immunity of plants by chitosan component of NPs. In our previous studies, we have reported Cu-chitosan NPs as an effective antibacterial and plant growth promotory agent.

In the present investigation, we report for the first time the efficacy of Cu-chitosan NPs to induce the Defence Related Enzyme in Soybean under *in vitro* and net house conditions. Our results convincingly establish Cu-chitosan NPs as a potent inducer of Defence Related Enzyme for effective control of Bacterial pustule disease of soybean.

2. MATERIALS AND METHODS

2.1 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 (22). 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 [26 and 22].

Soybean cultivars JS-335 susceptible towards bacterial pustules disease were selected. The seed lots to be used during the course of research were selected carefully by visual assessment to ensure the disease free and healthy vigour seeds. Highly infectious *Xanthomonas axonopodis pv.* glycine causing bacterial pustule disease of soybean, were procured.

Seeds were treated for 4h in different concentrations of Cu-chitosan NPs *viz.* 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. Pre-treated seeds were sown

In plastic pots filled with commercial coco soil at laboratory conditions under 16/8h light and dark condition. Foliar application of various concentrations of Cu-chitosan NPs as mention above was used after emergence of first trifoliate stage. Enzyme assay were conducted in leaves after 12, 48 and 72h of foliar application.

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 [7, 11].

Chart 1: Experimental details

Variety	JS-335

Number of treatments	9
T_1	Untreated control
T_2	Bulk chitosan
T_3	$CuSO_4$ (0.01%)
Cu-chitosan nanoparticles (NPs)	
T_4	Cu-chitosan NPs- 0.02 %
T_5	Cu-chitosan NPs- 0.04 %
T_6	Cu-chitosan NPs- 0.06 %
T_7	Cu-chitosan NPs- 0.08 %
T_8	Cu-chitosan NPs- 0.10 %
T_9	Cu-chitosan NPs- 0.12 %
Replication	03
Mode of experiment	*In Vitro* and Net house conditions

2.2 Measurements of enzyme activity:

Activity of antioxidant [superoxide dismutase (SOD) and peroxidase (POD)] and defence enzymes [phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO)] were estimated in 3rd leaf after 24 h of foliar spray of various treatments. For enzymes extraction, 0.2 g samples were homogenized in 5 ml of extraction Buffer (phosphate Buffer for SOD and PPO at pH 7.4 and 6.8, respectively; tris-HCl Buffer at pH 7.5 for POD and borate Buffer at pH 8.8 for PAL). The homogenates were centrifuged at 10,000×g for 20 min at 4 °C and supernatants were taken for enzymes assay. SOD (EC 1.15.1.1) activity was determined at 560nm, as reduction of nitro-blue tetrazolium (NBT) as an indicator of superoxide anion production39. POD (EC 1.11.1.7) activity was measured spectrophotometrically as described by Chance and Maehly by oxidation of guaiacol in the presence of hydrogen peroxide. Increase in absorbance at 470nm was recorded due to formation of tetra guaiacol. PPO (EC 1.10.3.1) was assayed according to Taneja and Sachar and activity was expressed as change in absorbance at 490nm. PAL (EC 4.3.1.5) was estimated as described by Moerschbacher where the deamination of L-phenylalanine to trans-cinnamic acid and ammonia was measured at 290nm. Activities of all the enzymes were expressed in µmol/min/g tissue

2.3 Disease assessment:

Disease incidence and disease severity was assessed in pot plants by using Bull and Koike scale as describe earlier [17]. 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).

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

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

Table 1: Rating scale for Bacterial pustule disease

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

3. RESULTS AND DISCUSSION

Chitosan nanoparticles have previously been reported as immune modulator through induction of antioxidant/defense enzymes activity in tea, maize and fnger millet plants [24 and 2]. Before conducting the pot and Net house experiments for defence responses and plant growth, in vitro antibacterial test was conducted to depict the degree of growth inhibition of Cu-chitosan NPs against *Xanthomonas axonopodis*. A considerable % control was noticed in the experiment. In our previous study, same batch of Cu-chitosan nanoparticles were found effective in inhibiting Pseudomonas syringae [26] and mycelial growth of Alternaria solani and Fusarium oxysporum in in vitro experiments [22].

3.1 Defence related enzyme activity SOD activity was recorded higher in Cu-chitosan NPs treatments at 12 hr. In addition, maximum activity was found in 0.06% of Cu-chitosan NPs which was 1.40 fold higher than control. At 48 hr Cu-chitosan NPs treatments showed higher activity compared to control. Similarly, at 72 hr 0.02 to 0.06 and 0.10% of Cu-chitosan NPs enhanced activity was recorded as compared to control and bulk chitosan treated plants. Maximum activity was found in 0.04% of Cu-chitosan NPs which was 1.32 fold higher than control (Fig. 1A).

At 12 hr and 48 hr POD activity was recorded higher in control but at 72 hr 0.02 and 0.06% concentration of Cu-chitosan NPs showed higher activity compared to control, bulk chitosan and $CuSO_4$ treated plants. In addition, maximum activity was found in 0.02% of Cu-chitosan NPs which was 1.38 fold higher than control (Fig. 1B).

PAL activity was significantly higher in 0.04 to 0.10% of Cu-chitosan NPs at 12 hr. At 48 hr 0.06% of Cu-chitosan NPs showed enhanced activity compared to control, bulk chitosan and $CuSO_4$ treated plants which was 2.29 fold higher than control. Similarly, at 72 hr 1.1–2 folds higher activity was recorded in 0.02 to 0.10% NPs treated plant leaves as compared to control and bulk chitosan treated plants (Fig. 1C).

PPO activity was recorded higher in 0.04 and 0.06% of Cu-chitosan NPs treatments at 12 hr. In addition, maximum activity was found in 0.06% of Cu-chitosan NPs which was 1.6 folds higher than control. At 48 hr 0.04 to 0.08% of Cu-chitosan NPs enhanced activity was recorded as compared to control, bulk chitosan and $CuSO_4$ treated plants. In addition, Maximum activity was found in 0.04 and 0.06% of Cu-chitosan NPs which was 1.30 fold higher than control. At 72 hr 0.02 to 0.08% of Cu-chitosan NPs treatments showed higher activity compared to control, bulk chitosan and $CuSO_4$ treated plants (Fig. 1D).

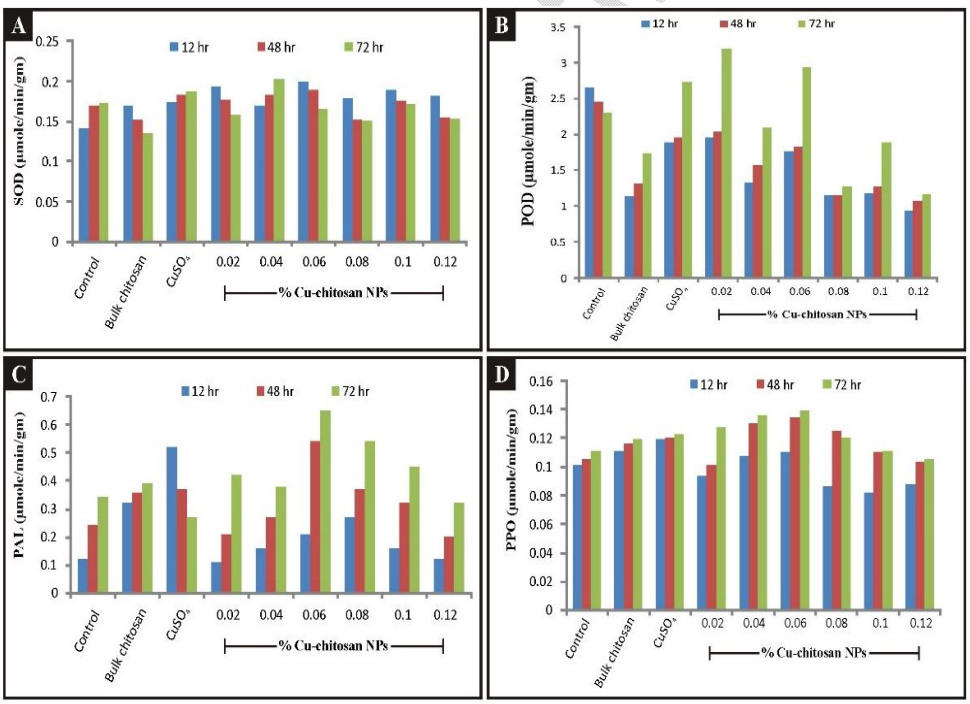

Fig 1. Effect of Cu-chitosan NPs on (A)SOD (B) POD (C)PAL and (D) PPO enzymes activity in soybean plant leaves after 12, 48 and 72 hr of foliar spray in pots (under laboratory condition). Control with water. Bulk chitosan, (0.01%) dissolved in 0.1% acetic acid. $CuSO_4$ (0.01%).

3.2 Defence related enzyme activity under net house condition SOD activity was significantly higher in 0.02, 0.06 and 0.10% of NPs (Fig. 2A). 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. 2B). The activity of PAL was also enhanced by NPs treatments (0.06 - 0.10%) as compared to control, bulk chitosan and CuSO4 treatments (Fig. 2C). Likewise, Cu-chitosan NPs treated plants leaves showed 1–2 folds increased PPO activity as compared to control and bulk chitosan treatment (Fig. 2D).

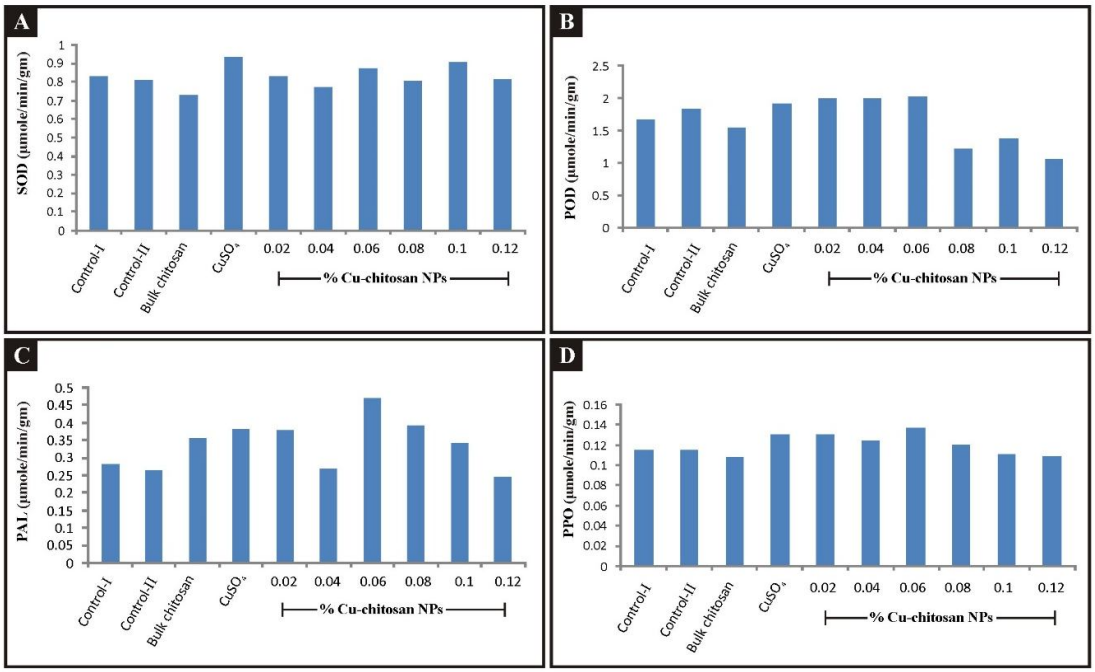

Fig 2. Effect of Cu-chitosan NPs on (A)SOD (B) POD (C)PAL and (D) PPO enzymes activity in soybean Plant leaves after 48 hr of foliar spray in pots (under laboratory condition). Control with water. Bulk chitosan, (0.01%) dissolved in 0.1% acetic acid. $CuSO_4$ (0.01%).

3.3 Disease occurrence With 4 hr 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)

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 П	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 П (water treated + inoculation). Chitosan dissolved in 0.1% acetic acid. PEDC = Percentage efficacy of disease control was calculated compare to control.

Three treatments of Cu-chitosan treatments (0.02, 0.06 and 0.10%) were found most effective in plant growth and disease control in pot (both laboratory and net house condition) experiments. Cu-chitosan NPs application to soybean plants significantly enhanced the activity of antioxidant enzymes (SOD and POD) and defence enzymes (PAL and PPO) from 1.1 to 2.29 folds.

4. CONCLUSION

Chitosan Nanoparticles have been investigated as a carrier for active ingredient delivery for various applications due to their biocompatibility, biodegradability, high permeability, cost-effectiveness, non-toxicity, excellent film forming ability and antimicrobial activities. Cu-chitosan NPs developed in this study were effective in controlling disease by enhancing the activity of different antioxidant [superoxide dismutase (SOD) and peroxidase (POD)] and defence enzymes [phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO)]. The potential of Cu-chitosan NPs is this study anticipated that developed NPs could be further exploited in large scale experiments.

5. STATISTICAL ANALYSIS

Statistical analysis of the data was performed with JMP software version 12. The significant difference 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.

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