Original Research Article

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

ABSTRACT

Aim: Assessment 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 (MBBT), RCA, Maharana Pratap University of Agriculture and Technology, Sub humid southern plain region of India between 2017-2019.

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

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

Conclusion: Cu-chitosan NPs developed in this study were effective in controlling disease by enhancing the activity of different defence related enzymes [polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL)], and antioxidant [peroxidise (POD) and superoxide dismutase (SOD)]. This could be an important nonmaterial research in the field of agriculture due to better compatibility of biodegradable Cu-chitosan nanoparticles with biological control. In this study, the effect of Cu-chitosan nanoparticles to boost defence responses against *Xanthomonas axonopodis* pv. *Glycine* were evaluated.

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

1. INTRODUCTION

"The continuous use of agrochemicals causes several adverse effects including, deterioration of soil health and increased resistance in plant pathogenic microbes and also affect the non-target organisms" [9] and [35]. "To deal with sustainable production and increasing challenges of food security, significant technological advancements and innovations have been made in these recent years in agriculture sector" [5, 14 and 33]. "To meet the increasing food demand of exploding global population through the uses of natural and synthetic resources such continuous agricultural innovations are crucial. 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 plentiful applications in agriculture sectors" [15 and 19].

Due to versatile, biodegradable, non-toxic and biocompatible nature chitosan 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]. "As compared to bulk chitosan, chitosan based nanoparticles have higher physiological and biochemical responses due to its high volume ratio and surface to surface charge" [20, 22 and 31]. "Hence, chitosan based Nanoparticles have been used for various applications in agriculture" [2, 21, 27 and 28). Recently, chitosan based NPs have been evaluated as potent inducer of defence related enzymes [polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL)], and antioxidant [peroxidise (POD) and superoxide dismutase (SOD)]. Transcript analysis of chitosan NPs treated plants showed that increased level of defence responses was due to high defence related genes expression. These finding supported the enhanced innate immunity of plants by chitosan component of Nanoparticles. In our previous studies, we have reported Cu-chitosan NPs as an effective antibacterial and plant growth promotory agent.

In this study, we reported 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 persuasively established Cu-chitosan Nanoparticles as a persuasive inducer of defence related enzyme for efficient control of Bacterial pustule disease of soybean.

2. Methods and Materials

2.1 Preparation and characterization of Cu-chitosan Nanoparticles: Ionic gelation method was followed to prepare Cu-chitosan nanoparticles. This method was developed in our laboratory. Characteristics of these synthesized NPs were same as we reported in our earlier publications [26 and 22].

The Soybean cultivars JS-335 which is susceptible towards bacterial pustules disease was being used during the course of research. The Seed lot were selected carefully by visual assessment to ensure the healthy and disease free vigour seeds. Highly infectious bacterial pustule disease causing agent *Xanthomonas axonopodis pv. glycines* were procured.

Seeds were treated for 4h in different concentrations of cu-chitosan nanoparticles *viz.* 0.02, 0.04, 0.06,0.08, 0.10 and 0.12 % w/v along with controls [bulk chitosan (0.01%), $CuSo_4$ (0.01%) and Untreated] in aqueous suspension. These 4 hr 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	
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%
Replications	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 production. 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" [3].

2.3 Disease Assessment: Disease severity and disease incidence was assessed in plants by using an earlier described scale by Bull and Koike [17]. By calculating the proportion of diseased plant in each treatment, disease incidence was determined. Disease severity was evaluated by rating the most severely damaged are on the plant on a scale of 0 to 5 (table 1). Further, the formula given by Chester (1959) and Wheeler (1969) was used to calculate the percentage efficacy of disease control (PEDC) and disease severity (DS).

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

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

Table 1: Rating scale for Bacterial disease

Description	Disease rating
Plant with no visible symptoms	0
A few individual lesions	1
Many individual lesions	2
Small patches of coalesced lesions	3

Medium sized patched of coalesced lesions	4
Large sized patched of coalesced lesions	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.

3. RESULTS AND DISCUSSION

"Previously, in maize, finger millet and tea plants, chitosan nanoparticles have been reported as immune modulator through induction of defence/antioxidant enzyme activity" [24 and 2]. *In vitro* antibacterial test was conducted to illustrate the degree of growth inhibition of Cu-chitosan NPs against *Xanthomonas axonopodis*, before conducting the pot and Net house experiments for disease responses. A considerable % control was noticed in the experiment. Same batch of Cu-chitosan NPs were found effective in our previous studies in inhibiting *Pseudomonas syringae* [2] and mycelia growth of *Alternaria solani* and *Fusarium oxysporum* in *in vitro* experiments [22].

3.1 Defence related enzyme activity SOD activity was found higher in Cu-chitosan NPs treatments at 12 hr. In addition, maximum activity was recorded in 0.06% of Cu-chitosan NPs which was 1.40 fold higher than control. At 48 hr Cu-chitosan Nanoparticles treatments showed higher activity compared to control. Similarly, at 72 hr 0.02 to 0.06 and 0.10% of Cu-chitosan nanoparticles enhanced activity was recorded as compared to bulk chitosan treated and control plants. Maximum activity was recorded in 0.04% of Cu-chitosan NPs which was 1.32 folds 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 Cuchitosan NPs showed higher activity compared to control, bulk chitosan and $CuSO_4$ treated plants. In addition, maximum activity was recorded 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 recorded 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 found in 0.02 to 0.10% NPs treated plant leaves as compared to control and bulk chitosan treated plants (Fig. 1C).

PPO activity was found higher in 0.04 and 0.06% of Cu-chitosan NPs treatments at 12 hr. In addition, maximum activity was recorded 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, $CuSO_4$ and bulk chitosan 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, $CuSO_4$ and bulk chitosan treated plants (Fig. 1D).

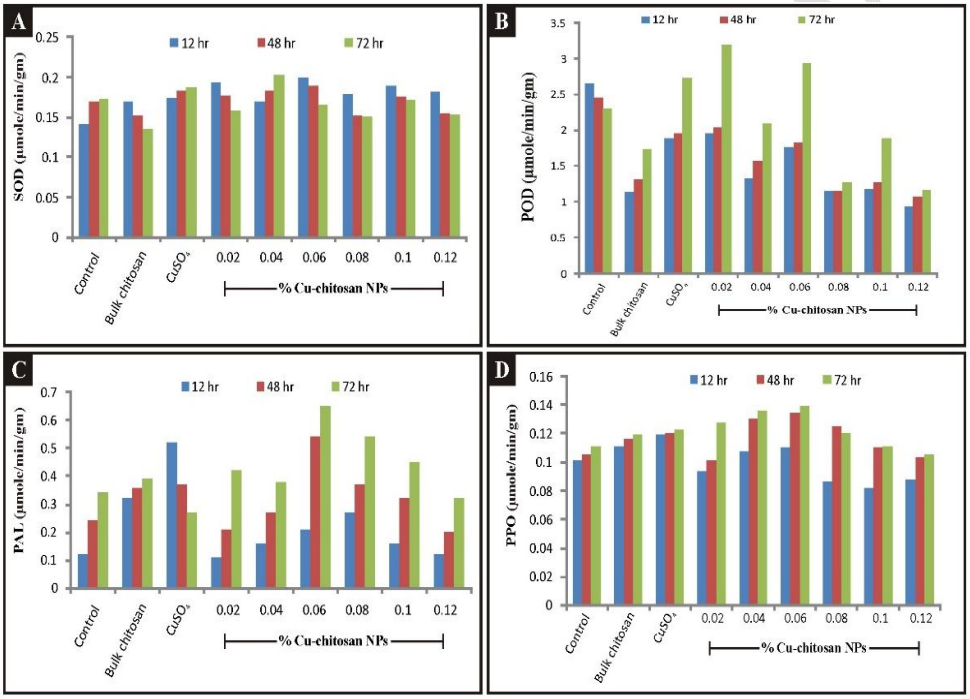

Fig 1. Effect of Cu-chitosan NPs on (A)SOD (B) POD (C)PAL and (D) PPO enzymes activity in soybean plant leaves after 12, 48 and 72 hr of foliar spray in pots (under laboratory condition). Control with water. Bulk chitosan, (0.01%) dissolved in 0.1% acetic acid. $CuSO_4$ (0.01%).

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 $CuSO_4$ 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)

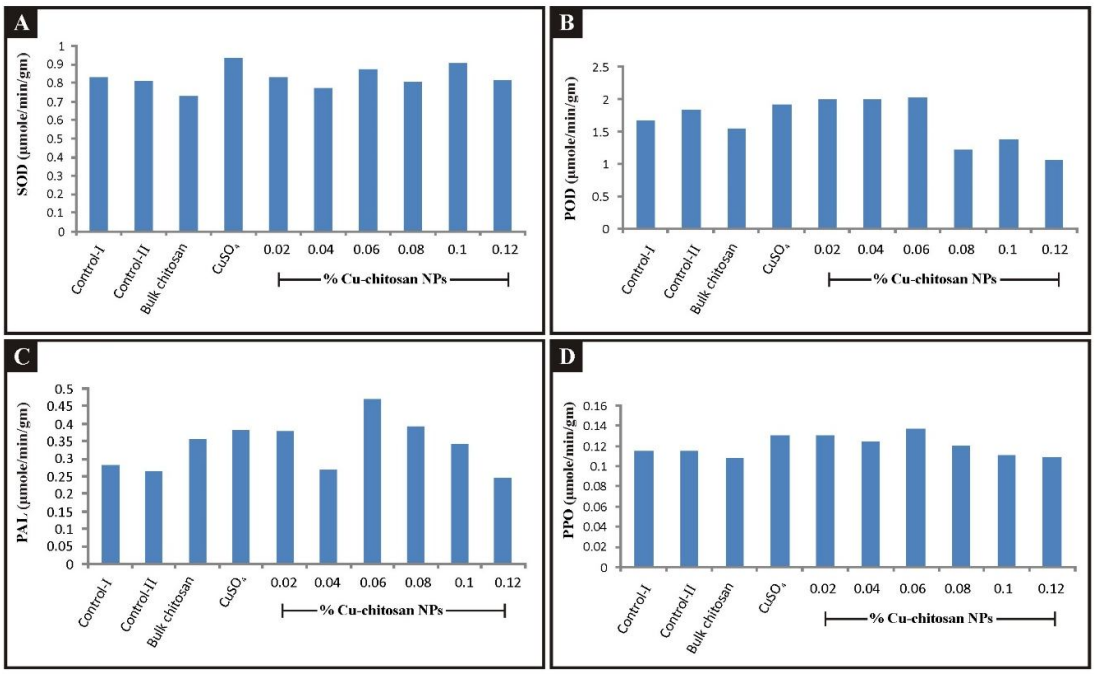

Fig 2. Effect of Cu-chitosan NPs on (A)SOD (B) POD (C)PAL and (D) PPO enzymes activity in soybean Plant leaves after 48 hr of foliar spray in pots (under laboratory condition). Control with water. Bulk chitosan, (0.01%) dissolved in 0.1% acetic acid. $CuSO_4$ (0.01%).

3.3 Disease occurrence After 4 hr of seed treatment followed by two foliar sprays, control plants showed average disease severity 64.30%. All plants treated with Cu-chitosan NPs (0.02 to 0.12%) showed significant antibacterial activity and express lower disease severity 50.0% to 33.3%. $CuSO_4$ showed 26.3% and 40.0% disease severity was found in bulk chitosan. PEDC was found maximum (49.7%) at 0.06% of Cu-chitosan NPs. Significantly higher PEDC value was recorded in 0.06% of Cu-chitasan NPs from all other treatments at statistical level (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 1	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}

Three treatments of Cu-chitosan treatments (0.02, 0.06 and 0.10%) were found most effective in 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 and defence enzymes from 1.1 to 2.29 folds.

4. CONCLUSION

Cu-chitosan NPs developed in present investigation were found effective in controlling the disease by enhancing the activity of the antioxidant (superoxide dismutase and peroxidase) and defence enzyme (phenylalanine ammonia-lyase and polyphenol oxidase). In this study, the potential of Cu-chitosan NPs anticipated that these nanoparticles could be further exploited in large scale experiments.

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