Original Research Article

Cu-Chitosan Nanoparticles Stimulated Defence responses in Soybean crop

ABSTRACT

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

Study design: Randomized block Method

Place and Duration of Study: Department of Molecular biology and Biotechnology, RCA, MPUAT, Udapur, between 2017-2019.

Methodology: To test the efficacy of Cu-chitosan Nanoparticles against Bacterial pustule disease, Lab and Net house experiments were conducted. Activity of antioxidant [superoxide dismutase (SOD) and peroxidase (POD)] and defense enzymes [phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO)] were estimated 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 defense 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 defense 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.

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 promotry agent [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 defense 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 promotry agent.

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

3.1 Defense related enzyme activity under *In Vitro* condition 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).

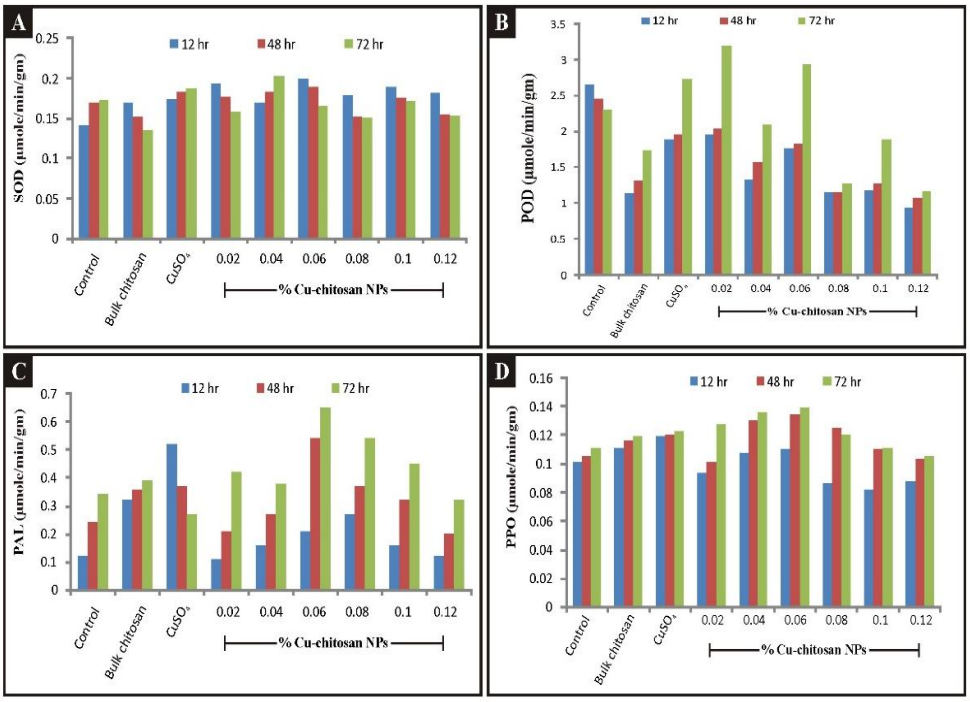

Fig 1. Effect of Cu-chitosan NPs on (A)SOD (B) POD (C)PAL and (D) PPO enzymes activity in soybean plant leaves after 12, 48 and 72 hr of foliar spray in pots (under laboratory condition). Control with water. Bulk chitosan, (0.01%) dissolved in 0.1% acetic acid. $CuSO_4$ (0.01%).

3.2 Defense 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).

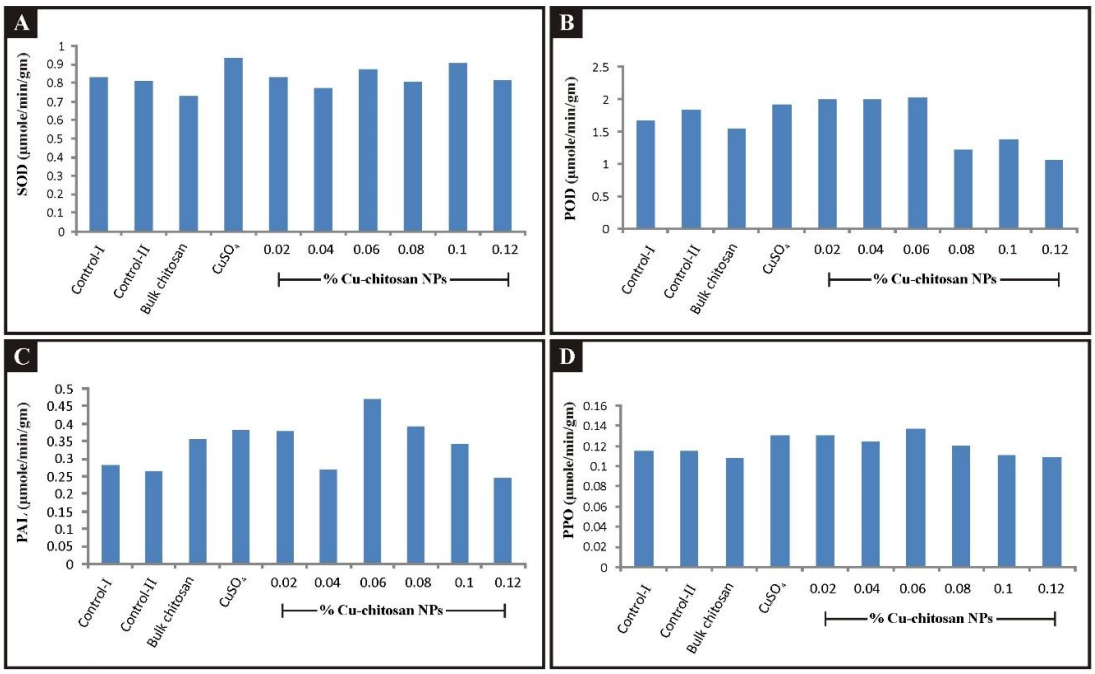

Fig 2. Effect of Cu-chitosan NPs on (A)SOD (B) POD (C)PAL and (D) PPO enzymes activity in soybean Plant leaves after 48 hr of foliar spray in pots (under laboratory condition). Control with water. Bulk chitosan, (0.01%) dissolved in 0.1% acetic acid. $CuSO_4$ (0.01%).

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 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 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 defense enzymes (PAL and PPO) from 1.1 to 2.29 folds.

4. 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.

5. REFERENCES

[1] Amborabé B-E, Bonmort J, Fleurat-Lessard, P & Roblin G. Early events induced by chitosan on plant cells. *J. Exper. Bot*. 2008; **59,** 2317–2324.

[2] Chandra S. et al. Chitosan nanoparticles: a positive modulator of innate immune responses in plants. *Sci. Rep.* 2015**; 5,** 15195.

[3] Chen Y W, Lee H V, Juan J C, Phang S M. Production of new cellulose nanomaterial from red algae marine biomass Gelidium elegans. *Carbohydr. Polym.* 2016; **151**, 1210–1219.

[4] Chester K S. How sick is the plant? In: Plant Pathology an Advances Traits (Eds. J.G. Horsfall and A.E. Diamond), *Academic Press, New York*. 1959; **1:** 199-242.

[5] Dwivedi S, Saquib Q, Al-Khedhairy A A, Musarrat J. Understanding the role of nanomaterials in agriculture. In Microbial Inoculants in Sustainable Agricultural Productivity; Singh, D.P., Singh, H.B., Prabha, R., Eds.; *Springer: New Delhi, India*. 2016; 271–288.

[6] Goy R C, Morais S T & Assis O B. Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on E. coli and S. aureus growth. *Revista Brasileira de Farmacognosia.* 2016; **26**, 122–127.

[7] Jung W J, Mabood F, Souleimanov A and Smith D L. Induction of defense-related enzymes in soybean leaves by class IId bacteriocins (thuricin 17 and bacthuricin F4) purified from Bacillus strains. *Microbiological Research*. 2011; **167:** 14-19.

[8] Kananont N, Pichyangkura R, Chanprame S, Chadchawan S and Limpanavech P. Chitosan specificity for the in vitro seed germination of two Dendrobium orchids (Asparagales: Orchidaceae). *Scientia Horticul*. 2010; **124**, 239–247.

[9] Kashyap P L, Xiang X and Heiden P. Chitosan nanoparticle-based delivery systems for sustainable agriculture. *Int. J. Biol. Macromol*. 2015; **77**, 36–51.

[10] Katiyar D, Hemantaranjan A and Singh B. Chitosan as a promising natural compound to enhance potential physiological responses in plant: a review. *Indian J. Plant Physiol*. 2015; **20**, 1–9.

[11] Khan W, Prithiviraj B and Smith D L. Chitosan and chitin oligomers increase phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities in soybean leaves. *Journal of Plant Physiology.* 2003; **160:** 859-863.

[12] Kim K H, Park J H, Kim M Y, Heu S and Lee S H. Genetic mapping of novel symptom in response to soybean bacterial leaf pustule in PI 96188. *Journal of Crop Science and Biotechnology*. 2011; **14 (2):** 119-123.

[13] Kong M, Chen X G, Xing K and Park H J. Antimicrobial properties of chitosan and mode of action: a state of the art review. *Int. J. Food Microbiol*. 2010; **144**, 51–63.

[14] Kou T J, Yu W W, Lam S K, Chen D L, Hou Y P, Li Z Y. Di erential root responses in two cultivars of winter wheat (Triticum aestivum L.) to elevated ozone concentration under fully open-air field conditions. *J. Agron. Crop Sci.* 2018; **204**, 325–332.

[15] Lv M, Liu Y, Geng J H, Kou X H, Xin Z H, Yang D Y. Engineering nanomaterials-based biosensors for food safety detection. *Biosens Bioelectron.* 2018; **106**, 122–128.

[16] Moerschbacher B M, Noll U M, Flott B E and Reisener H J. Lignin biosynthetic enzymes in stem rust infected, resistant and susceptible near-isogenic wheat lines. *Physiological and Molecular Plant Pathology.* 1988; **33:** 33-46.

[17] Odubanwo B O, Popoola A R, Ojo D K and Ganiyu S A. Yield performance of soybean accessions on field naturally infected with bacterial pustules (*Xanthomonas axonopodis* pv. *glycines*). *African Crop Science Society.* 2013; **11:** 309-315.

[18] Popova E, Domnina N, Kovalenko N, Sokornova S and Tyuterev S. Effect of chitosan and vanillin-modified chitosan on wheat resistance to spot blotch. *Appl. Biochemi. Microbiol*. 2016; **52**, 537–540.

[19] Prasad R, Bhattacharyya A and Nguyen Q D. Nanotechnology in sustainable agriculture: Recent developments, challenges, and perspectives. Front. Microbiol. 2017; **8**, 1014.

[20] Saharan V and Pal A. Chitosan Based Nanomaterials in Plant Growth and Protection. *Springer Briefs in Plant Sci*. 2016.

[21] Saharan V. In International Conference on Advances in Biotechnology (BioTech). Proceedings. 2014; **23**.

[22] Saharan V. Synthesis and in vitro antifungal efficacy of Cu–chitosan nanoparticles against pathogenic fungi of tomato. *Int. J. Biol. Macromol.* 2015; **75**, 346–353.

[23] Sathiyabama M and Manikandan A. Chitosan nanoparticle induced defense responses in fingermillet plants against blast disease caused by Pyricularia grisea (Cke.) Sacc. *Carbo. Poly.* 2016; **154**, 241–246.

[24] Sathiyabama M and Parthasarathy R. Biological preparation of chitosan nanoparticles and its in vitro antifungal efficacy against some phytopathogenic fungi. *Carbo. Polymers.* 2016; **151**, 321–325.

[25] Sathiyabama M, Bernstein N and Anusuya S. Chitosan elicitation for increased curcumin production and stimulation of defence response in turmeric (Curcuma longa L.). *Ind. Crops Prod.* 2016; **89**, 87–94.

[26] Swati, Choudhary M K, Joshi A and Saharan V. Assessment of Cu- Chitosan Nanoparticles for its Antibacterial Activity against Pseudomonas syringaepv. glycinea. *International Journal of Current Microbiology and Applied Sciences*. 2017; **6:**1335-1350.

[27] Swati, Joshi A and Saharan V. Cu- chitosan nanoparticles: As an antibacterial agent against bacterial pustule disease of Soybean [Glycine max (L.)]. *International Journal of Chemical Studies.* 2018; **6**:994-999.

[28] Swati, Joshi K and Joshi A. Cu-chitosan nanoparticle induced plant growth and antibacterial activity against bacterial pustule disease in soybean [Glycine max (L.)]. *Journal of Pharmacognosy and Phytochemistry.* 2020; **9:** 450-455.

[29] Taneja S R and Sachar R C. Induction of polyphenol oxidase in germinating wheat seeds. *Phytochemestry*. 1974; **13:** 2695-2702.

[30] Tilman D, Cassman K G, Matson P A, Naylor R and Polasky S. Agricultural Sustainability and Intensive Production Practices. *Nature*. 2002; **418**, 671–677.

[31] Van S N, Minh H D and Anh D N. Study on chitosan nanoparticles on biophysical characteristics and growth of Robusta coffee in green house. *Biocat. Agric. Biotechnol*. 2013; **2**, 289–294.

[32] Wheeler B E J. An Introduction to plant disease. *John Wiley and Sons Limited, London*. 1969; Pp. 301.

[33] Xiao M, Song F, Jiao J, Wang X, Xu H, Li H. Identification of the gene Pm47 on chromosome 7BS conferring resistance to powdery mildew in the Chinese wheat landrace Hongyanglazi. *Theor. Appl. Genet*. 2013; **126**, 1397–1403.

[34] Xing K, Zhu X, Peng X and Qin S. Chitosan antimicrobial and eliciting properties for pest control in agriculture: a review. *Agronomy Sustain. Develop.* 2015; **35**, 569–588.

[35] Zhan J, Thrall P H, Papaïx J, Xie L and Burdon J J. Playing on a pathogen's weakness: using evolution to guide sustainable plant disease control strategies. *Ann. Rev. Phytopathol*. 2015; **53**, 19–43.