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Cu-chitosan nanoparticle boost defense responses and plant growth in maize (*Zea mays* L.)

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In agriculture, search for biopolymer derived materials are in high demand to replace the synthetic agrochemicals. In the present investigation, the efficacy of Cu-chitosan nanoparticles (NPs) to boost defense responses against *Curvularia* leaf spot (CLS) disease of maize and plant growth promotory activity were evaluated. Cu-chitosan NPs treated plants showed significant defense response through higher activities of antioxidant (superoxide dismutase and peroxidase) and defense enzymes (polyphenol oxidase and phenylalanine ammonia-lyase). Significant control of CLS disease of maize was recorded at 0.04 to 0.16% of Cu-chitosan NPs treatments in pot and 0.12 to 0.16% of NPs treatments in field condition. Further, NPs treatments exhibited growth promotory effect in terms of plant height, stem diameter, root length, root number and chlorophyll content in pot experiments. In field experiment, plant height, ear length, ear weight/plot, grain yield/plot and 100 grain weight were enhanced in NPs treatments. Disease control and enhancement of plant growth was further enlightened through Cu release profile of Cu-chitosan NPs. This is an important development in agriculture nanomaterial research where biodegradable Cu-chitosan NPs are better compatible with biological control as NPs "mimic" the natural elicitation of the plant defense and antioxidant system for disease protection and sustainable growth.

Environmental contamination has become a challenging issue because of uncontrolled and rampant use of synthetic agrochemicals for plant growth and protection¹. The perpetual 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^{2,3}. Globally, crops are severely affected by diseases which lead to qualitative and quantitative losses in agriculture⁴. Consequently, potential emphasis needs to be concentrated on development of biomaterial based biodegradable agrochemicals for effective and safe application in crops. Chitosan, a versatile biomaterial that is of a non-toxic, biocompatible and biodegradable nature, is being exploited in agriculture^{5,6}. It is well recognized as an antimicrobial^{7,8}, immuno modulatory⁹⁻¹¹ and plant growth promotory agent^{12,13}. 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 charge¹⁴⁻¹⁶. Hence, chitosan based NPs have been used for various applications in agriculture including plant growth¹³⁻¹⁸. Recently, chitosan based NPs have been evaluated as potent inducer of antioxidant and defense enzymes^{17,19}. Transcript analysis of chitosan NPs treated plants depicted that increased level of defense responses was due to high expression of defense related genes. These findings supported the enhanced innate immunity of plants by chitosan component of NPs¹⁸. In our previous studies, we have reported Cu-chitosan NPs as an effective antifungal and plant growth promotory agent^{15,20}. Further studies revealed that application of Cu-chitosan NPs enhanced maize seedling growth by mobilizing reserve food through the enhanced activities of α -amylase and protease²¹. To comprehend the dynamic bioactivities of Cu-chitosan NPs, which makes them more bioactive to other chitosan based NPs, we ought to understand the physicochemical properties of these NPs. Cu-chitosan NPs demonstrates porous network in which Cu is

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entrapped in the pores¹⁵. We strongly reckon that the porous architecture of chitosan NPs slowly releases Cu from the nanostructures. Therefore, we presupposed that after inflowing of Cu-chitosan NPs to plant cells, the contact of Cu to cellular system is long lasting^{14,15,20}. As we acquainted, Cu is well established in plants as a key structural and catalytic component in various enzymes of electron transfer and redox reactions, thus, crucial for boosting plant growth^{22,23}. Therefore, sustained releases of Cu from NPs prove for accelerating various metabolic processes in plant growth during various development stages. Moreover, in acidic pH environment of target site, chitosan porous network dissolved and entrapped Cu release faster^{14,15}. Alongside, it has been envisaged that establishment of acidic pH during infection of plant pathogenic fungi, faster releases of Cu may wield strong fungicidal activity against fungal pathogens^{14,15,24}. Thus Cu-chitosan NPs expressed a far elevated and diverse bioactivity as compared to sole chitosan based NPs. Up to now, rudimentary studies have been performed to induce the plant innate system for plant defense and subsequent higher growth and yield by NPs applications, thus, need further study of Cu-chitosan NPs for its effect on plant growth and protection for its comprehensive application in crop. World-wide, maize is an important food crop but is prone to various fungal diseases like curvularia leaf spot (CLS) disease caused by *Curvularia lunata*, which alone causes yield loss up to 60%²⁵. Many strategies have been applied to control CLS disease using chemicals and other bio-agent but there is no report on evaluation of Cu-chitosan NPs against CLS disease in maize.

In the present investigation, we report for the first time the efficacy of Cu-chitosan NPs to induce the defense responses against CLS disease in maize, and to promote sustainable plant growth 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 CLS disease of maize and a plant growth promotory agent.

Results

Cu-chitosan NPs. The NPs used in present study had almost same characteristics of mean hydrodynamic diameter 361.3 ± 2.1 nm, zeta-potential $+22.1$ mV and 0.20 PDI value (Fig. S1) as reported previously in DLS study (Fig. S2)^{15,21}. DLS data verified the stability (sufficient repulsion between positively charged NPs) and monodispersed nature (low PDI) of NPs in aqueous. In our earlier study, these NPs were well characterized for various physico-chemical properties like interaction of chitosan to TPP and Cu by fourier transform infrared spectroscopy (FT-IR) (Fig. S2), internal architecture by transmission electron microscopy (TEM) (Fig. S3), external architecture by scanning electron microscopy (SEM) (Fig. S3) and elemental analysis by energy dispersive X-ray spectroscopy (EDX) (Fig. S4)^{15,21}. Based on the physico-chemical characterizations, a hypothetical model was proposed for understanding of structural and synthesis aspects of Cu-chitosan NPs (Fig. S5)¹⁵. Affinity of bulk chitosan towards metals, especially Cu, has been well described in literature and strong evidences suggest that Cu-chitosan exist as metal-polymer complex through C-N bonding^{26,27}. Further, Cu-chitosan nano-complex has also been broadly studied through FT-IR, TEM, SEM and EDX. FT-IR spectrum of Cu-chitosan NPs strongly evident that peaks at 1636 cm^{-1} (CONH₂) and 1550 cm^{-1} (NH₂) were sharper and shifted to 1631 and 1536 cm^{-1} which denotes Cu bonding with chitosan nanostructures (Fig. S2)^{15,20}. TEM micrographs have visibly described Cu deposition into highly porous network of chitosan nanomaterials (Fig. S3)¹⁵. SEM-EDX analyses further inferred the higher deposition of Cu into porous surface of Cu-chitosan NPs (Fig. S4)¹⁵. Higher zeta-potential, low PDI value and embedded Cu endowed Cu-chitosan NPs a highly biologically active nanomaterial^{14,15}. These laboratory synthesized stable NPs were used in present study to evaluate their *in vitro* antifungal activity, effect on antioxidant and defense enzymes, disease control, plant growth and yield promotion in maize (Table 1).

Cu release profile. Release of Cu from Cu-chitosan NPs was studied in the pH range 1 to 7 (Fig. 1). With decrease in pH from 3 to 1, release of Cu increased rapidly from 21.5 to 44.11% due to protonation of amino group of chitosan. At pH above 6, release of Cu drastically decreased (4.94%) due to deprotonation of amino group of chitosan (Fig. 1; Table S7). Release study was further continued at 4.5 pH with respect to time. Cu release increased slowly and steadily with time and at 96 h ~85% of Cu released from Cu-chitosan NPs (Fig. 1; Table S7). The release profile indicates that acidic pH expedited the Cu release and over time (at pH 4.5), a slow and sustained release of Cu from Cu-chitosan NPs was evident.

***In vitro* antifungal activity of Cu-chitosan NPs.** Cu-chitosan NPs comprehensively inhibited *in vitro* mycelial growth of *C. lunata*. Cu-chitosan NPs (0.12–0.16%) significantly inhibited mycelial growth from 50.0 to 52.7% as compared to all other treatments (Fig. S6 and Table S1).

Effect of Cu-chitosan NPs on activities of antioxidant and defense enzymes in pot experiment. To estimate the activities of antioxidant and defense enzymes, leaf samples were collected after 24 h of foliar treatments. Application of NPs substantially induced the enzyme activities in leaves. SOD activity was significantly higher in all the treatments of NPs (Fig. 2a; Table S8). Similarly, 1.5–2 folds higher POD activity was recorded in 0.04 to 0.16% NPs treated plant leaves as compared to control and bulk chitosan treated plants (Fig. 2b; Table S8). Likewise, Cu-chitosan NPs treated plants leaves showed 2–3 folds increased PAL activity as compared to bulk chitosan treatment (Fig. 2c; Table S8). The activity of PPO was also enhanced by NPs treatments (0.12 and 0.16%) as compared to control (water), bulk chitosan and CuSO₄ treatments (Fig. 2d; Table S8).

Effect of Cu-chitosan NPs on CLS disease in pot experiment. In pot experiment, symptoms of CLS disease initiated after 3–4 days of fungal inoculation in control plants. The early appeared small chlorotic spot gradually extended into large eye shaped lesion, leading to the formation of leaf necrosis (Fig. 3a). Contrarily, in NPs treated plants, the disease symptoms in the form of small lesions without chlorosis were visualized after 7–8 days of fungal inoculation (Fig. 3b). The spread and severity of disease was also slow in proceeding days. After 15 days of inoculation, data for DS and PEDC were recorded. DS decreased with increasing concentrations of NPs as compared to other treatments (Table 2; Table S3). Commercially available fungicide (0.01% Bavistin), used

Experiment	Analysis/method	Remarks
Synthesis of Cu-chitosan NPs	Ionic gelation method ¹⁵	Cu-chitosan NPs were synthesized
<i>In-vitro</i> Cu release	Using AAS ¹⁵	Cu release from Cu-chitosan NPs was evaluated with respect to pH and time
Pot experiment		
Antioxidant and defense enzymes assay	Methods described by Giannopolitis and Ries ⁵² ; Chance and Maehly ⁴⁹ ; Moerschbacher <i>et al.</i> ⁵¹ ; Taneja and Sachar ⁵⁰	Activities of SOD, POD, PAL and PPO were estimated
Chlorophyll content (a, b)	As described by Stangarlin <i>et al.</i> ⁴⁷	Chlorophyll content (a, b) was quantified
Disease assessment	Using 1 to 9 standard disease rating scale	DS and PEDC were calculated
Copper content in leaves	AAS method described by Adrian ⁴⁸	Cu content in leaves was determined
Field experiment		
Days to 50%, tasseling, silking, ear leaf senescence and number of leaves/plant, plant height, ear length, ear weight/plot, grain yield/plot and 100 grain weight	Maize descriptor ⁵³	Crop yield assessment
Statistical analysis	JMP version-12 using Tukey–Kramer HSD test	Significant difference between treatment ($p = 0.05$) were calculated

Table 1. Experimental outline.

as positive control, showed 29.3% DS. All the plants treated with 0.04 to 0.16% Cu-chitosan NPs showed significantly lower DS to an extent of 24.6–22.6%. The Cu-chitosan NPs at 0.04–0.16% significantly controlled CLS disease as depicted by higher value of PEDC (Table 2; Table S3).

Effect of Cu-chitosan NPs on plant growth in pot experiment. To evaluate the effect of NPs on plant growth, various growth characteristics namely plant height, stem diameter, root length, root number and chlorophyll content were recorded. Statistical analyses showed that Cu-chitosan NPs significantly enhanced the growth of maize plants in pot experiments as compared to control, bulk chitosan, CuSO₄ and fungicide treatments (Fig. 4). Significantly higher values of plant height, stem diameter, root length and root number were recorded in 0.01 to 0.12% NPs treated plants (Fig. 5a–d; Table S9). A significant increase in chlorophyll a and b content (10.58 to 16.22 mg/g and 0.58 to 1.03 mg/g) was recorded in 0.01 to 0.12% of NPs treatments. In CuSO₄ treatment, chlorophyll a and b content were found minimum (4.53 and 0.20 mg/g) followed by 0.16% NPs (6.81 and 0.35 mg/g) treatment (Fig. 5e,f; Table S9). To illustrate the possible association between plant growth and Cu, Cu content was estimated in treated plant leaves by AAS. Increasing concentrations of Cu-chitosan NPs (0.01–0.16%) showed increased Cu content (8.6–28.5 µg/g dry weight) in treated leaves (Table 3; Table S4). CuSO₄ (0.01%) treated plant leaves had 24.1 µg/g dry weight of Cu, whereas in control (water) and bulk chitosan treatment, same content was observed (Table 3; Table S4).

Effect of Cu-chitosan NPs on CLS disease and crop yield. Data for DS and PEDC were recorded after 20 days of inoculation. At 0.12 and 0.16% of NPs treatments, plant showed comparatively less disease severity to other concentration of NPs, control (water), bulk chitosan, CuSO₄ and fungicide (Table 4; Table S5). Likewise, disease control was also considerably higher at higher concentration of NPs i.e. 0.12 and 0.16% (Table 4; Table S5). In field experiment, days to 50% tasseling, silking ear leaf senescence and number of leaves per plant were not affected by NPs treatments and remain same as with other treatments. Plant height and ear length was marginally higher in NPs treatments, whereas, ear weight/plot, grain yield/plot and 100 grain weight were significantly higher in 0.12 to 0.16% of NPs treatments (Table 5; Table S6).

Discussion

Among foliar diseases, CLS is common in maize growing countries and first reported from North Carolina and Georgia²⁸. Disease prevalent in hot, humid areas and the severity of the disease depends on environmental conditions and susceptibility of maize lines. Infected leaves develop white circular spots with dark/ brown marginal rings, that often coalesce to form larger infected areas, subsequently affected yellow leaves dried, cause significant damage up to 60%²⁹. Chitosan NPs have previously been reported as immune modulator through induction of antioxidant/defense enzymes activity in tea and finger millet plants^{11, 19}. Before conducting the pot and field experiments for defense responses and plant growth, *in vitro* antifungal test was conducted to depict the degree of growth inhibition of Cu-chitosan NPs against *C. lunata*. A considerable % inhibition of mycelial growth was noticed against *C. lunata* in the experiment (Table S1). In our previous study, same batch of Cu-chitosan NPs were found effective in inhibiting mycelial growth of *Alternaria solani* and *Fusarium oxysporum* in *in vitro* experiments¹⁵. In present study, foliar application of Cu-chitosan NPs in pot experiments substantially induced antioxidant/defense enzyme activity in maize leaves. NPs treated plant leaves showed 4–6 fold higher activity of SOD as compared to bulk chitosan (Fig. 2a). The higher activity of SOD effectively converts highly toxic superoxide radicals into less toxic H₂O₂ species³⁰. A significantly higher activity of POD, a key enzyme to scavenge H₂O₂ into

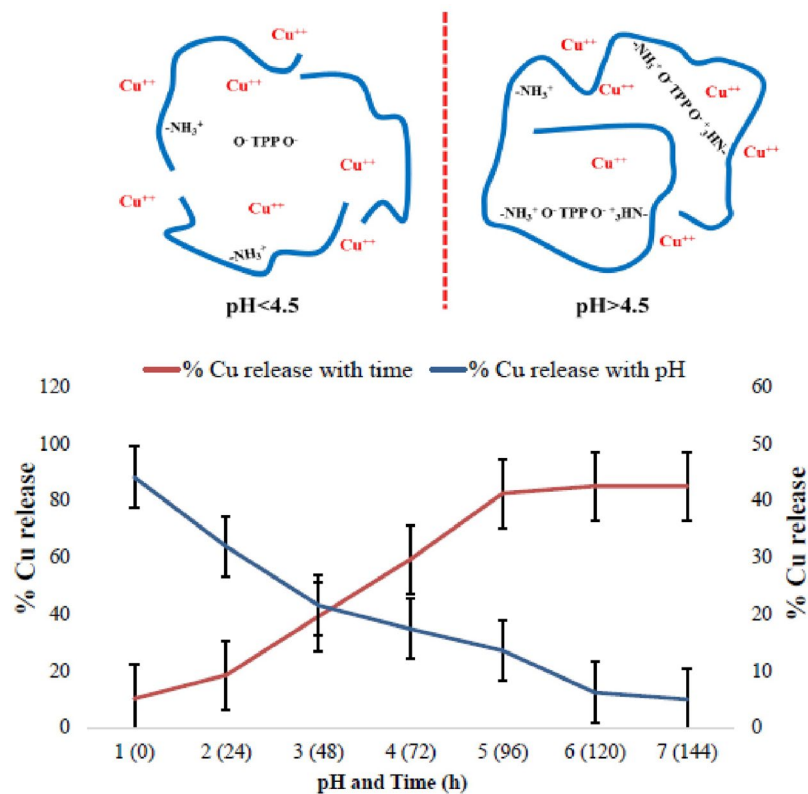


Figure 1. *In-vitro* Cu release from Cu-chitosan NPs at different pH and time. Each value is mean of triplicates and each replicate consisted of 3 samples.

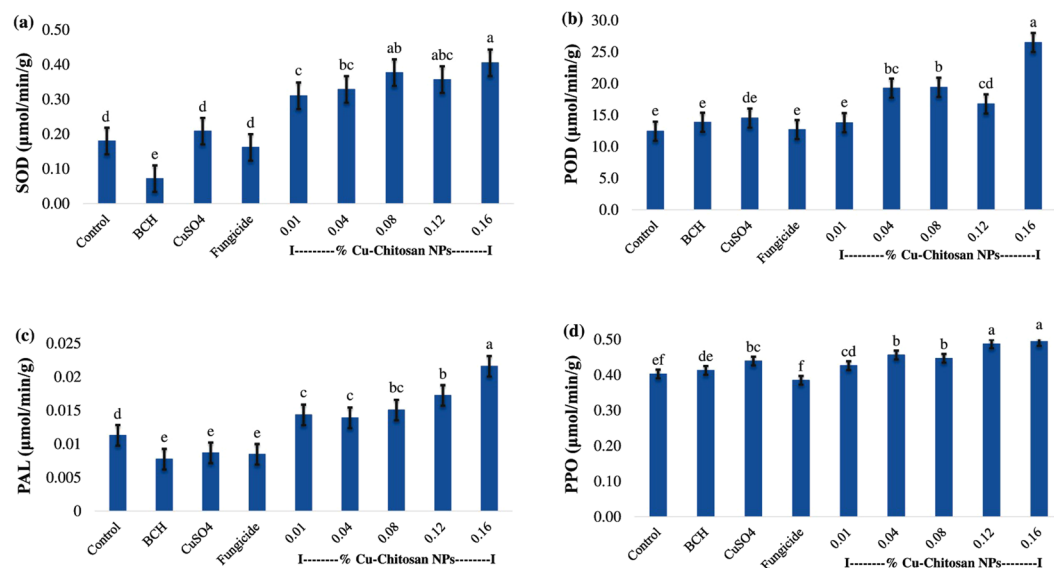


Figure 2. Effect of Cu-chitosan NPs on (a) SOD (b) POD (c) PAL (d) PPO enzymes activity in maize plant leaves after 24 h of foliar spray. Each value is mean of triplicates and each replicate consisted of 3 plants samples and same letter in the graph of each treatment is not significantly different at $p = 0.05$ as determined by Tukey–Kramer HSD, control with water. BCH (bulk chitosan, 0.01%) dissolved in 0.1% acetic acid. CuSO₄ (0.01%) and fungicide (0.01% of Bavistin).

H₂O and O₂, was also recorded in NPs treated leaves (Fig. 2b). The elevated activities of SOD and POD after NPs treatments might be responsible for balancing, degeneration, and scavenging of reactive oxygen species (ROS) to protect plant from oxidative stress during pathogen invasion.

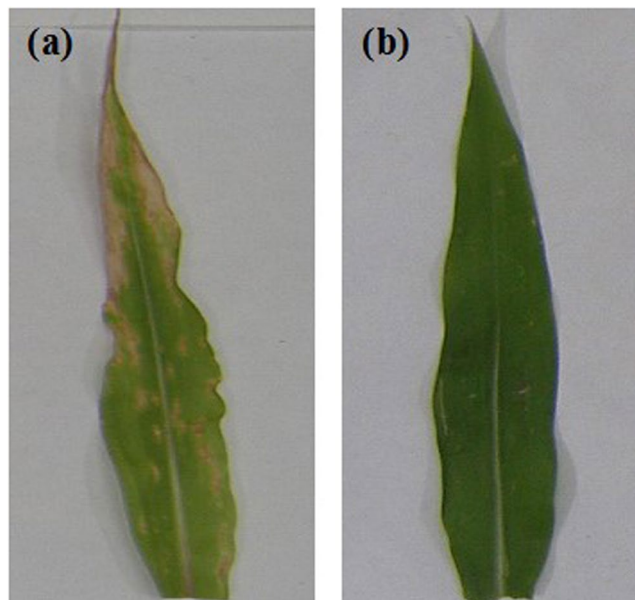


Figure 3. Symptoms of CLS disease on maize plant leaf in pot experiments (a) large necrotic lesion in control (b) micro lesions on Cu-chitosan NPs (0.16%) treated leaf.

Treatment (%)	DS (%) ^A	PEDC (%) ^A
Control (water)	44.00 ± 1.15 ^a	00.00 ± 0.00 ^e
BCH (0.01)	32.67 ± 0.66 ^b	25.72 ± 1.01 ^d
CuSO ₄ (0.01)	32.00 ± 1.15 ^{bc}	27.24 ± 2.31 ^{cd}
Fungicide (0.01)	29.33 ± 0.66 ^{bc}	33.31 ± 0.85 ^{bc}
Cu-chitosan NPs		
0.01	28.67 ± 0.16 ^c	34.83 ± 0.87 ^b
0.04	24.67 ± 0.32 ^d	43.86 ± 2.06 ^a
0.08	24.67 ± 0.21 ^d	43.93 ± 0.78 ^a
0.12	23.33 ± 0.26 ^d	46.97 ± 0.76 ^a
0.16	22.67 ± 0.56 ^d	48.48 ± 0.76 ^a

Table 2. Effect of Cu-chitosan NPs on CLS disease in pot condition. Disease data were recorded after 15 days of inoculation using 1 to 9 standard disease rating scale. ^AEach value is mean of triplicates and each replicate consisted of 3 plants samples. Mean ± SE followed by same letter is not significantly different at $p = 0.05$ as determined by Tukey–Kramer HSD. BCH (bulk chitosan) dissolved in 0.1% acetic acid and fungicide (0.01% of Bavistin).

In Cu-chitosan NPs treated plant leaves, PAL activity also persuaded from 46.15 to 66.66% and PPO activity increased from 3.05 to 16.39% as compared to bulk chitosan treatment (Fig. 2c,d). The increased activity of POD, PAL and PPO might be associated with production of suberin, melanin and lignin^{31,32} for cell wall strengthening which further acts as a mechanical barrier to invading plant pathogen^{31–34}.

To test the efficacy of Cu-chitosan NPs against CLS disease, pot and field experiments were conducted by inoculating highly sporulating inoculum of *C. luanta*. Sorghum grain medium was used to grow the fungal culture to achieve higher mycelial growth and sporulation and to maintain the desire pathogenicity of the fungal inoculum. The detailed methodology has been explained elsewhere³⁵. This method is widely adopted to develop CLS disease artificially in pot and field conditions. As already mentioned, sorghum grain medium gave excellent results to achieve adequate mycelial growth and sporulation as compared to other grain like wheat, barley, rice, maize etc. The content and type of sorghum carbon source may be expected more suitable for rapid mycelial growth and sporulation as compared to maize grain. In pot experiment, DS and PEDC were recorded to determine the efficacy of Cu-chitosan NPs against CLS disease. A significant control of CLS disease was recorded on Cu-chitosan NPs treatments (0.04–0.16%) as compared to others (Table 2). These Cu-chitosan NPs (0.08–0.12%) have previously been reported very effective against early blight and *Fusarium* wilt of tomato¹⁵. The defense response of Cu-chitosan NPs might be due to direct activity like (a) through membrane destruction by electrostatic interaction of chitosan with microbial cell surface⁶ (b) positively charged NPs could binds to DNA/RNA which affects transcription and translation process and inhibit fungal proliferation⁶. On another side, indirect activity might be exerted by chitosan through aroused plant immune response by enhanced activities of antioxidant and defense

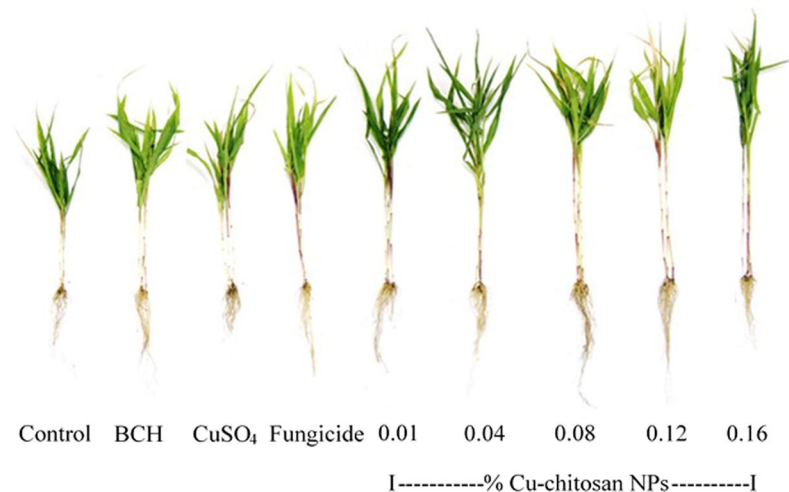


Figure 4. Effect of Cu-chitosan NPs on plant growth of maize in pot condition.

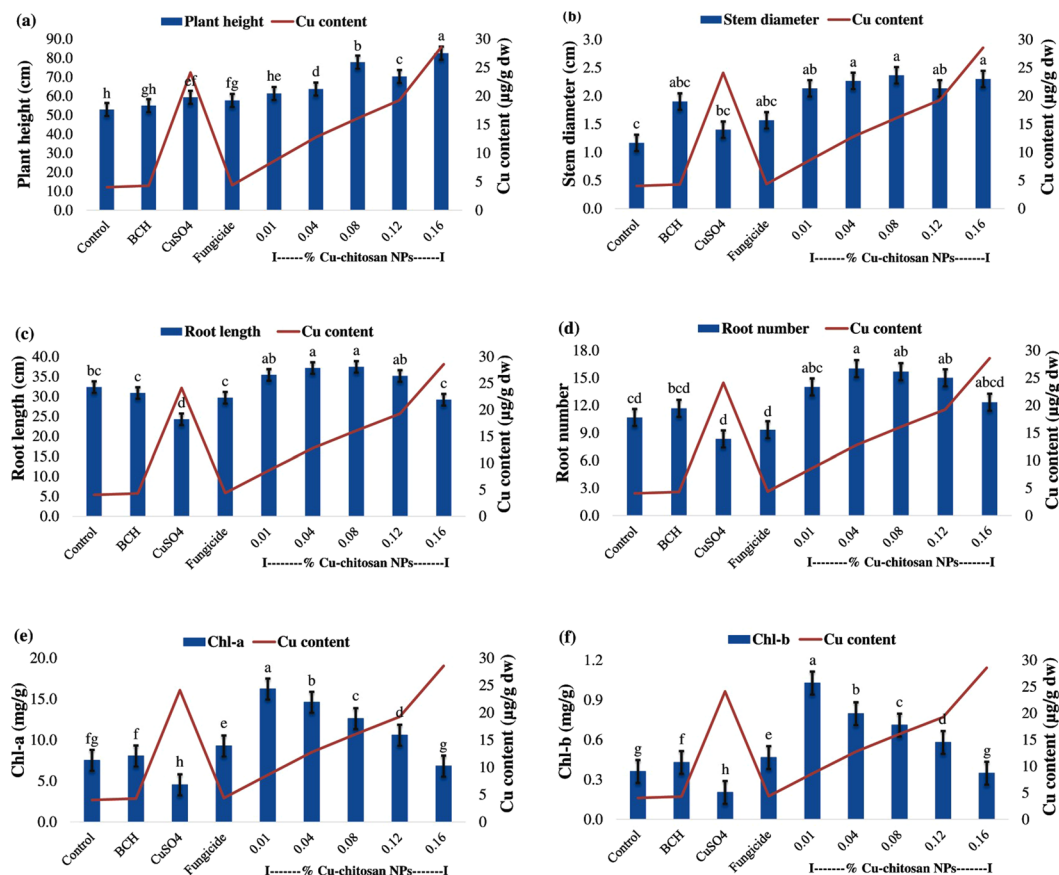


Figure 5. Effect of Cu-chitosan NPs on (a) plant height (b) stem diameter (c) root length (d) root number (e) chlorophyll-a and (f) chlorophyll-b content. Each value is mean of triplicates and each replicate consisted of 3 plants samples and same letter in the graph of each treatment is not significantly different at $p = 0.05$ as determined by Tukey–Kramer HSD, control with water. BCH (bulk chitosan, 0.01%) dissolved in 0.1% acetic acid. CuSO₄ (0.01%) and fungicide (0.01% of Bavistin).

enzymes³⁶. Furthermore, we foresee that Cu-chitosan NPs releases Cu rapidly in acidic pH (Fig. 1) which is created upon fungal infection and the released Cu may act weighty on the fungus¹⁵. Altogether Cu-chitosan NPs lead to abate *C. lumata* spreading and contributed resistance in maize plants against CLS disease through synergistic effect of chitosan and Cu^{15,24}.

Treatment (%)	Cu content ^A (µg/g dw)
Control (water)	4.02 ± 0.37 ^g
BCH (0.01)	4.28 ± 0.24 ^g
CuSO ₄ (0.01)	24.10 ± 0.67 ^b
Fungicide (0.01)	4.37 ± 0.37 ^g
Cu-chitosan NPs	
0.01	8.60 ± 0.47 ^f
0.04	12.77 ± 0.33 ^e
0.08	16.07 ± 0.13 ^d
0.12	19.25 ± 0.61 ^c
0.16	28.55 ± 0.50 ^a

Table 3. Cu content in maize leaves in various treatments. Data were recorded in 3rd leaf after harvest. ^AEach value is mean of triplicates and each replicate consisted of 3 plants samples. Mean ± SE followed by same letter is not significantly different at $p = 0.05$ as determined by Tukey–Kramer HSD. BCH (bulk chitosan, 0.01%) dissolved in 0.1% acetic acid and fungicide (0.01% of Bavistin).

Treatment (%)	DS (%) ^A	PEDC (%) ^A
Control (water)	64.26 ± 0.94 ^a	00.00 ± 0.00 ^d
BCH (0.01)	48.18 ± 0.43 ^b	25.00 ± 0.81 ^c
CuSO ₄ (0.01)	47.53 ± 0.61 ^{bc}	26.00 ± 1.66 ^{bc}
Fungicide (0.01)	46.99 ± 0.23 ^{bc}	26.82 ± 1.43 ^{bc}
Cu-chitosan NPs		
0.01	46.42 ± 0.31 ^{bc}	27.72 ± 0.98 ^{bc}
0.04	46.26 ± 0.16 ^{bc}	27.96 ± 1.22 ^{bc}
0.08	45.90 ± 0.45 ^{bc}	28.53 ± 0.09 ^{bc}
0.12	44.71 ± 0.80 ^{cd}	30.42 ± 0.28 ^{ab}
0.16	42.48 ± 1.08 ^d	33.88 ± 1.10 ^a

Table 4. Effect of Cu-chitosan NPs on CLS disease in field condition. Disease data were recorded after visible appearance of symptoms following 20 days of inoculation using 1 to 9 standard disease rating scale. ^AEach 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. BCH (bulk chitosan) dissolved in 0.1% acetic acid and fungicide (0.01% of Bavistin).

These NPs significantly enhanced seedling growth of tomato¹⁵ and maize by mobilizing reserved food through higher activities of α -amylase and protease²¹. To take the advantage of growth promotory effect of Cu-chitosan NPs (as reported in our previous study), maize seeds were treated with Cu-chitosan NPs followed by foliar spray in pot experiment. Statistical analyses showed that Cu-chitosan NPs notably increased plant height, stem diameter, root length, root number and chlorophyll content (Fig. 5). However, at higher concentrations of Cu-chitosan NPs (0.16%) and CuSO₄ (0.01%) treatment, chlorophyll content significantly decreased (Fig. 5e,f). It has previously been proposed that accumulation of Cu interferes with chlorophyll biosynthesis and cause deficiency of Mg and Fe^{37–39}. Concomitantly, root length and root number was also affected at higher concentrations of NPs (0.16%) and CuSO₄ (0.01%) (Fig. 5c,d). In AAS analyses, we quantified Cu content in treated plant leaves and allied it with plant growth characters (Table 3; Fig. 5). We disentangled that the trend of plant growth was virtually related with Cu content, and this is in line with our previous study²¹. The toxicity envisaged only on chlorophyll content, root length and root number at CuSO₄ (0.01%) and 0.16% NPs treatments could be endowed by elevated accumulation of Cu (28.5 and 24.1 µg/g, Table 3). The accumulated level of Cu in present study is more than toxic level of Cu in maize leaves which is reported to be 20 µg/g⁴⁰. Therefore, we expect that for conceivable plant growth, Cu uptake must be controlled to avoid its sudden exposure to plant cells²¹ which can be achieved by slow release of Cu from Cu-chitosan NPs (Fig. 1). In field experiment, disease control and yield was significantly influenced by NPs treatments. However in field, higher concentration of NPs (0.12 and 0.16%) showed significant disease control and galvanize grain yield. A sturdy conclusion could be towed from pot and field experiments that, application of Cu-chitosan NPs unquestionably control disease and boost plant growth and yield. Review of literature emphasized that during grain filling, an adequate level of antioxidant environment may contribute the mobilization of reserve food from leaf, stem etc to developing grain for uninterrupted grain filling^{41,42}. Studies have proved the antifungal activity of chitosan based NPs and its role in induction of defense enzymes¹⁴, however, the enhancement of growth and yield is not well understood. It is imperative to study further into reserve food mobilization and its interaction with NPs to optimize the application module of Cu-chitosan NPs for higher growth and yield of crop. Results in present study categorically claim that Cu-chitosan NPs act as antifungal agent, enhance the activities of antioxidant and defense enzymes which cohorts with plant growth and disease resistance in treated maize plants (Fig. 6).

Treatment (%)	Days to 50% tasseling	Days to 50% silking	Days to 50% ear leaf senescence	Number of leaves per plant ^A	Plant height (cm) ^A	Ear length (cm) ^A	Ear weight (kg/plot) ^B	Grain yield (kg/plot) ^B	100 grain weight (g) ^B
Control (water)	53.66 ± 0.33 ^a	55.66 ± 0.33 ^a	78.33 ± 0.88 ^{bc}	12.66 ± 0.38 ^{bc}	176.33 ± 1.83 ^c	19.66 ± 0.88 ^b	2.83 ± 0.09 ^{ab}	2.08 ± 0.03 ^{abcd}	25.55 ± 0.29 ^b
BCH (0.01)	54.33 ± 0.33 ^a	58.66 ± 0.33 ^a	77.67 ± 0.33 ^c	12.22 ± 0.96 ^{bc}	186.22 ± 1.3 ^b	21.00 ± 1.15 ^{ab}	2.69 ± 0.06 ^{abc}	2.26 ± 0.15 ^{abcd}	25.68 ± 0.30 ^b
CuSO ₄ (0.01)	54.33 ± 0.88 ^a	57.66 ± 0.33 ^a	80.67 ± 0.33 ^{abc}	11.33 ± 0.38 ^c	185.77 ± 2.43 ^b	22.66 ± 0.88 ^{ab}	1.89 ± 0.12 ^d	1.46 ± 0.18 ^d	25.09 ± 0.69 ^b
Fungicide (0.01)	54.00 ± 0.57 ^a	56.33 ± 0.88 ^a	79.33 ± 0.33 ^{abc}	11.22 ± 0.11 ^c	189.88 ± 2.99 ^{ab}	22.00 ± 1.52 ^{ab}	2.10 ± 0.09 ^{cd}	1.58 ± 0.06 ^{cd}	26.70 ± 0.41 ^b
Cu-chitosan NPs									
0.01	53.33 ± 0.33 ^a	57.00 ± 1.00 ^a	81.33 ± 0.66 ^{abc}	13.44 ± 0.22 ^{bc}	190.22 ± 1.82 ^{ab}	22.66 ± 0.88 ^{ab}	2.42 ± 0.12 ^{bcd}	1.87 ± 0.09 ^{abcd}	26.82 ± 0.37 ^b
0.04	53.33 ± 0.33 ^a	56.66 ± 0.66 ^a	82.00 ± 1.00 ^{ab}	13.77 ± 0.40 ^{ab}	194.00 ± 0.19 ^{ab}	22.66 ± 1.76 ^{ab}	2.37 ± 0.21 ^{bcd}	1.79 ± 0.30 ^{bcd}	25.77 ± 0.35 ^b
0.08	54.00 ± 0.57 ^a	57.00 ± 0.57 ^a	82.67 ± 1.33 ^a	15.88 ± 0.55 ^a	198.11 ± 2.45 ^a	26.33 ± 0.88 ^a	2.70 ± 0.03 ^{abc}	2.38 ± 0.15 ^{abc}	26.91 ± 0.53 ^b
0.12	53.66 ± 0.66 ^a	56.33 ± 0.88 ^a	80.67 ± 0.33 ^{abc}	12.66 ± 0.33 ^{bc}	198.00 ± 1.30 ^a	24.00 ± 1.00 ^{ab}	3.09 ± 0.21 ^a	2.61 ± 0.21 ^{ab}	29.23 ± 0.46 ^a
0.16	53.66 ± 0.66 ^a	57.00 ± 0.57 ^a	79.67 ± 0.88 ^{abc}	12.00 ± 0.38 ^{bc}	191.00 ± 1.07 ^{ab}	25.00 ± 1.00 ^{ab}	3.10 ± 0.03 ^a	2.69 ± 0.12 ^a	29.80 ± 0.59 ^a

Table 5. Effect of Cu-chitosan NPs on growth parameter of maize in field condition. Various growth parameters were recorded at 80 days^A and 95 days^B of crop. 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. BCH (bulk chitosan) dissolved in 0.1% acetic acid and fungicide (0.01% of Bavistin).

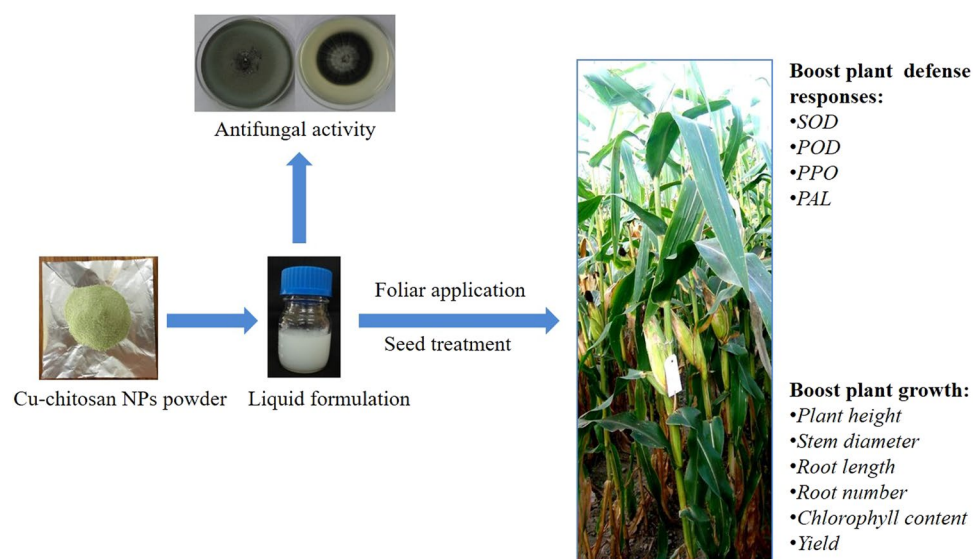


Figure 6. Application model of Cu-chitosan NPs induced defense responses and plant growth in maize.

Conclusion

Demand of food/feed crop free from synthetic components has exponentially increased in recent years to avert hazardous effects of synthetic additives and to evade the development of resistance in pathogens. A new approach is imperative to be adapted to strengthen plant innate immunity to cope with mutating plant pathogens, reduce chemical use and alongside sustained plant growth. Cu-chitosan NPs have been proven as a promising plant protection and growth promotory agent in our past and recent studies. Its unique ability to sustain the plant growth under disease conditions makes it a very effective and usable agent. These bio-based nanomaterials could be pivotal towards sustainable agriculture without harming ecosystem. The synthesized NPs have immense potential to be commercially explored for agriculture use.

Methods

The experiments performed in the study are summarized in Table 1 and details are in following headings.

Materials. 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 ‘Surya local’ were obtained from the Department of Plant Breeding and Genetics, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India. Inoculum of *Curvularia lunata* was received from Department of Plant Pathology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India.

Cu-Chitosan NPs. Cu-chitosan NPs were obtained as dry powder from the batch synthesized and used in our earlier study¹⁵. The obtained NPs were characterized for its mean hydrodynamic diameter, zeta-potential and polydispersity index (PDI) by dynamic light scattering (DLS) on Zetasizer ZS 90 (Malvern, UK) at 25 °C at a scattering angle of 90 ° in triplicate.

In-vitro Cu release profile. *In-vitro* experiments were conducted to study the effect of pH and time on the release of Cu from Cu-chitosan NPs. In brief, freeze dried Cu-chitosan NPs were dispersed in deionized water with pH adjusted in the range of 1 to 7. The contents were centrifuged at 10,000 × g for 10 min and supernatants were collected for further analysis. Similarly, in separate experiments, the NPs were dispersed at 4.5 pH for 0, 24, 48, 72, 96, 120 and 144 h followed by centrifugation at 10,000 × g for 10 min. The supernatants, thus obtained, from both the experiments were analyzed for Cu contents using atomic absorption spectrophotometer (AAS 4141 model, Electronics Corp. of India Ltd., India).

In vitro antifungal activity of Cu-chitosan NPs. Antifungal activity was evaluated by poisoned food technique^{20,43} using Cu-chitosan NPs (0.01, 0.04, 0.08, 0.12 and 0.16%, w/v), control, bulk chitosan (0.01%, w/v), CuSO₄ (0.01%, w/v) and commercially available fungicide (0.01% Bavistin, w/v). The treated plates were compared with the control to calculate the per cent inhibition of mycelia growth by using the formula given by Vincent⁴⁴.

$$\% \text{ Inhibition} = \frac{\text{Mycelial growth in control} - \text{mycelial growth in treatment}}{\text{mycelial growth in control}} \times 100$$

Pot experiment for disease assessment and plant growth. Seeds of disease susceptible maize cultivar ‘Surya local’ were surface sterilized with 10% sodium hypochlorite for 10 min and further treated for 4 h with different concentrations of Cu-chitosan NPs (0.01, 0.04, 0.08, 0.12 and 0.16%, w/v), control (water), bulk chitosan (0.01%, w/v), CuSO₄ (0.01%, w/v) and commercially available fungicide (0.01% Bavistin, w/v). The treated seeds were dried and sown in earthen pots filled with standard clay type soil in net house condition by following standard agronomic practices. The plants were subjected to foliar spray of same treatments as did with seeds until runoff at 35 days of sowing. After 10 days of foliar treatments, inoculum of *C. lunata*, prepared on sorghum seed medium³⁵, was inoculated on plants. In brief, *C. lunata* culture was seeded on medium papered using sorghum seeds and kept at 27 ± 1 °C for 15 days to achieve adequate mycelial growth and sporulation. Colonized grains were rinsed with water and conidial suspension was filtered through cheese cloth and diluted with water to a concentration of 5 × 10³ conidia ml⁻¹. The inoculum was sprayed on plants by backpack sprayers for disease development in maize plants. Disease assessment was performed after appearance of symptoms on leaves at 15 days of inoculation. Disease Severity (DS) was recorded on 1 to 9 standard disease rating scale. Further, the DS and per cent efficacy of disease control (PEDC) were calculated by using the formula given by Chester⁴⁵ and Wheeler⁴⁶.

$$DS = \frac{\text{Sum of all individual disease rating}}{\text{total number of plant assessed}} \times \text{maximum rating.}$$

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

The plants were harvested at maturity (95 days) to determine plant height, stem diameter, root length and root number. Chlorophyll a and b were quantified in 3rd leaf after 24 h of foliar spray⁴⁷. Cu content was also measured in 3rd leaf of treated plant after harvest using AAS as described earlier⁴⁸.

Measurement 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 560 nm, 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 Maehly⁴⁹ by oxidation of guaiacol in the presence of hydrogen peroxide. Increase in absorbance at 470 nm was recorded due to formation of tetra guaiacol. PPO (EC 1.10.3.1) was assayed according to Taneja and Sachar⁵⁰ and activity was expressed as change in absorbance at 490 nm. PAL (EC 4.3.1.5) was estimated as described by Moerschbacher *et al.*⁵¹ where the deamination of L-phenylalanine to trans-cinnamic acid and ammonia was measured at 290 nm. Activities of all the enzymes were expressed in μmol/min/g tissue.

Field experiment for disease assessment and crop yield. The field experiment was conducted in year 2016 (July to December) at research farm of Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India, (24.58° latitude, 73.70° longitude) on a standard clay type soil in randomized block design (RBD) with three replication. In each replication, seeds were grown in a plot (size 3.6 m², where row to row and plant to plant space was 0.6 m and 0.2 m) having three rows (each 3 m). Seed treatment, foliar application, artificial inoculation, assessments of DS and PEDC were performed as explained in pot experiments. The plants were maintained as per standard agronomic practices. Observation of days to 50% tasseling,

silking, ear leaf senescence and number of leaves/plant, plant height, ear length (at 80 days), ear weight/plot, grain yield/plot and 100 grain weight were recorded using maize descriptor.

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.

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Author Contributions

V.S. designed the study. R.C.C., R.V.K., S.K., A.P. and S.S.S. conducted the plant experiments and synthesis of nanomaterials. R.R. Characterize the synthesized nanomaterial and stability test. P.B., S.S.S., A.P. and V.S. provided the overall supervision of the project. V.S. wrote the manuscript and all the authors read and revise the manuscript, and all author approved the final version of the manuscript before submission.

Additional Information

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