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Preparation of Cu-chitosan nanoparticle and its effect on growth and enzyme activity during seed germination in maize

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Abstract

In present investigation, Cu-chitosan nanoparticle was prepared based on ionic gelation method. Prepared NPs were characterized for physicochemical properties using DLS and FTIR. Characterized NPs were evaluated for seedling growth, physiological and biochemical responses during maize seed germination. In Cu-chitosan NPs treatments, significant higher values of shoot length, seedling vigor index, fresh and dry weight were observed at 0.01-0.16% treated seeds. Similarly, at 0.04-0.12% treatment, root length was recorded maximum among all treatments. However, NPs treatment at 0.01-0.016%, per cent germination and root number were not statistically different but higher than control (water), CuSO₄ and bulk chitosan treatments. The activity of α -amylase and protease enzymes were recorded maximum at seventh and fifth days of germination and declined in proceeding days in all treatments. Moreover, in fifth days of germination, the increased activity of α -amylase was observed in NPs at 0.01-0.08% (0.90, 0.90 and 0.94 $\mu\text{mol}/\text{min}/\text{g dw}$) as compared to other treatments. Protease activity was reported higher in NPs at 0.01-0.04% (3.81 and 3.94 $\mu\text{mol}/\text{min}/\text{g dw}$) in fifth days of germination. Therefore, it can suggested that increased activity of α -amylase and protease is due to Cu-chitosan NPs, which could correlated with starch and protein content for higher seedling growth.

Keywords: Cu-chitosan, Seedling Growth, Hydrolytic enzyme, Maize

1. Introduction

Worldwide, cereal crops are exposed by uncontrolled use of synthetic agrochemicals for better yield potential and improved quality (Tilman *et al.*, 2002) [5]. Since past decade various alternative approaches has been explored in crops to enhance production by reducing environmental contamination and risk associated with human health (Kashyap *et al.*, 2015) [9]. Several polymers have been used in the field of agriculture either naturally occurring or synthetic for wide range of applications (Kashyap *et al.*, 2015; Saharan *et al.*, 2015) [9]. Among them, chitosan is a promising polymers consisting of glucosamine and N-acetyl glucosamine units (Saharan and Pal, 2016) [11]. In recent years, potential application of nanomaterials have been extensively studied to overcome the major problems associated with conventional methods in agriculture (Chen *et al.*, 2014) [3]. The search of such alternatives strategies, chitosan based nanoparticles (NPs) are known for safe, nontoxic, biocompatible and biodegradable (Saharan *et al.*, 2016) [12]. Due to unique properties chitosan can be blended with micronutrient including Cu, Zn, or alone. Among them, Cu is most important micronutrient which is essentially required for plant growth and protection. It also act as active components in several enzymes as well as participates in metabolism of carbohydrates, protein synthesis and gene expression (Choudhary *et al.*, 2017) [4]. Globally, maize is an important crop and has higher economic value as food grain and fodder crop. In plants, reduced nutrient uptake, uptake distribution and translocation is major limitation for the improved plant vigor and crop quality (Dordas, 2008; Deshpande *et al.*, 2017) [6, 5]. Therefore, the aim of present investigation was to synthesize Cu-chitosan NPs and synthesized NPs were characterize for size, polydispersity index value and charge. Further these NPs were assessed for seedling growth and hydrolytic enzymes including α -amylase and protease during *in vitro* seed germination. Thus, it is expected that Cu-chitosan NPs might be act as growth promoter through its immune elicitor activity in maize crop.

2. Material and methods

2.1 Materials

Chemicals for preparation of Cu-chitosan NPs including, chitosan, sodium tri-polyphosphate

(TPP) and CuSO₄ were purchased from Sigma-Aldrich (St. Louis, USA.). Other chemicals for enzyme assay were of analytical grade and procured from HiMedia (Mumbai, India). For seedling experiment, maize seeds of Suryal local cultivar were obtained from the Department of Plant Breeding and Genetics, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India.

2.2 Preparation and characterization of Cu-chitosan NPs

Cu-chitosan NPs were synthesized from method based on ionic gelation of chitosan with sodium triphosphate (TPP) and CuSO₄ (Saharan *et al.*, 2015) [14]. Briefly, 500 mg of chitosan was dissolved using 1% (v/v) acetic acid solution and stirred at 200 rpm until solution become clear. Further, TPP solution (5mg/ml) was prepared and added to the chitosan solution gradually in drop wise manner under continuous stirring condition at room temperature. Before completion of crosslinking reaction with TPP, CuSO₄ solution (2mg/ml) was added. Resulting solution was centrifuged at 10,000 rpm for 15 min at 4 °C followed by sonication to achieve Cu-chitosan NPs. Nanoparticles was dried using freeze drying and stored for further use. Synthesized NPs were characterized for physicochemical properties including particle size, charge, poly dispersity index value (PDI) and interaction between nanoparticulate system using dynamic light scattering and Fourier transform infrared.

2.3 Seedling bioassay

In present investigation 'Suryal local' cultivar of maize was selected to examine the effect of Cu-chitosan NPs on growth and α -amylase and protease enzymes activity during seedling growth and development. In brief, healthy maize seeds were selected and surface sterilized with 10% sodium hypochlorite (NaOCl) solution for 10 min followed by three times washing with deionized water. Further, sterilized seeds were treated for 4 h with Cu-chitosan NPs at different concentration 0.01, 0.04, 0.08, 0.012 and 0.16% w/v, bulk chitosan (0.01%), CuSO₄ (0.01%), fungicide (0.01%, Bavistin) and deionized water (control). The seeds were dried and placed in pre autoclaved Petri plates (90 × 15 mm) with moistened filter paper. Each treatment was consisted of three plates (triplicates) and each plates consisted of 10 seeds under aseptic condition. The Petri plates were incubated at 28 ± 2 °C in growth chamber. Regularly deionized water was applied to maintain moisture level for germination and growth. Data were recorded for percent germination, shoot/root length, root number, seedling length, seedling vigor index (SVI), fresh weight and dry weight were measured (ISTA, 1996). Seedling vigor index was calculated formula given (Abdul-Baki and Anderson, 1973) [1].

Seedling vigor index = (germination %) × (shoot length + root length)

2.4 Measurement of enzyme activity

The activity of α -amylase and protease were determined at different growth stages (0, 1, 3, 5, 7 and 9 days of germination). The samples were taken from germinating seeds and homogenized in sodium acetate buffer (100 mM, pH 4.7) for α -amylase and 100 mM phosphate buffer (pH 7.4) for protease at 4 °C. The homogenized samples were centrifuged

at 10,000 rpm for 15 min at 4 °C. The supernatant was taken for assay of enzymes. The activity of α -amylase was determined spectrophotometrically at 560 nm (Bernfeld *et al.*, 1955) [2]. Protease activity was measured at 620 nm absorbance using UV-visible spectrophotometer (Lowry *et al.*, 1951) [10]. The enzyme activity was expressed in μ mol/min/g dry weight of tissue.

2.5 Statistical analysis

Statistical analyses was performed using JMP software version 12 by Tukey-Kramer HSD test to determine significant differences among treatment at $p = 0.05$ level. Experiment was repeated twice, and each treatment consisted of three replicates. The results were expressed as mean ± SE (standard error).

3. Result and discussion

3.1 Cu-chitosan NPs

Cu-chitosan NPs were formed due to interaction between positively charged chitosan and amino group of negatively charged TPP followed by adding of CuSO₄. The average hydrodynamic size Cu-chitosan NPs was 400±10 nm with poly dispersity index value of 0.22 (Fig. 1). The particles exhibited surface charge of +34 mV (Fig. 2). Furthermore, FTIR analysis was carried out to confirm the interaction of chitosan with TPP and Cu. The sharper peaks attributed to the interaction of TPP with amino group of chitosan. The specific peaks at 1639 cm⁻¹ for amide group and 1540 cm⁻¹ for amino group were sharper from bulk chitosan (data not shown) which indicates the binding of Cu with chitosan in Cu-chitosan NP through redistribution of vibration frequencies (Fig. 3)

3.2 Effect of NPs on seedling growth

To evaluate the effect of Cu-chitosan NPs on seedling growth and development, seeds were treated with different concentration of treatment for 4 h. Data for seedling growth *viz.*, percent germination, shoot/root length, root number, seedling vigor index, fresh and dry weight were recorded. Results showed that Cu-chitosan NPs exhibited significant differences among all parameters measured for maize seedling growth (Table 1). Significant higher values of shoot length, SVI, fresh weight and dry weight were recorded at 0.01-0.16%, whereas, root length was recorded from 0.04 to 0.12% NPs treatments (Table 1). However, per cent germination and root number were not statistically different in treated seeds but higher in NPs treatment (0.01-0.16%) than the other treatments (Table 1). On the other hand, effect of bulk chitosan on all parameter was higher as compared to control (water), CuSO₄ and fungicide except shoot length, which was higher in control. Bulk chitosan exhibited satisfactory result but significantly lower growth than Cu-chitosan NPs. Among NPs treatments, various growth parameters at 0.01% conc. showed minimum seedling growth. Shoot length, root length, SVI and dry weight was drastically decreased at 0.01% of fungicide, which exhibited minimum growth promoting activity among all treated seeds. Therefore, it can be suggested that Cu-chitosan NPs at 0.01-0.16% exert growth promotory activity followed by bulk chitosan and CuSO₄. Whereas, fungicide at 0.01% showed growth inhibitory effect.

Table 1: Effect of Cu-chitosan NPs on seedling growth of maize.

Treatment (%)	% Germination ^A	Shoot length (cm) ^A	Root length (cm) ^A	Root number ^A	SVI ^A	Fresh weight (g) ^A	Dry weight (g) ^A
Control (water)	100±0.0 ^a	7.47±0.7 ^{abc}	7.64±0.7 ^{cd}	2.13±0.1 ^b	1511±131.7 ^c	0.70±0.04 ^{abc}	0.14±0.003 ^{cd}
BCH (0.01)	100±0.0 ^a	6.09±0.3 ^{bcd}	10.1±0.4 ^{bc}	3.72±0.1 ^{ab}	1619±55.0 ^{de}	0.75±0.02 ^{abc}	0.17±0.003 ^{abcd}
CuSO ₄ (0.01)	95.18±4.8 ^a	5.8±0.6 ^{cd}	9.45±0.9 ^a	3.72±0.4 ^{ab}	1525.39±124.5 ^{abcd}	0.68±0.01 ^{bc}	0.15±0.005 ^{bcd}
Fungicide (0.01)	100±0.0 ^a	4.3±0.1 ^d	6.98±0.1 ^d	2.23±0.5 ^b	1128±26.3 ^f	0.71±0.02 ^c	0.12±0.008 ^d
Cu-chitosan NPs							
0.01	100±0.0 ^a	8.29±0.8 ^{ab}	9.7±0.2 ^{bc}	4.00±0.4 ^a	1799±78.6 ^{bcd}	0.78±0.01 ^{ab}	0.17±0.003 ^{bcd}
0.04	100±0.0 ^a	8.10±0.4 ^{ab}	10.9±0.7 ^{ab}	4.11±0.1 ^a	1900±26.5 ^{ab}	0.75±0.02 ^{abc}	0.19±0.003 ^{ab}
0.08	100±0.0 ^a	8.19±0.2 ^{ab}	12.15±0.1 ^{ab}	4.27±0.2 ^a	2034±17.1 ^{abc}	0.78±0.05 ^{ab}	0.20±0.020 ^{ab}
0.12	100±0.0 ^a	8.5±0.4 ^{ab}	13.95±0.3 ^a	4.98±0.3 ^a	2245±72.2 ^a	0.83±0.04 ^a	0.22±0.023 ^a
0.16	100±0.0 ^a	9.33±0.3 ^a	8.14±0.2 ^{cd}	4.33±0.2 ^a	1747±22.5 ^{cde}	0.76±0.01 ^{ab}	0.19±0.006 ^{abc}

Data were recorded after 10 days. ^AEach value is mean of triplicates and each replicate consisted of 10 seedling. 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) and CuSO₄ (0.01%).

3.3 Effect of Cu-chitosan NPs on α -amylase and protease activity

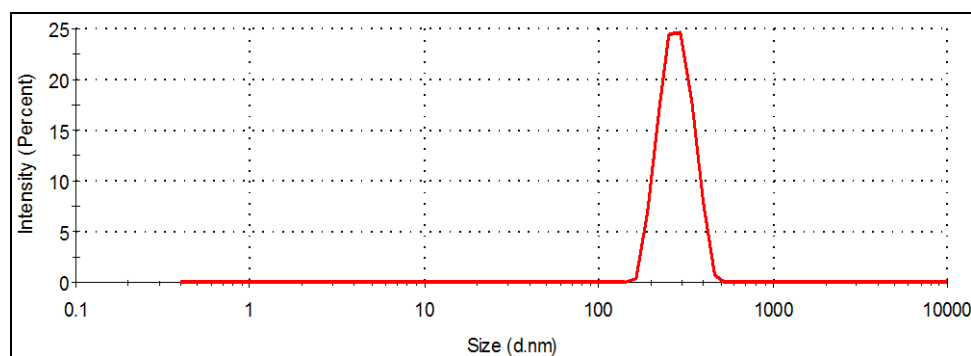
To determine the correlation between seedling growth and hydrolytic enzymes during seed germination and seedling development, enzyme activity of α -amylase and protease were measured at 0, 1, 3, 5, 7, and 9 days of germination (Fig 4-5). Initially, at 0 day of germination, the activity of both the enzymes were observed minimum in all treatments. The activity was increased from first days to seventh and fifth days for α -amylase and protease respectively and declined in proceeding days in all treatments. The maximum activity of α -amylase was recorded at seventh days in Cu-chitosan NPs at 0.01-0.08% (0.90, 0.90 and 0.94 $\mu\text{mol}/\text{min}/\text{g dw}$) as compared to other treatments (Figure 4). Among all other treatment, minimum activity was recorded in bulk chitosan, fungicide and control. On the other hand CuSO₄ induced comprehensively higher activity than bulk chitosan, fungicide and control (Figure 4). Similarly, the activity of protease was also increased by application of NPs in treated seeds. In all treatments, protease activity was observed maximum in fifth days and declined in remaining days. In fifth days of germination, maximum activity was found in NPs at 0.01-0.04% (3.81 and 3.94 $\mu\text{mol}/\text{min}/\text{g dw}$). Among NPs, lowest activity was observed at 0.16 followed by 0.12% treated seeds. However, CuSO₄ showed increased activity than bulk chitosan, control and fungicide (Figure 5). Therefore, result suggested that Cu-chitosan NPs significantly induced the activity of α - amylase and protease, which results enhanced

starch and protein content for seedling growth and development during germinating seeds.

To determine the growth and toxicity of metals or nanoparticles, germination assay is fundamental procedure in plants (Feizi *et al.*, 2012) [7]. However, few studies have been conducted for application of chitosan NPs for enhancing plant growth and protection (Saharan *et al.*, 2013) [13]. In plants, the role of Cu is well established as a micronutrient, structural component of various enzymes as well as proteins and participated in several metabolic reactions (Choudhary *et al.*, 2017) [4]. Moreover, Cu component of Cu-chitosan NPs may triggers metabolic reaction in germinating seeds and leads to the enhanced growth. In some previous studies the superiority of chitosan NPs over bulk chitosan has been reported and it can be suggested that chitosan NPs may enhancing activity in plants (Saharan *et al.*, 2015; 2016) [12, 14]. Therefore, from our result it can be assume that chitosan NPs substantial effect on cells of germinating seeds as they can easily passes along with encapsulated Cu and involved actively in metabolism processes during seed germination while, due to large size bulk chitosan with low surface area unable to pass into the seeds.

4. Conclusion

The present study suggested that Cu-chitosan NPs can significantly enhances maize seedling growth and development through Cu as micronutrient and chitosan as plant growth promoting activity. Further, these NPs also act as upregulation of α -amylase and protease enzymes for food mobilization during seed germination. The effect of NPs and subsequent response to seedling led to improvements in growth, plant vigor and quality. Therefore, seed priming with Cu-chitosan NPs may help to improve seedling growth and protection from plant diseases either seed borne or soil borne.

**Fig 1:** Size of Cu-chitosan NPs by intensity.

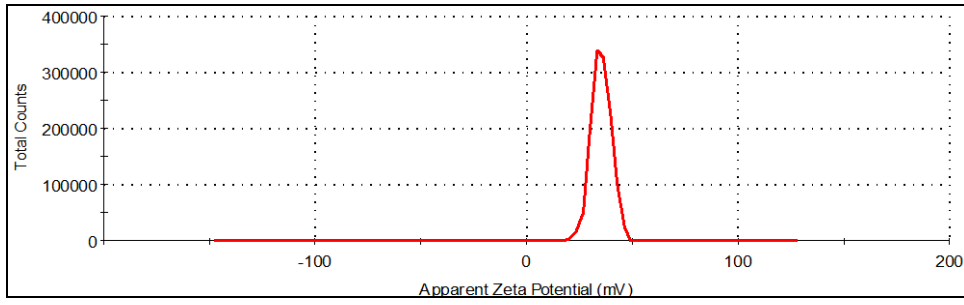


Fig 2: Zeta potential of Cu-chitosan NPs.

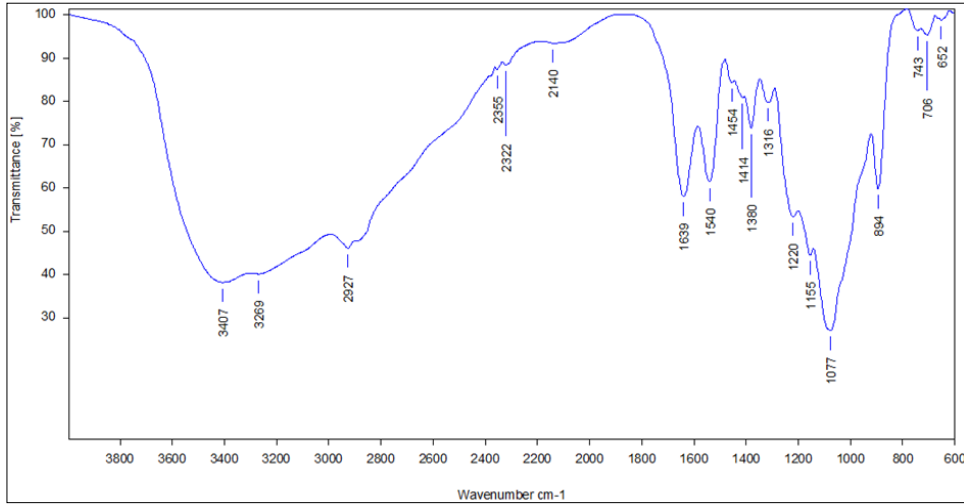


Fig 3: FTIR spectra of Cu-chitosan NPs.

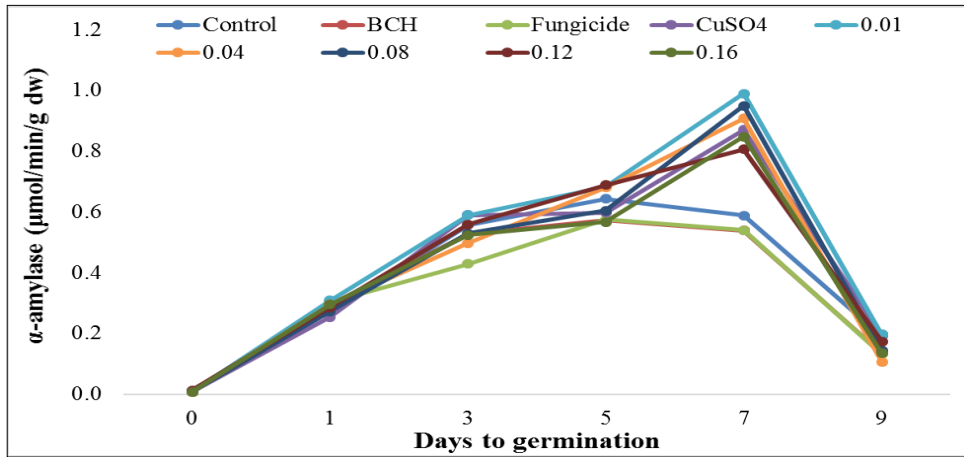


Fig 4: Effect of Cu-chitosan NPs on α -amylase activity.

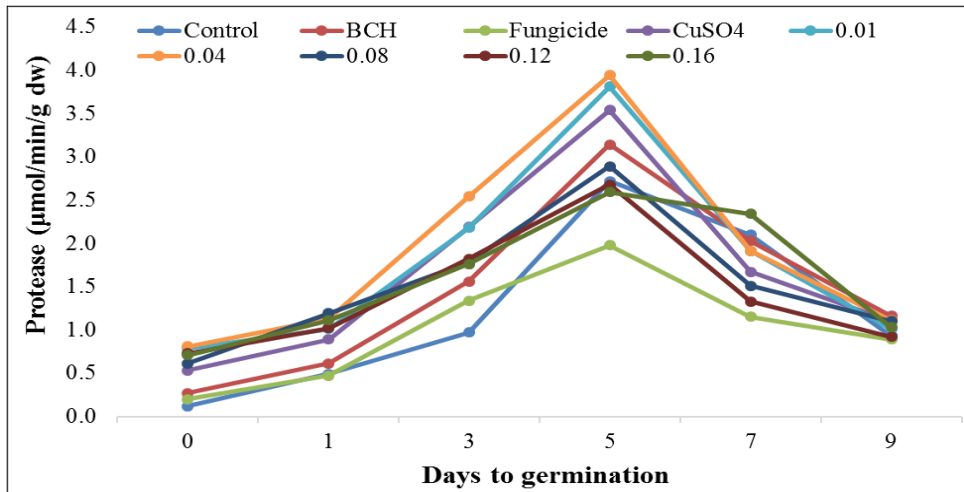


Fig 5: Effect of Cu-chitosan NPs on protease activity.

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