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Copper-Chitosan Nanoparticles Boost Plant Growth Stimulation and Induced Resistance against Fusarium Wilt of Banana (*Musa* sp.)

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Abstract

In banana cultivation, the search for biopolymer-derived compounds is seeking huge demand to replace chemicals harmful to the environment. In the present study, synthesized Cu-Ch Nps showed potential activity from 30 to 240 days after treatment in *in vivo* studies against *Fusarium* wilt of banana caused by Fusarium oxysporum f. sp. *cubense* (*Foc*). Cu-Ch Nps treated banana plants exhibited a growth promotor effect through enhanced chlorophyll stability index (CSI), chlorophyll (a and b) content, leaf succulence (LS), and also the vitamin C contents of banana fruits *in vivo* conditions. In the field experiments, plant height, the number of leaves, total hands/bunch of whole fingers, and bunch weight were found to enhance through Cu-Ch NPs (0.20%). Control of disease and plant growth enhancement was favored when Cu directly released from Cu-Ch NPs. Furthermore, Cu-Ch NPs treated plants showed significant defense responses in chitinase, peroxidase, phenylalanine ammonia lyase, and polyphenol oxidase enzymes. This gives better natural elicitation towards the plant defense and disease protection and hence enhances the sustainable growth of the banana crop.

Keywords: Cu-ChNPs; Chlorophyll; Chlorophyll Stability Index (CSI); leaf succulence (LS); vitamin C; Defense Enzymes

Introduction

Banana (Musa sp,) is one of the most popular fruits worldwide and stands second most important fruit crop in India. Banana contains important nutrients which are essential to human health. At present, India is the largest producer of bananas. According to FAO data, India produces 29 million tonnes of bananas annually, followed by China (11 million tonnes), the Philippines (7.5 million tonnes), and Brazil on average [1]. A substantial source of carbohydrates and vitamins, particularly B and C, are found in bananas. It is a significant fruit with high calcium, magnesium, potassium, and phosphorus [2]. The nutrients in bananas support several benefits, including stress relief and a decrease in cancer and cardiovascular disorders. Along with treating ulcers, it has also been said to prevent kidney stones [3]. Additionally, banana is known for antibacterial, anticancer, antifungal, antidiabetic, anti-inflammatory and antioxidant effects [4]. Among the fungal diseases of bananas, Fusarium wilt, also known as Panama wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is one of the most severe diseases, highly epidemic among banana plants if they are not protected properly [5]. It reduces the crop's physiological maturity, biomass and yield. The pathogen damages the banana crop at every stage of development, from seedling through fruiting.

With the use of nanomaterial, modern agriculture is evolving into precision agriculture, which is helping to maximize the output from these technologies. There are various ways to increase crop yield and crop nutraceutical quality with the technology that is now available. The use of nanotechnology to increase productivity is one of them [6,7]. Due to nanotechnology, disease management, genetic improvement, nutrient management, and plant growth promotion have been improved through nanomaterials such as nanoparticles, nano fertilizers, and nano pesticides [6]. Materials with at least one dimension smaller than 100nm are referred to as nanoparticles (NPs) and nanomaterials (NMs). This small size results in different properties from those displayed by most of the same composition material. In fact, cellular responses of different metallic elements such as Fe, Ag, Cu, Ce, and Ti when induced by ionic forms as opposed to nanometric forms [8]. The number of

studies on nanoparticles (NPs) in crop plants has expanded along with the development of nanotechnology [9]. Fertilizers are required to increase crop yield, but numerous studies have shown that they can reduce soil fertility by upsetting the mineral balance in the soil [10]. Pesticides, fertilizers and medicines are frequently sprayed and can flow off effectively. These insecticides and fertilizers are also produced at a very high cost, which needs no alteration. The use of nanoparticles in agriculture seeks to minimize production costs to optimize output, decrease product amounts for plant protection and reduce nutrient losses to boost yields [11]. More emphasis is being placed on chitosan-based nanotechnology in agrochemical delivery systems and research into utilizing chitosan as a carrier of active chemicals is ongoing. Molybdenum (Mo), Copper (Cu), Iron (Fe), Nickel (Ni), Manganese (Mn), and Zinc (Zn) are some of the micronutrients needed by plants, and they are required considerably in lesser quantities than macronutrients for the proper growth of crops [12].

In contrast, copper is a crucial metal for plants because it plays various roles in respiration and photosynthesis, including the movement of electrons [13,14]. On the other hand, copper has lower plant toxicity and is frequently employed as a fungicide against several infections. In addition, copper packed in NPs improves pathogen inhibition, the efficiency of fertilizer usage, and nutrient bioavailability when compared to other conventional ions and salts, leading to less environmental degradation [15]. Considering the beneficial effects reported, this study's has been emphasized to ascertain how the foliar application of copper nanoparticles (cu NPs) on the accumulation of bioactive compounds in banana fruits affected the positive effects on the increase of bioactive compounds as observed in other crops.

Materials and Methods

Crop Development

"Nanjangudrasabale" (NRB) banana cultivar was used for this experiment. After the transplant of NRB plants, flowering took place after 9 months after the crop was established under field conditions. The crop was maintained with a single stem till the end of the experiments from the transplant date to obtain fruits for the evaluations. A substrate composed of a mixture of perlite–peat moss (1-1) and N:P:K (20:20:20) was used accordingly [16].

Application of Treatments

The treatments consisted of foliar applications of five different Cu-ChNPs concentrations, as mentioned below. For positive control, SAAF fungicide was used, whereas distilled water was applied as a control. Cu-ChNPs applications were carried out during the development of the crop. The first application was made 30 days after the transplant, the second one at 60 DAT third treatment at 120 DAT, and the final treatment at 240 days. In total, 30 mL of solution was applied per plant, corresponding to different concentrations of Cu-ChNPs per plant in each treatment. The copper nanoparticles were synthesized in the Institute of Excellence (IOE), University of Mysore, Mysuru, Karnataka, India, following the methodology described by Vasanth. [17].

a. Control (dw)

b. Bulk Chitosan (BCH) (0.2%).

c. Copper sulfate($CuSO_4$)(0.2%).

d. Fungicide (SAAF) (0.2%).

e. Cu-ChNPs (0.01, 0.05, 0.1, 0.15 and 0.20mg/mL).

The plants were maintained as per standard agronomic practices. Observation days start from 30 days till the fruiting ends are considered. Meanwhile, disease symptoms, leaf senescence, number of leaves/plant, plant height, fruits/bunch, and weight/plant were preferred to record at maturity (365 days) time as suggested by Campbell and Madden, [18]. Field condition ware maintained to accord percent wilt index: external wilt symptoms were taken from 1 to 5 scales as per International Musa Program Testing (IMPT) rating.

Effect of Cu-ChNPs on fusarium wilt disease in field condition

After transferring treated plants (Nanjanagudurasabale, a susceptible banana cultivar) from pot to the field condition [17]. Plants were treated with fungal spores (5×10^3 conidia mL^{-1})inoculated on banana plants as per the proposed procedure [18]. After the 30th day of inoculation, symptoms were recorded for up to 240 days, and the treatment was continued every 30 days at intervals up to 180 days.Fusarium wilt, the disease was evaluated based on the yellowing, and wilted leaves were considered to calculate the disease severity according to the standard rating 0-5 grades [18]. Further, the Percentage Efficacy of Disease Control (PEDC) was calculated using the formula [19].

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

Plant physiological parameters

Cu-Ch Nps treated banana plants were evaluated for chlorophyll stability index (CSI), chlorophyll content (CC), leaf K (LK), and leaf succulence (LS) [20].

Chlorophyll content measurement

The chlorophyll content of the leaves of the banana plant was measured from the 30th, 60th, 90th, 120th,and 240th DAT. The banana leaves were extracted with the solution of (80% v/v) acetone, and the temperature was maintained at 4°C for 24h under dark which was followed by the reading using a spectrophotometer at different nm 647 and 665 nm as per the procedure [21].

Chlorophyll stability index measurement

The banana leaf samples were collected from 30th days up to 240 days. One gm of fresh banana leaves was taken and heated at 65 0C in 25 mL of distilled water using a water bath. The chloro-

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phyll content was extracted using 80% acetone in 25 mL quantity, followed by filtration using Whatman no. 1 filter paper. Filtered samples were read at 660 nm using a spectrophotometer. The chlorophyll stability index was calculated using the formula mentioned by [22].

$$CSI\left(\%\right) = \frac{Totalchlorophyll\ content\ \left(stressed\right)}{Totalchlorophyll\ content\ \left(control\right)} \times 100$$

Measurement of leaf succulence

Measurement of leaf area, and fresh and dry weight of leaves were subjected to oven drying at 70 oC for a period until the leaves get reduced its moisture content. The leaf succulence was recorded using the following equation as mentioned by [23].

$$LS = \frac{Fresh\ weight - Dry\ weight}{Leaf\ \ area}$$

Morphological parameters

The parameters attribute to plant height, the number of leaves, total hands/bunch of whole fingers, and bunch yield, such as yield per plant, were recorded during the maturity period [24].

Copper release through *in vitro* condition

In vitro experiments were conducted to study the effect of time and pH to check the release of copper content from Cu-ChNPs. The Cu-ChNPs were dissolved in distilled water, and pH was adjusted between the range of 3 to 9. Later the contents were centrifuged at 6000 rpm for 15 min, and supernatants were collected for further analysis. The supernatant was analyzed for copper contents using atomic absorption spectrophotometer (Bio-Rad) [25].

Evaluation of Mineral content in banana fruit

To evaluate the mineral content of banana fruit, uniform sized undamaged fruits were selected at stage six (light yellow) of the maturity stage according to the color scale of USDA [26]. Furthermore, fruits were peeled and kept at -20 °C for 72 h in a deep freezer. These samples were ground to a fine powder and used to perform further analysis [27].

Estimation of vitamin C content in banana fruit

The use of a meta-phosphoric acid-acetic acid extraction solution has been reported to efficiently extract 99% of ascorbic acid from banana fruit samples, according to [28].

Induction of defense enzymes and pathogenesis-related (PR) proteins

The expression of defense-related proteins for peroxidase (POD), chitinase, polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) activities was examined after 240 DAT and 300 DAT against Foc pathogen.

Protein extraction

Treated banana samples were collected at the stage of 240 days after treatment and 300 days after treatment. Fungicide (SAAF)was used as a positive control, and negative control was maintained by spraying plants with distilled water.

Chitinase (EC 3.2.1.14) assay

The chitin powder (5 gm) (Hi-media Laboratories Pvt. Ltd. India) was dissolved in HCl (60 ml) with constant stirring for 30minand kept for precipitation at 40C overnight. The filtered were used for further use [29].

Peroxidase (EC. 1.11.1.7) assay

Peroxidase activity was estimated in the banana leaf samples. The reaction mixture was prepared by using 3mL of pyrogallol solution and 0.5 mL of enzyme extract. The reading was taken at 430 nm, according to the slight modification method of Chance and Maehly [30].

Polyphenol oxidase (EC.1.10.3.2) assay

Polyphenol oxidase (PPO) activity was determined by preparing the reaction mixture (1.5mL of 0.1M sodium phosphate buffer (pH 6.5) and 100µL of the enzyme extract). 0.01M catechol was added to start the reaction According to the procedure of Taneja and Sachar [31]. The activity was expressed as a change in absorbance at 495nm per minute/g/of protein.

Phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) assay

The deamination of L-phenylalanine determined phenylalanine ammonia-lyase to trans-cinnamic acid, and ammonia was measured at 290nm according to the procedures prescribed by Moerschbacher [32]. All enzymes' activities were expressed in µmol/min/g tissue.

Results and Discussion

The formation of copper nanoparticles was confirmed through UV-visible spectroscopy at 590 nm due to surface plasmon resonance (SPR), according to our previous report [17] (Figure 1).

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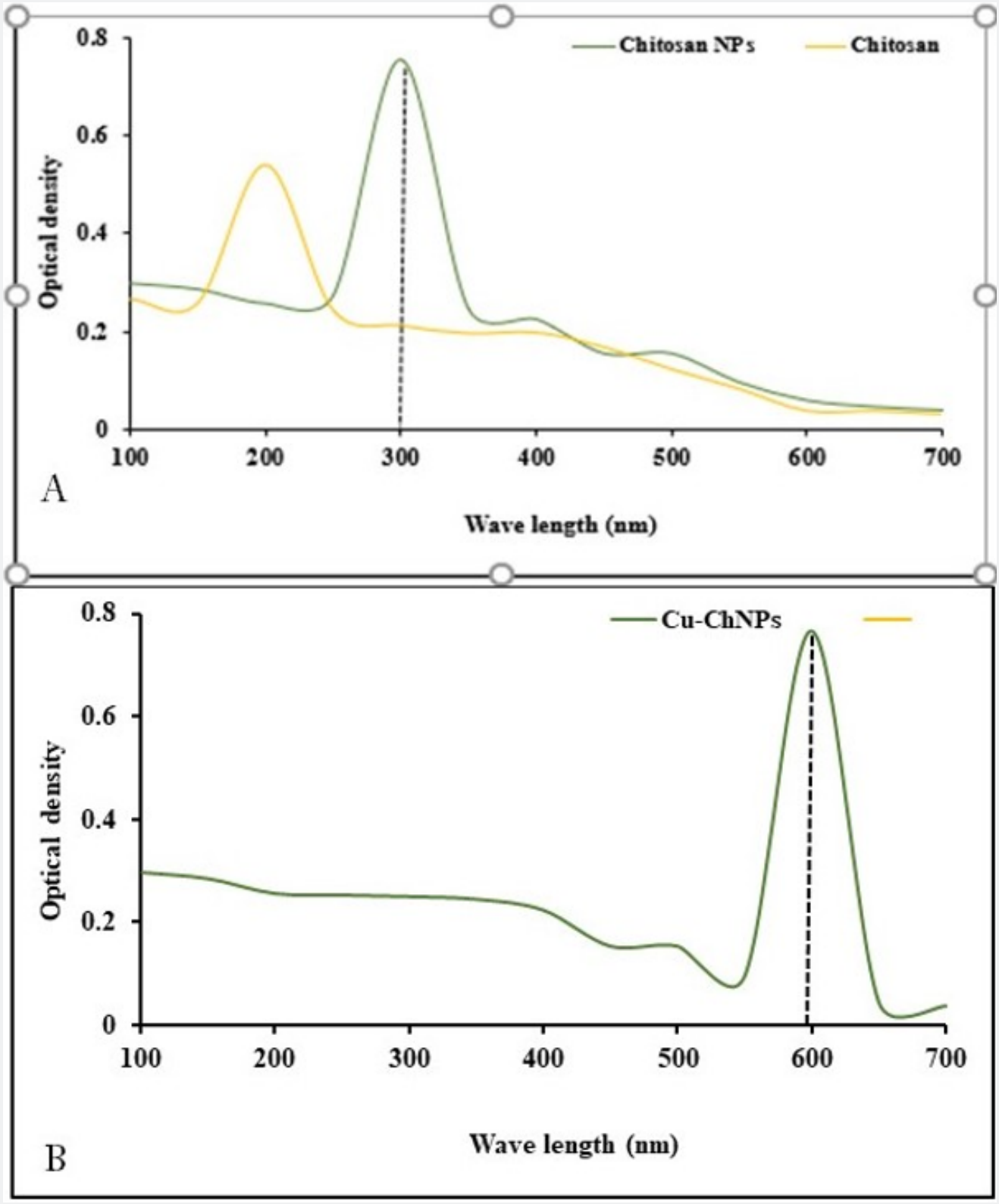

Figure 1: (A) UV-visible spectra indicate the chitosan NPs alone and bulk chitosan showing at 295nm. (B) Synthesized Ch-CuNPs at 590nm. Bulk chitosan shows peak at 190nm [17].

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Effect of Cu-ChNPs on Fusarium wilt disease in field experiment

After transferring treated plants (Nanjanagudurasabale, a susceptible banana cultivar) from pot to the field condition [17]. Banana plants were subjected to foliar spray by following standard agronomic conditions. After the 30thDAT of pathogen inoculation, symptoms were recorded. Treatments were continued every 30 days intervals up to 180 days, and results were recorded up to 240 days. The symptoms appeared with wilt symptoms which gradually extended to the entire banana plant. The maximum disease incidence was recorded in control plants which showed 98% disease symptoms at 240 DAT and chitosan alone treated plants. The maximum PEDC was recorded in fungicide treated banana plants (22%), followed by 0.20mg/mL of Cu-ChNPs (24%) when recorded at 240 DAT. The least PEDC was recorded in 0.01mg/mL followed by BCH alone (65 and 55%),as shown in Figure 2. It was noticed that Cu-ChNPs were an improved version of chitosan and fungicide alone when used, and also, the concentrations used for chitosan and fungicides were higher than NPs used in the experiments. This may be due to the chitosan biopolymer, which acts as a strong elicitor of plant defense mechanisms and has the highest antifungal activity when coated with copper NPs [33]. Furthermore, significant growth promotion and antifungal activity capabilities of Cu-ChNPs confirmed the result in agricultural crops [34,35].

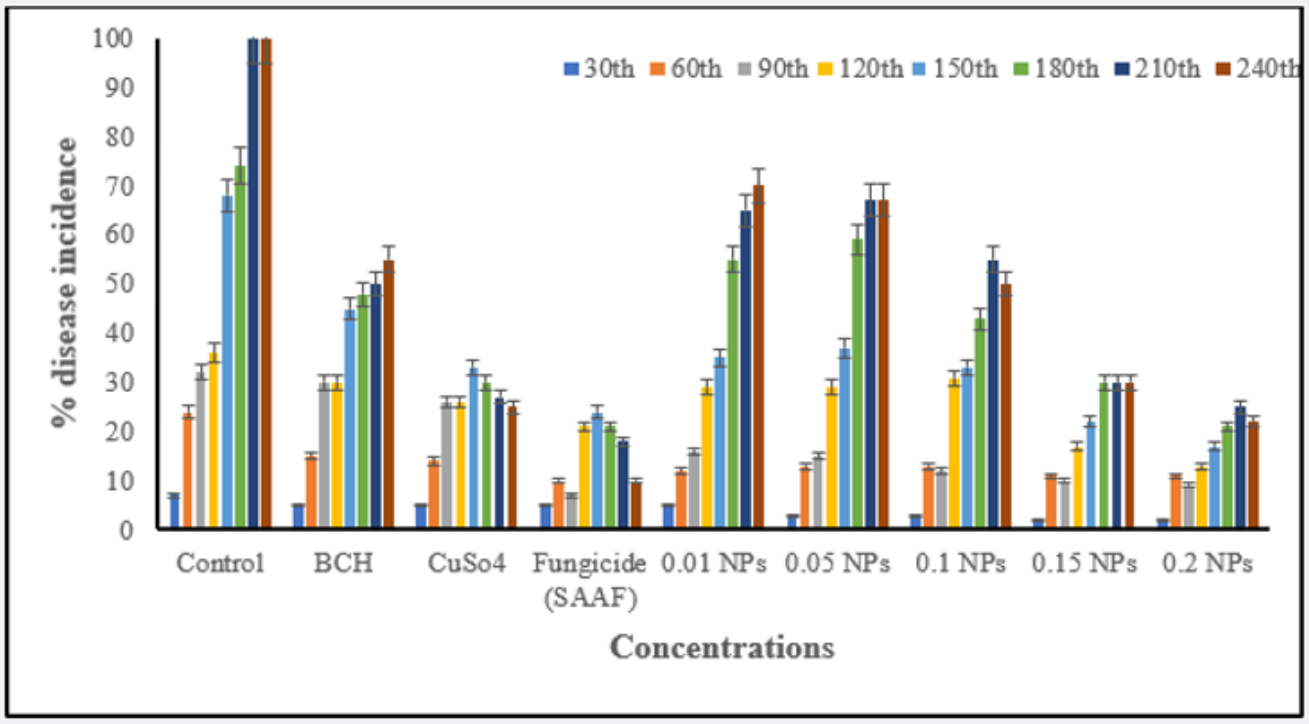

Figure 2: Effect of Cu-ChNPs on Fusarium wilt disease in field condition from 30 days to 240th day after treatment.

Disease data were recorded after 15 days of inoculation using 1 to 9 standard disease rating scale. Each value is mean of triplicates and each replicate consist of 3 plant samples and same letter in the graph of each treatment is not significantly different at p=0.05 as determined by Turkey-Kramer HSD, control with water, BCH (bulk chitosan 0.01% dissolved in acetic acid 0.1%), CuSO4 0.20%, Fungicide (SAAF 0.2%).

Plant physiological parameters

The data on CSI, CC and LS contents of treated banana plants were recorded accordingly. The CC of the banana plant was measured after 30th DAT up to 240th DAT. The data in Figure 3 revealed the effect of Cu-NPs on chlorophyll content. The highest Chl a content was observed in Cu-ChNPs at 150 DAT when compared to other treatments. Likewise, the highest Chl-b was recorded in Cu-ChNPs treated plants at 240 DAT, as shown in Figure 4. Stability and leaf succulence was reported, as shown in Table 1. The different treatments showed different effects on the physiological attributes of the banana fruits. The different concentrations of Cu-ChNPs were designed according to the response of the banana plants to the lower concentration. The CSI and LS contents showed a marked decrease from 36.57 to 21.54% in control, whereas increased CSI was observed in 0.2mg/mL NPs treated plants which showed 50.40 at 30th DAT reaches to 58.08% at 240th DAT, respectively. The treatment with fungicide (SAAF) showed 46.78% at 30th DAT, which increased up to 56.54% on 240th DAT, respectively.

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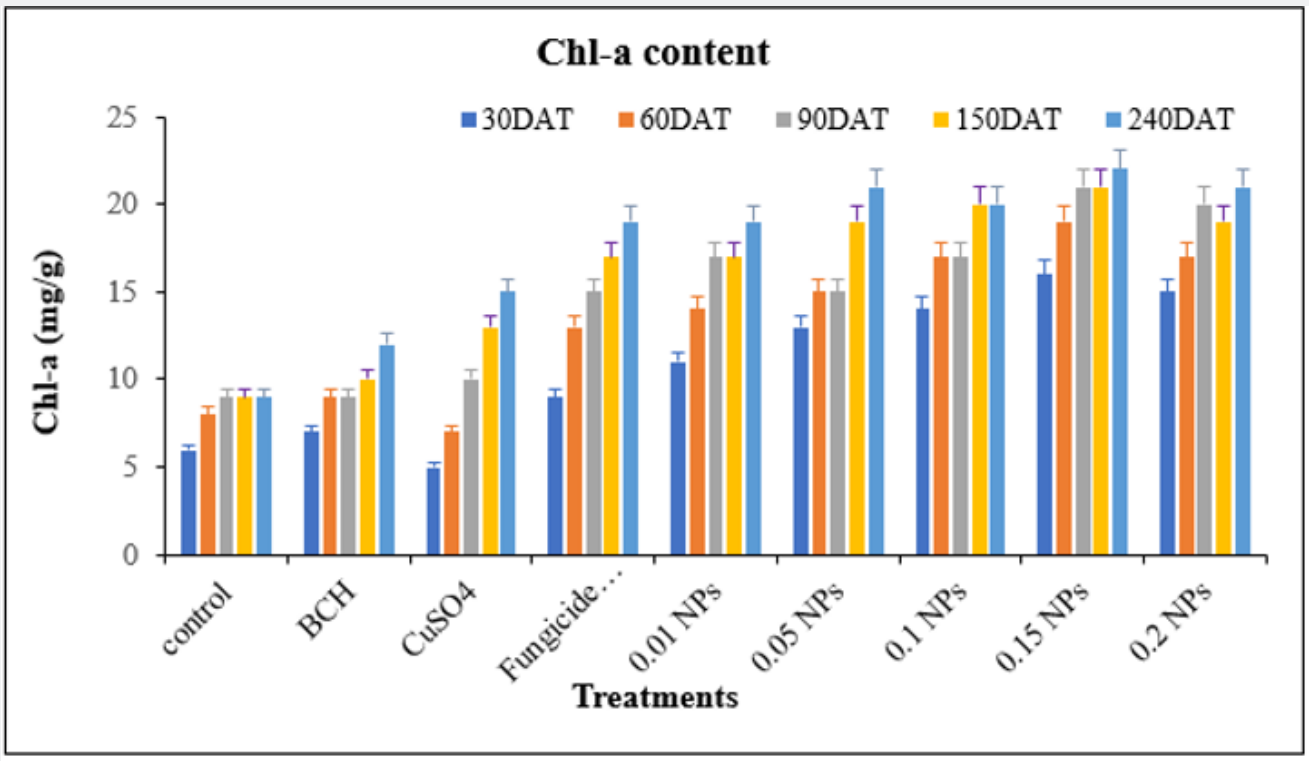

Figure 3(a): Effect of Cu-ChNPs on Chl-a content.
Each value is the mean of triplicates, and each replicate consisted of 3 plant samples and the same letter in the graph of each treatment is not significantly different at p=0.05 as determined by Turkey-Kramer HSD, control with water, BCH (bulk chitosan 0.01% dissolved in acetic acid 0.1%), CuSO4 0.20%, Fungicide (SAAF 0.2%).

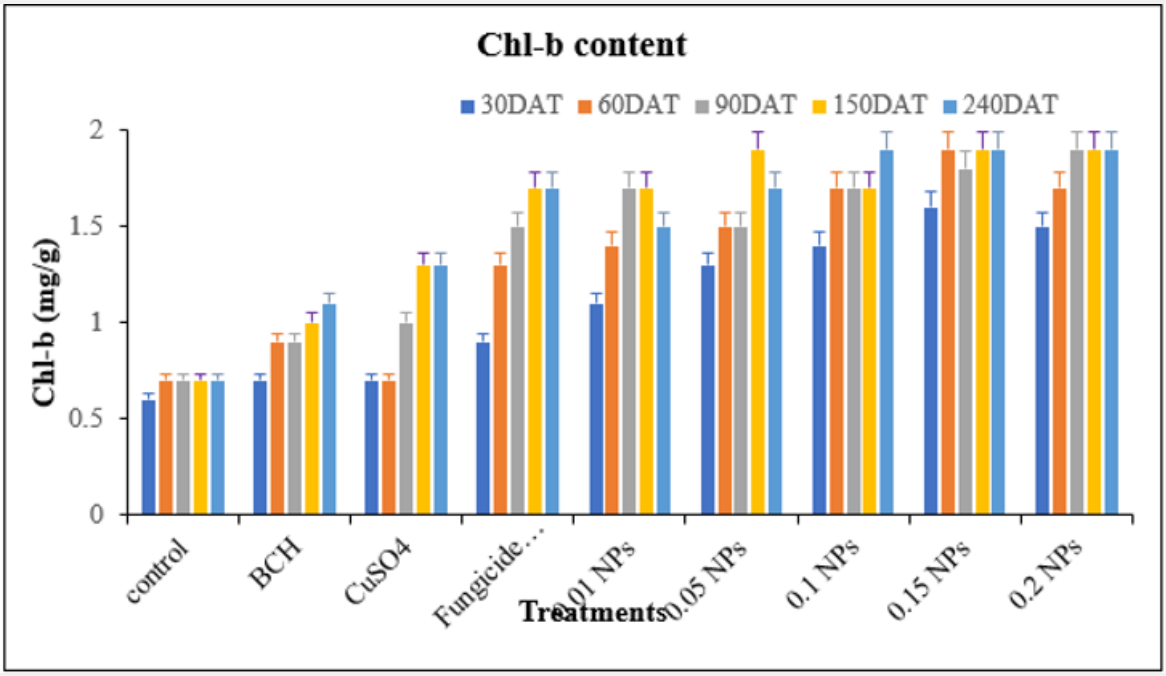

Figure 3(b): Effect of Cu-ChNPs on Chl-a content.
Each value is the mean of triplicates, and each replicate consisted of 3 plant samples and the same letter in the graph of each treatment is not significantly different at p=0.05 as determined by Turkey-Kramer HSD, control with water, BCH (bulk chitosan 0.01% dissolved in acetic acid 0.1%), CuSO4 0.20%, Fungicide (SAAF 0.2%).

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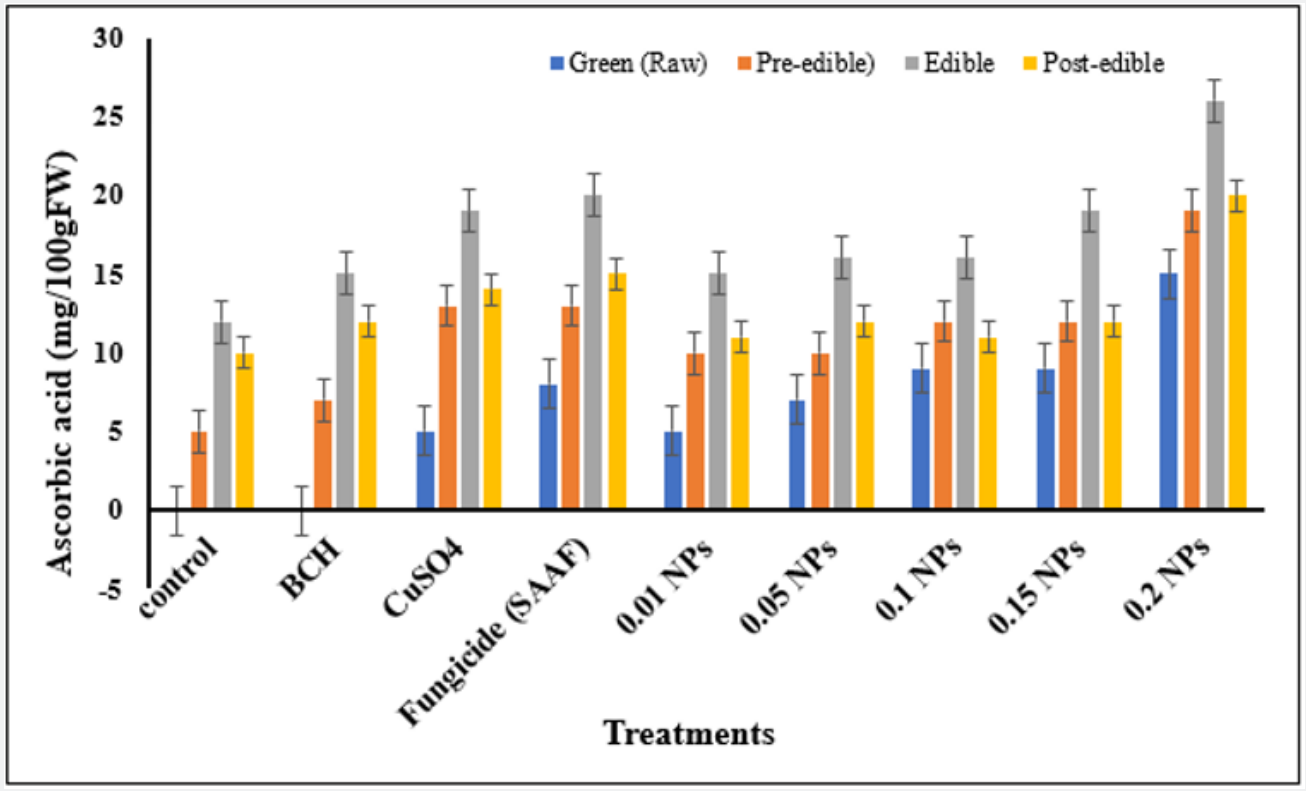

Figure 4: Effect of Cu-ChNPs on vitamin C at different ripening process.
Each value is mean of triplicates and each replicate consisted of 3 plants samples. Each treatment is not significantly different at p = 0.05.

Table 1: Effect of Cu-ChNPs on chlorophyll stability index and leaf succulence.

Treatments	Chlorophyll stability index (%)					Leaf succulence (mg/cm²)				
	30th DAT	60th	90th	150th	240th	30th	60th	90th	150th	240th
Control	36.57	33.01	31.01	33.45	21.54	6.23	7.4	7.8	7	5.6
BCH	40.23	44.1	46	47.21	47.65	7.2	8.2	8.5	9.5	6.5
$CuSo_4$	44.54	45.78	45.02	48.45	50.89	8.9	9.6	9.9	11.3	8.8
Fungicide (SAAF)	46.78	48.25	48.87	54	56.54	10.5	12.5	12	13.5	11.2
Cu-ChNPs										
0.01 NPs	45.25	46.25	45.45	48.23	48.98	7.45	8.5	8	10.5	8.5
0.05 NPs	44.09	45.89	47.56	48.45	48.05	7.89	8.9	8.5	10.8	7.9
0.1 NPs	46.56	46.89	49.54	49.78	50.45	8.2	9.2	8.9	11.3	8.3
0.15 NPs	47.54	46.54	47.87	49.45	50.78	8.2	9.6	9.3	12.5	9.8
0.2 NPs	50.4	51.51	52.45	55.32	58.08	9.4	12.2	11.8	13.2	13

Each value is the mean of triplicates, and each replicate consisted of 3 plant samples and the same letter in the table of each treatment is not significantly different at p=0.05 as determined by Turkey-Kramer HSD, control with water, BCH (bulk chitosan 0.01% dissolved in acetic acid 0.1%), CuSO4 0.20%, Fungicide (SAAF 0.2%). The growth of banana plants increased remarkably, shown in the form of physiological parameter changes when treated with 0.20mg/mL Cu-ChNPs used. At the same time, the Cu-ChNPs plants exhibit better stress tolerance represented by physiological traits. The CSI was found maximum through the usage of 0.20mg/mL NPs, showed 58%, and also improved the LS, 13.0 g/cm² at 240th DAT. Whereas positive control fungicide treatment showed 56.54% and LS showed 11.2 g/cm² at 240th DAT. This proves that banana plants treated with 0.20mg/mL NPs showed improved CSI, and LS content when compared to other treatments. Some of the previous reports revealed that copper contributes to both micronutrients as well as disease management [36]. Plastocyananin is an important component of copper in photosynthetic electron transport chains in the form of Cu^{2+} which helps plants to tolerate stress conditions [37]. The treatment with NPs can im-

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prove the electron exchange efficiency in the cells, which reduces the formation of reactive oxygen species (ROS) by arresting electron leakage [38,39].

Significant differences were observed in the parameter studies, as shown in Table 2. The Cu-ChNPs treated plants showed an increase in plant height, a total number of leaves, total bunches, total fingers/hand, and also an increase in bunch weight. This indicates that the application of Cu-ChNPs necessarily generates Cu content in the plants and increases the plant height and other parameters. An accumulation of Cu in the fruits. Control treatment showed 10ft height, with 06 leaves without bunch due to fusarium wilt disease. The application of 0.20% NPs showed increased plant height with 10.6 ft, an increase in the number of leaves, with a total number of 11 leaves, when recorded after 300 DAT. Highest number of fingers/hand was also observed with 15 fingers/hand was recorded at 340 DAT. Finally, the bunch weight was found to be 15.5kg and was recorded after 360 DAT compared to different treatments of NPs, as shown in Table 2. Whereas positive control SAAF (fungicide) showed a plant height of 10.5ft, the total number of 11 leaves was recorded after 300 DAT. A total number of fingers/hands observed with 15 fingers/hands was noticed at 340 DAT. The bunch weight was found to be 15.0kg and was recorded after 360 DAT. This may be due to the fact that chitosan directly delivers Cu that can penetrate tissues and move through the phloem to other organs, increasing the plant height and weight of the bunch [34]. In this study, applied as a foliar spray, the Cu-ChNPs were directly accumulated inside the leaves. Considering this, the application of Cu-ChNPs did not produce any accumulation of nanoparticle residues inside the banana fruits, and thus, there is no risk of consumption of the fruits [34].

Table 2: Effect of Cu-ChNPs on different parameter studies at different time intervals.

Quantitative Parameters					
Plant height		**Number of leaves**	**Total hands/bunch**	**Total fingers/hand**	**Bunch weight (Kg)**
Treatment	300 DAT	300 DAT	300 DAT	340 DAT	360DAT
Control	10 ft	6	---	---	----
BCH	10.2	10	9	14	8
CuSo4	10.3	10	9	14	14
Fungicide (SAAF)	10.5	11	9	15	15
Cu-ChNPs					
0.01 NPs	10.3	10	9	14	13
0.05 NPs	10.3	10	9	14	13
0.1 NPs	10.5	10	9	14	12
0.15 NPs	10.6	10	9	14	13.5
0.2 NPs	10.6	11	11	15	15.5

Plant height, number of leaves, total hands/bunch, total fingers, and bunch weight. Each value is the mean of triplicates, and each replicate consisted of 3 plant samples and the same letter in the table of each treatment is not significantly different at p=0.05 as determined by Turkey-Kramer HSD, control with water, BCH (bulk chitosan 0.01% dissolved in acetic acid 0.1%), CuSO4 0.20%, Fungicide (SAAF 0.2%).

Fruit Quality

The concentration of vitamin C in 100g of banana fruit pulp was higher at 0.20mg/mL NPs treated showed 14mg in the raw green banana fruit, 16mg in semi-ripened banana fruit, and the highest level of 25mg at the ripened stage, followed by decreasing the concentration of vitamin C to 18mg at post ripened stage. The treatment with fungicide showed 7mg in the raw green banana fruit, 7mg in semi-ripened banana fruit, and the highest level of 18mg at the ripened stage, followed by a decrease in vit C of 16mg. The control showed 11mg/100g pulp at the edible stage, whereas BCH alone showed 13mg at the edible ripened stage as shown in Figure 4. Lokesh [40] reported the highest content of carotenoids such as alpha-carotene and beta-carotene in banana fruit. Furthermore, Abhishek [41] reported the highest content 45mg/100g of vitamin C content in Nanjangud rasabale fruit variety when compared to other varieties. Juarez Maldonada. [42] reported that the application of Cu NPs + chitosan increased the firmness of banana fruits by 9%. This is consistent with the results as shown in the present study. The NPs may be translocated through the vascular tissues with the subsequent dissolution to Cu^{2+} ions gives rise to the observed lignification and may increase the carotenoid substances in the banana fruit pulp [43].

***In vitro* copper release**

The release of copper from Cu-ChNPs was studied in the pH range from 3 to 9 with a decrease in pH from 3 to 5, the release of Cu increased rapidly at pH 7 when compared with 6 and 8 pH. pH 7 showed the highest Cu release of 78% as shown in Figure 5. This is due to the protonation of the amino group of chitosan and helps the Cu to release into the delivery system rapidly, as shown in Figure 6. Cu release increased from pH 6 to 8 from Cu-Ch NPs, and slow, sustained release of Cu was observed at pH 9 and pH 4. Choudhary et al. [25] reported a pH with a decrease in pH from 3 to 1 and an increase in the release of Cu rapidly from 21.5 to 44.11 at pH 7 to 8.

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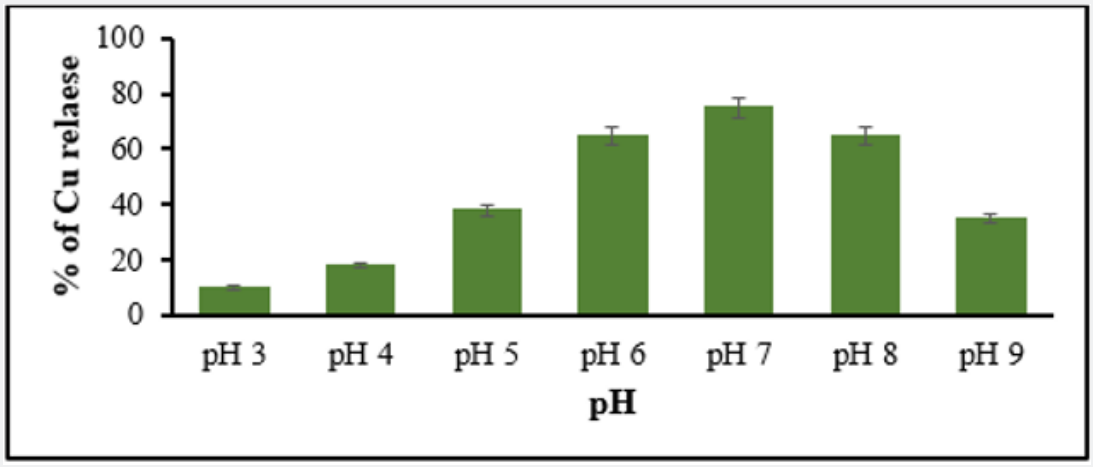

Figure 5: *In-vitro* Cu release from Cu-chitosan NPs at different pH. Each value is mean of triplicates and each replicate consisted of 3 plants samples. Each treatment is not significantly different at p = 0.05.

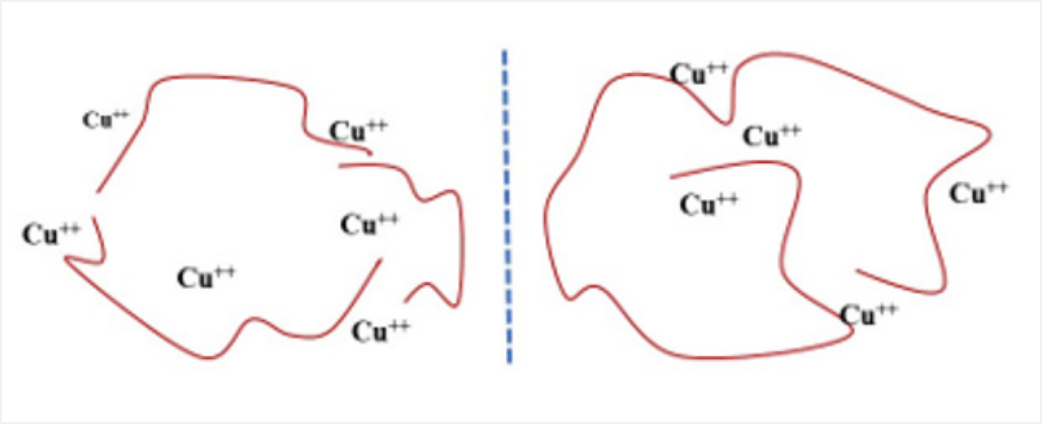

Figure 6: Schematic diagram of the release of cu release from chitosan.

However, at higher concentrations of Cu-ChNPs (0.20) and $CuSO_4$ treatment were significantly increased due to the accumulation of Cu (48.11 and 51.02) at 240DAT as shown in Table 3. Whereas BCH showed very less accumulation of Cu (3.73) and fungicide treatment showed (34.13) Cu at 240DAT, respectively.

Table 3: Effect of Cu-ChNPs on Cu release in banana plants. Each value is mean of triplicates and each replicate consisted of 3 plants samples. Each treatment is not significantly different at p = 0.05.

Treatments	Cu (µg/g)				
	30th DAT	60th	90th	150th	240th
Control (dw)	1.33±0.33^{g}	1.38±0.33^{g}	1.33±0.33^{g}	3.24±0.21^{g}	3.08±0.21g
Bulk Chitosan (BCH)	1.45±0.45^{g}	1.97±0.45^{g}	2.45±0.45^{g}	3.23±0.65^{g}	3.73±0.65g
Copper sulfate ($CuSO_4$)	5.60±0.40^{b}	7.77±0.40^{b}	19.52±0.40^{b}	35.78±0.06^{b}	48.11±0.06b
Fungicide (SAAF)	4.30±0.55^{c}	5.24±0.55^{c}	15.09±0.55^{c}	24.33±0.45^{c}	34.13±0.45c
Cu-chitosan NPs (mg/mL)					
0.01	2.32±0.55^{f}	3.08±0.55^{f}	5.55±0.55^{f}	12.22±0.64^{f}	20.22±0.64f
0.05	2.47±0.50^{e}	3.42±0.50^{e}	5.77±0.50^{e}	18.47±0.50^{e}	24.33±0.50e
0.1	2.92±0.55^{d}	4.84±0.55^{d}	8.12±0.55^{d}	21.23±0.67^{d}	27.27±0.67d
0.15	3.65±0.45^{c}	5.45±0.45^{c}	10.21±0.45^{c}	25.65±0.33^{c}	33.05±0.33c
0.2	5.85±0.55^{a}	7.15±0.55^{a}	21.21±0.55^{a}	38.45±0.05^{a}	51.02±0.05a

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Effect of Cu-ChNPs on the activities of plant defense enzymes under field conditions

The plant defense enzyme activities in Cu-ChNPs treated plants were examined on the 30th day after treatment, and the 240th day after treatment were recorded. Chitinase activity was recorded for Cu-ChNPs at different concentrations, and $CuSO_4$ treated plants. Chitinase activity was significantly Cu-ChNPs (0.20) treated plants showed 1.5 folds higher chitinase activity at 72 h was recorded when compared to fungicide-treated plants at 30th DAT as shown in Figure 7A. Applications of bulk chitosan, $CuSO_4$ treated alone, and 0.01, 0.05 0.10 treated NPs did not show much activity. Similarly, the control treated with distilled water does not show any activity. At 240th DAT, chitinase activity was decreased to 1.0 fold in Cu-ChNPs (0.20) treated plants 72h. Whereas fungicide treated and other treated plants also showed gradual decrease in activity when compared to 30th DAT as shown in Figure 8A Chitinase induction was elicited in banana-treated nanoparticles plants with Cu might provide more protection at the initial level at 30th DAT and might provide higher crop protection against fusarium disease at the initial stage. Kouzai [44] reported that chitinase proteins are involved in the defense response of the host plant against phytopathogens. Likewise, Cu-ChNPs (0.20) treated plants showed 2-3 folds increased peroxidase activity when compared to fungicide-treated plants at 30th DAT as shown in Figure 7B. Whereas 1.5 to 2.0 folds increased, peroxidase activity was recorded at 72h in 240th DAT as shown in Figure 8B. Similarly, 1.0-1.5 folds peroxidase were recorded in fungicide treated plants as compared to BCH, and $CuSO_4$ treatments. Peroxidase is one of the important enzymes involved in defense enzymes which converts H_2O_2 scavenging radicles, which are highly toxic converts to H_2O_2 and O_2. The elevated activities of peroxidase after the treatment with Cu-ChNPs (0.20) treatments might be responsible for balancing, degeneration, and reactive oxygen species (ROS), protecting plants from oxidative stress during pathogen invasion [45]. Likewise, Cu-ChNPs treated plants showed 1.5 to 2.0 folds increased PAL activity at 30th DAT as compared to fungicide and other treatments, as shown in Figure 7C. While PAL showed 1.0-1.5 folds increased activity at 72h in 240th DAT when compared with fungicide, bulk chitosan treated, $CuSO_4$ and NPs with (0.01. 0.05 and 0.10) as shown in Figure 8C.

PAL might be associated with the accumulation of copper in the cell wall and the responses of phenylalanine ammonia-lyase (PAL). This catalyzes L-phenylalanine to cinnamic acid, synthesizing suberin and lignin in the phenylpropanoid pathway, which further acts as a mechanical barrier to invading plant pathogens [46,47]. In contrast, the activity of PPO was also enhanced by Cu-ChNPs treated plants at 30th DAT as shown in Figure 8D. The activity showed 2.0-2.5 folds higher at 72h at 240th DAT when compared to fungicide, bulk chitosan treated, $CuSO_4$, and NPs with (0.01. 0.05 and 0.10) as shown in Figure 8D. The PPO might be associated with the production of suberin and lignin, which is responsible for the strengthening of the cell wall [48]. Previously in *in vitro* experiments Cu-ChNPs treated plants found effective in inhibiting the mycelial growth of *fusariumoxysporum* sp. *cubense* (*foc*) and in pot experiments, substantially induced antioxidant and defense enzyme activity in banana plants treated with foc [17].

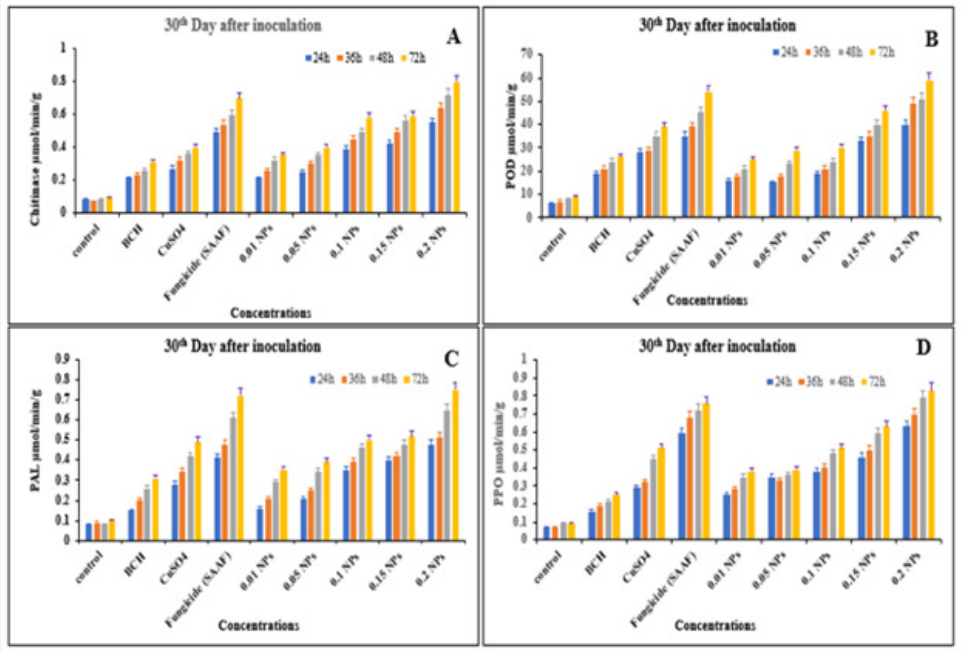

Figure 7: Effect of Cu-ChNPs on defense enzymes in banana plants challenged with the pathogen.
(A) Chitinase (B) POD (C) PAL (D) PPO enzymes activity in banana plant leaves after 30 DAT. Each value is mean of triplicates and each replicate consisted of 3 plants samples. Each treatment is not significantly different at p = 0.05.

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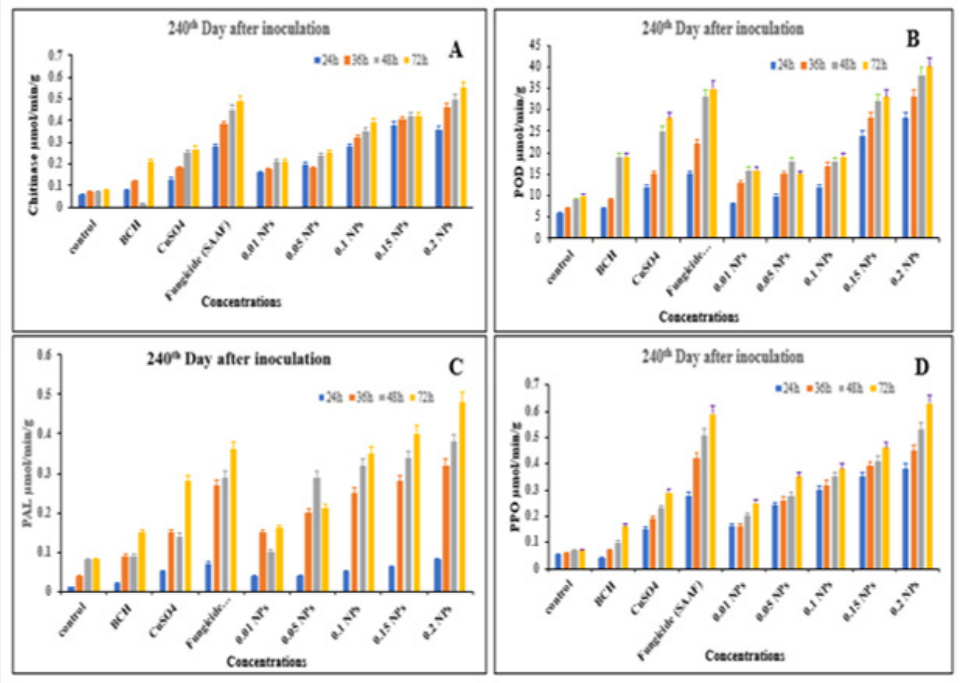

Figure 8: Effect of Cu-ChNPs on defense enzymes in banana plants challenged with the pathogen.
(A) Chitinase (B) POD (C) PAL (D) PPO enzymes activity in banana plant leaves after 30 DAT. Each value is mean of triplicates and each replicate consisted of 3 plants samples. Each treatment is not significantly different at p = 0.05.

Huge demand for food crops free from chemical components has exponentially increased in recent years to avoid the hazardous effects of chemical components and to evade the development of resistance to pathogens. A new approach is more important to strengthen plant innate immunity to manage plant pathogens. These NPs will also reduce chemical use and alongside enhances sustained plant growth. Cu-ChNPs at 0.20% has been proven as a promising plant protectant to manage plant pathogens and also enhances growth promotory agent in our past and recent studies against fusarium wilt. An increase in the physiological parameters such as chlorophyll content and leaf succulence, also indicated the growth-promoting effects of improving the crop yield. These bio-based nanomaterials could be pivotal toward sustainable agriculture without harming the ecosystem. The biopolymer-derived NPs have immense potential to be commercially explored for agriculture use.

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