Research Article

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Chitosan Nanoparticles Modulate Growth Promotion and Oxidative Stress Tolerance in Rice (*Oryza sativa L.*)

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ABSTRACT

Green nanotechnology provides innovative approaches to sustainable agriculture by creating eco-friendly nanomaterials with biostimulatory properties. In the current research, chitosan nanoparticles (ChNPs) were synthesised from crustacean shrimp shells through an ionic gelation process and further their characterization was examined. The scanning electron microscopy (SEM) revealed ChNPs were spherical in shape and size ranges from 16 to 24 nm. UV–visible spectroscopic analysis revealed the characteristic absorption behaviour of NPs which confirms the ChNPs synthesis and further X-ray diffraction (XRD) study confirmed semi-crystalline nature of the synthesized ChNPs. Fourier transform infrared spectroscopy (FTIR) confirmed the involvement of functional groups in ChNPs synthesis. The rice seedlings and pot-grown rice plants exposed to different concentrations (0, 10, 20, 30, 40, 50, and 60%) of ChNPs indicated significant increases in protein, proline, chlorophyll and carotenoids contents suggesting modulations in physiological and biochemical traits. The ChNPs concentration dependent increased in antioxidant enzymes such as superoxide dismutase, catalase, peroxidase, glutathione peroxidase, and ascorbate peroxidase were recorded in seedlings and plants treated with ChNPs. Moreover, the DPPH radical scavenging assay (77.9±3.1 % inhibition) confirmed the ChNPs improves antioxidant potential. These findings demonstrate that, ChNPs improves morphological, physiological, and biochemical traits and antioxidant defense systems in *Oryza sativa*. Therefore, ChNPs are shown to have promise as environmentally sound nanobiostimulants that can help to achieve sustainable crop production while also representing a new way of enhancing plant resilience and productivity.

Keywords: Chitosan nanoparticles; *Oryza sativa*; Growth promotion; Antioxidant activity; Stress tolerance.

1. INTRODUCTION

Nanotechnology primarily focuses on the design, creation, and analysis of particles at the nanoscale. The tiny particles are known as nanoparticles, and they act as a single entity depending on their characteristics and mode of transport (Sonawane *et al.* 2022). Owing to their small size, nanoparticles can more efficiently and directly enter the body and reach specific cell or tissues. Nanoparticles serve as contrast agents in medical imaging and as transport agents for drug and gene delivery in vivo (Jha and Mayanovic 2023). Nanoparticles can also carry and deliver pharmaceutical drug molecules in a shorter timeframe and with less discomfort in the body, and they also aiding in the detection and treatment of various diseases (Shelke *et al*. 2022).

In addition, chitosan is a linear polyamine with several free amine groups that are easily accessible for crosslinking. Its cationic characteristic enables ionic crosslinking with multivalent anions. ChNPs are a novel form of medication that has been made possible by recent advancements in nanotechnology (Huang and Yang, 2004). Compared to bulk chitosan, these nanoparticles have improved surface area, bioavailability, reactivity, potential to increase absorption, and effectiveness even at lower doses due to their more effective interaction with plant cells or tissues. For the reasons mentioned above, ChNPs are an appealing nano-biostimulant option for the application in agricultural improvement (Boonlertnirun *et al.* 2008).

According to previous literature, in the present study biosynthesized ChNPs are of considerable interest in medicine, environmental fields, and agriculture, especially for enhancing seed germination, stress tolerance, and antioxidant defence mechanisms in other crop and medicinally important plants. Consequently, the environment-friendly generation of ChNPs constitutes a promising and optimistic approach to producing sustainable nanomaterials that are more effective and safer for the environment (Aranaz *et al.* 2021). Chitosan, a naturally occurring polysaccharide obtained from the deacetylation of chitin, has attracted focused interest due to its biocompatibility, biodegradability, and non-toxicity. It has gained significant recognition due to its elicitor effects in

plants, antifungal properties, and antibacterial activities (Krishna *et al.* 2010). Numerous morphological, physiological, and biochemical processes, such as seed germination, growth rate, stress tolerance, and nutrient absorption, are improved in plants treated with chitosan and ChNPs. Chitosan is highly suitable for the preparation of nanoparticles intended for controlled drug delivery and drug release. ChNPs and their preparation methods are simple and mild; they offer versatile administration options and have attracted increased interest as a drug delivery carrier (Tiyaboonchai, 2003).

Enhancement in rice (*Oryza sativa*) grain production is a crucial staple crop for over 50% of the global population. It is crucial for the stability of food supplies and rural livelihoods, especially in Asian countries (Brunel *et al.* 2008). Abiotic factors like drought, salt, and extreme temperatures, as well as biotic factors like pests, and weeds, constantly threaten rice production worldwide. These restrictions drastically restrict the productivity and quality of agricultural products. As a result, using cutting-edge, environmentally friendly agricultural techniques is essential to improving rice development and production (Tiyaboonchai, 2003). Despite extensive study on the beneficial effects of chitosan on a variety of crops, nothing is known about how ChNPs affect *Oryza sativa* growth and development (Nguyen *et al.* 2013). According to Saharan *et al.* (2016), maximizing the use of ChNPs in rice production requires analysing a number of traits like germination rate, seedling vigor, plant growth and height, biomass accumulation, and stress tolerance. Divya *et al.* (2022) reported that the effectiveness of ChNPs as a growth promoter in improving the yield and biological activity of rice has been examined. For instance, 1.0 mg/ml of ChNPs was applied to seed, soil, foliar and combination treatments and the growth and yield parameters were assessed. The combination treatment of seed, soil and the foliar application of ChNPs was found to be most proficient. Therefore, the current research is to assess how biosynthesized ChNPs affect the growth, physiological characteristics, and biochemical responses of *Oryza sativa* during both the seedling and mature stages. Furthermore, the research seeks to assess their potential as a nano-biostimulant that is environmentally sustainable and can enhance growth performance and resilience to stress.

2 MATERIALS AND METHODOLOGY

2.1 Plant Materials and Chemicals

The certified rice seeds were procured from the Agriculture College Shivajinagar, Pune, Maharashtra. Shrimp shells were brought from the local fish market (Ravet, Pune), the shells were carefully cleaned in order to get rid of contaminants and traces of organic waste. In order to remove moisture and stop the growth of microbes, the cleaned shells were subsequently dried in a hot air oven at 80°C for 2-3 days. After proper drying, the Shrimp shells were crushed into fine powder using a mechanical grinder. Then, the dried powder was used for deproteinization, demineralization and deacetylation processes. Shrimp shells powder material was treated with 2.0 N sodium hydroxide (NaOH) (HiMedia, Mumbai, India) at a concentration of 12 g/ml (w/v). The mixture was heated for four hours at 70°C using a water bath (Steelmet Industries, Mumbai, India). Once the reaction was completed, the solid residue was filtered out and repeatedly cleaned with double-distilled water until the pH becomes neutral (pH 7.0). After deproteinization, the mixture was dried before being processed further.

The deproteinized product was treated with 10% hydrochloric acid (HCl, HiMedia, Mumbai, India) at room temperature for 4 hours at a ratio of 14 ml/g. In order to remove extra HCl and soluble salts, the solid residue was carefully cleaned with distilled water until a pH became neutral (pH 7.0). The material was dried after demineralization prior to further processing. The chitin was treated with 35% NaOH at a ratio of 14 mL per gram in order to deacetylation. The reaction mixture was kept at room temperature while being constantly stirred magnetically for 75 hrs. Once the reaction was finished, the deacetylated product was filtered and repeatedly cleaned with distilled water until the pH was 7.0 to eliminate alkali residues. The final product was cleaned, dried, and purified. The final product chitosan was subsequently used for further characterization.

2.2 Biosynthesis of Chitosan Nanoparticles

ChNPs were synthesized by using ionic gelation process, which is based on the electrostatic interaction between positively charged chitosan and negatively charged tripolyphosphate (TPP) (Himedia, Mumbai, India). In order to produce homogenous chitosan solution, 200 ml of distilled water, 1% of acetic acid (v/v) were used to dissolve 200 mg of extracted chitosan powder. The acidic media allows chitosan to fully dissolve by protonating its amino ($–NH_2$) groups. As an additional cross-linking agent, TPP (mg/ml) solution was made with distilled water. To create chitosan nanoparticles, 5 mL of chitosan solution was mixed with 2.0 mL of TPP solution drop-wise while being continuously stirred at 1000 rpm using a magnetic stirrer. To ensure uniform chitosan nanoparticle synthesis, the reaction containers were incubated at room temperature for 1 hour. As a result of ionic interactions between the negatively charged phosphate groups of TPP and the protonated amino groups of

chitosan, which leads to synthesis of stable chitosan nanoparticles. The constant stirring prevented the nanoparticles from aggregating and promoted appropriate dispersion. Centrifugation was used to separate the nanoparticles at 20000 rpm for 30 minutes.

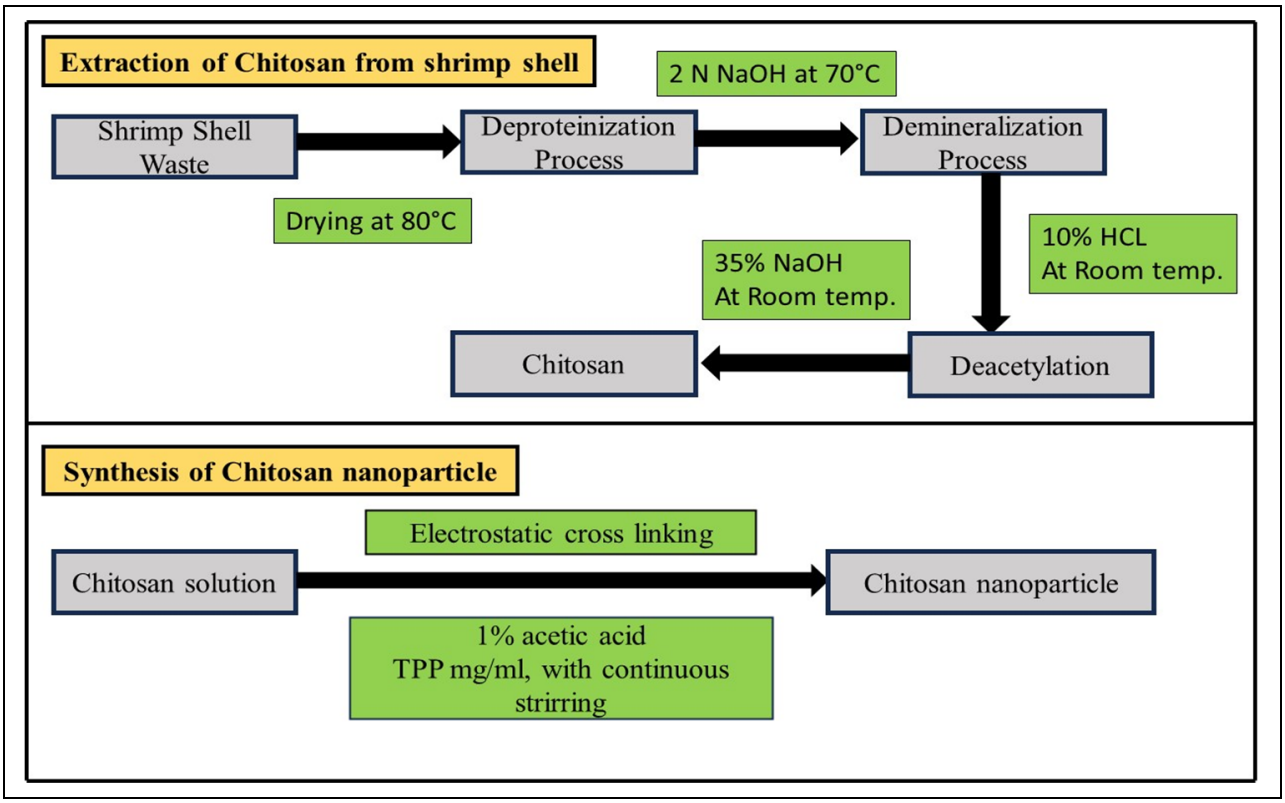


Fig. 1: Extraction of pure chitosan from shrimp shells and synthesis of ChNPs

2.3 Characterization of Chitosan Nanoparticles (ChNPs)

To examine the morphology and size distribution of the nanoparticles, Scanning Electron Microscopy (SEM, Nova NanoSEM NPEP303) imaging was performed at an accelerating voltage of 15 kV and under high vacuum conditions. Chitosan NPs were analysed using FTIR using a Bruker FTIR (Bruker Corporation, Germany) over the range of 4000–500 cm^{-1} and a resolution of 4 cm^{-1} to identify its functional groups and confirm its structure. The suspension of ChNPs was scanned through the wavelength range of 200–500 nm. X-ray diffraction (XRD, Rigaku Corporation, Ultima-IV, Japan) with a Cu Kα radiation source (λ = 1.5406 Å) operated at 40 kV and 40 mA was used to analyse the crystalline nature of chitosan NPs. The synthesized ChNPs were analysed for their optical properties using a UV–Visible spectrophotometer (Shimadzu, Japan).

2.4 Treatment of ChNPs to Seeds

Stock solutions of ChNPs were prepared in 6 different conical flasks and placed in a rotary shaker (Steelmet, Mumbai, India) for 3 days. The concentrations were varied from 0.0%, 10%, 20%, 30%, 40%, 50%, and 60% of ChNPs were represented as T0, T1, T2, T3, T4, T5 and T6. After 3 days, all conical flasks were removed from the rotary shaker, and 5.0 g of rice seeds were added to each conical flask. Seeds were treated with 10% of ChNPs for 60 min, 20% of ChNPs, 30% of ChNPs, 40% of ChNPs, 50% of ChNPs, and 60% of ChNPs were dissolved in 100 ml of distilled water in a conical flask. For control, the seeds were treated in 100 mL of distilled water for 60 min in conical flask separately. To evaluate the growth response of rice after germination, morphological parameters such as root length, shoot length, biomass (both fresh and dry weight), and seedling viability were assessed.

2.5 Physiological Analysis of *Oryza sativa* Plantlets Treated with ChNPs

2.5.1 Chlorophyll Content

The modified acetone method of Arnon's (1949) was used to estimate the chlorophyll content from leaf tissue of rice (Porra and Scheer, 2019). Fresh leaf tissue (0.5 g) was homogenized in 80% acetone using mortar pestle. The homogenized mixture was

centrifuged. The absorbance of supernatant was measured using spectrophotometer at 645 and 663 nm against blank of 80% acetone.

2.5.2 Carotenoids Content

The carotenoid content in fresh leaf tissue of rice was estimated using the method outlined by Yi *et al.* (1996). Fresh leaf tissue (0.5 g) was homogenized and extracted using 10 ml of 80% acetone. A spectrophotometer was used to measure the absorbance of the reaction mixture at 480 and 510 nm. Total carotenoid content from leaf tissue was estimated and the results were expressed as mg/g fresh weight.

2.5.3 Proline Content

The proline content was analysed using the technique of Bates *et al.* (1973) as described by Sadasivam and Manickam (1996). 0.5 g of fresh leaf tissue was homogenized in 3% sulfosalicylic acid, treated with ninhydrin and glacial acetic acid, heated at 100 °C, and the chromophore was extracted using 4.0 ml of toluene. Absorbance of the reaction mixture was measured at 520 nm, and proline content was determined from a standard curve, and results were expressed as µmol g^{-1} fresh weight of leaf tissue.

2.5.4 Protein Content

The protein content is estimated by using Lowry method (Lowry *et al.* 1951). Fresh plant tissue (1.0 g) was homogenized in phosphate buffer and centrifuged at 10000 rpm. The supernatant was treated with alkaline copper reagent and Folin–Ciocalteu reagent. The absorbance of the reaction mixture was measured at 660 nm, and the protein content was determined from a standard curve of BSA (HiMedia, Mumbai, India). The results of protein content were expressed as mg g^{-1} fresh weight.

2.6 Antioxidant Assays

2.6.1 Superoxide Dismutase (SOD) (EC 1.15.1.1)

Superoxide dismutase (SOD) activity was assessed in fresh plant tissue of rice using modified method of Sarker and Oba, (2018). To obtain the SOD enzyme extract, 0.1 g of fresh plant tissue of rice was homogenized in 250 mL of cold potassium phosphate buffer (pH of 7.0) using a mortar pestle. The enzyme extract was centrifuged at 10,000 rpm for 10 minutes at 4°C. Potassium phosphate buffer (2.45 ml), 300 µl of 100 mM methionine, 50 µl of 10 mM riboflavin, 50 µl of EDTA, 100 µl of 750 mM nitroblue tetrazolium (NBT) solution, and 100 µl of enzyme extract were collectively used as the reaction mixture for analysis. The absorbance of the reaction mixture was measured at 560 nm using spectrophotometer. The results were expressed as units per gram of fresh weight of tissue.

2.6.2 Catalase (CAT) (EC 1.11.1.6)

The catalase activity was determined from leaf tissue of rice using the modified method of Laxmi *et al.* (2018). To obtain the enzyme extract, 0.1 g of fresh plant tissue was homogenized in 0.067 M (pH 7.0) phosphate buffer and centrifuged at 1-4°C. The sediment was stirred using a cold phosphate buffer. Supernatant was used for the catalase activity, and enzyme extract (devoid of H_2O_2) was used as a blank. 3.0 ml of H_2O_2-PO_4 buffer and 100 µl of enzyme extract were used in the reaction mixture. The absorbance of the reaction mixture was measured at 240 nm using spectrophotometer. According to Luck (1965) catalase activity was measured in µmol of H_2O_2 degraded per minute per gram of fresh weight of leaf tissue.

2.6.3 Peroxidase (POX) (EC 1.11.1.7)

The peroxidase activity was measured in fresh plant tissue using the modified method of Wu *et al.* (2022). To obtain the enzyme extract, 0.1 g of fresh plant tissue was homogenized in 0.1 M phosphate buffer (pH 7.0) and centrifuged at 18,000 rpm for 15 minutes at 5°C. The reaction mixture included 30 µl of hydrogen peroxide, 50 µl of guaiacol solution and 100 µl of enzyme extract. A spectrophotometer was used to measure the increase in absorbance at 436 nm for 3 minutes. The change in absorbance per minute per gram of fresh weight was recorded and used to express enzyme activity.

2.6.4 Glutathione Peroxidase (GPX) (EC 1.11.1.9)

In order to prepare the enzyme extract, fresh plant tissue (0.1 g) was homogenized in 0.4 M sodium phosphate buffer (pH 7.0), and centrifuged at 10,000 rpm for 15 minutes at 4°C. 400 µl of phosphate buffer, 100 µl of sodium azide, 200 µl of reduced glutathione, 500 µl of enzyme extract, 100 µl of H_2O_2, and 2000 µL of distilled water were collectively used as the reaction mixture. The reaction mixture containing tubes was incubated at 37°C for 30 minutes. 500 µl of 10% Trichloroacetic acid centrifuged mixture. Save the supernatant and add 1000 µl of 5, 5'-Dithiobis (2-nitrobenzoic acid) Ellman's reagent (DTNB) and 3 ml of Na_2HPO_4 were added to the supernatant. The absorbance of the reaction mixture was measured at 412 nm using a spectrophotometer. The activity of glutathione peroxidase was measured in µg of GSH used per minute per gram of fresh weight.

2.6.5 Ascorbate peroxidase (APX) Assay (EC 1.11.1.11)

The Sadasivam and Manickam (1996) method was used to measure the activity of ascorbate peroxidase (APX). To obtain the enzyme extract, fresh plant tissue (0.1 g) was homogenized in ice-cold potassium phosphate buffer using a mortar and pestle and the enzyme extract was centrifuged at 12,000 rpm for 15

minutes at 4°C temperature. 800 µl of 50 mM phosphate buffer (pH 7.0), 100 µl of 0.5 mM ascorbate, 50 µl of 0.1 mM EDTA, 50 µl of enzyme extract, and 50 µl of 0.1 mM hydrogen peroxide were all included in the reaction mixture. The oxidation of ascorbate caused a drop in absorbance at 290 nm, which was recorded in 1 minute. The unit of measurement for APX activity was µmol of ascorbate oxidized per minute per gram of fresh weight of tissue.

2.6.6 DPPH (2,2-diphenyl-1-picrylhydrazyl)

In the DPPH radical scavenging assay, gallic acid served as a standard antioxidant to assess and contrast the antioxidant potential of the test samples (enzyme extract). A stock solution of gallic acid was made in methanol and then diluted to achieve various concentrations for assay. 100 µL aliquot of each concentration of gallic acid was combined with 3.0 mL of DPPH solution and incubated in the dark for 30 minutes at room temperature. After incubation, the reduction of absorbance was measured at 517 nm using a UV–Visible spectrophotometer (Baliyan *et al.* 2022).

2.7 Statistical Analysis

A completely randomized design (CRD) was used in all experiments. All tests were carried out three times, and the results are presented as mean ± standard deviation (SD). To assess the significance of differences between control and treated groups, statistical analysis was conducted using a t-test. A probability level of $p<0.05$ was regarded as statistically significant.

3. RESULTS

3.1 Synthesis and Characterization of ChNPs

The chitosan was obtained from a natural source, chitin present in shrimp shells. The extracted chitosan was used for the synthesis of ChNPs. Synthesis of ChNPs was achieved through using TPP. The structure of synthesized ChNPs was analyzed, and particle dimensions (varying from 16 to 25 nm) were measured using SEM analysis. Characterization of ChNPs was performed using SEM, FTIR, XRD, and UV–Visible spectroscopic analysis.

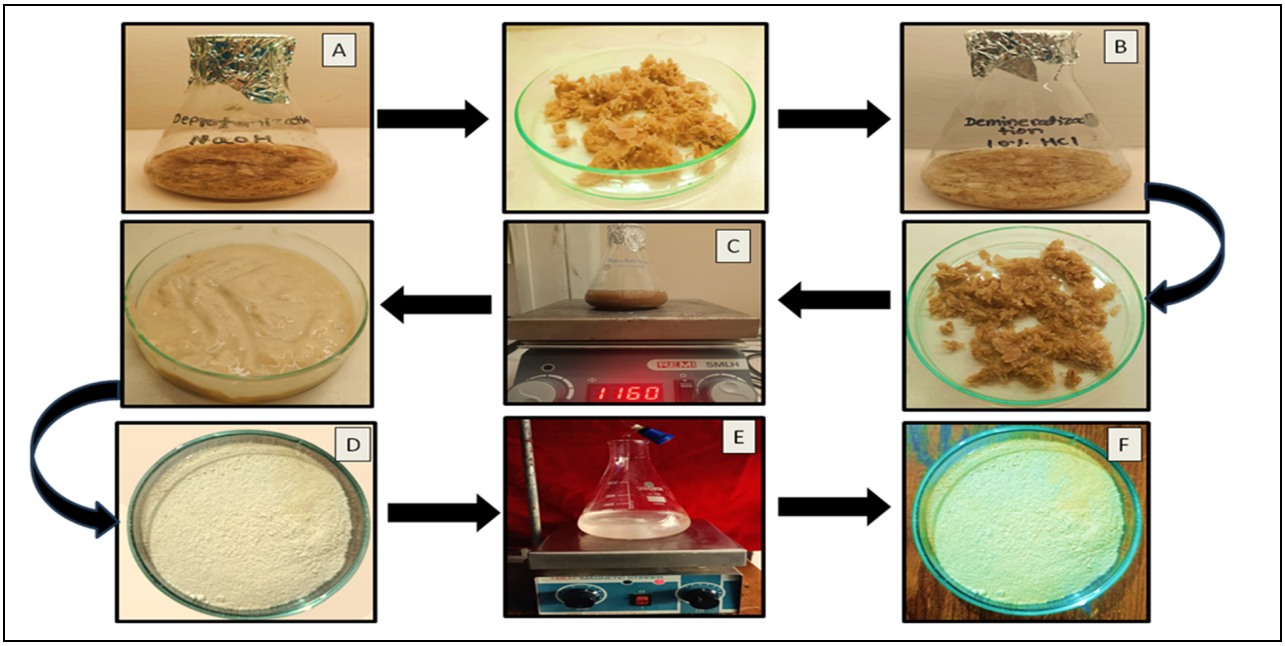


Fig. 2: The process of obtaining chitosan from shrimp shells and synthesis of ChNPs is depicted in sequential steps, (A) Removal of protein from shrimp shells using NaOH, (B) Demineralization of deproteinized material, (C) Ch was extracted from chitin through deacetylation process, (D) It showed extracted Ch from chitin, (E) Synthesis of ChNPs by using TPP and (F) Synthesized ChNPs.

3.1.1 SEM and EDS Analysis of ChNPs

The SEM analysis showed that the nanoparticles were mostly spherical in shape with slight agglomeration and a size varying between an average of 16 and 24 nm. The presence of carbon (C), oxygen (O), nitrogen (N), Al, Si, P, Ca, and Na were confirmed by EDS spectra, which indicate a high-purity chitosan-based organic matrix without detectable impurities.

3.1.2 FTIR Analysis of ChNPs

The FTIR analysis was performed for the characterization of the synthesized ChNPs. The FTIR spectra displayed characteristic absorption bands of chitosan, such as a broad peak around 3438 cm^{-1} linked to O–H and N–H stretching vibrations, a peak at approximately 2872 cm^{-1} associated with C–H stretching, and amide I and II bands at about 1651 and

1551 cm^{-1} that confirm the presence of residual acetyl groups and amine functionality. The presence of extra peaks in the ranges of 1422–1375 cm^{-1} and 1151–1026 cm^{-1}, which correspond to C–N stretching and C–O–C vibrations of the glycosidic backbone, confirms the structural integrity of ChNPs.

3.1.3 XRD and UV–Visible Spectroscopic Analysis of ChNPs

The XRD pattern showed a distinct semi-crystalline peak at approximately 19–20° (2θ) which corresponds to the (110) plane of chitosan, with broadening of the peak suggesting nanoscale crystalline material. Minor peaks were indicated as partial crystallinity and interactions among polymer chains, which align with standard JCPDS data. UV–Visible spectroscopy demonstrated a strong absorption band in the UV region (200–250 nm) with negligible absorption in the visible range, indicating no chromophoric impurities and confirming the successful synthesis and purity of ChNPs.

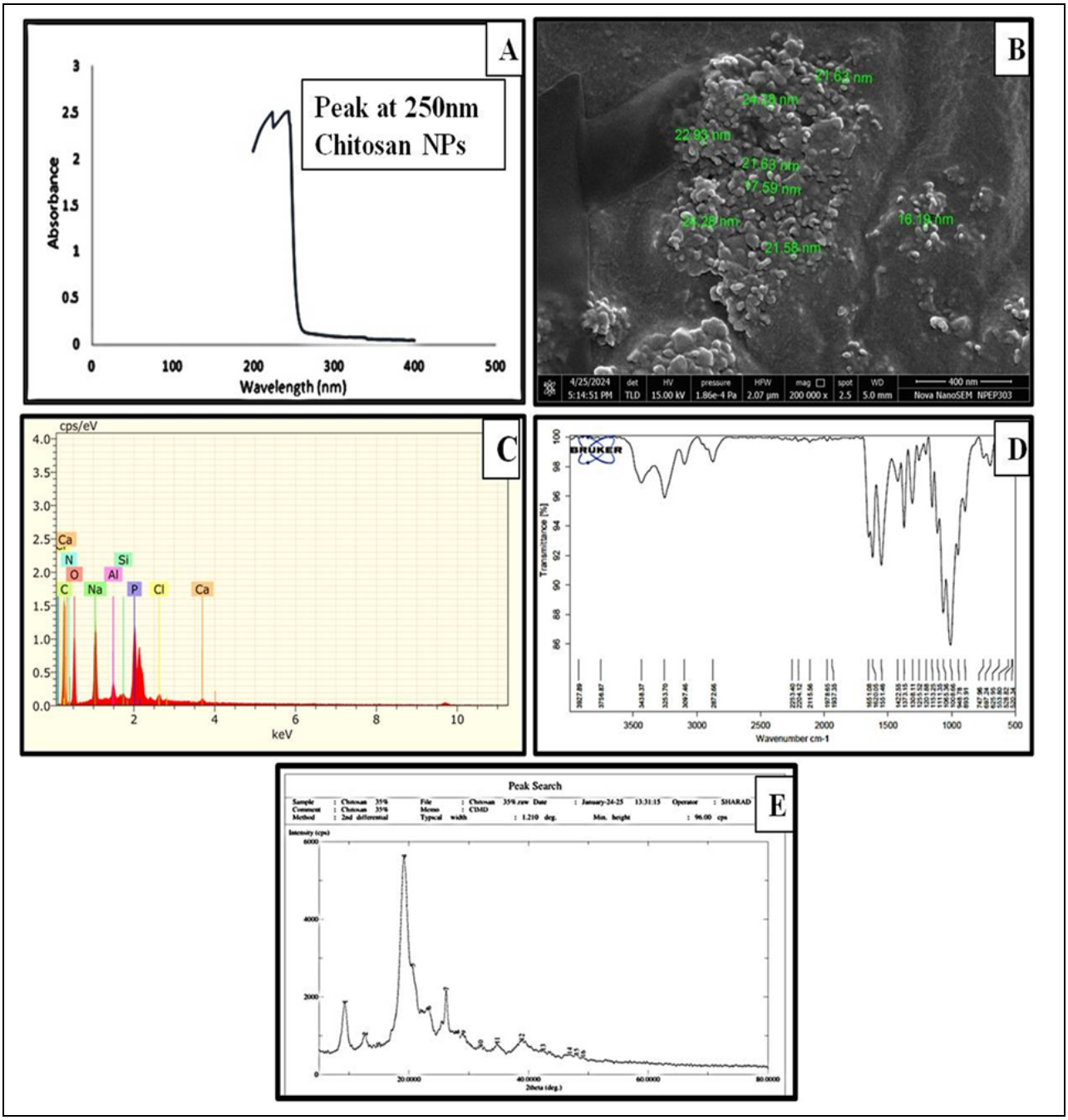


Fig. 3: Characterization of synthesized ChNPs, (A) UV–Visible spectrum visualized at 250 nm that confirms ChNPs synthesis, (B) SEM analysis of ChNPs sized from 16 to 25 nm, (C) EDS analysis confirms the existence of elements, (D) FTIR spectrum confirms the synthesis of ChNPs and (E) XRD showed the presence of semi-crystalline structure of ChNPs

3.2 Morphological Parameters of Rice Seedlings Treated with ChNPs

ChNPs treatment significantly improved the morphological characteristics of *Oryza sativa* seedlings as compared to the control as shown in Table 1. Control seedlings showed minimal growth, characterized by short roots (1.8±0.5 cm) and shoots (2.5±0.4 cm), low biomass accumulation, and low germination rate (6.6±0.9%) which reflects weak seedling vigor. Chitosan NPs-treated seedlings improved in a dose-dependent manner. Root length (50.9±2.6 cm at 50% ChNPs) and shoot length (67.7±2.4 cm at 60% ChNPs) grew significantly, and it was accompanied by increased fresh (0.84±0.06 g) and dry weight (0.21±0.04 at 50% ChNPs), indicating enhanced biomass production. The seed germination rate (96±2.9%) and seedling viability (96±2.9%) significantly modulated in rice seeds were treated with ChNPs at a concentration of 60%. Therefore, overall findings suggest that ChNPs, especially at a concentration of 50% and 60%, were found most effective in enhancing seedling growth, improving biomass accumulation and seed germination in seedlings of *Oryza sativa*.

Table 1. Morphological characters of *Oryza sativa* seedlings treated with ChNPs

Treatment of ChNPs	Root Length (cm)	Shoot Length (cm)	Fresh Weight (g)	Dry Weight (g)	Viability	% Germination
Control	1.8±0.5e	2.5±0.4d	0.046±0.004e	0.012±0.003d	0.66±0.04d	6.6±0.9e
10%	25.6±1.6d	61.2±2.8ab	0.60±0.05c	0.15±0.02b	8.0±0.7b	80±2.3c
20%	28.6±2.1d	47.2±2.0c	0.66±0.06b	0.17±0.03b	8.3±0.6b	83±2.7c
30%	43.1±2.3b	63.5±2.7a	0.58±0.03c	0.15±0.01b	9.0±0.9a	90±2.8b
40%	36.5±1.9c	50.6±2.2c	0.49±0.02d	0.12±0.02c	7.0±0.4c	70±2.6d
50%	50.9±2.6a	56.7±1.8bc	0.84±0.06a	0.21±0.04a	9.6±0.8a	96±3.0a
60%	49.1±2.3a	67.7±2.4a	0.81±0.04a	0.20±0.03a	9.6±0.9a	96±2.9a

Data are mean ± SE scored and followed by same letters are not significantly different at $p \leq 0.05$ level by Duncan's multiple range test (DMRT).

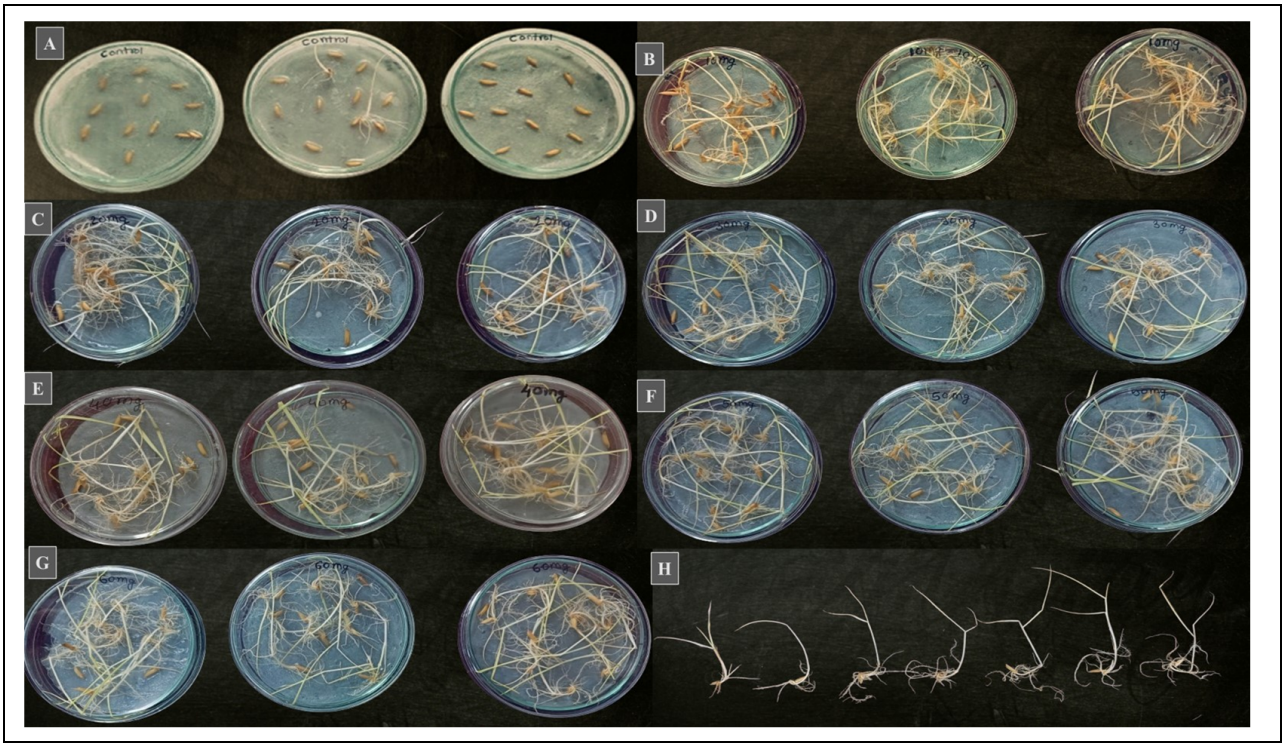


Fig. 4: Impact of ChNPs on seed germination, seedling growth and development at 10% to 60% concentrations of ChNPs, (A) Seeds showed restricted germination and seedling with reduced root and shoot length at the control condition, (B–G) Petriplates with improved germination, root and seedling growth and development at 10%-50% ChNPs concentrations, while 60% (ChNPs) diminished seed germination and seedlings growth and (H) Control (0% ChNPs)

3.3 Physiological and Biochemical Parameters of Rice Seedlings Treated with ChNPs

3.3.1 Protein Estimation

The protein content was estimated in ChNPs (10-60%) and control *Oryza sativa* seedlings. The protein content progressively enhanced in ChNPs rice seedlings as compared to the control rice seedlings. The control value was 0.012±0.002 mg g^{-1}, whereas treated seedlings rose from 0.013±0.002 mg g^{-1} at 20% ChNPs to 0.028±0.003 mg g^{-1} at 60% ChNPs. The standard deviation values across treatments were relatively small and consistent, which indicates the experiment was reproducible and confirms a clear dose-dependent enhancement of protein content in seedlings of rice (Fig. 5-A).

3.3.2 Proline Content

The proline content in control seedlings was 0.0080±0.0006 mg g^{-1}, while the most effective seedling treatment (40%) demonstrated a significantly elevated proline level of 0.0105±0.0007 mg g^{-1} of protein. Even though the standard deviation values were similar, the greater mean proline content in treated seedlings demonstrates a clear improvement over the control, which indicates the use of ChNPs significantly enhances proline accumulation in rice seedlings (Fig. 5-B).

3.3.3 Carotenoids Content

The accumulation of carotenoids in seedlings was found to increase with treatments of ChNPs (10, 20, 30, 40, 50, and 60%) in comparison to the control. The control seedlings exhibited a carotenoid content of 4.8±0.6 mg g^{-1}. Whereas seedlings treated with ChNPs exhibited elevated carotenoid content (11.2 ± 0.7 mg g^{-1}) at a concentration of 60% ChNPs, which was more than double that of the control (Fig. 5-C).

3.3.4 Chlorophyll Content

Data on the effect of different concentrations of ChNPs on total chlorophyll content in *Oryza sativa* petriplates-grown seedlings is presented in Figure 5-D. Compared to the control, treatments of ChNPs led to a notable increase in chlorophyll pigments in seedlings. Total chlorophyll content in the control was relatively lower, particularly chlorophyll a was found 4.0±0.6 mg g^{-1}, chlorophyll b was found 10.5±0.9 mg g^{-1}, and total chlorophyll was noted 14.0±1.2 mg g^{-1} in rice seedlings. Seedlings treated with the optimal concentration of ChNPs (60%) showed significantly higher pigment levels, with chlorophyll a (12.5±1.0 mg g^{-1}), chlorophyll b (22±1.5 mg g^{-1}), and total chlorophyll (35±2.0 mg g^{-1}) being recorded. The results of the analysis of chlorophyll content suggest that the content was increased with increase of concentrations of ChNPs. Compared to control (14.0±1.2 mg g^{-1}), the content of total chlorophyll was progressively increased with treatment of 30% and 50% ChNPs. More or less similar decline in the chlorophyll content was observed with the treatments of other concentrations of ChNPs. Both 30% and 40% treatments of ChNPs were highly significant over the similar treatments of 50% and 60% ChNPs.

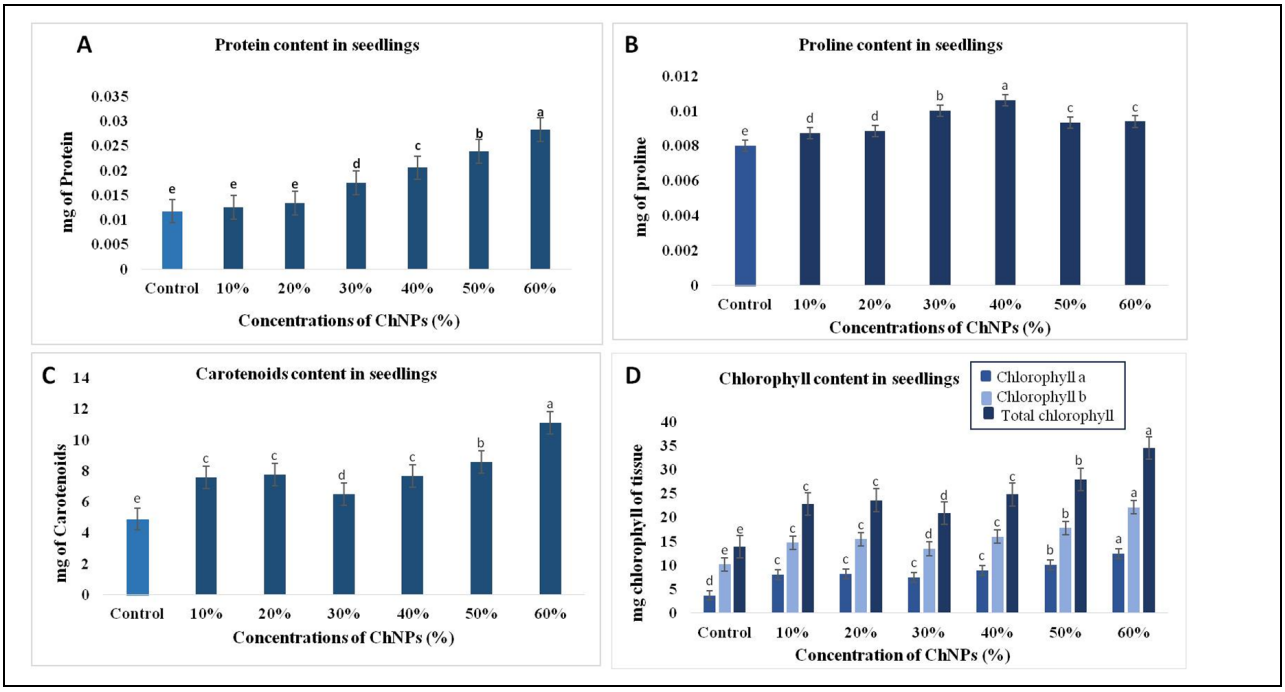


Fig. 5: Impact of ChNPs on physiological and biochemical parameters in rice seedlings, (A) Protein content, (B) Proline content, (C) Carotenoids content and (D) Total chlorophyll content in seedlings

Data are mean ± SE scored and followed by the same letters are not significantly different at $p \leq 0.05$ level by Duncan's multiple range test (DMRT).

3.4 Antioxidant Assays

3.4.1 Superoxide Dismutase (SOD)

When compared to the control, the activity of superoxide dismutase (SOD) was exhibited a clear dose-dependent increase in seedlings treated with ChNPs. Treated seedlings demonstrated a progressive increase in SOD activity, rising from approximately 220 mM min^{-1} mg^{-1} protein at 10% to 360 mM min^{-1} mg^{-1} protein at 60% ChNPs, while the control exhibited the lowest activity at about 180 mM min^{-1} mg^{-1} protein. All treatments (10, 20, 30, 40, 50, and 60%) with ChNPs showed a significant increase in SOD activity as compared to control rice seedlings (Fig. 6-A).

3.4.2 Catalase (CAT)

Catalase activity in the rice seedlings treated with the ChNPs showed significantly greater than that of the control seedlings (Fig. 6-B). The control rice seedlings exhibited lower catalase activity of 120±40 mM min^{-1} mg^{-1} protein that suggesting a restricted antioxidant effect. The optimal treatment (60% ChNPs) to seedlings showed significantly higher catalase activity, 620±65 mM min^{-1} mg^{-1} protein. The ChNPs-treated seedlings increased catalase activity, indicating that the seedlings have improved hydrogen peroxide detoxification and a more robust antioxidant defence system compared to the untreated control.

3.4.3 Peroxidase (POX)

Peroxidase enzyme activity in the control seedlings exhibited the lowest peroxidase enzyme activity, suggesting a minimal antioxidant response. The rice seedlings treated with 50% ChNPs yielded the best seedling performance, achieving the highest peroxidase activity (79.7±3.1 mM min^{-1} mg^{-1} protein). This activity indicates that the maximum activation of the antioxidant defence system in comparison to the control rice seedlings (Fig. 6-C).

3.4.4 Glutathione Peroxidase (GPX)

As a measure of baseline antioxidant activity, the control group had the lowest glutathione peroxidase activity (0.062±0.003 mM/min/mg protein). GPX activity was increased in seedling treatments of ChNPs in a concentration-dependent manner showing the greatest and most efficient response (0.15±0.04 mM/min/mg protein) was recorded at 60% ChNPs (Fig. 6-D).

3.4.5 Ascorbate Peroxidase (APX)

The control rice seedlings showed the lowest ascorbate peroxidase activity, 6±0.4 mM/min/mg protein group. The rice seedlings treated with 60% ChNPs showed noticeably significant APX activity (15±1.2 mM/min/mg protein). The seedlings treated with all concentrations of ChNPs (10, 20, 30, 40, 50, and 60%) showed increased activity of APX enzyme in rice. The best response of APX activity was recorded at 60% ChNPs treated rice seedlings, which suggested enhanced antioxidant effect (Fig 6-E).

3.4.6 DPPH

The control rice seedlings exhibited limited antioxidant capacity with low DPPH (Fig. 6-F) radical scavenging activity (3.34±0.6% inhibition) with a higher IC50 value (>100 µg/mL). The ChNPs-treated seedlings showed concentration-dependent increase in DPPH activity with a small standard deviation and a decreased IC50 (45–50 µg/mL) at the highest concentration and activity reached at 62.3±2.9% inhibition. Therefore, the best antioxidant response was recorded with seedlings treated with the highest concentration (60% ChNPs), whereas the control was the least effective baseline.

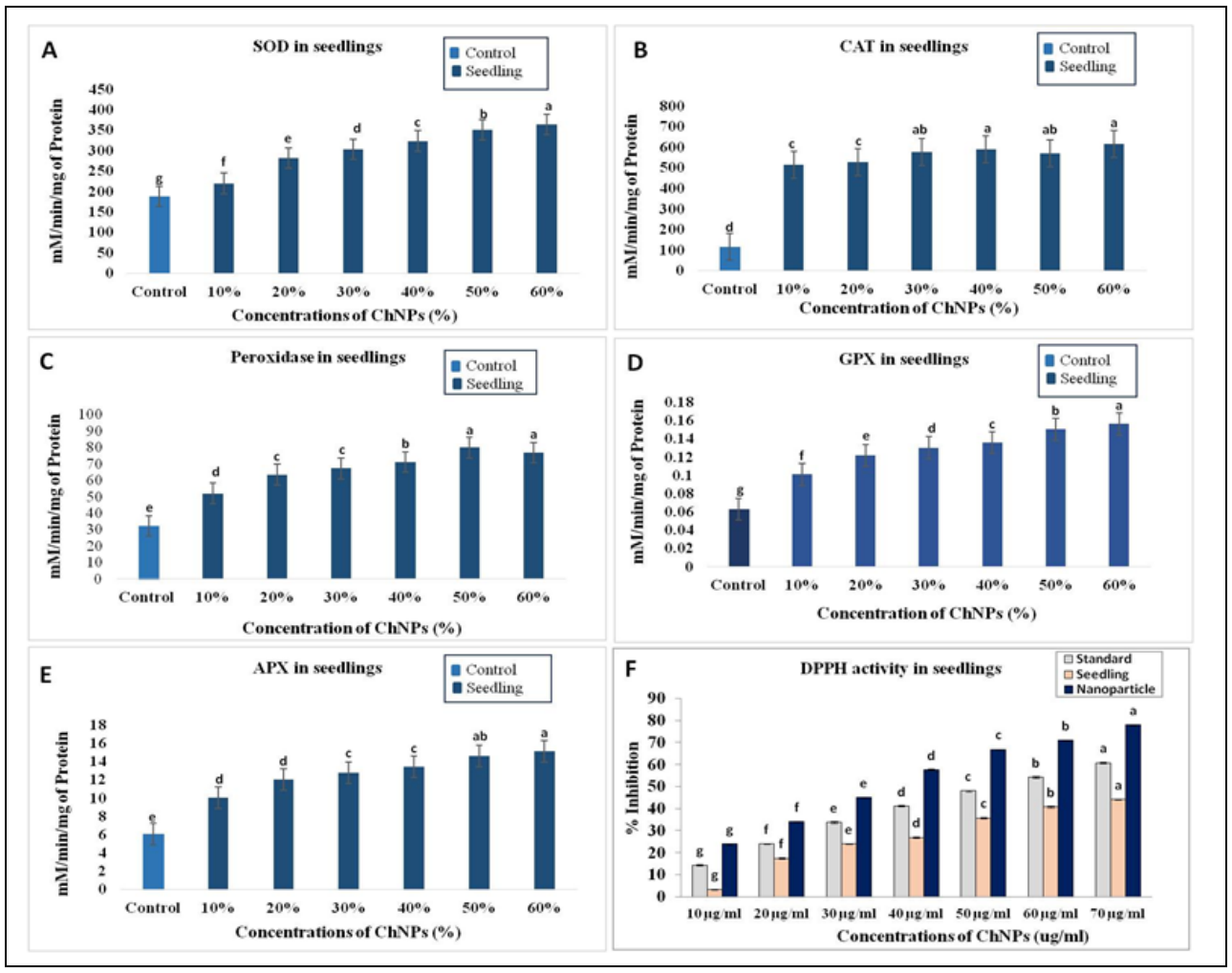


Fig. 6: Effect of ChNPs on antioxidant enzymes activities in seedlings, (A–E) Antioxidant enzymes measured at 10–60% of ChNPs in seedlings, (A) Superoxide dismutase activity, (B) Catalase activity, (C) Peroxidase activity, (D) Glutathione peroxidase activity, (E) Ascorbate peroxidase activity and (F) DPPH activity

3.5 Morphological Characters of ChNPs Pot-Grown Rice Plants

The treatment with ChNPs were affected the development and biomass production of *Oryza sativa* plants in a concentration-dependent manner (Table 2). The control plants exhibited moderate growth (root length- 10.4±1 cm and shoot length- 27.2±1.5 cm) and reduced fresh (0.76±0.03 g) and dry weight (0.15±0.003 g).The ChNPs exposed plants showed the most significant improvements in root (12.6±0.9 cm at 40%) and shoot length (39.1±2.9 cm at 40% ChNPs), maximum fresh (3.11±0.5 g at 30%) and dry biomass (0.62±0.09 g at 30% ChNPs), and enhanced viability (7.0±0.9%) at 30% ChNPs. Root growth and viability were diminished at lower concentrations (10% and 20%), whereas higher concentrations (50% and 60% ChNPs) preserved biomass accumulation. Overall, the data indicate that ChNPs at concentrations of 30% and 40% are optimal for promoting vegetative development and biomass accumulation in *Oryza sativa* pot-grown plants.

3.6. Physiological and Biochemical Parameters of ChNPs Potted Rice Plants

3.6.1 Protein Content

The pot-grown rice plants treated with ChNPs affects the protein content in plants as compared to the control (Fig. 8-A). The control showed the lowest protein content (0.011±0.003 mg) suggested that basal metabolic activity in untreated conditions of rice. In plants treated with 60% ChNPs a significant increase in protein content (0.027±0.003 mg).

Table 2. Morphological characters of *Oryza sativa* pot-grown plants treated with ChNPs

Treatment of ChNPs	Root Length (cm)	Shoot Length (cm)	Fresh Weight (g)	Dry Weight (g)	% Viability
Control	10.4±1b	27.2±1.5c	0.76±0.03c	0.15±0.003d	4.6±0.4d
10 %	4.5±0.2d	28.5±1.6c	2.3±0.6b	0.46±0.05b	5.0±0.6c
20 %	3.7±0.4d	23.5±0.9d	2.13±0.3b	0.42±0.04c	3.3±0.3e
30 %	9.1±0.6b	34.8±2.1b	3.11±0.5a	0.62±0.09a	7.0±0.9a
40 %	12.6±0.9a	39.1±2.9a	3.08±0.7a	0.61±0.06a	6.0±0.7b
50 %	12.5±0.7a	33.9±1.9b	2.43±0.4b	0.48±0.03b	5.0±0.6c
60 %	7.5±0.6c	31.4±1.7b	3.03±0.6a	0.60±0.08a	5.0±0.5c

Data are mean ± SE scored and followed by same letters are not significantly different at $p \leq 0.05$ level by Duncan's multiple range test (DMRT).

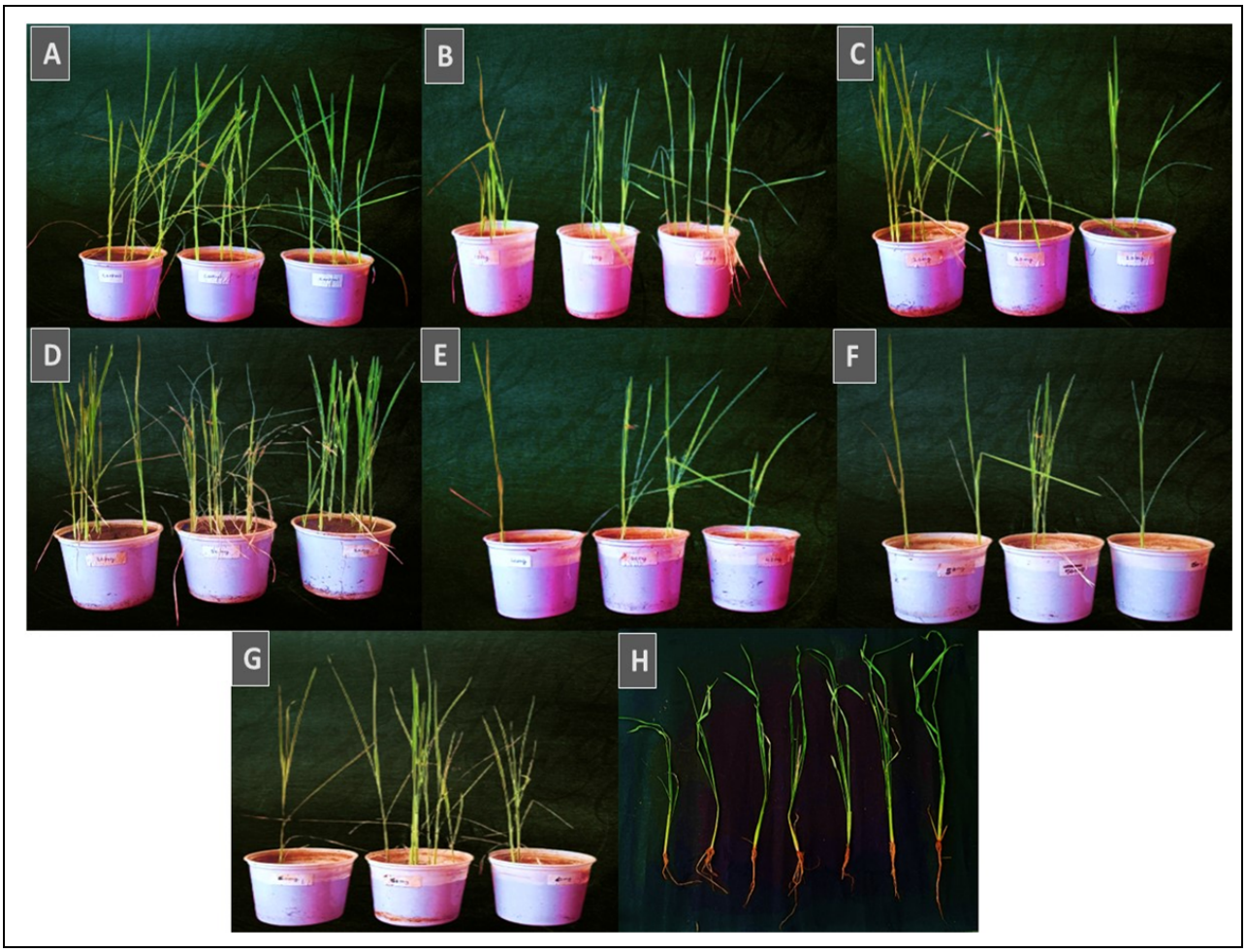


Fig. 7: Effect of ChNPs on pot-grown rice plants, (A–H) Typical photographs demonstrating the growth response of *Oryza sativa* plants under various treatments of ChNPs (0, 10, 20, 30, 40, 50, and 60%)

3.6.2 Proline Content

The proline content was estimated in control and ChNPs-treated pot-grown rice plants. The control plants showed a proline content (0.003±0.0008 mg of proline), indicating a significant decrease than ChNPs plants. The plants were performed best and increased proline content (0.012±0.0012 mg) at a concentration of 60% ChNPs treatment. The examined rice plants treated with 60% ChNPs showed a significant increase in proline content compared to the control.

3.6.3 Carotenoids Content

The rice plants treated with ChNPs (10, 20, 30, 40, 50, and 60%) showed a clear increase in carotenoids

content as compared to control (0.0%). The control plants were exhibited a carotenoid content of 8±1.2 mg which corresponds to the control pigment level. The rice plants treated with 10% ChNPs showed a marked increase in carotenoid content (20±1.5 mg) as compared to the control plants.

3.6.4 Chlorophyll Content

Rice plants treated with ChNPs (10, 20, 30, 40, 50, and 60%) exhibited a marked increase in total chlorophyll content when compared to the control group (0%). The control plants showed lower pigment levels such as chlorophyll-a (14±1.2 mg g^{-1} tissue), chlorophyll-b (16±1.4 mg g^{-1} tissue), and total chlorophyll (30±2.5 mg g^{-1} tissue) which reflect basal chlorophyll contents. Among all the treatments, the 10% ChNPs concentration elicited the best plant response, resulting in the significantly increased levels of chlorophyll, comprising chlorophyll-a (23±1.5 mg g^{-1} tissue), chlorophyll-b (36±2.0 mg g^{-1} tissue), and total chlorophyll (60±3.0 mg g^{-1} tissue). The substantial rise in chlorophyll pigments at the optimal concentration indicates that ChNPs effectively boost photosynthetic pigment synthesis and enhance photosynthetic efficiency when compared to the untreated controls.

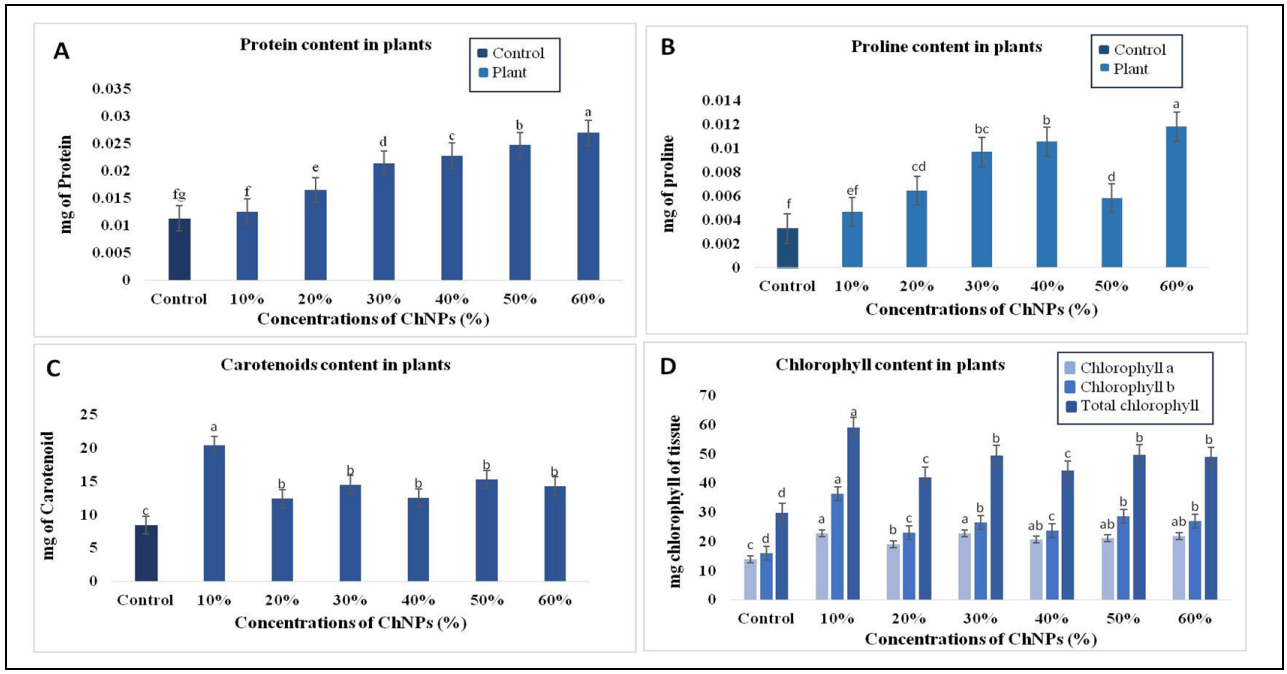


Fig. 8: Influence of ChNPs on physiological characteristics of plants, (A) Protein content, (B) Proline content, (C) Carotenoids content and (D) Total chlorophyll content

Data are mean ± SE scored and followed by same letters are not significantly different at $p \leq 0.05$ level by Duncan's multiple range test (DMRT).

3.7 Antioxidant Assays

3.7.1 Superoxide Dismutase (SOD)

Plants treated with ChNPs exhibited a marked activation of the antioxidant enzyme system, as evidenced by significant rises in SOD enzyme activities as compared to the control and corroborated by standard deviation values. Compared to the control, there was a distinct increase in the activities of SOD enzymes in plants treated with ChNPs. In (Fig. 9-A), the control plants showed a lower SOD activity (110±20 mM min^{-1} mg^{-1} protein) that reflected a basal superoxide scavenging effect. The most effective SOD activity (310 ± 30 mM min^{-1} mg^{-1} protein) in rice plants' response was noted at a concentration of 60% ChNPs. Such a trend indicates a vigorous activation of the antioxidant defense system associated with SOD enzyme. An enzyme like superoxide dismutase (SOD) converts harmful radicals into hydrogen peroxide, which is further detoxify with the use of ChNPs. Maintaining redox homeostasis and counteracting reactive oxygen species (ROS) was achieved with the treatment of ChNPs to rice.

3.7.2 Catalase (CAT)

Catalase activity was examined in ChNPs-treated plants of rice (Fig 9-B). Whereas the control plants were showed only minimal catalase activity (120±25 mM min^{-1} mg^{-1} protein). The plants showed significant CAT enzyme activity (650±43 and 680±50

mM min^{-1} mg^{-1} protein) at treatments of 50% and 60% ChNPs, respectively. The evident significant difference between control and treated plants, together with standard deviation ranges that are within acceptable limits, verifies a significant improvement in the detoxification of hydrogen peroxide. In comparison to the control, plants treated with ChNPs exhibited a marked activation of the CAT antioxidant enzyme system, as evidenced by significant rises in CAT enzyme activities. H_2O_2, a converted form of ROS, is further detoxified by an enzyme like catalase (CAT). Antioxidant defence in plants was achieved with the action mechanisms of reduction in malondialdehyde (MDA) levels, maintaining membrane stability and photosynthesis processes.

3.7.3 Peroxidase (POX)

The control plants showed low levels of Peroxidase (POD) activity (14±3 mM min^{-1} mg^{-1} protein) that suggested a limited basal oxidative defence system. The most effective POD enzyme response was noted in ChNPs exposed rice plants at a concentration of 60% ChNPs that exhibited the increased POD activity (42±4 mM min^{-1} mg-¹ protein) which implying an improved detoxification of reactive oxygen species (ROS) (Fig. 9-C). The antioxidant effect of ChNPs was measured by determining its ability to reduce cellular oxidative damage and minimize the induction of the antioxidant defense system.

3.7.4 Glutathione Peroxidase (GPX)

Glutathione peroxidase activity was assessed in ChNPs-exposed rice plants (Fig. 9-D). The control rice plants showed minimal GPX activity (0.03±0.006 mM min^{-1} mg^{-1} protein). The treatments of ChNPs (50% and 60%) to pot-grown rice plants showed a significant increase in response of GPX activity (0.08±0.01 mM min-¹ mg^{-1} protein) as compared to control. The induction of the glutathione-dependent antioxidant pathway is consistent as suggested by the treatments of 10% - 60% ChNPs.

3.7.5 Ascorbate Peroxidase (APX)

ChNPs-exposed rice plants exhibited a significant increase in APX activity (Fig 9-E). The control (0% ChNPs) plants showed lower APX activity (2.5±0.6 mM min^{-1} mg^{-1} protein), whereas the treatments of 50% and 60% ChNPs achieved significantly maximum APX activity (7.8±0.8 mM min^{-1} mg^{-1} protein) as compared to the control ones. The distinct separation of control and treated plants was coupled with standard deviation values that are within acceptable limits, which validates ChNPs enhance APX-associated enzyme antioxidant defences and improve plants' tolerance to oxidative stress. Key enzyme Ascorbate peroxidase (APX) converts harmful radicals into hydrogen peroxide (H_2O_2) at the tissue and cellular level.

3.7.6 DPPH Assay

The DPPH radical scavenging activity displayed a gradual increase among standard, plant extract, and ChNPs-exposed plant samples that signifies an enhancement in antioxidant potential in rice plants. The control (standard) showed the lowest DPPH scavenging activity (60.7±2.9 % inhibition) which indicates a baseline capacity for radical scavenging. The plants treated with 70 µg/ml ChNPs were showed steady increase in DPPH activity (77.9±1.6% inhibition) that resulted in superior free-radical scavenging efficiency. The significantly higher DPPH radical scavenging activity was recorded in rice plants-treated with ChNPs over control plants.

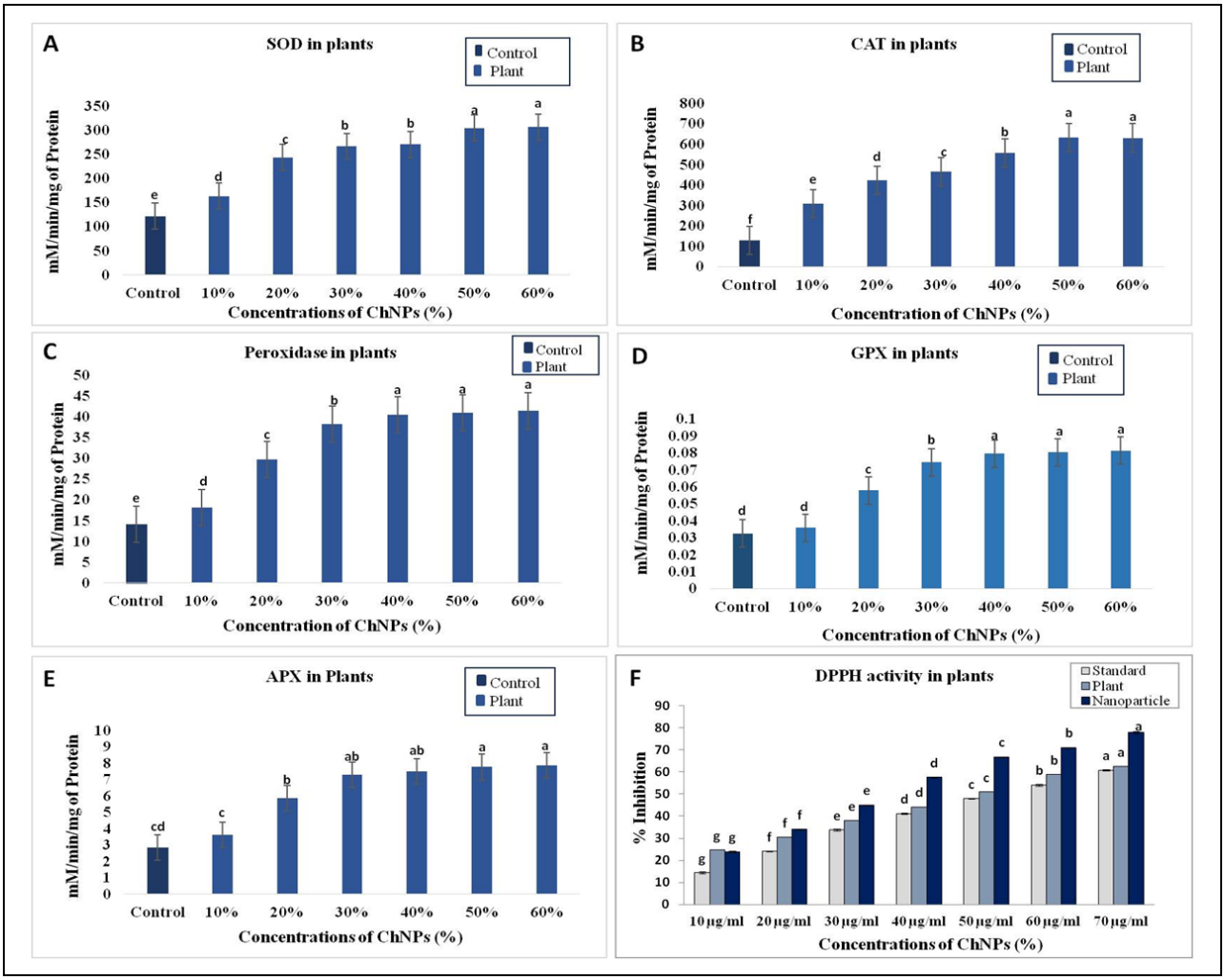


Fig. 9: Effect of ChNPs on antioxidant enzyme activities in *Oryza sativa* plants, (A–E) Activities of antioxidant enzymes measured at 10–60% of ChNPs in plant samples, (A) Superoxide dismutase activity, (B) Catalase activity, (C) Peroxidase activity, (D) Glutathione peroxidase activity, (E) Ascorbate peroxidase activity and (F) DPPH activity.

4. DISCUSSION

ChNPs were effectively produced through ionic gelation employing tripolyphosphate (TPP) as a cross-linking agent. The ionic gelation method consisted of dissolving chitosan in acetic acid, adding it drop-wise to TPP with magnetic stirring, then centrifuging and freeze-drying the mixture (Vaezifar *et al.* 2013). The characterization confirmed that the formation of ChNPs was achieved through SEM analysis showing spherical-shaped particles and a size measuring from 16–24 nm with slight aggregation; EDS identified the presence of C, O, and N, confirming the purity of the synthesized ChNPs. Similar studies were performed by Khanmohammadi *et al.* (2015). FTIR spectra exhibited characteristic functional groups of chitosan (Chakraborty *et al.* 2018); XRD revealed a semi-crystalline structure (Elhussieny *et al.* 2020), and UV–Visible analysis indicated a strong absorption peak at 250 nm that is consistent with the presence of ChNPs in the reaction mixture (El *et al.*, 2022). For biological application, stock solutions of ChNPs with different concentrations (10–60%) were created, sonicated, purified, and utilized for rice seed treatment. Before sowing, the seeds were disinfected with $HgCl_2$ and immersed in ChNPs solutions for 60 minutes each.

The systematic approach assured uniform exposure and facilitated the assessment of the impact of ChNPs nanoparticles on seed germination and growth of plants (Liu *et al.* 2022), the similar results were screened in the present study. The protein content was found to increase progressively in plants treated with the concentration of ChNPs (60%) in comparison to the control. This indicates improved nitrogen uptake and metabolic activity of plants. Guan *et al.* (2009) reported chitosan-based treatments have also resulted in comparable protein enhancement in Zea mays and Triticum aestivum. In the present study, the protein content showed an increase with higher concentrations of ChNPs. For instance, in Tomato, Mungbean, and Maize, a comparable increase in soluble protein was

recorded with chitosan promoting nitrogen metabolism and the production of defence proteins, which verifies that the ChNPs boosts metabolic activity in plants (El Amerany *et al.* 2020). The protein content, as illustrated in the current graph, increased steadily with higher concentrations of 60% ChNPs in comparison to the control. Likewise, in grapevine, the use of ChNPs increased protein accumulation compared to control plants. As a result, the two studies show that protein synthesis is stimulated and stress tolerance mechanisms are increased in plants as a result of treatment with NPs (Panahirad *et al.* 2025). Proline content have also been observed to increase after treatment of ChNPs in grapevines subjected to heavy metal stress and in drought-stressed Salvia abrotanoides, with the increase of proline content being associated with better osmotic adjustment and antioxidative defence mechanisms (Attaran *et al.* 2022). The increase in proline accumulation under ChNPs treatment suggests the induction of stress tolerance in plants. Similar results were noted in Ocimum ciliatum and Ocimum basilicum, where the use of chitosan led to a considerable increase in proline accumulation serving as an osmoprotectant. In our study, the rice plants performed best and increased proline content (0.012±0.0012 mg) at a concentration of 60% ChNPs treatment. This corroborates the function of ChNPs as stress elicitors (Pirbalouti *et al.* 2017). As the ChNPs concentration rises the carotenoids content depicted in the current study showed a marked increase compared to the control plants. This suggests improved carotenoid pigment production and superior antioxidant defence. Chitosan treatment has been shown to cause similar concentration-dependent increases in carotenoids in rice, maize, and tomato, confirming its contribution to the enhancement of photosynthetic pigment accumulation (Sangwan *et al.* 2023). Chlorophyll accumulation was significantly enhanced by seed priming with chitosan. Pigment enhancement was greater at optimal concentrations of ChNPs while excessive levels provided no additional benefit (Salachna and Zawadzińska, 2014), which corresponds with the current observation that higher concentrations, like 10% ChNPs showed maximum accumulation (30±2.5 mg g^{-1} tissue) of chlorophylls. At optimal concentrations, there was a significant increase in Chlorophyll a, b, and total chlorophyll. Similar results were observed in coffee plants treated with ChNPs, which exhibited enhanced photosynthetic efficiency. This trend is consistent with earlier studies on rice, maize, and tomato, in which chitosan treatment led to a significant increase in chlorophyll content. This improvement is usually ascribed to enhanced nitrogen metabolism, chloroplast development, and safeguarding of the photosynthetic system. Consequently, the trend shown in our study aligns with findings reported previously (Salachna and Zawadzinska, 2014).

Antioxidant enzymes like SOD, CAT, POX, GPX, and APX activities in rice seedlings treated with ChNPs imply an improved antioxidant potential during the initial stages of seedling development and in mature plants. Similar results were recorded in the study conducted by Alenazi *et al.* (2024). Particular antioxidant enzyme activities SOD, CAT, and POX in rice seedlings confirm that chitosan priming enhances after germination under stress condition which is consistent with a similar antioxidant enzyme activity pattern observed in the current study (Ling *et al.* 2022). As discussed in the results, activities of GPX, and APX were increased progressively in the plants treated with 60% ChNPs. Mulaudzi *et al.* (2022) reported that the increased enzyme levels in treated Sorghum seedlings suggest that ROS detoxification has been enhanced and antioxidant defence enhanced as a result of ChNPs treatment. GPX and APX exhibit a clear dose-dependent increase in activities compared to the control. In our study, the treatments of ChNPs (50% and 60%) to pot-grown rice plants showed a significant increase in response of GPX activity (0.08±0.01 mM min^{-1} mg^{-1} protein) as compared to control (0.0%). Because these enzymes are crucial for eliminating H_2O_2 and safeguarding cells against oxidative damage, and the increase in the antioxidant activity verifies that treating rice seedlings with ChNPs strengthens their antioxidant defence mechanisms (Champaneria *et al.* 2024). The graph showed that the concentration-dependent increase in DPPH activity was highest at the higher treatment level compared to the control rice seedlings and pot-grown plants. The control rice plants exhibited the lowest antioxidant effect. This indicates that ChNPs enhanced free radical scavenging activity in rice. Similar to the present study, a dose-dependent increase in DPPH activity been reported in *Anethum graveolens* treated with chitosan (Mashraqi, 2023). In *Solanum lycopersicon*, treatments with chitosan and metal-based NPs led to a notable increase in SOD activity compared to untreated controls, which indicates improved ROS scavenging and stress tolerance. In a comparable way, in Figure 9-A, the SOD activity is minimal in the control (110–130 mM/min/mg protein) and increases gradually (350 – 362 mM/min/mg protein) in rice seedlings treated with increasing ChNPs concentration (50 and 60%), respectively, which is nearly 2 to 2.5 times greater than that of the control. The activation of the antioxidant defence mechanisms by the treatments of ChNPs is evidenced by both findings (Quitadamo et al. 2021; Shelke *et al.* 2022). The distinct catalase (CAT) activity was recorded dose-dependent escalation, increasing from 120–150 mM/min/mg protein in the control to around 620–650 mM/min/mg protein at 50–60% ChNPs treatment. For example, chitosan treatments have also been found to enhance CAT levels in Hibiscus syriacus. The results corroborate the conclusion that using NPs enhances antioxidant defence through improved detoxification of ROS by enhanced catalase activity (Cao *et al.* 2025). Peroxidase (POX) is

a crucial antioxidant enzyme in plants that reduces hydrogen peroxide to detoxify reactive oxygen species and limit oxidative damage to plant tissues. ChNPs are serving as biotic elicitors and are recognized for boosting peroxidase activity as well as that of other defence enzymes. The rise in POX activity observed after chitosan treatment in stressed tobacco (*Nicotiana tabacum*) plants indicates the activation of systemic resistance and the reinforcement of the antioxidant defence mechanisms (Mukarram *et al*. 2023). Moreover, in tomato plants, chitosan and ChNPs formulations increased the expression and activity of peroxidase and similar oxidative stress enzymes, leading to improved ROS scavenging (Narasimhamurthy *et al.* 2022). Ascorbate peroxidase (APX) is a crucial enzyme in the ascorbate–glutathione cycle that neutralizes hydrogen peroxide (H_2O_2) and shields plant cells from oxidative damage during stress conditions. It is known that GPX activity in plants increases after elicitor treatments, including the use of ChNPs which activate antioxidant defence mechanisms during oxidative stress. The similar results on GPX activity in the present study were achieved with the treatment of ChNPs to rice plants at the seedling and plant levels. Research carried out on tomato plants has demonstrated that both chitosan and ChNPs boost GPX activity and other ROS-scavenging enzymes while increasing glutathione levels, thereby reinforcing the glutathione–ascorbate routes. The observed increase in GPX activity after treatment with ChNPs in the present study corresponds with these findings (Hernández-Hernández *et al.* 2018). The presence of APX in various cellular compartments facilitates an efficient scavenging of ROS and maintenance of cellular redox potential. Increased APX activity during abiotic stress demonstrates a stimulated antioxidant defense mechanism that is associated with plant stress tolerance and physiological stability (Li, 2023).

A recent study of Anethum graveolens extract encapsulated in ChNPs showed that DPPH radical scavenging activity increased with concentration, as higher inhibition percentages were noted with greater doses of NPs. This suggests that incorporating ChNPs improves antioxidant efficacy (Mashraqi, 2023). In the present investigation, the rice plants treated with 70 µg/ml ChNPs showed a steady increase in DPPH activity (77.9±1.6% inhibition) that resulted in superior free-radical scavenging efficiency. Aouadi *et al.* (2024) reported that the treatments of chitosan–ZnO and chitosan–ZnO/PVP nanocomposites increased the DPPH free radical scavenging efficiency, improving plants, resulting in inhibition rates that are higher and radical scavenging that is more effective than in the case of less complex NPs formulations. Hence, the current research exhibited the growth promotaion, physiological parameters, biochemical modulations, and antioxidant effects in rice crop at seedling and plant stages.

5. CONCLUSION

This study emphasizes the promise of ChNPs as a biologically sourced nanomaterial that possesses advantageous characteristics, namely, biodegradability, biocompatibility, and low toxicity, that render them appropriate for use in agriculture. The findings show that treatment with ChNPs had a beneficial effect on seed germination, early seedling development, and physiological performance in *Oryza sativa*, suggesting their involvement in the modulation of plant developmental processes. ChNPs application led to a greater accumulation of proteins and proline at the biochemical level, indicating enhanced metabolic activity and osmotic adjustment under experimental conditions. It is noted that a rise in chlorophyll and carotenoid levels indicates enhanced photosynthetic efficiency and photoprotective capacity. Moreover, the substantial increase in antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) suggests a bolstered antioxidant defence system that can reduce oxidative damage caused by reactive oxygen species (ROS) and preserve cellular redox balance. It is important to point out that the degree of these reactions was more marked during the seedling stage than in pot-grown mature plants. This implies that the effectiveness of ChNPs may depend on the developmental stage and physiological status. Moreover, the concentration-dependent effects noted in this study highlight the necessity of optimizing nanoparticle dosage to attain desired outcomes while avoiding inadvertent physiological stress. Further confirmation of the increased antioxidant potential in plants treated with ChNPs came from the DPPH radical scavenging assay. However, these results should be viewed as indicative rather than definitive proof of in vivo antioxidant effectiveness. The study offers useful insights into the physiological and biochemical responses of rice to ChNPs, but it is mainly restricted to controlled experimental conditions. A systematic evaluation of the long-term effects, field-level performance, persistence of nanoparticles, and potential ecological implications is still needed.

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AUTHOR CONTRIBUTIONS

MM performed sample collection, laboratory experiments, analysis, data interpretation, and wrote the first draft of the manuscript. HS and DS supervised the research work and contributed to the study design and critical revision of the manuscript. MR, SM and PS assisted in data interpretation and revision of the manuscript. All authors read and approved the final manuscript.

COMPETING INTEREST

All authors have no conflicts of interest.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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REFERENCES

Alenazi, M. M., El-Ebidy, A. M., El-shehaby, O. A., Seleiman, M. F., Aldhuwaib, K. J. and Abdel-Aziz, H. M. M., Chitosan and Chitosan Nanoparticles Differentially Alleviate Salinity Stress in *Phaseolus vulgaris* L. plants, *Plants*, 13(3), 398 (2024). https://doi.org/10.3390/plants13030398

Aouadi, A., Hamada, S. D., Rebiai, A., Achouri, A., Soulef, B., Fatma, M. A. M., Pohl, P., Ahmad, S. F., Attia, S. M., Hamada, A. S., Ararem, A. and Mohammed, M., Introducing the antibacterial and photocatalytic degradation potentials of biosynthesized chitosan, chitosan–ZnO, and chitosan–ZnO/PVP nanoparticles, *Sci. Rep.,* 14, 14753 (2024). https://doi.org/10.1038/s41598-024-65579-z

Aranaz, I., Alcántara, A. R., Civera, M. C., Arias, C., Elorza, B., Heras, C. A. and Acosta, N., Chitosan: An Overview of Its Properties and Applications, *Polym.,* 13(19), 3256 (2021). https://doi.org/10.3390/polym13193256

Attaran, D. S., Karimian, Z., Mostafaei, D. M. and Samiei, L., Chitosan nanoparticles improve physiological and biochemical responses of *Salvia abrotanoides* (Kar.) under drought stress, *BMC Plant Biol.,* 22(1), 364 (2022). https://doi.org/10.1186/s12870-022-03689-4

Baliyan, S., Mukherjee, R., Priyadarshini, A., Vibhuti, A., Gupta, A., Pandey, R. P. and Chang, C. M., Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of *Ficus religiosa*, *Mol.,* 27(4), 1326 (2022). https://doi.org/10.3390/molecules27041326

Bates, L. S., Waldren, R. P. and Teare, I. D., Rapid determination of free proline for water stress studies, *Plant Soil*, 39, 205–208 (1973). https://doi.org/10.1007/BF00018060

Boonlertnirun S., Boonraung, C. and Suvanasara, R., Application of Chitosan in Rice Production, *J. Met. Mater. Miner*., 18, 47–52 (2007).

Brunel, F., Vacron, L., David, L., Domard, A. and Delair, T., A Novel Synthesis of Chitosan Nanoparticles in Reverse Emulsion, *Langmuir*., 24(20), 11370–11377 (2008). https://doi.org/10.1021/la801917a

Cao, Y., Yan, R., Sun, M., Guo, J. and Zhang, S., Effects of exogenous chitosan concentrations on photosynthesis and functional physiological traits of hibiscus under salt stress, *BMC Plant Biol*., 25(1), 419 (2025). https://doi.org/10.1186/s12870-025-06424-x

Chakraborty, P., Mustafa, V. and Abraham, J., Synthesis and Characterization of Chitosan Nanoparticles and Their Application in Removal of Wastewater Contaminants, *Nat. Environ. Poll. Technol.*, 17(2), 469-478(2018).

Champaneria, P., Sonawane, H., Shelke, D., Chambhare, M., More, K., Math, S. and Zaware, B., Synthesis of *Inonotus rickii* metabolite-loaded chitosan nanoparticles and its biological activities, *Plant Nano Biol.,* 9, 10092 (2024). https://doi.org/10.1016/j.plana.2024.100092

Divya, K., Thampi, M., Vijayan, S., Shabanamol, S., and Jisha, M. S. Chitosan nanoparticles as a rice growth promoter: evaluation of biological activity, *Archiv. Microbio*., 204(1), 95 (2022). https://doi.org/10.1007/s00203-021-02669-w

El Amerany, F., Meddich, A., Wahbi, S., Porzel, A., Taourirte, M., Rhazi, M. and Hause, B., Foliar Application of Chitosan Increases Tomato Growth and Influences Mycorrhization and Expression of Endochitinase-Encoding Genes, *Int. J. Mol. Sci.,* 21(2), 535 (2020). https://doi.org/10.3390/ijms21020535

El, N., El, A., Shiha, A. M., Mahrous, H. and Mohammed, A. B. A., Green synthesis of chitosan characterization and antibacterial efficacy against multi drug resistant biofilm - forming Acinetobacter baumannii, *Sci. Rep*., 12(1), 19869 (2022). https://doi.org/10.1038/s41598-022-24303-5

Elhussieny, A., Faisal, M., Everitt, N. M., Fahim, I. S., Avenue, N. and Avenue, N., Experimental Investigation of Chitosan Film Reinforced by Chitin Fibers and Chitin Whiskers Extracted from Shrimp Shell Waste, *J. Engg. Sci. Technol*., 15(4), 2730–2745 (2020).

Guan, Y., Hu, J., Wang, X. and Shao, C., Seed priming with chitosan improves maize germination and seedling growth in relation to physiological changes under low temperature stress, *J. Zhejiang University–Sci. B,* 10(6), 427–433 (2009). https://doi.org/10.1631/jzus.B0820373

Hernández-Hernández, H., González-Morales, S., Benavides-Mendoza, A., Ortega-Ortiz, H., Cadenas-Pliego, G. and Juárez-Maldonado, A., Effects of Chitosan–PVA and Cu Nanoparticles on the Growth and Antioxidant Capacity of Tomato under Saline Stress, *Mol.,* 23(1), 178 (2018). https://doi.org/10.3390/molecules23010178

Huang, H. and Yang, X., Synthesis of chitosan-stabilized gold nanoparticles in the absence/presence of tripolyphosphate, *Biomacromol*., 5(6), 2340–2346 (2004). https://doi.org/10.1021/bm0497116

Jha, R. and Mayanovic, R. A., A Review of the Preparation, Characterization, and Applications of Chitosan Nanoparticles in Nanomedicine, *Nanomater.,* 13(8), 1302 (2023). https://doi.org/10.3390/nano13081302

Khanmohammadi, M., Elmizadeh, H. and Ghasemi, K., Investigation of Size and Morphology of Chitosan Nanoparticles Used in Drug Delivery System Employing Chemometric Technique, *Iran. J. Pharma. Res*., 14(3), 665–675 (2015).

Krishna, S. P., Amareshwar, P. and Chakravarty, P., Chitosan nanoparticles as a drug delivery system, *Res. J. Pharm. Biol. Chem. Sci*., 1(3), 474–484 (2010).

Laxmi, S., Chambhare, M. R., Kadlag, S., Ahire, M. L. and Nikam, T. D., Herbicide effects on pigments and antioxidant enzymes of *Portulaca oleracea* L., *J. Pharmac. Phytochem*., 7(1), 2024-2031 (2018).

Ling, Y., Zhao, Y., Cheng, B., Tan, M., Zhang, Y. and Li, Z., Seed Priming with Chitosan Improves Germination Characteristics Associated with Alterations in Antioxidant Defense and Dehydration-Responsive Pathway in White Clover, *Plants,* 11(15), 2015 (2022). https://doi.org/10.3390/plants11152015

Liu, H., Zheng, Z., Han, X., Zhang, C., Li, H. and Wu, M., Chitosan Soaking Improves Seed Germination of Platycodon Grandiflorus and Enhances Its Growth, Photosynthesis, Resistance, Yield, and Quality, *Horticult*., 8(10), 943 (2022). https://doi.org/10.3390/horticulturae8100943

Lowry, O. H., Rosenbrough, N. J., Far, A. L. and Randall, R. J., Protein measurement with the Folin-phenol reagent, *J. Biol. Chem.*, 193, 265–275 (1951).

Mashraqi, A., Induction role of chitosan nanoparticles to Anethum graveolens extract against food-borne bacteria, oxidant, and diabetic activities in vitro, *Front. Microbiol*., 14, 1209524 (2023). https://doi.org/10.3389/fmicb.2023.1209524

Mashraqi, A., Induction role of chitosan nanoparticles to Anethum graveolens extract against food-borne bacteria, oxidant, and diabetic activities in vitro, *Front. Microbiol*., 14, 1209524 (2023). https://doi.org/10.3389/fmicb.2023.1209524

Mukarram, M., Ali, J., Dadkhah-Aghdash, H., Kurjak, D., Kačík, F. and Ďurkovič, J., Chitosan-induced biotic stress tolerance and crosstalk with phytohormones, antioxidants, and other signalling molecules, *Front. Plant Sci.,* 14, 1217822 (2023). https://doi.org/10.3389/fpls.2023.1217822

Mulaudzi, T., Nkuna, M., Sias, G., Doumbia, I. Z. and Njomo, N., Antioxidant Capacity of Chitosan on Sorghum Plants under Salinity Stress, *Agricult.*, 12(10), 15441 (2022). https://doi.org/10.3390/agriculture12101544

Narasimhamurthy, K., Udayashankar, A. C., De Britto, S., Lavanya, S. N., Mostafa, A., Soumya, K., Shekar, H. S., Srinivas, C. and Jogaiah, S., Chitosan and chitosan-derived nanoparticles modulate enhanced immune response in tomato against bacterial wilt disease, *Int. J. Biol. Macromol*., 220, 223-237 (2022). https://doi.org/10.1016/j.ijbiomac.2022.08.054

Nguyen, V. S., Dinh, M. H. and Nguyen, A. D., Study on chitosan nanoparticles on biophysical characteristics and growth of Robusta coffee in green house, *Biocatal. Agric. Biotechnol*., 2(4), 289–294 (2013). https://doi.org/10.1016/j.bcab.2013.06.001

Panahirad, S., Dadpour, M., Kulak, M., Vita, F. and Gohari, G., Selenium-coated chitosan nanoparticles (CTS- Se NPs) improve grapevine (*Vitis vinifera* cv. Sultana) performance grown under lead (Pb) toxicity, *BMC Plant Biol*., 25(1), 1178 (2025). https://doi.org/10.1186/s12870-025-07192-4

Pirbalouti, A. G., Malekpoor, F., Salimi, A. and Golparvar, A., Exogenous application of chitosan on biochemical and physiological characteristics, phenolic content and antioxidant activity of two species of basil (*Ocimum ciliatum* and *Ocimum basilicum*) under reduced irrigation, *Scientia Horticult*., 217, 114–122 (2017). https://doi.org/10.1016/j.scienta.2017.01.031

Porra, R. J. and Scheer, H., Towards a more accurate future for chlorophyll a and b determinations: the inaccuracies of Daniel Arnon's assay, *Photosyn. Res.*, 140(2), 215-219 (2019). https://doi.org/10.1007/s11120-018-0579-8

Quitadamo, F., De Simone, V., Beleggia, R., Trono, D., Chitosan-Induced Activation of the Antioxidant Defense System Counteracts the Adverse Effects of Salinity in Durum Wheat. Plants (Basel), 3, 10(7), 1365 (2021). https://doi.org/10.3390/plants10071365.

Sadasivam, S. and Manickam, A., Biochemical Methods, New Age Int. Ltd, New Delhi, 2, 124-126 (1996).

Saharan, V., Kumaraswamy, R. V., Choudhary, R.C., Kumari, S., Pal, A., Raliya, R. and Biswas, P., Cu-Chitosan Nanoparticle Mediated Sustainable Approach to Enhance Seedling Growth in Maize by Mobilizing Reserved Food, *J. Agric. Food Chem*., 64(31), 6148–6155 (2016). https://doi.org/10.1021/acs.jafc.6b02239

Salachna, P. and Zawadzińska, A., Effect of Chitosan on Plant Growth, Flowering and Corms Yield of Potted Freesia, *J. Ecol. Engg*., 15(3), 97–102 (2014). https://doi.org/10.12911/22998993.1110223

Sangwan, S., Sharma, P., Wati, L. and Mehta, S., Effect of chitosan nanoparticles on growth and physiology of crop plants, *Elsevier,* 99–123 (2023). https://doi.org/10.1016/B978-0-323-91933-3.00011-8

Sarker, U. and Oba, S., Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of Amaranthus tricolor, *Sci. Rep.*, 8(1), 16496 (2018). https://doi.org/10.1038/s41598-018-34944-0

Shelke, H., Gupta, S., Mahesh, N., Ram, P., Anand, K., Deepak, S., Mahadev, C. and Hiralal, S., Mycosynthesis of Nanoparticles and Their Potential Application in Pharmaceutical Bioprocessing, *Nanotech. Life Sci.,* 425–442 (2022). https://doi.org/10.1007/978-3-030-82918-6_17

Sonawane, H., Shelke, D., Chambhare, M., Dixit, N., Math, S., Sen, S., Borah, S. N., Islam, N. F., Joshi, S. J., Yousaf, B., Rinklebe, J. and Sarma, H., Fungi-derived agriculturally important nanoparticles and their application in crop stress management - Prospects and environmental risks, *Env. Res.,* 212, 113543 (2022). https://doi.org/10.1016/j.envres.2022.113543

Tiyaboonchai, W., Chitosan nanoparticles: a promising system for drug delivery, *Asian Hea. Sci. Technol. Rep.,* 11(3), 51-66 (2003).

Vaezifar, S., Razavi, S., Golozar, M. A., Karbasi, S., Morshed, M. and Kamali, M., Effects of Some Parameters on Particle Size Distribution of Chitosan Nanoparticles Prepared by Ionic Gelation Method, *J. Cluster Sci.*, 24(3), 891–903 (2013). https://doi.org/10.1007/s10876-013-0583-2

Wu, L., Jiang, Q., Zhang, Y., Du, M., Ma, L. and Ma, Y., Peroxidase Activity in Tomato Leaf Cells under Salt Stress Based on Micro-Hyperspectral Imaging Technique, *Horticulturae,* 8(9), 813 (2022). https://doi.org/10.3390/horticulturae8090813

Yi, Q., Jiapaer, G., Chen, J., Bao, A. and Wang, F., Different units of measurement of carotenoids estimation in cotton using hyperspectral indices and partial least square regression, *ISPRS J. Photogram. Remote Sens*., 91, 72-84 (2014). https://doi.org/10.1016/j.isprsjprs.2014.01.004