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Chitosan Nanoparticles as Seed Priming Agents to Alleviate Salinity Stress in Rice (*Oryza sativa* L.) Seedlings

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Abstract: Nanoparticle-based seed priming has opened new avenues in crop science due to their plant growth promoting potential. Similarly, biopolymers such as chitosan (CS) are widely studied as seed priming agents due to the biodegradable and biocompatible nature, ability to enhance germination percentage and overall seedling health. Therefore, priming with chitosan nanoparticles (CNPs) is a promising tool to enhance overall plant health. Here, we studied the effect of nanopriming with CNPs or CS (50 µg/mL) on morphological, physiological, and biochemical parameters of rice seedlings, grown in salinity stress conditions NaCl (0–250 mM). CNPs were synthesized using an ionic gelation method and characterized by scanning electron microscopy (50–100 nm), zeta potential analyser (Particle size distribution–373.5 ± 3.7 nm; polydispersity index- > 0.4; zeta potential–45.3 ± 2.5 mV) and profilometry (300–1500 nm hydrodynamic height). Morphological, physiological, and biochemical responses of rice seedlings grown from seeds primed with either CNPs or CS showed a positive effect on germination, seedling vigour, biochemical and antioxidant responses. Seeds primed with CNPs and CS demonstrated significantly higher germination potential and seedling vigour compared to control hydro-primed seeds when grown under increasing NaCl concentrations. These outcomes highlight that CNPs and CS can be used as potential seed priming agents to alleviate salinity stress in rice seedlings. However, further studies are warranted to understand the effect of CNPs and CS seed priming on the overall growth and development of rice plants as well as rice yield.

Keywords: chitosan; chitosan nanoparticles; nanopriming; NaCl; salt stress alleviation

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1. Introduction

Rice (*Oryza sativa* L.) is the third most significant grain staple, serving more than half of the world's population daily, with a yearly production of more than 514 million metric tons in 2020/2021 [1]. It is rich in carbohydrates and includes small amounts of proteins, fat and vitamin B complexes such as riboflavin, niacin and thiamine [2]. Recent predictions suggest that future climatic alterations will have a significant impact on rice cultivation [3]. Dynamic and stressful abiotic environmental conditions such as salt, cold, drought, heavy metals and organic pollutants largely resulting from anthropogenic activities negatively influence the germination of rice seeds, plant growth, nutritive content and subsequent productivity [4,5]. Of these, soil and water salinity are among the major bottlenecks to achieving high quality and yield in rice in most parts of the world.

Saline water (above 0.6 M NaCl) that runs into rice paddy fields increases the salinity of the soil [6]. Irrigation with salty groundwater and the release of seawater onto coastal agricultural areas contributes to the accumulation of salts in arid/semiarid regions due to ion leaching. This adverse effect of soil salinity on rice cultivation results in reduced seed germination [7], generation of reactive oxygen species (ROS) [8], physiological and biochemical alterations [9] and modifications to the transcriptome [10]. Various stress

alleviators are being employed to reduce the salinity stress or boost plants' immunity against biotic and abiotic stresses, particularly salinity [11]. Amongst these alleviators, nanomaterials constitute a new class that can provide immunity against various biotic and abiotic factors by priming plants' immune responses [12,13]. Moreover, unlike conventional fertilizers, the fundamental economic benefit of using biodegradable nanomaterials are low cost and application in small quantities [14].

Nanoparticle-based fertilizers are emerging as novel solutions to enhance plant growth, render climate resilience and induce pathogen resistance in plants [1,15]. Similarly, seed priming with nanoparticles is an innovative strategy to enhance germination and seedling vigour [16]. The impact of nanoparticles as fertilizers or seed priming agents on the physiological and molecular parameters of rice has been extensively reviewed earlier [1,17]. However, to date, there has been a lack of exploration of polymeric nanoparticles as seed priming agents, particularly biopolymeric forms such as chitosan nanoparticles (CNPs), which are yet to be explored in rice cultivation [18]. CNPs are biocompatible and biodegradable, which makes them a sustainable and desirable option for basic and advanced agricultural applications [19,20]. In this regard, nanopriming with CNPs would be an interesting alternative to prevent undesirable outcomes such as phytotoxicity associated with other nanomaterial types.

Chitosan (CS) is processed from chitin, which is the second most abundant biopolymer on earth after cellulose. Chitosan is well-known for its biodegradability, biocompatibility and non-toxic properties, and is available in various forms, including powders, liquid solutions and beads [21]. More recently, CS has been used in the fabrication of hydrogels [22], drug-carriers [23] and the synthesis of biodegradable CNPs [20]. Owing to their natural origin and amenability to fabrication, CS and CNPs are extensively studied for applications in agriculture; in particular, CNPs have been used to deliver fertilizers, hormones and nutrients to plants [24].

Apart from a few reports, CNPs have been rarely explored as nanopriming agents. Li et al. [25] tested CNPs as seed priming agents to enhance germination and seedling vigour in *Triticum aestivum* L. Furthermore, Divya et al. [26] showed that brief treatment of rice seeds with CNPs elicits increased germination capacity and promotes growth. However, CNPs are not being explored as nanopriming agents to alleviate salinity stress in rice seedlings. Therefore, in the present study, we aim to explore the potential of CS and CNPs as seed priming agents to alleviate salinity stress in rice seedlings by analysing their physiological, biochemical and antioxidant responses. This is the first report that investigates the salt stress alleviating role of CNPs on rice seedlings that are germinated at varying salt concentrations.

2. Materials and Methods

2.1. Plant Material and Chemicals

The rice seeds (*Oryza sativa* L., cv. Calrose–brown rice) were purchased from Sunrice, Ricegrowers, Ltd., Leeton, New South Wales, Australia. The chitosan and trisodium polyphosphate (TPP) used for nano-chitosan (CNPs) synthesis were procured from Sigma Aldrich, Castle Hill, NSW, Australia.

2.2. Preparation and Characterization of CNPs

The CNPs were prepared as described previously [20]. Briefly, CS solution (0.5% w/v) was prepared by dissolving CS 0.5 (mg/mL) in aqueous acetic acid (0.5% v/v) and the mixture was stirred overnight on magnetic stirrer adjusted to 250 rpm at 25 ± 2 °C. For the synthesis of CNPs, 3 mL TPP (0.25% w/v) was added dropwise to the 15 mL CS solution kept on magnetic stirrer for 30 min adjusted to 700 rpm at 25 ± 2 °C. The mixture was centrifuged at 11,337× g for 20 min and the pelleted CNPs were further used for physiochemical characterization, which involved scanning electron microscopy (SEM), dynamic light scattering (DLS) and profilometry. Surface morphology of nanoparticles was visualized using scanning electron microscopy (SEM; JEOL 7800F, Tokyo, Japan) as

described earlier [20]. The average hydrodynamic size, zeta potential and polydispersity index of CNPs (50 µg/mL) dissolved in MilliQ water were determined using dynamic light scattering (Malvern Zetasizer Nano ZS, Cambridge, UK) at 25 °C. Profilometry was performed by drop-casting CNPs on a glass slide and subsequent visualization under profilometer (3D laser confocal microscope, LEXT OLS4100 Olympus, Tokyo, Japan).

2.3. Sterilization, Seed Priming and Growth Conditions

The rice seeds were washed twice with distilled water. Then washed with Tween 80 (1% v/v) for 5 min, followed by repeated rinses with distilled water. Later, the seeds were washed with ethanol (70% v/v) for 2 min. The seeds were then sterilized with sodium hypochlorite (0.4% v/v) for 2 h. The seed priming was performed as described by Li, He, Xie, Wang, Bose, Sun, Hu and Yin [25]. Then, the seeds were soaked in sterile distilled water (control), and water supplemented with CS and CNPs at a concentration of 50 µg/mL, which was chosen based on previous reports [25]. After 24 h, the seeds were washed with autoclaved distilled water under sterile conditions. The seeds were blotted on sterile tissue paper and the those with intact embryos were transferred to the Petri plates (10 cm) containing N6D media (Phytotechnology laboratories LLC., Lenexa, KS, USA) with agar and varying concentrations of NaCl (0, 50, 100, 150, 200, 250 mM). The Petri plates were then incubated in a growth chamber adjusted at 27 ± 1 °C under dark conditions. After 7 days, the seedlings were used to measure physiological indexes and biochemical parameters as described in subsequent sections.

2.4. Measurement of Physiological Indexes

Physiological indexes such as germination percentage, root/shoot length and ratio, fresh weight and seedling vigour were calculated by measuring length and weight of root and shoot, as well as calculating the root/shoot ratio compared to plant fresh weight, respectively [25].

2.5. Enzyme Assays

Fresh plant (root/shoot) samples (500 mg) were homogenized manually in chilled mortar at 4 °C in 1.5 mL of cold 50 mM sodium phosphate buffer (pH 7.8) with 0.1 mM EDTA. The mixture was then centrifuged at 11,269× g for 20 min at 4 °C, and the resulting supernatant (enzyme solution) was used for enzyme assays.

2.5.1. Catalase

Catalase activity was determined by reduction in the absorbance at 240 nm for 5 min, following the decomposition of H_2O_2 [27]. The 3 mL reaction mixture consisted of 2.98 mL of 50 mM sodium phosphate buffer (pH 7.8) and 20 µL of enzyme extract. The reaction was started by adding 2.5 µL of 30% H_2O_2 [28,29]. The molar extinction coefficient of H_2O_2 ($3.6 \times 10\ M^{-1}cm^{-1}$) was used to calculate enzyme activity in terms of mM/min/mg of protein.

2.5.2. Peroxidase

The POD activity was determined as an increase in absorbance at 470 nm for 2 min. The 3 mL reaction mixture contained 2.47 mL of 50 mM sodium phosphate buffer (pH 7.8), 20 µL of enzyme extract and 500 µL of 30 mM guaiacol [30]. The reaction was started by adding 5 µL of 30% H_2O_2.

2.5.3. Superoxide Dismutase

SOD activity is measured by its ability to prevent the photochemical reduction of nitro blue tetrazolium (NBT) [31]. The 3 mL reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.8), 5 mM EDTA (pH 7.5), 130 mM Methionine, 7.5 mM NBT and 100 µL enzyme extract. The reaction was started by adding 2 mM riboflavin [32], and the absorbance was measured at 560 nm after 15 min of incubation. One unit of SOD activity

was defined as the amount of enzyme required for 50% inhibition of the photochemical reduction of NBT and was expressed as mM/min/mg of protein.

2.6. Estimation of Protein and Carbohydrate Content

For the estimation of protein content, 200 mg of tissue sample was macerated using a mortar and pestle in 2 mL of phosphate buffer (0.1 M, pH 7.2), with the homogenate centrifuged at 11,337× *g* for 15 min at 4 °C. The supernatant was used to determine protein content by following the Bradford [33] method. Bovine serum albumin was used a protein standard.

For the estimation of total reducing sugar content, Miller's method involving DNSA reagent was followed [34]. To 1 mL of seedling (200 mg) extract macerated using a mortar and pestle in water, 1 mL of dinitrosalicylic acid (DNSA (1 g DNSA dissolved in 20 mL NaOH with the help of a magnetic stirrer)) reagent was added. The solution was incubated in water bath for 5 min. When deep reddish yellow colour developed, 1 mL of 40% Rochelle salt (sodium potassium tartrate [30 g of sodium potassium tartrate dissolved in 50 mL dH_2O)] solution was added. The absorbance was measured at 510 nm and to estimate the total reducing sugar, a glucose standard curve was used.

2.7. Chlorophyll and Carotenoid Estimation

The chlorophyll and carotenoid contents in rice seedlings were analysed according to the method descried by Salah et al. [35] Briefly, 0.1 g shoot tissue was macerated in 2 mL of ethanol (95% *v*/*v*). The homogenate was centrifuged at 704× *g* for 20 min. The absorbance of the supernatant was measured at 665 nm (A_{665}) and 649 nm (A_{649}). Chlorophyll content was calculated as

$$Chl\ (a) = 13.95 \times Abs.665 - 6.88 \times Abs.649$$

$$Chl(b) = 24.95 \times Abs.665 - 7.83 \times Abs.649$$

$$Chl(a+b) = Chl(a) + Chl(b)$$

$$Chl\ content\left(\frac{\text{mg}}{\text{g}} fresh\ weight\right) = \frac{Chl(a+b) \times Extraction\ liquid\ volume \times Dilution}{Fresh\ weight(\text{g})}$$

$$Carotenoids = \frac{1000 \times Abs.470 - 2.05 \times Chla - 104 \times Chlb}{245}$$

2.8. Statistical Analysis

Each experiment was replicated thrice (three biological replicates) and data were expressed in terms of means ± standard deviation (SD). The significance of difference between control and elicitation treatments was obtained through a one-way ANOVA using Duncan's multiple range test (DMRT) in IBM SPSS statistics 27 (version 1.0). Significance was considered when $p \leq 0.05$.

3. Results and Discussion

3.1. Preparation and Characterization of CNPs

The CNPs were prepared using the ionic gelation method [20]. The -NH_2 groups of CS are protonated under acidic conditions which results in the formation of -NH_3^+ groups. The NH_2 groups on CS undergo protonation in acidic medium [25]. Negatively charged phosphate groups of TPP become electrostatically attracted to the NH_3^+ groups on CS to produce ionically crosslinked CNPs. The morphology and particle size using SEM showed spherical CNPs which were in the size range of 50 to 100 nm (Figure 1A). Profilometry revealed that the hydrodynamic height of CNPs was between 300 and 1500 nm (Figure 1B). The particle size distribution, polydispersity index and zeta potential values of CNPs were 373.5 ± 3.7 nm, >0.4 and 45.3 ± 2.5 mV, respectively (Figure 1C). The particle size variation in SEM, profilometry and DLS is due to the principle behind these techniques, as SEM needs the sample to be in dry state, whereas profilometry and DLS measure the particles when they are in their hydrodynamic state.

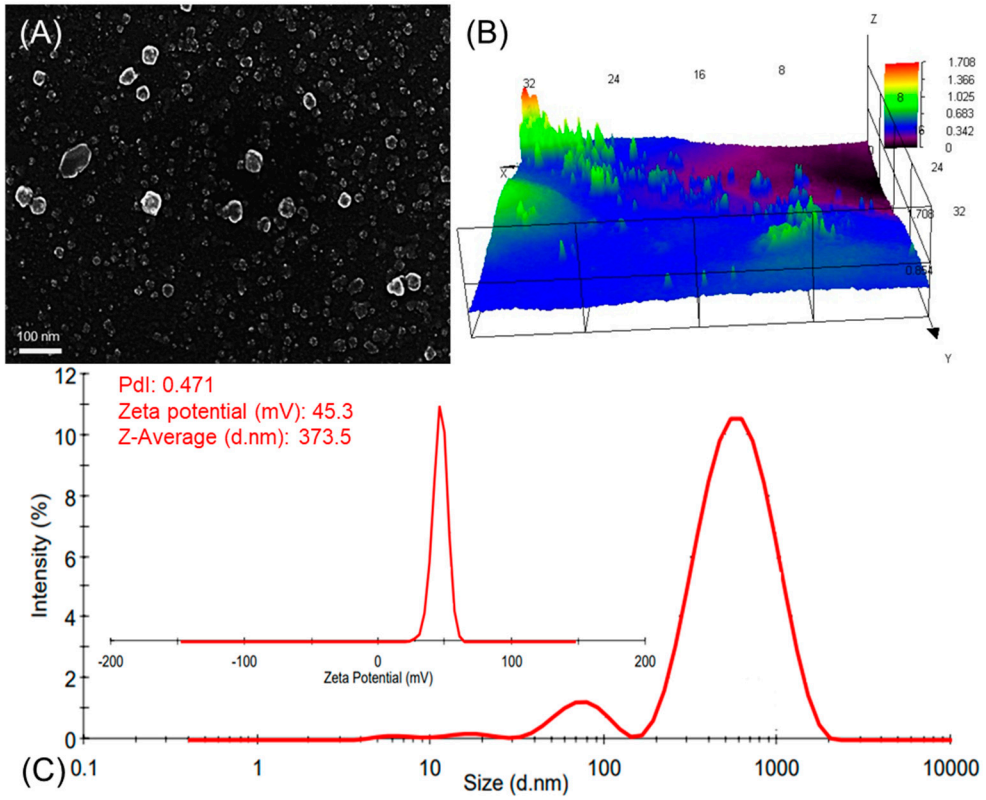

Figure 1. Morphological details of CNPs: (**A**) Scanning electron micrograph (50-100 nm). (**B**) Profilometry mapping revealing hydrodynamic height of CNPs (300-1500 nm). (**C**) Polydispersity index with zeta measurements.

CNPs are becoming widely explored in the field of agriculture and are also studied as plant hormone/nutrient carriers [36]. In this study, CNPs were synthesized by the ionotropic gelation method as it is very economic, simple and requires less equipment and time. The synthesized CNPs were found to be monodispersed as the PDI ranged between 0.3 to 0.4 (with a value of >0.7 indicating heterogenicity of nanoparticles). As the PDI of CNPs prepared was less than 0.7, this indicates there is minimal chance agglomeration of CNPs, rendering them suitable for agricultural applications [36]. The significance of zeta above 30 mV indicates an increased state of mutual repulsion of the CNPs in the medium, suggesting good stability during storage. Analysis of shelf-life and elicitation capacity of CNPs on rice seeds was examined by Divya, Vijayan, Nair and Jisha [26] who reported their effectiveness for a period of seven months, which can be attributed to a high zeta potential (>+30 mV or <−30 mV) and low PDI (<0.7). Higher zeta potential and lower PDI values are often used as indicators of good colloidal stability and monodispersion of nanoparticles in a solution.

3.2. Effect of Priming on Germination of Seed and Seedling Vigour

In our study, we analysed the effect of CS and CNPs priming on the germination and physiological indexes of rice grown under different salt concentration regimes (0–250 mM and was compared with control hydropriming). After the beginning of the experiment, seed germination was observed daily up to the 7th day. Seeds were considered germinated after radical formation of a minimum of 2 mm. Both CNPs and CS priming were found to enhance overall germination percentage compared to control hydropriming (Figure 2A). The CS priming showed significantly higher germination potential compared to CNPs priming at 150 mM NaCl concentration. Whereas, the CNPs priming showed significantly higher germination potential compared to CS priming at 200 mM NaCl concentration. Under salinity stress (50 to 200 mM NaCl), the seedling vigour of CNPs and CS primed

seeds significantly increased compared to control hydro-primed seeds (Figure 2B). At higher NaCl concentration (200 mM) the vigour index of CNPs treated seeds is significantly higher than the CS treated seeds. It is also worth noting that CS priming also improved performance of seeds grown without salt stress. Figure 2C represents the experimental data of rice germination post-priming under different NaCl concentrations.

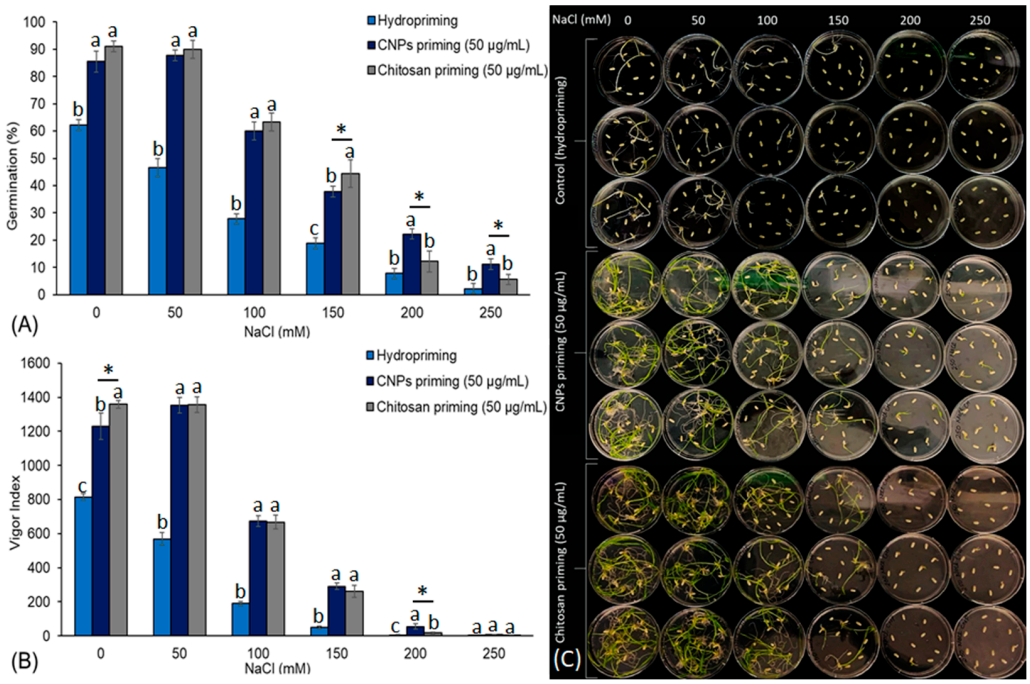

Figure 2. Effect of chitosan nanoparticles (CNPs) and chitosan (CS) priming on (**A**) germination percentage, and (**B**) seedling vigour index of rice seedlings grown under salinity stress. (**C**) Representative photographs of rice germination post-priming under different NaCl concentrations. Different letters indicate statistical significance between control and priming treatments, and "*" shows a significant difference between treatments, i.e., (CNPs and CS), which was obtained through a one-way ANOVA using Duncan's multiple range test (DMRT) in IBM SPSS statistics 27 (version 1.0). Significance was considered when $p \leq 0.05$.

3.3. Chlorophyll and Carotenoid Content

High salinity levels have detrimental effects on photosynthetic activity in plants. The chlorophyll a, chlorophyll b and total chlorophyll levels showed a gradual decrease with increasing salinity stress in control hydropriming groups. A similar trend was recorded in CNPs and CS priming sets (note: due to insufficient tissue, data could not be obtained for seedlings grown under 200 and 250 mM NaCl concentration). In the case of chlorophyll a (Figure 3A), no significant difference was recorded between CNPs- and CS-primed seedlings under no and 50 mM NaCl concentrations, whereas a significant difference was observed between them at 100 and 150 mM NaCl concentrations. For chlorophyll b (Figure 3B), CS priming showed significant increase compared to CNPs priming under no NaCl concentration, whereas the trend reversed under salinity stress, i.e., chlorophyll b content was significantly higher in CNPs-primed seedlings compared to those primed with CS. Whereas, for total chlorophyll content (Figure 3C), a significant difference was observed for no salt and 50 mM NaCl concentration, while for 100 and 150 mM no significant difference was recorded.

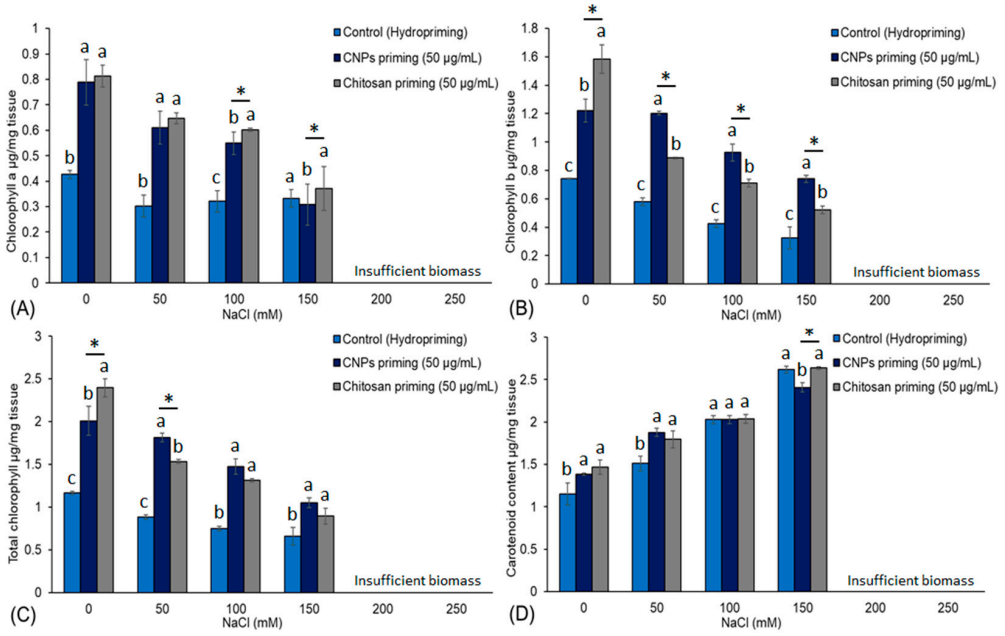

Figure 3. Effect of chitosan nanoparticles (CNPs) and chitosan (CS) priming on (**A**) chlorophyll a, (**B**) chlorophyll b, (**C**) total chlorophyll and (**D**) carotenoid content of rice seedlings grown under salinity stress. Different letters indicate statistical significance between control and priming treatments, and "*" shows a significant difference between treatments; i.e., (CNPs and CS), which was obtained through a one-way ANOVA using Duncan's multiple range test (DMRT) in IBM SPSS statistics 27 (version 1.0). Significance was considered when $p \leq 0.05$.

The carotenoid content in the seedlings showed a significant increase (Figure 3D), i.e., both CNPs- and CS-primed seedlings showed significant increase in carotenoid content compared to control hydro-primed seedlings without NaCl and at 50 mM NaCl. However, at 100 mM NaCl the carotenoid content was approximately similar in all CNPs, CS and control hydropriming, whereas, at 150 mM NaCl a significant decline in carotenoid content was observed in CNPs-primed seedlings compared to CS and control hydro-primed seedlings.

The measurement of the photosynthetic pigment chlorophyll is a cost-efficient and quick technique that can be useful in evaluating the fitness of plants, especially under stressful conditions. Plant growth is intimately related with photosynthetic pigments and rate of photosynthesis [37]. Under higher salt concentrations, the accumulation of Na^+ and Cl^- ions increased, which hinders the process of chlorophyll synthesis by influencing the activity of some chlorophyll synthesizing enzymes containing Fe^{3+} [38]. The photosynthetic pigments such as chlorophyll a, b and total chlorophyll content were significantly decreased in salt-stressed rice seedlings (Figure 3A–C), and similar results were also reported earlier in rice and *Pisum sativum* [39,40]. Contrary to the findings presented here, Li et al. reported that CNPs and CS priming of *Triticum aestivum* L. reduced the chlorophyll content [25].

Like chlorophyll, salinity stress can affect other leaf pigments such as carotenoids, which can alter the photosynthetic efficiency [41]. Carotenoids present in the chloroplasts play a significant role in the mechanisms shielding the photosynthetic apparatus against various hazardous environmental factors [42]. They scavenge the ROS produced in stress conditions, mitigating the effect of stress in plants [43]. Seedlings from CNPs- and CS-primed seeds grown without salt stress showed an increase in carotenoid content, which suggests that enhancement of carotenoid can help the seedlings grown under salt stress to deal with ROS species and maintain a high germination rate and seedling vigour chlorophyll content compared to seedlings from control hydro-primed seeds.

3.4. Antioxidant Enzymes

The activities of antioxidant scavengers CAT, POD and SOD (Figure 4) showed an overall increasing trend across the NaCl concentrations. Although the increase was gradual, the activities of CAT, POD and SOD in CNPs- and CS-primed seedlings in 50 to 150 mM NaCl concentrations were less compared to control hydro-primed seedlings. It was observed that CNPs priming demonstrated better salt stress alleviation potential compared to CS priming, which can be attributed to reduced enzymatic activities.

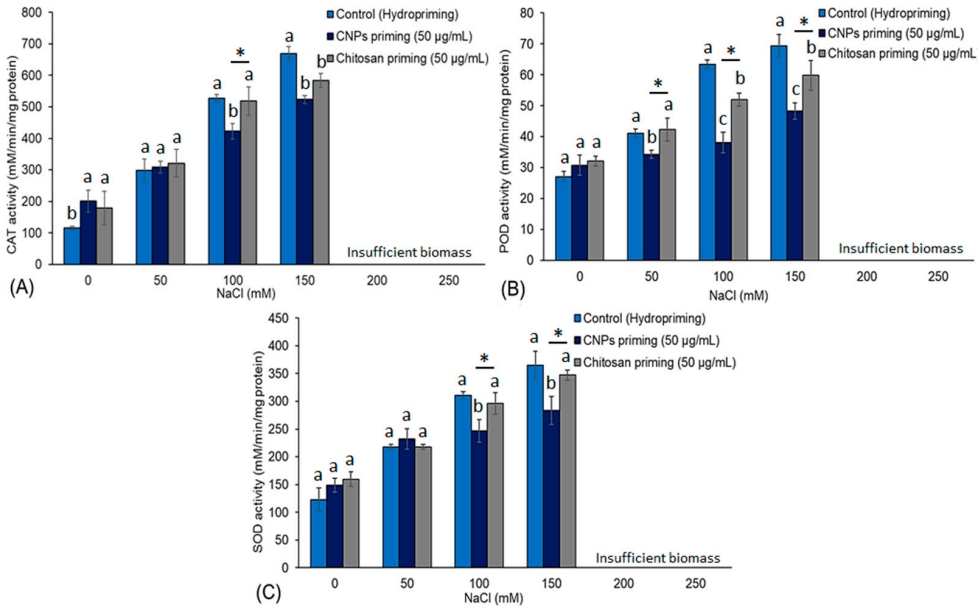

Figure 4. Effect of chitosan nanoparticles (CNPs) and chitosan (CS) priming on antioxidant enzymes (**A**) catalases (CAT), (**B**) peroxidases (POD) and (**C**) superoxide dismutase (SOD) of rice seedlings grown under salinity stress. Different letters indicate a statistical significance between control and priming treatments, and "*" shows a significant difference between treatments; i.e., (CNPs and CS), which was obtained through a one-way ANOVA using Duncan's multiple range test (DMRT) in IBM SPSS statistics 27 (version 1.0). Significance was considered when $p \leq 0.05$.

Plants activate both non-enzymatic antioxidants (carotenoids, proline and reducing sugars) and enzymatic antioxidants (such as CAT, POD and SOD) to scavenge the toxic effects of ROS generated from salinity stress to prevent cells from oxidative damage. The antioxidant enzymes are especially essential for the maintenance of the equilibrium between ROS generation and its destruction [44]. The increase in antioxidant enzymes in the absence of salt stress (0 mM NaCl) in CS- and CNPs-primed seedlings suggest that priming enhances the activities of CAT, POD and SOD. The enhanced activities of these enzymes under priming conditions are likely to help reduce the salinity stress (50–150 mM NaCl). In a study performed on Broad beans, priming with 0.1% CNPs showed reduction in the activities of CAT, APX, POX and PPO [45]. However, an increase in their activities was recorded when the seeds were primed with lower concentrations of CNPs, i.e., 0.05%.

3.5. Total Protein and Total Reducing Sugar Content

In this experiment, the total protein content was found to be decreased with increasing salinity stress (Figure 5A). However, the seedlings grown from CS- and CNPs-primed seeds showed a significant increase in protein content compared to respective hydro-primed control only at 100 mM and 150 mM NaCl concentrations.

Similar to protein content, reducing sugar content was found to be increased in CS- and CNPs-primed seedlings compared to control hydro-primed seedlings (Figure 5B). The difference was not prominent among CS- and CNPs-primed seedling when grown under 0 to 150 mM NaCl.

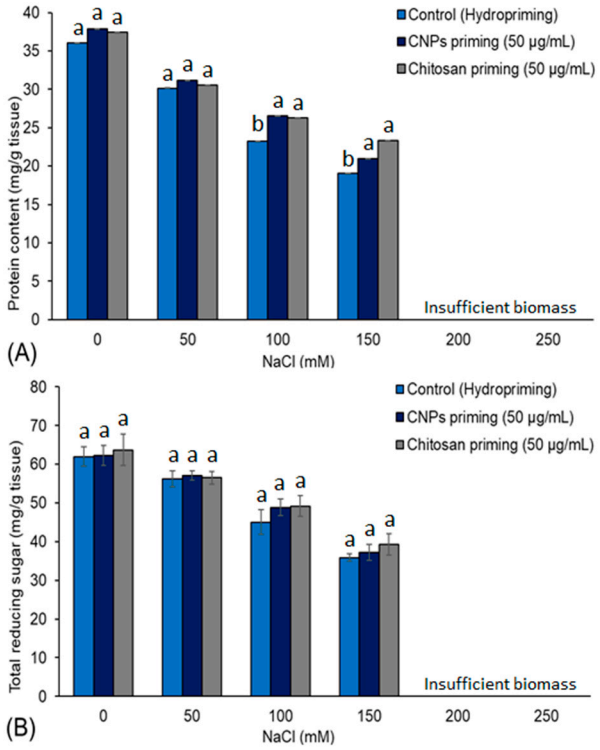

Figure 5. Effect of chitosan nanoparticles (CNPs) and chitosan (CS) priming on (**A**) total protein content, and (**B**) total reducing sugar content of rice seedlings grown under salinity stress. Different letters indicate a statistical significance between control and priming treatments; i.e., (CNPs and CS), which was obtained through a one-way ANOVA using Duncan's multiple range test (DMRT) in IBM SPSS statistics 27 (version 1.0). Significance was considered when $p \leq 0.05$.

Protein content is an important physiological parameter that is evaluated to understand the plant growth, development and metabolism. Measurement of protein content can provide information on protein synthesis, degradation and metabolic processes. In this experiment, both CS and CNPs treatment of seeds resulted in the increase in protein content. Similarly, a study performed on mung beans primed with CS and CNPs showed an increase in protein content with an increase in salinity stress [44]. Though not in rice and under normal conditions (no salinity stress), wheat seeds primed with 50 µg/mL CS showed enhancement in protein content compared to control [25]. However, wheat seeds primed with 50 µg/mL CNPs showed reduction in protein content compared to control. Therefore, looking at these studies and current outcome, it can be concluded that differences exist between how different species respond to the CS and CNPs priming.

To defend against adverse environmental effects such as salinity stress, plants adopt several mechanisms. Increasing the production of organic osmolytes such as sugars is one major systemic response to minimize salt-induced osmotic stress. Consistent with this, the reducing sugar content was found to be higher in CS- and CNPs-primed seedlings when grown under high salt stress, although the increase in reducing sugar was not prominent between the seedlings grown at lower concentrations of NaCl, i.e., 0 to 100 mM.

Priming is an agricultural practice conducted to improve seed germination. In this process, the hydration level within seeds is increased so that the metabolic activity vital for seed germination is accelerated, but radicle emergence is prevented [46]. There are many factors associated with the effects of seed priming such as temperature, humidity, type and concentration of the priming agent, but the type of priming agent used is particularly crucial. Several seed priming agents from synthetic to natural are used to initiate seed germination. Nanopriming is a novel strategy that employs nanoscale materials as seed priming agents. Here, we have explored the potential of biocompatible CNPs and compared it with CS for rice seed priming as well as salt stress alleviation post-germination.

Previous studies have reported that treatment of maize with a high concentration of CS (500 µg/mL) promoted maize seedling length, fresh weight and vigour index [47]. Similarly, 0.5 µg/mg CS enhanced pearl millet germination and vigour, as well as alleviation of biotic stress caused by *Sclerospora graminicola* [48]. Similar outcomes were observed for *Zea mays*, *Pisum sativum* and *Brassica rapa* treated with CS [49]. In concurrence with the current findings, Songlin and Qingzhong [50] have earlier reported an increase in seed germination percentage in CS-coated rice seeds when grown in salinity stress (50, 100 and 150 mM). In addition, CS priming also improved maize germination and other physiological parameters under low temperature stress [46]. In the case of CNPs, Divya, Vijayan, Nair and Jisha [26] reported that brief treatment (120 min) of rice seeds with 1 mg/mL enhanced the growth rate. Similarly, Li, He, Xie, Wang, Bose, Sun, Hu and Yin [25] reported that *Triticum aestivum* seeds treated with a low concentration of CNPs (5 µg/mL) was more effective in promoting growth as compared to an equal concentration of CS and higher concentration of CNPs. Laboratory studies showed that CNPs seed treatment significantly enhanced pearl millet seed germination percentage and seedling vigour compared to the hydro-primed control [51]. Furthermore, a similar enhancement in the germination percentage was recorded in broad beans primed with CNPs [45]. Collectively, these studies are focused on CS or CNPs priming and not their effect on germination under salinity stress; however, Zayed et al. [52] have shown that the priming of *Phaseolus vulgaris* with CNPs provided an overall increase in seed germination and seedling vigour. The above-mentioned studies have reported that a high concentration of CS exhibits positive effects on seed germination and plant growth but Li et al. found that seed priming with a high concentration of CNPs and CS, i.e., 100 µg/mL had negative or no growth effects on wheat [25]. It has also been observed that other natural oligo- and polysaccharides exhibit positive physiological activity, such as enhanced growth and seedling vigour in the range of low concentrations, and show inhibitory activity at high concentrations [46,53]. It has been previously reported that high concentrations of oligosaccharides such as oligochitosan can induce cell apoptosis in plants [54]. Therefore, in consideration of previous studies and the outcome of this investigation, we can hypothesize that it is essential to optimize the concentration and timing of seed priming to achieve high growth rates under salinity stress. In a similar study performed on wheat, it was observed that adsorption capacity of CNPs on seeds is significantly higher than CS [25]. The analysis of the SEM images of wheat seeds incubated with CS revealed a smooth coat texture, whereas the ones incubated with CNPs had a rough texture. According to the authors, CNPs promoted wheat growth by increasing the adsorption of CS on the seed surface. However, they suggested that the actual adsorption mechanism needs further investigation. In the present study, it can be hypothesized that the CS priming directly predisposes the seeds to CS, whereas CNPs dissociate slowly into the individual constituents, i.e., CS and TPP, based on an increase in pH (as CNPs are synthesized under acidic pH and the CNPs solution for priming consists of water, which has near neutral pH, i.e., 7) [20,55].

4. Conclusions and Prospects

The current study concludes that increased salinity levels lead to impaired seed germination and seedling development in rice. CNPs and CS seed priming modulated the salinity stress in rice by improving the seedling's development for thriving in both normal growth conditions and under salinity stress. Furthermore, the CNPs and CS priming of seeds helped in retaining high levels of chlorophyll and carotenoid pigments in the seedlings grown under salinity stress. CNPs and CS priming also mitigated the damage to rice seedlings under NaCl stress by enhancing SOD, CAT and POD activities. Although priming with both CNPs and CS showed positive results, the major difference between them was their effectiveness at different salt concentrations, i.e., CNPs priming performed better at higher NaCl concentrations, whereas CS was superior at lower concentrations. In summary, it has been demonstrated that rice seed priming with CNPs and CS is an effective strategy to reduce the influence of salinity stress on rice seedlings.

Future investigations are needed to understand the underlying molecular mechanism (functional genomics and proteomics) of action as to how the CNPs or CS improve seed parameters and enhance the health of seedlings, particularly if they are to be used commercially. Additionally, there is a need to test different concentrations of these priming agents and optimization of treatment time should be evaluated. Analysis of possible CS and CNPs internalization into plant cells post-priming is also required to assess the health and safety aspects. The effect of these priming agents on the expression of housekeeping genes is also essential to understanding potential adverse events that may occur inside the cellular machinery. As CS and CNPs are known to inhibit microbial growth, they can also be tested for increasing the germination percentage of rice seeds infested with fungal or bacterial spores, which are known to reduce and impact germination and seedling vigour, respectively. Lastly, field trials of plants treated with the priming agents are warranted, especially under saline conditions, along with cost-benefit analysis of the technology to farmers.

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