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EFFECT OF SEED TREATMENT WITH CHITOSAN ON THE GROWTH OF RICE (*Oryza sativa* L.) SEEDLINGS cv. INCA LP-5 IN SALINE MEDIUM

Efecto del tratamiento a las semillas con quitosana en el crecimiento de plántulas de arroz (*Oryza sativa* L.) cultivar INCA LP-5 en medio salino

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ABSTRACT. The chitosan is widely used in agriculture for having a strong fungistatic effect, also it has other properties such as extending the post harvest life of fruits and vegetables and stimulating the plant growth, among others. However, the use of this biopolymer for inducing salinity tolerance has been few studied, so the objective of this work was to check if chitosan-treated seeds are able to reverse the effects caused by salinity in the rice seedling growth and some biochemical indicators associated to this response. To do this, rice (*Oryza sativa* L.) seeds variety INCA LP-5 were treated for 24 hours with different concentrations of chitosan (0, 100 and 500 mg L^{-1}). The germinated seeds were transferred to pots containing diluted Hoagland nutritive solution supplemented or not with NaCl 100 mmol L^{-1} and they were placed in a growth chamber with controlled conditions. The growth and biochemical indicators were evaluated eleven days after stress treatment. Seeds treated with chitosan 100 mg L^{-1} stimulated shoot length and dry matter in saline medium grown seedlings and lowered malondialdehyde and increased proline levels. Both chitosan concentrations enhanced the activities of catalase and peroxidase enzymes, although a higher effect was obtained with chitosan 500 mg L^{-1}.

Key words: rice, growth, chitosan, salt stress

RESUMEN. La quitosana es usada ampliamente en la agricultura por su efecto fungistático, además alarga la vida postcosecha de frutos y vegetales y estimula el crecimiento de las plantas. Sin embargo, ha sido poco estudiado el uso de este biopolímero en la inducción de tolerancia a la salinidad, por lo que el objetivo de este trabajo fue comprobar si el tratamiento a las semillas con quitosana revertía los efectos inhibitorios del estrés salino en el crecimiento de plántulas de arroz, así como en algunos indicadores bioquímicos asociados a esta respuesta. Para ello se trataron semillas de arroz (*Oryza sativa* L.) variedad INCA LP-5, durante 24 horas, con diferentes concentraciones de quitosana (0, 100 y 500 mg L^{-1}). Las semillas germinadas se transfirieron a potes, a los que se les adicionó solución nutritiva Hoagland diluida, suplementada o no con NaCl 100 mmol L^{-1} y se colocaron en un cuarto de crecimiento con condiciones controladas. Las evaluaciones de crecimiento y los indicadores bioquímicos se realizaron a los once días después de establecido el estrés. El tratamiento a las semillas con la concentración de 100 mgL^{-1} de quitosana estimuló la longitud y la masa seca de la parte aérea de las plántulas crecidas en medio salino, así como disminuyó los niveles de malondialdehído e incrementó los de prolina. En cuanto a la actividad enzimática ambas concentraciones de quitosana estimularon las enzimas catalasas y peroxidasas, siendo el efecto más notable con la concentración de 500 mg L^{-1}.

Palabras clave: arroz, crecimiento, quitosana, estrés salino

INTRODUCTION

Chitosan is a safe and biodegradable compound mainly obtained from the exoskeleton of crab, shrimp and lobster (1, 2). It is used for its potentialities in different fields including biotechnology, agriculture, food and pharmaceutical industries (3, 4, 5).

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Chitosan has been widely used in agriculture mainly because of its antifungal and antimicrobial activity (6, 7). It is able to stimulate the enzyme production related to defensive responses in plants, such as chitinases, glucanases and phenylalanine ammonia lyase (PAL) (8). Besides, it has been found that these compounds, depending on their concentration and degree of acetylation, are able to activate PAL enzyme and other resistance components induced in plants (9).

Salinity is an abiotic stress which causes low productivity in most crops around the world (10). Such stress decreases germination and provokes an uneven emergence of seedlings, thereby reducing population density, an aspect that affects crop establishment. On the other hand, salinity is known to inhibit plant growth caused, at a first phase, by water uptake reduction in roots, which is called osmotic stress or water stress phase (11).

There is little information about the use of chitosan to relieve the adverse effects of salinity on plant growth and there is not any background knowledge in rice crop. Therefore, the aim of this study was to evaluate whether seeds treated with chitosan were able to reverse the adverse effects of salinity on rice seedling growth of variety INCA LP-5, as well as to evaluate some biochemical indicators associated to this answer.

MATERIALS AND METHODS

Rice (*Oryza sativa* L.) seeds from variety INCA LP-5 were treated for 24 hours by different concentrations (0, 100 and 500 mg L^{-1}) of a chitosan polymer with the following characteristics: molecular mass 93.3 kDa and 31.69% degree of acetylation. Subsequently, the treated seeds were placed in Petri dishes with distilled water for its germination in the dark at 25±2 °C. After 48 hours, germinated seeds were transferred to pots, to which 50 mL of a diluted Hoagland nutrient solution was added (1:2), supplemented or not with 100 mmol NaCl L^{-1}. Then, 20 seeds per pot and six pots per treatment were used, that is, 120 seedlings. Pots were placed in a growth chamber at a 12-hour photoperiod and a temperature of 24±0.2 °C.

Eleven days later, the lengths of roots and aerial parts were measured in 25 plants per treatment, and five samples of five plants each were formed to evaluate the dry mass of aerial parts and roots. At this very moment, some biochemical determinations were performed. Thus, 0.25 g fresh tissue were taken of each treatment and macerated in liquid nitrogen, adding 2.5 mL phosphate buffer 100 mmol L^{-1}, pH 7 and then centrifuged at 10,000 rpm for 20 minutes at 4 °C. The supernatant obtained allowed to estimate lipid peroxidation by measuring malondialdehyde (MDA) (12), the enzyme activity of total peroxidases (13) and catalases (14), and total soluble proteins by MicroLowry method (15).

Moreover, for determining proline amino acid, 0.25 g fresh tissue were taken of each treatment and macerated in liquid nitrogen, adding 10 mL water at 100 ºC. Proline content was quantified by Bates method (16), using L-proline (Sigma) as a standard.

The experiment was repeated twice with similar performance. Results from one replicate are presented. Data were statistically processed by using a Single Classification Variance Analysis and means were compared by Tukey Multiple Range test at $p<0.05$.

RESULTS AND DISCUSSION

Figure 1 shows the results of variable performance in rice seedling growth of cv. INCA LP-5, cultivated both in nutrient solution alone and in another one supplemented with NaCl. As it can be seen, the presence of 100 mmol L^{-1} NaCl decreased significantly the length and dry mass of the aerial part; however, it had no effects on roots. Chitosan concentration of 100 mg L^{-1} had significant stimulation on the length and dry mass of the aerial part of those seedlings whose seeds were treated with chitosan (Figure 1A and C). The treatment of 100 mg L^{-1} decreased root dry mass in stressed seedlings (Figure 1D).

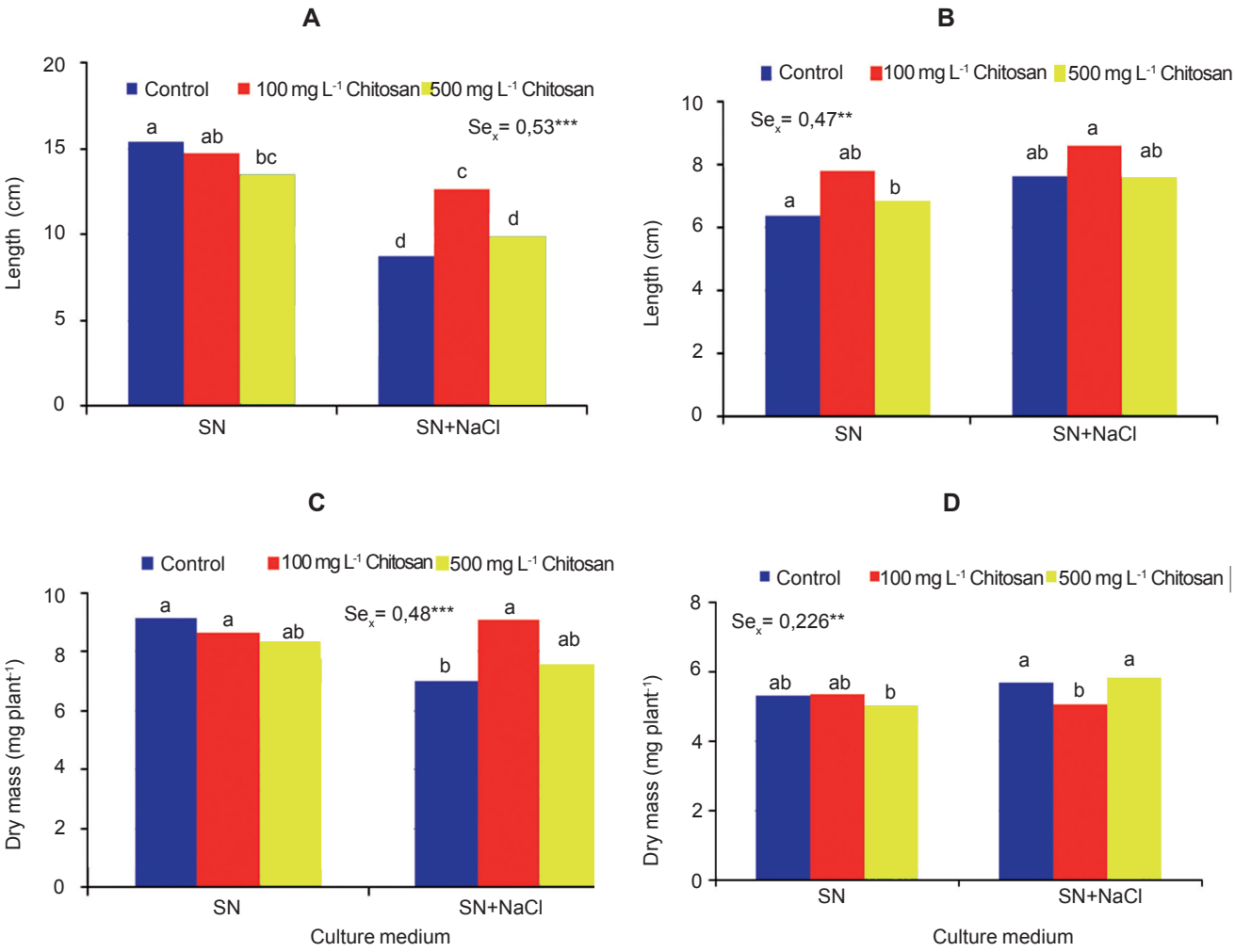
It is interesting to remark that seeds treated with chitosan had no effect on seedling growth in nutrient solution. This result could be due to the concentrations used, as it has been observed that growth is induced by lower concentrations.

Chitosan not only has effects against pathogenic fungi, but also it generally has growth-promoting effects, although it has been so far hardly studied. Some authors point out that crop yields are higher when seeds are treated with chitosan. Rice yield increased 20 to 30 % after foliar spraying and seed treatment with chitosan, respectively (17, 18).

When analyzing chitosan treatments applied to seeds, a positive effect of this polymer was observed on rice seedling growth of variety INCA LP-5 in saline medium. The treatment of 100 mg L^{-1} was the best, since it fully and partially reversed the negative impact of salt on the dry mass and length of the aerial part, respectively (Figure 1).

Previous studies found similar performance in Isabgol (*Plantago ovata* Forsk) plants, where chitosan (0.2 %) induced shoot and root lengths, as well as root dry mass under salt stress conditions (19). With chitosan applications to soybean seedlings under salt stress conditions, a higher dry mass was also observed (20).

Similarly, safflower (*Carthamus tinctorius* L.) seeds treated with chitosan increased seedling growth under water deficit conditions (20).



Common letters do not differ according to Tukey test (p≤ 0.05) n= 6
A. Length of the aerial part. B. Root length. C. Dry mass of the aerial part. D. Root dry mass

Figure 1. Influence of chitosan treatment on some variables of rice seedlings of cv. INCA LP-5 grown in nutrient solution supplemented (SN+NaCl) or not (SN) with NaCl 100 mmol L^{-1}

Zeng and Luo reported something similar, as they found that seeds coated with chitosan enhanced germination rate and plant growth of wheat crop under drought conditions (21).

Other authors have reported that corn seeds treated with chitosan at 0.50 % improved germination rate and the length and dry mass of seedling roots and shoots under low temperature stress conditions (22).

Malondialdehyde (MDA) is one of the final low-molecular-mass products from lipid hydro peroxide decomposition, which is the most commonly used compound to measure lipid peroxidation in cell membrane.

As shown in Figure 2A, seedlings grown in nutrient solution with NaCl showed higher MDA levels. This increased MDA concentration to various stresses is widely described in literature (23, 25) and it is a direct consequence of the action of reactive oxygen species (EAO).

Seeds treated with both chitosan concentrations decreased MDA levels in stressed seedlings. Similar results have been reported in sunflower plants (26). Besides, it has been observed that MDA content decreased in tomato seedlings foliarly sprayed with 150 mg L^{-1} chitosan and subjected to salt stress conditions (27). Also, oligochitosan pretreatment with 0.0625 % reduced MDA levels in wheat plants grown under saline conditions (28).

Likewise, chitosan concentrations (up to 0.5 %) decreased MDA levels of safflower seedlings subjected to osmotic stress (20). At low temperature conditions, chitosan treatment also decreased MDA content in corn plants (22).

Higher plants may accumulate L-proline (Pro) in response to various environmental stresses. In this experiment, salt stress increased this amino acid concentration in rice seedlings over the control.

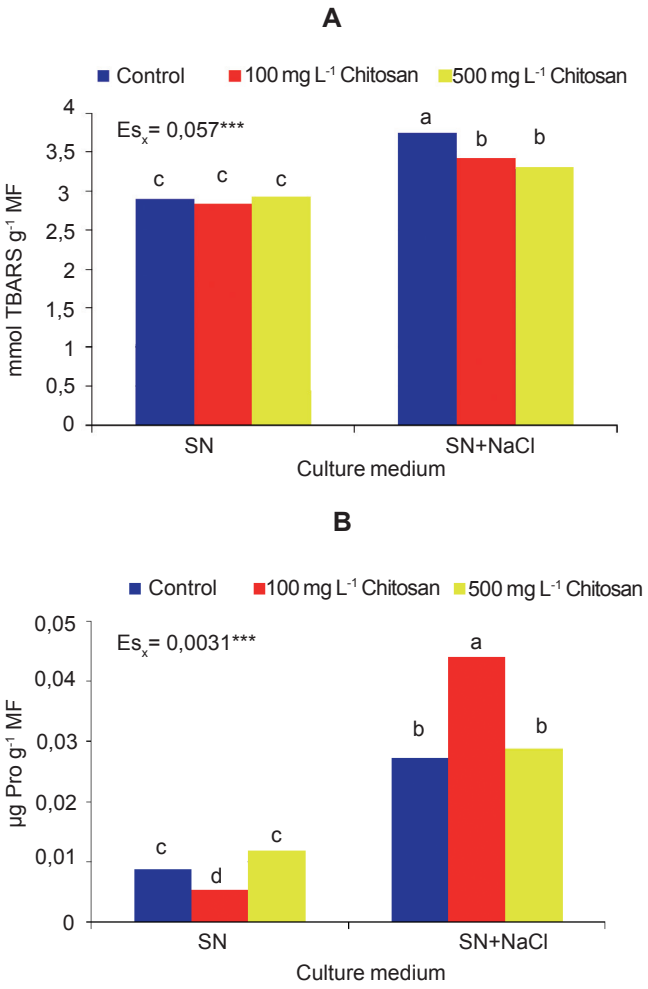


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Figure 2. Influence of chitosan treatment at malondialdehyde (A) and proline (B) levels on rice seedlings of cv. INCA LP-5 grown in nutrient solution supplemented (SN+NaCl) or not (SN) with 100 mmol L^{-1} NaCl

Chitosan treatment at a concentration of 100 mg L^{-1} increased these levels even more. This same concentration significantly decreased this indicator in unstressed seedlings.

Although the exact role of proline on salt stress is controversial, since not always increases of this amino acid have been correlated with a higher stress tolerance (29), in recent years it has been shown that the exogenous application of proline provides a protective action against oxidative damage induced by salt stress (30, 32).

Furthermore, it has been shown that proline accumulation stabilizes membranes and subcellular components including complex II of mitochondrial electron transport chain. Studies have suggested proline as final acceptor of free radicals (33) and redox potential stabilizer by resupplying $NADP^+$ supplies (34).

Results of this study showed that chitosan treatment increased proline levels under saline conditions; however, only the concentration of 100 mg L^{-1} significantly increased this indicator in relation to the control treatment. Increased proline has been found in wheat and tomato plants subjected to saline stress (27, 28), whereas seeds treated with chitosan had an opposite effect in sunflower plants (26).

Total protein concentration decreased with salt stress and chitosan treatment worsened this performance (Figure 3A). Contrary to this result, it was found that treatment with low chitosan concentrations in safflower seedlings subjected to water stress increased soluble protein concentration (20).

It is interesting to observe how the polymer treatment at the concentration of 500 mg L^{-1} in unstressed seedlings also decreased protein content. Similar results were recorded in safflower seedlings derived from chitosan-treated seeds (20).

Among the key enzymes removing toxic oxidants are peroxidases and catalases. Figure 3 (B and C) shows that, in the case of the control treatment, salt stress decreased peroxidase enzyme (POX) activity and had no effect on catalases (CAT).

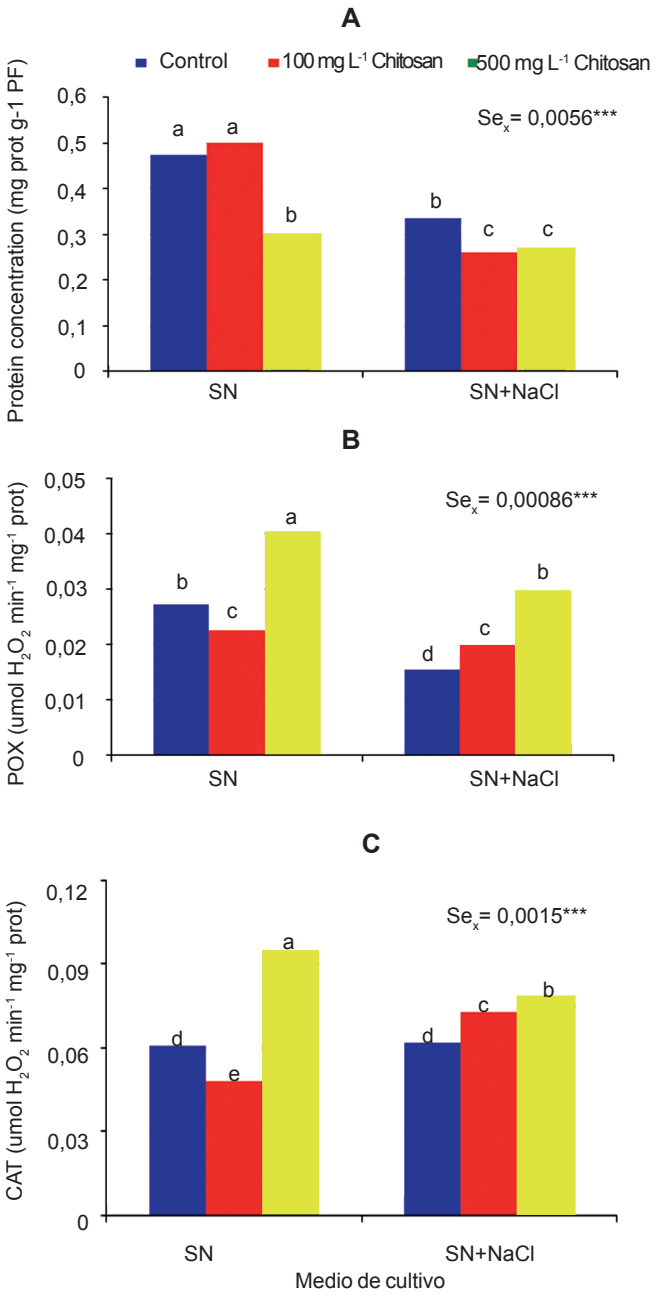
Chitosan treatment increased the activity of both enzymes in stressed seedlings and its effect depended on the concentration. It is interesting to point out that the concentration of 100 mg L^{-1} decreased both enzymatic activities in unstressed seedlings; however, the concentration of 500 mg L^{-1} significantly increased enzyme activity. Perhaps, it is because a higher concentration could put the cell on the alert, strengthening defenses.

There are numerous studies about salt stress effects on plant antioxidant enzymes, but they are sometimes contradictory.

Regarding peroxidases, salt stress increases this enzyme activity in rice plants (23, 35, 36). Peroxidase activity of potato calluses increased under salt stress conditions, but at higher concentrations (150 mmol L^{-1} NaCl) this activity decreased (37). Similarly, in cotton, it has been reported that POX increases in salt-tolerant crops and decreases or remains constant in salt-sensitive crops (38, 40).

However, salinity decreased POX enzyme in wheat, whereas increased MDA levels and root superoxide production (41), similar to this study.

Concerning catalase enzyme, something similar occurs because some authors have recorded stimulation of this enzyme activity through activating *Cat2*, and *Cat3* (42) genes. Nevertheless, others found CAT decreased in rice leaves by NaCl treatment (43, 44). On the other hand, it has also been observed that this enzyme does not vary in rice seedlings under salt stress (45).



Common letters do not differ according to Tukey test (p≤ 0.05) n= 6

Figure 3. Influence of chitosan treatment on total soluble protein concentration (A), peroxidase (B) and catalase (C) enzyme activities of rice seedlings of cv. INCA LP-5 grown in nutrient solution supplemented (SN+NaCl) or not (SN) with 100 mmol L^{-1} NaCl

So far as the stimulation of CAT enzymatic activity by chitosan treatment is concerned, similar results were obtained in tomato seedlings sprayed with chitosan and subjected to salt stress (27). Besides, increases in POX, CAT and superoxide dismutase (SOD) enzymatic activities were found in wheat seedlings treated with oligochitosan and subjected to stress (28).

However, under normal conditions, POX and CAT activities were stimulated in sunflower seedlings after chitosan treatment, but these same concentrations reduced both enzyme activities under saline conditions (26).

Furthermore, it was found that chitosan treatment stimulates antioxidant enzyme activity in plants subjected to other kinds of stress, such as low temperatures in corn plants (22) and water stress in wheat plants (21).

Recently, chitosan has received special attention for its ability as an antioxidant, which depends on the molecular mass and degree of acetylation (46). Several studies have confirmed that chitosan may have potential as a free radical trapper (46, 47). This compound can catch the radical hydroxyl and superoxide and it has been reported for its protective DNA properties (48). The mechanism by which chitosan acts could be attributed to its structure with a large number of available hydroxyl and amino groups reacting with active EAO oxygen species (49).

In general, chitosan treatment activated POX and CAT antioxidant enzymes in seedlings grown in saline medium, thereby lipid peroxidation decreased in the membrane. Moreover, although chitosan also increased proline levels in seedlings grown in saline medium, only the concentration of 100 mg L^{-1} induced an increase which differed statistically from the other treatments, coinciding with the concentration that reversed growth inhibition caused by salinity in the aerial part of seedlings, suggesting the importance of this metabolite under these conditions, as it is known that proline can act as an antioxidant and as osmotically active solute.

Taking into account that these are the first results from chitosan effect on rice seedling growth in saline medium, particularly in cv. INCA LP-5, it is suggested to keep on deepening into these studies, especially by considering the importance of this variety in our rice production and the constant enlargement of salinity-affected areas.

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