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SPIROSTANIC ANALOGUES OF BRASSINOSTEROIDS ENHANCE THE RICE (Oryza sativa L.) CV. INCA LP-7 SEEDLING GROWTH UNDER NACL STRESS

Análogos espirostánicos de brasinoesteroides estimulan el crecimiento de plántulas de arroz (*Oryza sativa* L.) cv. INCA LP-7 sometidas a estrés por NaCl

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ABSTRACT. Spirostanic analogues of brassinosteroids are synthesized, compounds in Cuba and some of their formulations have demonstrated to enhance the growth when plants grow under saline stress. Recently, a new analogue (Biobras-25) which should have a good biological activity has been synthesized. For this reason, the objective of this paper was to evaluate rice seedling growth under NaCl stress by fourteen days, when the seeds were treated with Biobras-25 or Biobras-16. Two experiments were performed, in the first one, rice seeds of cv. INCA-17 were soaked in BB-25 or BB-16 solutions (0; 0,005; 0,05 y 0,5 mg L^{-1}) and after germination, they were placed in pots which contained Hoagland nutritive solution with the addition or not of NaCl 0,1 mol L^{-1} and remained during fourteen days in a growth room. At the end of the experiment, root and shoot length and dry mass were evaluated to 25 plants by treatment. Later, a second experiment was performed with the best concentrations of both formulations and, also, the growth indicators described above, leaf proline, a, b and total chlorophylls and total carotenes were determined. Results showed that both analogues exhibited a distinct behavior. Biobras-25 not only enhanced significantly seedling growth under NaCl stress but also increased significantly pigment concentration and decreased the proline content in leaves.

Key words: plant growth regulators, pigments, proline, salt stress

RESUMEN. Los análogos espirostánicos de brasinoesteroides son compuestos sintetizados en Cuba y algunas de sus formulaciones han demostrado estimular el crecimiento cuando las plantas son sometidas a estrés salino. Recientemente, se ha sintetizado un nuevo análogo (Biobras-25), que debe poseer una buena actividad biológica. Por tal motivo, el objetivo de este trabajo fue evaluar el crecimiento de plántulas de arroz sometidas a estrés por NaCl, durante catorce días, cuando sus semillas fueron tratadas con Biobras-25 o Biobras-16. Se montaron dos experimentos, en el primero las semillas de arroz cv. INCA LP-7 se sumergieron en soluciones de 0; 0,005; 0,05 y 0,5 mg L^{-1} de BB-25 o BB-16 y después de germinadas se colocaron en potes que contenían solución nutritiva Hoagland diluida suplementada o no con NaCl 0,1 mol L^{-1} y se mantuvieron durante catorce días en un cuarto de crecimiento. Al final del experimento se evaluaron 25 plantas por tratamiento, la longitud; la masa seca de raíces y la parte aérea. El segundo experimento se montó con las mejores concentraciones de ambas formulaciones y, además, de los indicadores de crecimiento descritos anteriormente, se determinaron las concentraciones de prolina, clorofilas a, b, totales y carotenos totales en las hojas. Los resultados mostraron que ambos análogos exhibieron un comportamiento diferencial, destacándose el BB-25, que no sólo estimuló el crecimiento de las plántulas sometidas a estrés por NaCl, sino que además, estimuló significativamente, la concentración de los pigmentos y disminuyó la concentración de prolina en las hojas.

Palabras clave: reguladores del crecimiento, pigmentos, prolina, estrés salino

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INTRODUCTION

Salt stress is a limiting factor for growth and crop productivity factor. The harmful effects of this stress on growth and productivity of plants to osmotic and ionic stress severely depresses various physiological and biochemical processes, such as photosynthesis, synthesis of protein and energy and lipid metabolism must (1). In the case of rice, it has been reported that culture sensitivity to salt varies with the growth stage, the stance phase being one of the most sensitive (2).

There are several strategies for reversing the adverse effects caused by salt stress in this crop. These include: the use of tolerant varieties and use of bioactive products, within which some growth regulators include, such as brassinosteroids (3) and analogs thereof (4).

It is well documented that brassinosteroids protect plants when they are grown under salt stress (5-7). Specifically in the cultivation of rice, several authors have reported the positive influence of exogenous application of these compounds in these stress conditions (8-10). However, it is still little available information regarding the protection induced by brassinosteroids analogues synthesized in Cuba. There results the effectiveness of certain concentrations of spirostanic analogs BB BB-6 and-16 in stimulating the growth of seedlings seven days of age cultivars INCA LP-7 and J-104 (11). Similar results were reported with BB-16 when seedlings cv J-104 were subjected to salt stress for eleven days (12). However, it is not known whether this effect BB-16 is maintained by extending the exposure time of seedlings to NaCl, much less the effect the new analog called biobras-25 can exercise in the growth of rice seedlings in these conditions.

For all the above, the objective of this study was to evaluate whether treatment with seeds biobras-16 or with biobras-25 stimulates the growth of rice seedlings cv. INCA LP-7 under stress by NaCl for fourteen days.

MATERIALS AND METHODS

Seeds of rice (*Oryza sativa* L.) cv. INCA LP-7 (cultivar recommended for salt-affected soils) (13) were treated for 24 hours with different concentrations (0, 0.005, 0.05 and 0.5 mg L^{-1}) Biobras-16 and Biobras -25.

Biobras-16 (BB-16) has as active ingredient a espirostánico brassinosteroid analogue, ID-31, while the Biobras-25 (BB-25) has as active ingredients one colestánico brassinosteroid analogue (65%) and an analog espirostánico brassinosteroid, the DI-31 (35%). Both formulations were provided by the Center for Studies of Natural Products Faculty of Chemistry of the University of Havana.

Once treatment is completed to seeds (200 per treatment) with different solutions, they were placed in Petri dishes with distilled water in the dark at 25 ± 2 ° C to promote germination. At 48 hours, the germinated seeds were transferred to pots, to which are added 50 × 10-3 L Hoagland nutrient solution diluted (1: 1) supplemented or NaCl 0.1 mol L-1. six pots per treatment and 20 seedlings per pot, ie, 120 seedlings were used per treatment and these were kept in a growth room (average of 25 ± 2 ° C average temperature, photoperiod of 12 hours and an average relative humidity of 70 %) for fourteen days. Growth indicators were assessed at 25 seedlings per treatment, at the end of the experiment and these were: length (cm) and dry mass of the aerial part and roots (mg plant-1).

With treatments that led to the best response of seedling growth after fourteen days under stress by NaCl, we proceeded then to mount another similar experiment with these concentrations only.

For this experiment, seeds (250 per treatment) were treated for 24 hours with water, BB-16 0,05 mg L^{-1} and BB-25 0,005 mg L^{-1}. They over this period were placed in Petri dishes with distilled water in a growth chamber at 26 ± 2 °C to promote germination. After 48 hours, the germinated seeds were transferred to pots and placed in the growth chamber following the methodology described in the previous experiment; with the difference that in this case ten pots were used per treatment. Similar to the above in the previous experiment, evaluations of seedling growth fourteen days with the percentages of reduction that salinity caused in each of the indicators through the following formula were calculated were made:

Percentage reduction (%) = (1-VSN + NaCl / VSN) * 100

where:

$V_{SN + NaCl}$ is the value of the average indicator of seedlings grown in nutrient solution supplemented with NaCl.

V_{SN} is the value of the average indicator of seedlings grown in the nutrient solution.

Furthermore, the leaves following biochemical tests were performed:

Chlorophylls a, b, total and total Carotene

To determine the concentration of chlorophylls (chlorophyll a, total by) and carotenes, three samples were used for treatment of 4 x 10-5 kg of leaf tissue, they were immersed in acetone 1x10-2 L (80% v / v) and kept in the dark for 24 hours (14). After this time the absorbance at 440, 665 and 649 nm for carotenes, chlorophyll a and b, respectively read. For calculating concentrations chlorophylls the following equations (14) were used:

$$C_a = (11,63\, A_{665} - A_{649})/MF$$

$$C_b = (20,11\, A_{649} - A_{665})/MF$$

$$C_{a+b} = (6.45\, A_{665} + 17.72\, A_{649})/MF$$

where:
A_{649}, A_{665} - absorbance at 649 and 665 nm.
MF grams of fresh tissue mass

The concentration of carotenes totals determined using a standard curve β-carotene and he expressed based on fresh tissue mass.

Proline content

Proline content was determined according to the method most widely used internationally (15). Three samples of 0, 25 g of leaf tissue were frozen in liquid nitrogen and homogenized with 1 x10^{-2} L of distilled water at 100 °C. Subsequently the samples were filtered. Then, 2 × 10^{-3} L of extract was placed in a test tube and reacted with 2 x 10,03 L of glacial acetic acid and 2 x 10^{-3} L ninhydrin at 100ºC for one hour. The reaction was stopped when the tubes were placed on ice bath. The chromophore containing proline was extracted with 4 × 10^{-3} L of toluene. Proline was quantified in a UV / Visible spectrophotometer (Genesys 6, Thermo Electron Corporation) at 520 nm, using L-proline (Sigma) for the calibration curve. Each sample was made two spectrophotometric readings.

Statistical analysis

The data obtained were analyzed by ANOVA simple classification. In cases where significant difference between treatments was found, the comparison of means was performed by multiple range test of Tukey HSD p≤0,05 (16).

RESULTS AND DISCUSSION

The effects of treatments rice seeds with different concentrations of BB-16 and BB-25 indicators of seedling growth, fourteen days after being placed in nutrient solution Hoagland (SN) or nutrient solution supplemented with NaCl 0,1 mol L^{-1} (SN + NaCl) can be seen in Table I. As the lowest concentration (0.005 mg L^{-1}) of both products is appreciated stimulated the growth of the aerial part (length and dry mass) and the length of roots when the seedlings grown in nutrient solution.

It is known that brassinosteroids play an essential role in the growth and development of plants (17); however, it is important to determine, depending on the mode of application, what should be the concentration which causes growth stimulation. For example, recently, mustard seed treatments were performed with 24-epibrassinolide extracted from the crop and found that concentrations of 10^{-9} and 10^{-11} mol L^{-1} were the stimulated plant growth, not and the 10-7 mol L^{-1} (18). In this work the lowest concentration used, equal to 10-8 mol L^{-1}, it was the most effective, suggesting evaluated in future studies, lower concentrations in order to determine the most appropriate concentration to stimulate initial growth of rice seedlings of this cultivar under normal conditions.

In the presence of NaCl, BB-16 and BB-25 behaved differently, as treatment with BB-25 0.005 mg L^{-1} completely reversed the inhibition salt resulted in root growth and partially induced in length of the aerial part. Moreover, salinity did not affect the dry mass of the aerial part; however, seedlings from treatments BB-25 stimulated this indicator, achieving significantly higher values to control plants grown without the presence of salt. However, the BB-16 0,05 mg L^{-1} only completely reversed the radical growth.

The results presented in this paper show that biobras-25 was more effective than biobras-16 to reverse the adverse effects of NaCl causes stress in rice seedlings cv. INCA LP-7, after fourteen days of treatment.

Table I. Influence of seed treatment with different concentrations of BB-16 or BB-25 in some indicators of growth of rice seedlings cv. INCA LP-7-stressed NaCl fourteen days

Treatments	Growth medium	Root length (cm)	Length of aerial part (cm)	Dry mass of roots (mg planta^{-1})	Dry mass of aerial parta (mg planta^{-1})
Control	SN	6,7 bcd	14,7 de	5,0 cde	7,9 efg
BB-16 0,5 mg L^{-1}		6,3 bcde	14,4 e	4,8 e	7,7 fg
BB-16 0,05 mg L^{-1}		**7,7 a**	15,5 cd	**5,8 ab**	8,1 cde
BB-16 0,005 mg L^{-1}		6,6 bcd	**16,4** bc	**6,2 a**	**8,8 c**
BB-25 0,5 mg L^{-1}		5,9 def	**18,1 a**	5,4 bcd	**10,0 a**
BB-25 0,05 mg L^{-1}		6,4 bcde	**16,7 b**	5,5 bc	**8,7 cd**
BB-25 0,005 mg L^{-1}		7,0 abc	**17,1 b**	**5,9 ab**	**9,7 ab**
Control	SN+NaCl	5,5 ef	9,1 hi	3,5 g	7,1 g
BB-16 0,5 mg L^{-1}		5,7 ef	10,0 gh	4,0 fg	7,8 efg
BB-16 0,05 mg L^{-1}		**6,9 abc**	8,7 i	**4,4 ef**	7,1 g
BB-16 0,005 mg L^{-1}		5,3 f	10,0 gh	4,0 fg	7,9 efg
BB-25 0,5 mg L^{-1}		5,4 f	**12,1 f**	**5,0 cde**	**9,9 a**
BB-25 0,05 mg L^{-1}		6,1 cdef	**11,0 g**	**4,7 e**	**8,9 bc**
BB-25 0,005 mg L^{-1}		**7,2 ab**	**12,1 f**	**4,9 de**	**10,2 a**
SEx		0,28*	0,36*	0,20**	0,28*

It is known that the exogenous application of brassinosteroids stimulates the growth of rice plants when subjected to salt stress and for this have employed various modes of application, within which is treating the seeds with and without the presence of NaCl (8-10, 19), although it should be noted that the most commonly used concentrations, generally have been higher than 10-6 mol L^{-1}, which is equivalent to 0,5 mg L^{-1}. However, the existing information relating to the protection of brassinosteroid analogues synthesized in Cuba, is scarce, using analogs known as biobras-6, biobras-16 and MH-5 (11, 20) and generally, cv. most studied has been J-104, which is a sensitive cultivar.

A tolerant cultivar, a new formulation (Biobras-25) and also NaCl treatment was extended fourteen days previously unstudied aspects; hence the importance of these preliminary results was used in this work. It should be noted that, in all cases, the most effective concentration of the analogs (10-8 10-7 mol L^{-1}) was less than the reported natural brassinosteroids for. Therefore, concentrations of 0.05 and 0.005 mg L^{-1} BB-16 and BB-25, respectively to execute another experiment and assess whether the growth response was associated or not with some biochemical indicators were selected plants.

Table II shows the percentages of reduction caused salinity indicators of growth of seedlings of different treatments studied are presented. The Biobras-25 was able to reverse more effectively than the Biobras-16 inhibition in the growth of rice seedlings caused by the presence of NaCl 100 mmol L^{-1} for fourteen days.

Given that is well documented that salinity exerts influence on photosynthesis and especially in photosynthetic pigments and the positive effect that the exogenous application of brassinosteroids exert on these indicators (5, 21-23), plus it is known proline is a metabolite which acts as osmotic protector, membrane stabilizer and trapping active oxygen species (24), in Figure 1 the results of the influence are presented treatment to seeds with Biobras-16 and Biobras-25 they exercised in the concentration of these indicators in the leaves of rice seedlings cv. INCA LP-7 grown in nutrient solution with or without the addition of NaCl.

It can be seen that the presence of NaCl did not affect the photosynthetic pigment concentration in leaves and Biobras-16 treatment did not affect this response. However, the seedlings whose seeds were treated with Biobras-25 exhibited a pigment concentration in leaves was significantly superior to that shown for control treatment grown nutrient solution.

Table II. Influence of brassinosteroids analogues biobras-16 and biobras-25 in the percentages of reduction, stress induced by NaCl for fourteen days, in some indicators of growth of rice seedlings cv. INCA LP-7

Tratamientos	Longitud de las raíces	Longitud de la parte aérea	Masa seca de las raíces	Masa seca de la parte aérea
Control	0	37,4	21,5	32,1
Biobras-16 (0,05 mg L^{-1})	5,7	19,4	21,3	0
Biobras-25 (0,005 mg L^{-1})	0	10,4	1,9	0

This behavior of the pigments can be associated with cultivating tolerance to salt stress, as it has been reported that in tolerant rice cultivars when subjected to this kind of stress there is an increased activity of the enzyme fructose 1,6 - bisphosphatase in leaves and roots, a high concentration of sugars and delayed degradation of chlorophylls (25).

Moreover, it has been shown that exogenous application of brassinosteroids reverses inhibition salinity causes the photosynthetic pigment concentration (5, 22, 23, 26). In this case, this only occurred when the seeds were treated with biobras-25 and especially chlorophyll a, total chlorophylls and carotenoids. These photosynthetic pigments are responsible for capturing light energy (chlorophylls) and protect the photosynthetic apparatus of photooxidation (carotenes), dissipating excess light absorbed by pigments, hence the importance of the BB-25 increases the concentration of these pigments in saline conditions.

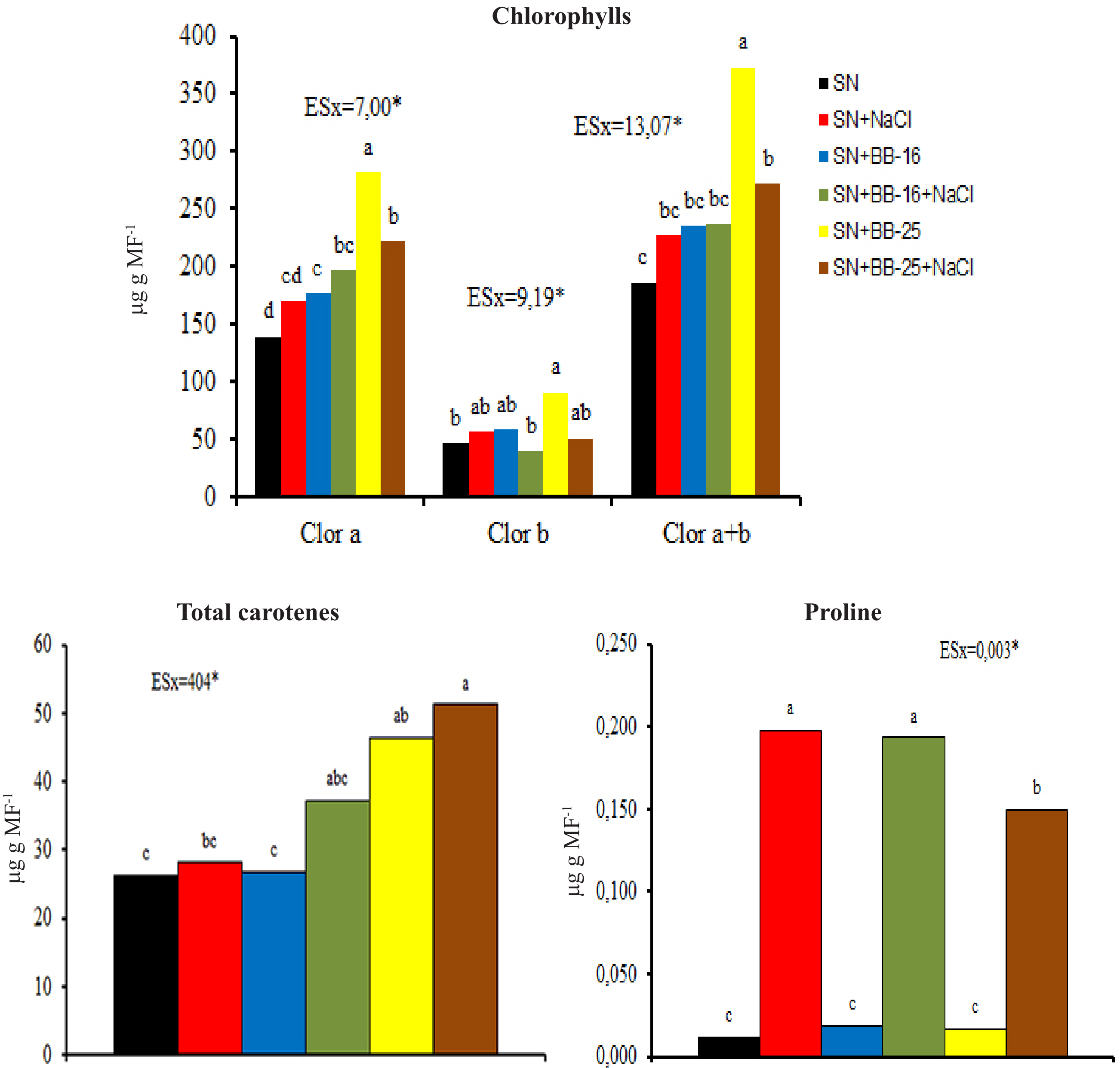
As proline, it is increasing the salt stress produced in this indicator is seen; however, only treatment Biobras-25 could significantly reduce this increase. It should be noted that the results reported in international literature regarding the effects of exogenous application of brassinosteroids in the concentration of proline in plants subjected to salt stress are variables, because while some authors have found that brassinosteroids increase further proline concentration when plants are subjected to salt stress (5, 26), others have reported the opposite (9, 21, 27), arguing that if the increase of proline is a response to stress, brassinosteroids to protect the same against it, cause a decrease in the synthesis of this metabolite. Moreover, in an experiment where a foliar spray with 24-epibrassinolide, prior to treatment with NaCl in rice seedlings was performed was found that proline acted as a free radical scavenger and as a protective membrane, rather than an osmolyte (27).

In this connection, it has been suggested that proline is synthesized predominantly in glutamate salt stress conditions and γ-glutamyl kinase P5CS represents the limiting step of the reaction rate of this pathway activity. P5CS expression is strongly induced by salt stress, suggesting that P5CS in proline biosynthesis plays a protective role under stress conditions. On the other hand, in *Arabidopsis thaliana*, drought and salt stress differentially activate the expression of two genes P5CS: AtP5CS1 (At2g39800) and AtP5CS2 (At3g55610). AtP5CS1 is responsible for proline accumulation during salt stress and drought. AtP5CS1 expression is activated by a signal translation pathway ABA-dependent and modulated by light and brassinosteroids. This could suggest a possible mechanism by which brassinosteroids reduce the adverse effects of salinity (28).

In this case, the Biobras-25 decreased the concentration of proline; however, the mechanism used this brassinosteroid analogous to exert their action is not known.

In this work the positive effect analogs brassinosteroids exert on the growth of rice seedlings under NaCl stress was confirmed. However, they should continue research on this issue, since the results that have been obtained to date with brassinosteroid analogues synthesized in Cuba, limited to evaluate the effects of exogenous applications exert on plants, making it necessary to determine the mechanisms that these analogs used for protection are.

Special attention should be paid to new analogue called biobras-25 for their potential to be used as protector of rice plants grown in saline.



Means with same letters do not differ significantly by multiple range test of Tukey HSD p≤0,05 (16)

Figure. Effect of two brassinosteroid analogues in concentrations chlorophylls, carotenes and proline in leaves of rice seedlings cv. INCA LP-7 grown in Hoagland nutrient solution supplemented or not with NaCl 100 mmol L^{-1} for fourteen days

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