Turkish Journal of Biology

Volume 31 | Number 3 Article 3

1-1-2007

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Turk J Biol
31 (2007) 141-146

Effect of 28-Homobrassinolide on Salinity-Induced Changes in *Brassica juncea*

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Received: 19.07.2006

Abstract: Seedlings of *Brassica juncea* Czern & Coss cv. Varuna generated from seeds soaked in 0, 50, 100, or 150 mM NaCl for 6 h were treated with 0, 10^{-10}, 10^{-8}, or 10^{-6} M 28-homobrassinolide (HBL) after 14 days of growth. Plants that received only NaCl treatment exhibited a decrease in nitrate reductase and carbonic anhydrase activity, chlorophyll content, and P_N 60 days after sowing (DAS), and decreased seed yield at harvest (140 DAS). Subsequent treatment with HBL significantly increased all of the above parameters. The 10^{-8} M concentration of HBL generated the best response and also overcame the detrimental effects of NaCl more effectively when NaCl treatment was at the 50 mM level. Tissue proline concentration exhibited a different pattern, increasing in response to both NaCl and HBL treatment; 10^{-8} M of HBL interacted with 150 mM NaCl most significantly and increased the concentration of proline more than all other treatments. The study results suggest that HBL can be considered an important plant protector under NaCl stress conditions.

Key Words: Carbonic anhydrase, chlorophyll, nitrate reductase, photosynthesis, seed yield

Abbreviations: CA: carbonic anhydrase; DAS: days after sowing; HBL: 28-homobrassinolide; NR: nitrate reductase; P_N: photosynthetic rate

Brassica juncea'da Tuz ile Uyarılmış Değişiklikler Üzerine 28-homobrassinolid'in Etkisi

Özet: *Brassica juncea* Czern ve Coss cv. Varuna tohumları 0, 50, 100 ve 150 mM NaCl'de 6 saat bekletildikten sonra toprağa ekildi. 14 gün sonra bitkilere 0, 10^{-10}, 10^{-8} ya da 10^{-6} M 28-homobrassinolide (HBL) verildi. Ekimden 60 gün sonra NaCl muamelesi yapılan bitkilerde nitrat redüktaz ve karbonik anhidraz aktivitelerinde, klorofil muhtevasında ve P_N'de azalma görüldü. HBL ile muamele yukarıdaki bütün parametrelerde önemli artışa neden oldu. 10^{-8} M dahi HBL derişimi en iyi gelişime neden olmuş ve NaCl'nin zararlı etkisinin azalmasını sağlamıştır. Doku prolin gelişimi ise farklı reaksiyon göstermiştir. NaCl ve HBL muamelesindeki artış 10^{-8} M HBL ile 150 mM NaCl cevabı artırmış ve diğer muamelelerde artışlar gözlenmiştir. Sonuç olarak, bu çalışma ile HBL'nin strese karşı bitki için önemli bir koruyucu olduğu söylenebilir.

Anahtar Sözcükler: Karbonik anhidraz, klorofil, nitrat redüktaz, fotosentez, tohum verimi

Introduction

Brassinosteroids (BRs) have recently attained the status of plant hormones. They are ubiquitous in the plant kingdom and are reported to occur in all plant parts, including roots, and have the capability of long-distance transport (1). More than 35 years of research related to BRs has revealed that they elicit a wide spectrum of physiological processes in plants (2). In the recent past, researchers worldwide have focused their efforts on assessing the role of BRs in plants subjected to various stresses. It was concluded that BRs ameliorate various biotic and abiotic stresses (1).

Treatment of *Oryza sativa*, *Lycopersicon esculentum*, *Zea mays*, *Cucumis sativus*, *Triticum estivum*, and *Bromus inermis* with BRs increased their resistance to temperature stress. BRs treatment countered drought

stress in sugar beet and moisture stress in *Triticum estivum* (2). BRs also activated an antioxidative defense system in *Oryza sativa* seedlings grown under NaCl stress conditions (3).

In this study, the ability of 28-homobrassinolide (HBL) to modify the activity of certain enzymes and photosynthesis in *Brassica juncea* plants pre-treated with NaCl was investigated to explore possible remedial measures to counter salinity stress.

Materials and Methods

The seeds of *Brassica juncea* Czern & Coss cv. Varuna were obtained from the National Seed Corporation Ltd., New Delhi, India. Healthy seeds (around 1500) were surface sterilized with 5% sodium hypochlorite solution followed by repeated washing with double distilled water (DDW). The surface-sterilized seeds were then soaked in 0, 50, 100, or 150 mM NaCl solutions in petri plates for 6 h (duration based on earlier experiments). The soaked seeds were sown in earthen pots (25 cm in diameter) at the rate of 10 seeds per pot, and filled with sandy loam soil and farmyard manure in a 9:1 ratio. Thinning was performed 7 days after sowing (DAS) and 3 plants were maintained per pot. At 14 DAS the seedlings were treated with 0, 10^{-10}, 10^{-8}, or 10^{-6} M of HBL solution through their roots. These hormone solutions were percolated through roots in the soil at the rate of 30 cm^3 per plant. The plants were grown in a net house under natural environmental conditions. The average temperature, humidity, and day/night photoperiod were 22 ± 2 °C, 60%, and 10/14 h, respectively. The plant samples were collected 60 DAS to assess chlorophyll content, CA activity, P_N, NR activity, and proline content in leaves. Plants were harvested 140 DAS to assess seed yield.

NR activity (E.C. 1.6.6.1) was determined in fresh leaf samples using the procedure described by Jaworski (4). This method is based on the reduction of nitrate to nitrite, whose values were estimated calorimetrically. CA (E.C. 4.2.1.1) was assayed according to Dwivedi and Randhawa (5). Fresh leaf samples (200 mg) were cut into small pieces in 0.2 M cysteine hydrochloride solution. These pieces were transferred to test tubes containing phosphate buffer (pH 6.8). Solutions of sodium bicarbonate (0.2 M) and bromothymol blue were added to the reaction mixture. CO_2 liberated during catalytic action of CA on $NaHCO_3$ was estimated by titrating the reaction mixture against 0.05 N HCl using methyl red as an indicator. Total chlorophyll content was extracted in 80% acetone and estimated according to Mackinney (6). P_N was measured in fully expanded leaves with a portable photosynthesis system (LI-COR-6200, Lincoln, Nebraska, USA). Proline was extracted in 3% sulphosalicyclic acid solution and its concentration estimated as described by Bates et al. (7). At harvest, 4 plants representing each treatment were randomly selected for assessment of yield. Pods were collected and crushed, and the seeds were separated and weighed to record yield per plant. Each observation was repeated 4 times. Standard errors due to replicates were calculated (8).

Results and Discussion

Plants grown from seeds that received NaCl treatment prior to sowing exhibited a significant decrease in both NR and CA activity in leaves (Tables 1 and 2). Moreover, the decrease was proportional to the concentration of NaCl in the seed immersion medium; however, HBL, irrespective of its concentration, enhanced the activity of both these enzymes, compared to both water- and NaCl-treated controls. The 10^{-8} M concentration generated the best response, increasing NR and CA activity 17.6% and 30.7%, respectively, over the water-treated control. Furthermore, the 10^{-6} M and 10^{-8} M HBR treatments also significantly ameliorated the detrimental effects generated by 50 mM NaCl treatment, where the values were higher than those receiving 50 mM NaCl alone.

NR activity is determined by several external and internal factors. Campbell (9) has highlighted at least 4, which include (a) the availability of substrate (NO_3) at the level of cytoplasm; (b) the level of functional NR; (c) the activity level of functional NR; (d) the overall metabolic state of the plant. Salinity was found to affect nitrate uptake in at least 2 ways: by direct competition of chloride with nitrate, and at the membrane level and/or membrane proteins by changing plasmalemma integrity (10). This may have led to restricted nitrate influx, thus decreasing substrate availability. Since nitrate (substrate) is a key regulator of NR (11), the activity of NR decreased in response to saline stress. Moreover, the degradation/inactivation, and reduction in gene expression and NR-protein synthesis in response to NaCl stress (12) may be another cause of lower NR activity. Contrary to the above effect of NaCl, BRs favor uptake of

Table 1. The effect of HBL applied to roots on salinity-induced changes in chlorophyll (mg g^{-1}), P_N [µmol (CO_2) m^{-2} S^{-1}], and NR activity (µmol kg^{-1} leaf fresh mass) of 60-day-old *Brassica juncea* plants. (Data are the mean of 4 replicates ± SE).

NaCl (mM)	HBL (M)	Chlorophyll	P_N	NR activity
Control (0)	Control (0)	1.96 ± 0.15	17.63 ± 1.65	426 ± 26
	10^{-10}	2.12 ± 0.12	20.45 ± 1.78	439 ± 36
	10^{-8}	2.35 ± 0.18	26.65 ± 2.58	501 ± 29
	10^{-6}	2.32 ± 0.19	27.02 ± 2.35	491 ± 31
50	Control (0)	1.78 ± 0.11	16.41 ± 1.69	413 ± 21
	10^{-10}	1.90 ± 0.14	17.70 ± 1.84	426 ± 23
	10^{-8}	2.20 ± 0.16	21.00 ± 2.05	464 ± 25
	10^{-6}	2.22 ± 0.17	21.30 ± 2.11	451 ± 27
100	Control (0)	1.63 ± 0.10	15.23 ± 1.48	369 ± 19
	10^{-10}	1.80 ± 0.14	16.80 ± 1.68	389 ± 21
	10^{-8}	1.95 ± 0.19	20.15 ± 2.05	421 ± 23
	10^{-6}	1.92 ± 0.20	20.23 ± 1.93	423 ± 29
150	Control (0)	1.42 ± 0.11	14.50 ± 1.39	305 ± 18
	10^{-10}	1.65 ± 0.13	15.90 ± 1.59	323 ± 23
	10^{-8}	1.80 ± 0.12	18.45 ± 1.93	380 ± 24
	10^{-6}	1.74 ± 0.18	18.80 ± 1.78	377 ± 17

Table 2. The effect HBL applied to roots on salinity-induced changes in CA activity [mol (CO_2) kg^{-1} fresh mass S^{-1}] and proline content (mg g^{-1} fresh mass) of 60-day-old *Brassica juncea* plants, and seed yield (g $plant^{-1}$) at harvest. (Data are the mean of 4 replicates ± SE).

NaCl (mM)	HBL (M)	CA activity	Proline content	Seed yield
Control (0)	Control (0)	1.78 ± 0.11	4.50 ± 0.29	7.24 ± 0.54
	10^{-10}	1.88 ± 0.23	4.63 ± 0.36	8.13 ± 0.65
	10^{-8}	2.30 ± 0.18	5.24 ± 0.28	9.35 ± 0.34
	10^{-6}	2.39 ± 0.11	5.31 ± 0.28	9.42 ± 0.63
50	Control (0)	1.69 ± 0.10	5.23 ± 0.32	6.21 ± 0.29
	10^{-10}	1.77 ± 0.11	5.36 ± 0.28	7.16 ± 0.38
	10^{-8}	1.96 ± 0.15	5.92 ± 0.34	8.30 ± 0.45
	10^{-6}	2.04 ± 0.14	5.84 ± 0.28	8.32 ± 0.51
100	Control (0)	1.53 ± 0.10	5.98 ± 0.31	5.56 ± 0.36
	10^{-10}	1.68 ± 0.15	6.56 ± 0.28	6.69 ± 0.74
	10^{-8}	1.82 ± 0.14	7.12 ± 0.36	7.89 ± 0.38
	10^{-6}	1.76 ± 0.18	7.00 ± 0.39	7.94 ± 0.52
150	Control (0)	1.31 ± 0.11	6.63 ± 0.35	5.43 ± 0.63
	10^{-10}	1.58 ± 0.14	7.31 ± 0.39	6.56 ± 0.52
	10^{-8}	1.77 ± 0.17	7.89 ± 0.45	7.80 ± 0.36
	10^{-6}	1.71 ± 0.15	7.92 ± 0.52	7.76 ± 0.38

NO_3 (13) and have a profound influence on transcription and/or translation (14). These 2 processes could have led to increases in available substrate and the level of functional NR, which are among the regulatory factors described by Campbell (9). Therefore, the activity of NR increased in the HBL-treated plants. Moreover, the favorable effect of HBL on plants grown from seeds pre-treated with NaCl could be a cumulative expression of the ameliorative character of BRs in stressed plants (15).

CA catalyzes the reversible interconversion of CO_2 and HCO_3^- in plants, whose level is regulated by photon flux density, CO_2 concentration, and availability of zinc (16). Salinity stress is reported to cause stomatal closure, thereby decreasing CO_2 partial pressure (17). The fall in CO_2 levels in NaCl pre-treated plants seems to be the cause of the decrease in CA activity (Table 2). The increased level of CA in plants receiving HBL alone or a follow-up treatment with NaCl is probably an expression of the involvement of BRs in transcription and/or translation (14). The application of HBL also increased CA and NR activity in *Cicer arietinum* pre-stressed with NaCl (18).

The content of chlorophyll pigment and the P_N in the leaves of plants grown from NaCl-soaked seeds decreased proportionately with the concentration of NaCl in the soaking medium (Table 1); however, HBL increased both parameters. The 10^{-8} M and 10^{-6} M concentrations generated a comparable response. The 10^{-8} M concentration increased the chlorophyll content by 19.9% and the P_N by 50.9% over the water-treated control. Moreover, the 2 highest concentrations of HBL neutralized the effect of salinity, and values were comparable with those of the 50 mM NaCl-treated control.

The decrease in chlorophyll content in plants grown from NaCl-treated seeds may be the consequence of the activation of chlorophyllase (19), the enzyme that degrades chlorophyll, which is activated by various stresses. Furthermore, HBL treatment may have increased the chlorophyll content due to the activation of genes responsible for chlorophyll biosynthesis since the role of BRs in transcription and/or translation is well documented (14). Rubisco, the key enzyme that determines the P_N in plants, is regulated by a number of factors, including CO_2 concentration (20). Salinity stress may have decreased CO_2 availability by inducing stomatal closure (17); therefore, partly inhibiting rubisco activity (21) and, consequently, the P_N (Table 1). Moreover, the decrease in CA activity (Table 2) and lowered quantity of chlorophyll pigment (Table 1) may be the other reasons that the P_N decreased. In contrast to the above, BRs were found to activate rubisco (22), which may have activated the genes encoding the enzymes involved in photosynthesis. This may have resulted in an increase in the P_N in HBL-treated plants, whether soaked in water or NaCl solution; other causes may have increased chlorophyll content and elevated CA level (Tables 1 and 2). Similarly, BRs increased the chlorophyll content and P_N in other plants (22).

Proline content increased in response to both NaCl and HBL treatments (Table 2). Treatment of the plants with 150 mM NaCl alone generated maximum proline, a concentration that was 47.3% higher than the water-treated control. Moreover, 10^{-6} M HBL was the most effective concentration and significantly interacted with all levels of NaCl, most effectively with the 150 mM level. This combined treatment resulted in better performance than all other treatments and increased proline 76.0% higher than the water-treated control.

Increased proline is a general response of plants to various stresses, including salt stress (21). It has been assumed that the accumulation of organic solutes, including proline, in response to salt stress was involved in protection mechanisms, such as restoration of cell volume and turgor, reduction of cell damage induced by free radicals, and protection and stabilization of enzymes and membrane structure (23). Accumulation of proline is a highly regulated process, which is in turn controlled by both synthesis and degradation. Proline is synthesized from glutamate that is catalyzed to Δ^1-pyrroline-5-carboxylate (P5C) by the enzyme Δ^1-pyrroline-5-carboxylate synthetase (Δ^1P5CS), which is subsequently converted to proline by the Δ^1-pyrroline-5-carboxylate reductase (Δ^1P5CR) enzyme. However, it is degraded to P5C by proline dehydrogenase (ProDH) and further to glutamate by pyrroline-5-carboxylate dehydrogenase (P5CDH) (24). The activity of Δ^1P5CS and Δ^1P5CR (the enzymes of proline biosynthesis) were reported to increase, and that of ProDH to decrease, in cowpea grown under water stress (25). Similarly, Kishor et al. (26) reported an over-expression of the gene coding P5CS and repression of ProDH in some transgenic plants. We think that the principal reason for the accumulation of proline in plants pre-stressed with NaCl is a physiological

drought caused by NaCl (20), which may have activated the genes of proline biosynthesis and repressed those of its degradation. Similar results were reported for *Cicer arietinum* (21) and *Oryza sativa* (27). Moreover, the increase in proline levels in plants treated with HBL may have been the result of HBL's favorable impact on the activation of genes that are responsible for proline biosynthesis, thus offering more tolerance to saline stress. Brassinosteroids are also reported to increase the content of proline in rice subjected to low temperature stress (1-5 °C) (28) and sorghum grown under moisture stress (29).

Soaking seeds in NaCl prior to sowing also reduced the seed yield of the resulting plants at harvest. The maximum NaCl concentration (150 mM) had the most severe impact, resulting in a yield 25.0% less than that of the control; however, follow-up treatment with HBL neutralized the effect of NaCl. The moderate concentration (10^{-8}M) was the most effective, closely followed by the 10^{-6} M concentration, particularly in cases in which NaCl was at the minimum (50 mM) level.

The yield of any crop is determined by a number of factors. Physiological factors include the water relations of the plant, nutrient availability, photosynthesis and translocation of photoassimilates, hormonal balance, and, primarily, the attachment of leaves and flowers to the plant body. It is well documented that salinity elevates the level of abscisic acid and ethylene (30), which are accelerators of senescence (31). This may have resulted in leaves and flowers falling prematurely, degradation of chlorophyll (Table 1), and, consequently, decreased P_N (Table 1). NaCl stress also disrupts the translocation of assimilates (27) and decreases enzyme activity (Tables 1 and 2). All of these factors may have contributed to poor seed yield in NaCl-treated plants. BRs, on the other hand, have a favorable effect on plants: they delay senescence (1), increase chlorophyll pigment concentration and the P_N (22), facilitate translocation of assimilates (32), enhance enzyme activity (Tables 1 and 2) (22), and elevate the level of protein (14). All these factors may have countered the effect of salinity and improved physiological processes so as to ameliorate the detrimental effects of saline stress. Brassinosteroids also improved the yield of wheat grown under moisture stress (33) and chickpea grown under NaCl stress (18).

Acknowledgments

We thank Prof. John Pichtel, Natural Resources and Environmental Management, Ball State University, Muncie, Indiana, USA, for reviewing the manuscript. Financial assistance (SR/FTP/LS-A-37/2002) received from the Department of Science & Technology, Govt. of India, New Delhi, India is gratefully acknowledged by S. Hayat.

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