BIOLOGIA PLANTARUM 47 (1): 67-70, 2003/4

Influence of a brassinosteroid analogue on antioxidant enzymes in rice grown in culture medium with NaCl

M. NÚÑEZ*, P. MAZZAFERA**[1], L.M. MAZORRA*, W.J. SIQUEIRA*** and M.A.T. ZULLO***

*Departamento de Fisiología y Bioquímica Vegetal, Instituto Nacional de Ciencias Agrícolas, San José de las Lajas, CP 32 700, La Habana, Cuba**
*Departamento de Fisiologia Vegetal, IB, CP 6109, Universidade de Campinas, 13083-970, Campinas, SP, Brasil***
*Centro de Recursos Genéticos Vegetais, Instituto Agronômico, CP 28, 13001-970, Campinas, SP, Brasil****

Abstract

We studied the effects of a polyhydroxylated spirostanic brassinosteroid analogue (BB-16) on the activities of antioxidant enzymes in rice seedlings grown *in vitro* in culture medium supplemented with NaCl. Seedlings were grown in medium with 75 mM NaCl and 0.001 or 0.01 mg dm^{-3} BB-16 for 16 d or 3-d-old seedlings were exposed for 4 d to 0, 0.001 or 0.01 mg dm^{-3} BB-16 then further grown in medium with 75 mM NaCl without BB-16. Seedlings exposed to 0.01 mg dm^{-3} BB-16 for 16 d showed significant increase in the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) and a slight increase in ascorbate peroxidase (APX). On the other hand, 4-d exposure to BB-16 only increased SOD and CAT activities at concentration 0.001 mg dm^{-3}. GR activity was not altered by this BB-16 treatment. These results indicated that BB-16, which is structurally modified in the lateral chain in relation to natural brassinosteroids, changes the activity of key antioxidant enzymes, which might confer tolerance to saline stress.

Additional key words: ascorbate peroxidase, catalase, glutathione reductase, *Oryza sativa*, saline stress, superoxide dismutase.

Introduction

Reactive oxygen species (ROS) singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl (OH^-) are highly reactive, destroying lipids, nucleic acids and proteins (Foyer *et al.* 1994). Therefore their contents in the cells are controlled by an antioxidant defense system that includes several enzymes (ascorbate peroxidase, glutathione reductase, superoxide dismutase and catalase) and non-enzymic compounds (ascorbate, reduced glutathione, tocopherol, carotenoids and flavonoids). However, under stresses, such as drought, heavy metals, heat and chilling temperatures, salinity, air pollution, wounding, senescence and pathogen infection (Azevedo *et al.* 1998, Del Rio *et al.* 1998, Manchandia *et al.* 1999), the production of ROS is increased usually with an concomitant increase in antioxidants, suggesting that the antioxidant defense system may have a general role in the acquisition of tolerance by plants (Vitória *et al.* 2001).

Brassinosteroids (BRs) are naturally occurring plant growth regulators (Clouse and Sasse 1998), and among several stresses (Khripach *et al.* 1999) it has been reported that they might confer tolerance against salt stress (Takematsu and Takeuchi 1989). BR analogues have been assessed as plant growth regulators in field trials (Kamuro and Takatsuto 1999, Ramirez *et al.* 2000) and it has been shown that they might induce antioxidant responses in tomato under stress conditions (Mazorra and Núñez 2000, Mazorra *et al.* 2002). However, it is still

Received 28 April 2002, *accepted* 5 September 2002.
Abbreviations: APX - ascorbate peroxidase; BB-16 - biobras-16 brassinosteroid analogue; CAT - catalase; GR - glutathione reductase; SOD - superoxide dismutase; ROS - reactive oxygen species.
Acknowledgements: Support from the Third World Academy of Sciences and Brazilian National Research Council (CNPq, grant 170363/01-5) are gratefully acknowledged.
[1] Corresponding author; fax: (+55) 19 3788 6210, e-mail: pmazza@unicamp.br

unknown whether brassinosteroid analogues with little structural modification from natural brassinosteroids may protect plants against different kinds of stresses.

In this study the ability of a polyhydroxylated spirostanic brassinosteroid analogue to modify the activity of some key antioxidant enzymes in rice seedlings grown in culture medium with NaCl was investigated.

Materials and methods

Sterilized rice (*Oryza sativa* L. cv. J-104) seeds were germinated in Petri dishes containing MS solid medium (Murashige and Skoog, 1962) supplemented with 100 mg dm^{-3} inositol, 10 mg dm^{-3} thiamine, 2.5 mg dm^{-3} pyridoxine, 1.85 mg dm^{-3} nicotinic acid, 30 g dm^{-3} sucrose and 0.6 % purified agar. The culture medium pH was adjusted to 5.8 before autoclaving (20 min, 121 °C, 1.2 kg cm^{-2} pressure). The seeds were maintained under 16-h photoperiod (48 μmol m^{-2} s^{-3}, from cool white fluorescent lamps) and temperature 26 ± 2 °C.

Biobras-16 (BB-16), a commercial formulation with a polyhydroxylated spirostanic brassinosteroid analogue as active substance, $C_{27}H_{42}O_5$, Mr 446, was supplied by the Centro de Estudios de Productos Naturales de la Universidad de la Habana.

Six-d-old seedlings grown in the Petri dishes were transferred to culture tubes containing 15 cm^3 of MS basal solid culture medium supplemented with 75 mM NaCl and BB-16 (0, 0.001 and 0.01 mg dm^{-3}). The tubes with seedlings (12 per treatment) were maintained at irradiance of 48 μmol m^{-2} s^{-3} and 25 ± 2 °C over a period of 16 d. At the end of this period, the shoots were collected for enzyme extraction.

In a further experiment, 3-d-old seedlings were transferred to Petri dishes containing 25 cm^3 MS basal solid medium supplemented with BB-16 (0, 0.001, 0.01 mg dm^{-3}) for a period of 4 d. After that, seedlings (12 per treatment) were transferred to culture tubes containing 15 cm^3 MS basal solid medium supplemented with 75 mM NaCl but without BB-16. The light and temperature conditions were the same as described above and the shoots were collected for enzyme extraction 14 d later.

Plant tissue (0.5 g) was frozen and ground in liquid N_2 in mortar and pestle and homogenized in 5 cm^3 of extraction buffer (25 mM HEPES, pH 7.8, 0.2 mM Na_2EDTA). The homogenate was centrifuged (15 000 *g*, 4 °C, 20 min) and the supernatant was collected and stored at -20 °C for further enzyme analysis. Catalase (CAT; EC 1.11.1.6) and glutathione reductase (GR; EC 1.6.4.2) were assayed according Cakmak *et al.* (1993), and ascorbate peroxidase (APX; EC 1.11.1.11) and total superoxide dismutase activity (SOD; EC 1.15.1.1) according to Nakano and Asada (1981) and Giannopolitis and Ries (1977), respectively. Assays for CAT and SOD activities and SOD isoenzyme classification in native PAGE gels were also carried out (Vitória *et al.* 2001). Proteins in the extracts were determined according to Bradford (1976)

Results and discussion

Preliminary studies showed that 75 mM NaCl in the culture medium caused a 10 % reduction in the fresh mass of rice seedlings (data not published), suggesting that at this salt concentration a mild saline stress was induced. Therefore, this saline concentration was used to investigate the effects of BB-16 on the activity of key antioxidant enzymes.

Table 1. Activities [U g^{-1}(f.m.)] and specific activities [U g^{-1}(protein)] of antioxidant enzymes in seedlings germinated with BB-16. Means ± SE, $n = 12$.

	Treatments	CAT	SOD	APX	GR
Experiment 1	control	1.70 ± 0.22	0.110 ± 0.020	2.25 ± 0.75	16.25 ± 1.70
(activities)	0.001 mg dm^{-3} BB-16	3.90 ± 0.68	0.110 ± 0.010	2.50 ± 0.48	12.85 ± 0.44
	0.010 mg dm^{-3} BB-16	4.50 ± 0.57	0.190 ± 0.030	3.82 ± 0.83	23.75 ± 1.50
Experiment 2	control	14.10 ± 1.06	0.060 ± 0.005	7.30 ± 0.80	14.70 ± 1.22
(activities)	0.001 mg dm^{-3} BB-16	16.88 ± 1.35	0.180 ± 0.015	10.70 ± 0.82	13.90 ± 0.83
	0.010 mg dm^{-3} BB-16	24.72 ± 1.13	0.180 ± 0.004	6.32 ± 1.09	16.98 ± 1.33
Experiment 2	control	1.61 ± 0.12	0.007 ± 0.0006	0.83 ± 0.09	1.68 ± 0.14
(specific activities)	0.001 mg dm^{-3} BB-16	2.14 ± 0.17	0.023 ± 0.002	1.36 ± 0.10	1.76 ± 0.10
	0.010 mg dm^{-3} BB-16	2.06 ± 0.09	0.015 ± 0.0004	0.53 ± 0.09	1.42 ± 0.11

In the first experiment, the activities of all enzymes analyzed increased at the highest BB-16 concentration of 0.01 mg dm^{-3} (Table 1). CAT activity already increased at 0.001 mg dm^{-3} BB-16.

A different response was observed when the activities of the antioxidant enzymes were studied in rice seedlings derived from germinated seeds previously exposed to BB-16 and later grown in saline medium (Table 1). CAT activity only increased significantly at 0.01 mg dm^{-3} while SOD increased at both BB-16 concentrations (Table 1). APX only increased at 0.001 mg dm^{-3} and no response was observed for GR activity. To verify whether the observed changes in these antioxidant enzymes resulted from BB-16-induced modifications in the protein content, the specific activity was also calculated in this experiment (Table 1). When enzymatic activity was expressed as specific activity [U mg^{-1}(protein)] the response to BB-16 treatments was not completely different, but SOD activity at 0.01 mg dm^{-3} was reduced and CAT activity for both BB-16 concentrations became similar, but still greater than the control treatment.

Interestingly, higher CAT and APX values were observed in the controls from the second experiment. These differences might be due to seedling handling but also to the developmental stage of the seedlings used in experiments 1 and 2. CAT activity was much higher in three day germinated barley seeds than in three-week-old seedlings (Azevedo *et al.* 1998).

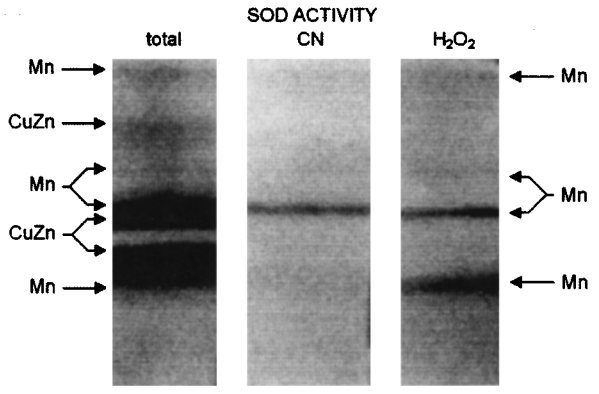

Fig. 1. Superoxide dismutase activity staining and isoenzymes classification in rice shoot extract. Gel slices were incubated with either KCN or H_2O_2 or no inhibitors (total activity). The negative display is presented for better visualization of the activity bands.

Activity staining of CAT and SOD in PAGE did not reveal any difference in the banding pattern among treatments (data not shown). A single activity band was observed for CAT and at least seven SOD isoenzymes were observed, some of very low activity. SOD isoenzyme classification using H_2O_2 and KCN allowed the identification of three CuZn-SOD (inactivated by KCN and H_2O_2) and four Mn-SOD (resistant to both inhibitors). Two CuZn-SOD accounted for the majority of the SOD activity in the gel (Fig. 1).

Previous reports showed that exogenous application of BRs modified antioxidant enzyme activity (Li and Van Staden 1998a,b). Chen *et al.* (1997) found that application of homobrassinolide increased SOD and peroxidase activities and decreased membrane lipid peroxidation in rice. Furthermore, Mazorra and Núñez (2000) and Mazorra *et al.* (2002) demonstrated that the effect of BRs and their analogues on antioxidant activity was dependent on concentration.

NaCl stress may impose an increased oxidative stress and treatments with brassinosteroids stimulated the growth of rice plants in field trials under saline conditions (Takematsu and Takeuchi 1989), therefore it was expected that rice seedlings treated with BB-16 were likely to show an enhanced antioxidant response in the saline growth medium. Indeed, our results showed variations in the activity of key antioxidant enzymes in rice seedlings grown either in the presence of BB-16 and NaCl (experiment 1) or pre-treated with BB-16 and then grown in NaCl without BB-16 (experiment 2). Therefore, H_2O_2 produced by SOD activity or by the presence of NaCl in the culture medium was removed by CAT in the peroxisome or by APX of the ascorbate-glutathione antioxidant cycle in the chloroplasts (Foyer *et al.* 1997). Since only CAT showed higher activity in the presence of BB-16, APX activity may play a minor role in the BB-16 induced detoxifying process and/or H_2O_2 is probably produced in higher concentrations in the peroxisome.

GR activity maintains the pool of glutathione in the reduced state, which in turn reduces dehydroascorbate to ascorbate. Increased expression of GR enhances tolerance to oxidative stress (Noctor and Foyer 1998). the GR activity was investigated to address the question of whether BB-16 in the saline medium causes change in the glutathione pool. An increase in GR activity was observed at 0.01 mg dm^{-3} BB-16 concentration in the first experiment supporting the fact that BB-16 may be an effective protection to saline stress.

The present study is evidence that developmental stage, concentration and period of time of brassinosteroid application are very important parameters in the study of the effects of BRs in plants. In addition, in the second experiment it was shown that BB-16 induced a durable effect, implying that BB-16 might trigger cellular signals that lead to long-term effects on antioxidant enzymes. Alternatively, BB-16 in its metabolically active forms, could be stored in the plant tissue, which activate or inhibit the activity of these enzymes. In addition, these results also showed that similarly to natural BRs, BB-16 was able to stimulate the activities of key antioxidant enzymes. Since it is known that the rice variety used in the present study (J-104) is sensitive to saline stress (M.C. González, personal communication) the BB-16 antioxidant induced activity needs to be confirmed in field trials with plants growing in saline soil.

References

Azevedo, R.A., Alas, R.M., Smith, R.J., Lea, P.J.: Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. - Physiol. Plant. **104**: 280-292, 1998.

Bradford, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 255-260, 1976.

Cakmak, I., Strback, D., Marschner, H.: Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. - J. exp. Bot. **44**: 127-132, 1993.

Chen, S.N., Li, J.N., You, H.L., Zhu, H.J., Qin, Z.B., Hong, G.M., Shen, Y.G.: The effect of a compound inducing cold resistance and homobrassinolide on the chilling resistance of plateau rice. - Acta bot. yunnanica **19**: 184-190, 1997.

Clouse, S.D., Sasse, J.M.: Brassinosteroids: essential regulators of plant growth and development. - Annu. Rev. Plant Physiol. Plant mol. Biol. **49**: 427-451, 1998.

Del Rio, L.A., Pastori, G.M., Palma, J.M., Sandalio, L.M., Sevilla, F., Corpas, F.J., Jimenez, A., Lopez-Huertas, E., Hernandez, J.A.: The activated oxygen role of peroxisomes in senescence. - Plant Physiol. **116**: 1195-1200, 1998.

Foyer, C.H., Decourvieres, P., Kunert, K.J. Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. - Plant Cell Environ. **17**: 507-523, 1994.

Foyer, C.H., Lopez-Delgado, H., Dat, J.F., Scott, I.M.: Hydrogen peroxide and gluthatione-associated mechanisms of acclimatory stress tolerance and signalling. - Physiol. Plant. **100**: 241-254, 1997.

Giannopolitis, C.N., Ries, S.K.: Superoxide dismutases. I. Occurrence in higher plants. - Plant Physiol. **59**: 309-314, 1977.

Kamuro, Y., Takatsuto, S.: Practical application of brassinosteroids in agricultural fields. - In: Sakurai, A., Yokota, T., Clouse, S.D. (ed.): Brassinosteroids: Steroidal Plant Hormones. Pp. 223-241. Springer-Verlag, Tokyo 1999.

Khripach, V.A., Zhabinskii, V.N., de Groot, A.E.: Brassinosteroids. A New Class of Plant Hormones. - Academic Press, San Diego 1999.

Li, L., van Staden, J.: Effects of plant growth regulators on the antioxidant system in callus of two maize cultivars subjected to water stress. - Plant Growth Regul. **24**: 55-66, 1998a.

Li, L., van Staden, J.: Effects of plant growth regulators on drought resistance of two maize cultivars. - South Afr. J. Bot. **64**: 116-120, 1998b.

Manchandia, A.M., Banks, S.W., Gossett, D.R., Bellaire, B.A., Lucas, M.C., Millhollon, E.P.: The influence of α-amanitin on the NaCl-induced up-regulation of antioxidant enzyme activity in cotton callus tissue. - Free Rad. Res. **30**: 429-438, 1999.

Mazorra, L.M., Núñez, M.: Brassinosteroid analogues differentially modify peroxidase activity, superoxide dismutase activity and protein content in tomato seedlings. - Cult. Trop. **21**: 29-34, 2000.

Mazorra, L.M., Núñez, M., Hechavarria, M., Coll, F., Sánchez-Blanco, M.J.: Influence of brassinosteroids on antioxidant enzymes activity in tomato under different temperatures. - Biol. Plant. **45**: 593-596, 2002.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant. **15**: 473-497, 1962.

Nakano, Y., Asada, K.: Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. - Plant Cell Physiol. **22**: 867-880, 1981.

Noctor, G., Foyer, C.H.: Ascorbate and glutathione: keeping active oxygen under control. - Annu. Rev. Plant Physiol. Plant mol. Biol. **49**: 249-279, 1998.

Ramirez, J.A., Centurion, O.M.T., Gros, E.G., Galagovsky, L.R.: Synthesis and bioactivity evaluation of brassinosteroid analogs. - Steroids **65**: 329-337, 2000.

Takematsu, T., Takeuchi, Y.: Effects of brassinosteroids on growth and yields of crops. - Proc. Jap. Acad. **B 65**: 149-152, 1989.

Vitória, A.P., Lea, P.J., Azevedo, R.A.: Antioxidant enzymes responses to cadmium in radish tissues. - Phytochemistry **57**: 701-710, 2001.