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Technical Note

EFFECT OF THE BRASSINOSTEROID ANALOGS BB-6 AND MH-5 ON PROTEINS METABOLISM IN SUGARCANE SOMATIC EMBRYOGENESIS [1/]

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RESUMEN

Efecto de los análogos de brasinoesteroides B6 y MH-5 sobre el metabolismo de las proteínas durante la embriogénesis somática en caña de azúcar. El B6 y el MH-5, fueron evaluados en caña de azúcar *(Saccharum* spp.), y su efecto correlacionado con el metabolismo de las proteínas. Callos con estructuras embriogénicas fueron cultivados con 2 concentraciones de BB-6 y MH-5 (0,001 y 0,01 mg l^{-1}, respectivamente). Los controles fueron un tratamiento sin hormonas y otro con NAA. La producción de embrioides no mostró diferencias entre los controles y los BRs en la concentración más alta. Los BRs influenciaron los niveles de proteínas solubles, proteínas de almacenamiento y los de prolina libre. Cambios en la prolina libre podrían indicar que los BRs participan en respuestas de estrés.

ABSTRACT

Brassinosteroid analogs (BRs), BB-6 and MH-5, were evaluated during sugarcane *(Saccharum* spp.) somatic embryogenesis, and their effect correlated to protein metabolism. Calli with embryogenic structures were cultured on a differentiation medium, supplemented with 2 concentrations of BB-6 and MH-5 (0.001 and 0.01 mg l^{-1}, respectively). A free hormone treatment and one with NAA were used as controls. Regarding somatic embryos production, no differences were found between control treatments and both BRs at high concentration. Both BRs influenced total soluble proteins, storage proteins, and free-proline levels. Changes in free-proline levels can be an indication that BRs may be involved in stress responses.

INTRODUCTION

Brassinosteroids (BRs) are recognized as a new kind of growth regulator, able to influence different physiological plant processes at very low concentrations. Then, BRs can affect plant elongation, cellular division, vascular development and reproduction (Núñez and Robaina 2000, Bishop and Yokota 2001, Friedrichsen and Chory 2001, Mussig and Altmann 2001). The biological activity of BRs sometimes resemble those of auxins, ethylene or gibberellins. In particular, interactions between auxin and BRs have been discussed. Mandava (1988) and Kim *et al.* (2000) demonstrated that BRs are involved in root gravitropism in an IAA-dependent manner. Also, BRs affect the biosynthesis of other hormones and enhance

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the ethylene production (Mandava 1988). Interestingly, 12-oxophytodienoate reductase-3 involved in jasmonate biosynthesis is upregulated by BRs, suggesting a relation between BRs and jasmonate signalling (Schaller *et al.* 2000). It should be noted that some sterol/BR mutations were recovered from genetic screens for other hormones, e.g. the *fk* mutant was observed in a screen for cytokinin mutants (Jang *et al.* 2000) and the *sax1* mutation in a screen for ABA/auxin mutants (Ephritikhine *et al.* 1999a). Overall, these results indicate that information can be gained using BR and other hormone mutants to dissect the interactions of hormone response in plant metabolism.

BRs could act synergistically with auxins or replace them. Biddington (1992) described synergisms of BRs with auxins and the stimulation of ethylene biosynthesis. Bais and Ravishankar (2002), mentioned changes in polyamine titres in response to pathogen infections and as an adaptation of plants to stress. Brassinolide at 0.005 mg l^{-1} was suitable for callus growth and shoot regeneration when used at 0.2 mg l^{-1} of IAA and 3.0 mg l^{-1} of BA on calli of *Spartina patens (Lu et al. 2003)*.

Dwarf and de-etiolated phenotypes of some Arabidopsis mutants were rescued by application of BRs (Bishop and Yokota 2001). Abe (1989) reported that BRs not only increased yield of sugarcane in the field, but also tolerance to stress and diseases, and decrease damages caused by herbicides. BRs are active at extremely low concentrations (0.1-0.001 mg l^{-1}). They can stimulate roots growth without causing any plant distortions. The effect on vegetative growth is particularly strong under adverse growing conditions (e.g. suboptimal temperature and salinity); therefore, BRs can be called "stress hormones". Also, they have shown a low residual toxicity (Núñez 1999).

There are few reports about biochemical events in plants when BRs are applied; thus, this study can provide an important contribution on the use of these new plant regulators in plant biotechnology. Our hypothesis is that BRs influence sugarcane morphogenesis through nitrogen metabolism. The objective of this study was to evaluate the effect of 2 spirostanic analogs of brassinosteroids, BB-6 and MH-5 on sugarcane somatic embryogenesis, and correlate their effect with protein metabolism.

MATERIALS AND METHODS

Plant material

Segments of immature inflorescences (3-5 mm long) of sugarcane (*Saccharum spp.*) var. CP52-43, a hybrid from CP43-64 and CP38-34, Canal Point, FL, USA were used for callus production.

Callus induction

Callus induction was initiated in Petri dishes containing 25 ml of MS (Murashige and Skoog 1962) medium, supplemented with arginine (50 mg l^{-1}) and 2,4-Dichlorofenoxyacetic acid (2,4-D, 13.5 μmol l^{-1}), for 21 days. After callus formation, they were maintained in culture medium with 4.5 μmol l^{-1} of 2,4-D. Agar agar as gelling agent was used. pH was adjusted to 5.7 before autoclaving. Calli were maintained in dark at 25°C.

Calli selection and treatments

Two-month old embryogenic callus were selected for each treatment, based on their morphology (Taylor *et al.* 1992). The differentiation medium used was the one described by Tapia *et al.* (1999). Treatments consisted of: a hormone free and MS basal medium with NAA (1 mg l^{-1}) as controls; BB-6 (0.001 mg l^{-1}); BB-6 (0.01 mg l^{-1}); MH-5 (0.001 mg l^{-1}); and MH-5 (0.01 mg l^{-1}).

Calli were placed in darkness for 7 days and then in culture chambers under fluorescent light with 16 h light at 50 μmol $m^{-2}s^{-2}$ photosynthetic photon flux density (PPF). Number of embryoids per 50 mg of callus, and their classification

as globular, early scutellar, late scutellar, and "germinated" stages, were evaluated at 21 days. An embryoid was considered germinated when both root and leaf were elongated.

Total soluble protein concentration was measured at 0, 7, 14 and 21 days. At 14 days, the time of maximum protein concentration, storage proteins and free-proline were determined. Five Petri dishes with 8 calli each were used per treatment. The experiment was repeated 3 times.

Biochemical analyses

Total soluble proteins. 50 mg of callus were crushed in liquid nitrogen using a glass rod in an Eppendorf tube containing borate-saline buffer pH=8.5 (Altherr *et al.* 1993). Extract was centrifuged at 48000 xg and 4°C for 1 h, and then stored at -20°C until determination. Quantification was carried out using BSA (1.0 mg l^{-1}) as binding agent (Bradford 1976).

Storage proteins. The procedure followed was the one described by Osborne (1924) starting with 250 mg of callus. Quantification was carried out as mentioned above.

Free-proline. 100 mg of callus were crushed in an Eppendorf tube with the aid of a glass rod following Bates *et al.* (1973).

Statistical analysis

The experiment was set up in a completely randomized design. Data were processed by Statgraphics (Version 2.0 for Windows). Statistical differences were determined using ANOVA and the Tukey Test. Percent data were transformed by means $2\arcsin[(x/100)^{0.5}]$ equation.

RESULTS

Figure 1 shows the behavior of free-proline in different treatments with BR analogs and both controls. The free-proline concentration was higher in the hormone free treatment, compared to the NAA, whereas both BRs reduced proline levels, especially at the lowest concentration of MH-5 (Figure 1); a treatment showing the higher percentage of embryoids in late stages. Low free-proline concentration correlates with total embryoids production.

The influence of the different treatments on embryoids production is shown in table 1. Even though, the highest concentration of BR evaluated produced more embryoids at 21 days than the control treatment, but less than the NAA treatment (hormone usually used for callus induction in sugarcane), no statistical differences were found. However, statistical differences were

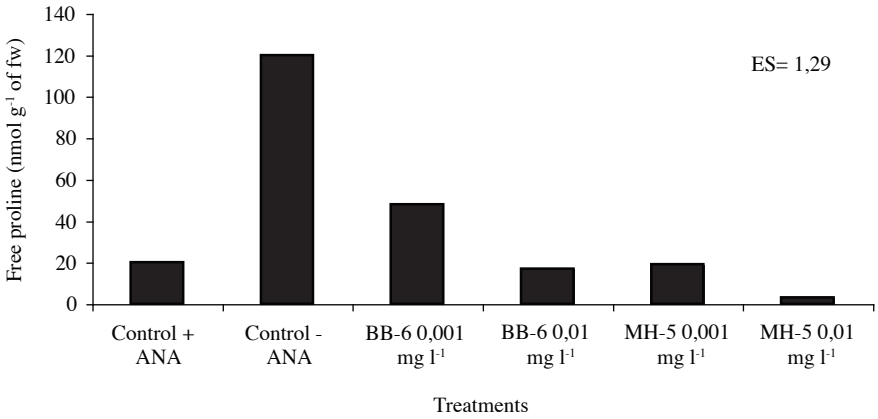

Fig. 1. Effect of BR analogs -BB-6 and MH-5- on free proline levels in calli of sugarcane *var.* CP-5243.

Table 1. Effect of the BR analogs BB-6 and MH-5 on sugarcane embryogenic callus

Treatments	Number of Embryoids*	Globular	Early Scutellar	Late Scutellar	Germinated
Control	151.6a	13.6b	29.1b	34.5ab	74.4b
NAA (5.37 μmol)	191.9a	15.0b	29.3b	40.3a	107.3a
BB-6 (0.001 mg l^{-1})	119.3b	14.2b	20.4bc	26.5b	58.2bc
BB-6 (0.01 mg l^{-1})	172.9a	16.2b	20.6bc	30.4b	105.7a
MH-5 (0.001 mg l^{-1})	102.0b	10.8b	17.2c	25.8b	48.2c
MH-5 (0.01 mg l^{-1})	183.7a	25.0a	43.8a	47.2a	67.0bc
SE	15.03	3.02	4.06	4.63	10.16
Significance	*	*	*	*	*

* Number of embryoids=embryoids per 50 mg of callus
Different letters in the same row indicate statistical differences at $p<0.05$).

observed between controls and MH-5 at 0.01 mg l^{-1}, the later producing the highest numbers of globular, early, and late scutellar embryoids. Germinated embryoids in the NAA and BB-6 (0.01 mgl^{-1}) indicates the early induction of root differentiation.

Table 2 is showing the behavior of the total soluble proteins of embryoids. These proteins concentration declined during differentiation in all treatments; although at 14 days -the beginning of embryoids differentiation- there was an increase with MH-5 and BB-6 (0.01 mg l^{-1}). At 21 days soluble proteins concentration was lower than the control treatment. As mentioned before, these treatments also showed the highest number of embryoids.

Storage proteins percentages are shown in table 3. In relation to albumins, the lowest concentration of both BRs induced the biggest

Table 2. Total soluble proteins (mg g^{-1} of fresh mass) in sugarcane callus treated with the BR analogs BB-6 and MH-5

Treatments	Time (days)			
	0	7	14	21
Control	199.03	129.87	123.91bc	65.54a
NAA (1 mg l^{-1})	199.03	130.88	108.87d	60.33a
BB-6 0.001 mg l^{-1}	199.03	129.73	116.34d	48.11b
BB-6 0.01 mg l^{-1}	199.03	129.63	155.17a	45.99b
MH-5 0.001 mg l^{-1}	199.03	121.79	133.45b	62.06a
MH-5 0.01 mg l^{-1}	199.03	125.89	143.02a	40.13bc
SE		7.02	4.06	4.63
Significance	NS	NS	*	*

Different letters in the same row indicate statistical differences at $p<0.05$).

Table 3. Storage proteins in callus of sugarcane treated with the BR analogs BB-6 and MH-5.

Treatments	Albumins	Globulins	Prolamins	Glutelins
Control	26.61±0.5	14.75±0.1	18.43±0.5	39.63±0.4
NAA (1 mgl^{-1})	45.41±0.6	16.51±0.2	14.22±0.6	17.43±0.6
BB-6 0.001 mgl^{-1}	66.12±1.1	7.86±0.1	8.35±0.4	16.20±0.3
BB-6 0.01 mgl^{-1}	54.77±0.3	22.61±0.4	7.91±0.3	11.68±0.2
MH-5 0.001 mgl^{-1}	52.99±0.3	18.40±0.3	7.91±0.2	13.29±0.3
MH-5 0.01 mgl^{-1}	38.58±0.5	27.77±0.4	7.91±0.3	19.23±0.4

Values are expressed in percentage ± SE

values, the opposite was observed in the case of globulins. Compared to both controls, prolamin levels decreased in all BRs treatments; a similar situation was observed for glutelins, except for MH-5 at 0.01 mg l^{-1} (Table 3).

DISCUSSION

Brassinosteroids are able to influence several physiological plant processes, including cell division and elongation, biomass accumulation, reproduction, vascular development, etiolation, interaction with other hormones and environmental factors, and ethylene biosynthesis induction (Sasse 1990). They may also have an effect in developing insects and fungi resistance (Sasse 1997).

Applications of brassinosteroids as 'anti-stress hormones', have been done by Takeuchi (1992) in rice, Wilen *et al.* (1995) in bromegrass for temperature stress, and Vasyukova *et al.* (1994) for disease stress. Since Hare *et al.* (1999) consider proline accumulation a mechanism of stress resistant; several authors stated that proline accumulation in plant tissues is the result of a decline in proline degradation, an increment in its biosynthesis, and proteins hydrolysis (Yoshiba *et al.* 1997, Hare and Cress 1997, Hare *et al.* 1999).

In rice, application of 24-epibrassinolide showed a decrease in electrolytes flow during freezing at 1-5ºC, indicating a reduced low temperature injury, most likely due to an improvement of the membrane stability and osmo-regulation. The hormone also provoked a decrease of malondialdehyde and reduced the superoxide dismutase activity; while the ATP and proline levels increased (Wang and Zeng 1993).

Our results imply that the BRs studied were involved in differentiation and maturation of sugarcane somatic embryos, caused by a decrease in proline synthesis. These results corroborate the hypothesis of the 'anti-stress' action of this new plant growth regulators. Since the percentage of embryoids reaching late stages did not exceed 50% in any treatment (Table 1). This can be the result of the action mode of these growth regulators, and a lack of knowledge of application time and the conditions required for the BRs's expression during differentiation in Gramineae.

Based on these results we consider that BRs BB-6 and MH-5 influenced proteins metabolism, in particular storage proteins, which are considered as an important nitrogen reserve in somatic and zygotic embryos.

Albeit, to our knowledge, these results constitute the first evidence of the effect of BRs on somatic embryogenesis in sugarcane, more detailed studies are recommended to determine the genetic origin of the protective attributes of BRs, as well as their role in proteins metabolism,

and of course to better understand their effect on stress tolerance mechanisms.

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