Short communication
BRASSINOSTEROID ANALOGUES DIFFERENTIALLY MODIFY PEROXIDASE ACTIVITY, SUPEROXIDE DISMUTASE ACTIVITY AND PROTEIN CONTENT IN TOMATO SEEDLINGS

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ABSTRACT. To explore if brassinosteroid (BR) analogues can affect the antioxidant capacity of tomato seedlings, the effect of exogenous application of two brassinosteroid analogues, BB6 and MH5, on peroxidase activity (POX), superoxide dismutase activity (SOD) and protein content three and six days after BR treatment was studied. Leaves from 15-day-old tomato seedlings were sprayed with MH5 (0.01 and 0.05 ppm) and BB6 (0.01 and 0.05 ppm). The changes on SOD and POX activity largely depended on the dosis applied and the type of analogue used. Total protein content was differentially changed by both MH5 and BB6; however, it depended less on the dosis applied and the analogue used. Results strongly suggest that these BR analogues may differentially regulate the antioxidant capacity of tomato seedlings through changes in several enzymatic components of this system depending on the dosis and structure of each analogue used.

Key words: brassinosteroids, antioxidant, peroxidase, seedlings, tomato

RESUMEN. Para explorar si análogos de brasinoesteroides pueden afectar la capacidad antioxidante de plantas jóvenes de tomate, se estudió el efecto de la aplicación exógena de dos análogos de brasinoesteroides, BB6 y MH5, en la actividad peroxidasa (POD), superóxido dismutasa (SOD) y el contenido de proteínas totales tres y seis días después de la aplicación. Se asperjaron hojas de plantas jóvenes de tomate de 15 días de edad con los análogos MH5 (0.01 y 0.05 ppm) y BB6 (0.01 y 0.05 ppm). Los cambios en la actividad de las enzimas estudiadas dependieron de las dosis aplicadas y el tipo de análogo utilizado. Ambos análogos modificaron diferencialmente el contenido de proteínas totales, aunque las variaciones fueron menos dependientes de la concentración y el análogo empleado. Los resultados sugieren que estos análogos cubanos de brasinoesteroides podrían regular diferencialmente la capacidad antioxidante de las plantas jóvenes de tomate a través de cambios en algunos componentes enzimáticos del sistema antioxidante, dependiendo de la dosis y la estructura de cada análogo utilizado.

Palabras clave: brasinoesteroides, antioxidante, peroxidasa, plántulas, tomate

INTRODUCTION

Since the discovery of brassinolide more than 40 natural analogues, collectively called brassinosteroids (BRs), have been isolated and characterized (1). BRs are ubiquitously distributed in the plant kingdom, and when exogenously applied at nanomolar to micromolar levels, they exhibit a wide spectrum of physiological effects, including promotion of cell elongation and division, enhancement of tracheary element differentiation, retardation of abscission, enhancement of gravitropic-induced bending, promotion of ethylene biosynthesis and enhancement of stress resistance (2). These responses depend on the brassinosteroid concentration used in the assay. Although BRs have been proposed to be considered a new class of plant hormones (3), their specific physiological functions and essential roles in plant growth have remained obscure. However, their importance in plant growth and development has recently gained acceptance, mainly due to the isolation of Arabidopsis and pea mutants that are defective in steroid biosynthesis (4).

One mechanism that may be involved in the resistance to many types of stress is the activity of the antioxidant pathway. The enzymes in this pathway include superoxide dismutase, peroxidase and catalase, as well as those of the ascorbate-glutathione cycle. High levels of these enzymes have been found in response to heat, chilling, salt, drought and wounding, as well as to oxidative stress. The overproduction of antioxidant enzymes under different kinds of stress may suggest that these proteins have a general role in the acquisition of tolerance by plants (5).

The aim of the present investigation was to study if the brassinosteroid spirostanic analogues synthesized in Cuba, MH5 and BB6, affect enzymatic components of the antioxidant system in tomato seedlings.

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MATERIALS AND METHODS

Growth and BR analogue treatment of plant. Tomato seeds (*Lycopersicon esculentum,* Mill), variety Amalia, were pregerminated over three days on Petri dishes under room temperature. Tomato seedlings were then grown on organic matter:zeolite (1:1) over 15 days in a growth cabinet at 25±2ºC (16 hours light) and 18±2ºC (8 hours dark). BR analogues were supplied by the Laboratory of Natural Products, Faculty of Chemistry, University of Havana, as 1000 ppm stock solutions in absolute ethanol. 15-day-old tomato seedlings were sprayed with MH5 (0.01 and 0.05 ppm) and BB6 (0.01 and 0.05 ppm). About 2 mL were applied per plant, keeping the leaves completely wet. Controls were sprayed with distilled water adjusted to optimal concentration of ethanol. Levels of SOD and POX activity were measured in young leaves of tomato seedlings during a 6-day-period after spraying with MH5 and BB6 (0.01 and 0.05 ppm). Protein content was also measured three and six days after analogue treatment.

Chemicals. Pirogallol and bovine serum albumin were obtained from Sigma Chemical Co. All other chemicals were of analytical reagent grade.

Enzyme extraction. Leaf tissue (0.25 g) was homogenized in 2 mL of 50 mM Tris-HCl (pH 7.5) containing $MgCl_2$ 8 mM, EDTA 3 mM and ß-Mercaptoethanol 2 % (v/v). The homogenate was centrifuged at 4°C for 15 min. at 20000 g. The supernatant was collected in two samples of 1 mL and was stored at -20°C until enzymatic activity and protein determinations were performed.

Superoxide dismutase assay. Superoxide dismutase activity was assayed according to the method of Beauchamp (6). SOD activity in extracts of leaf tissue was spectrophotometrically recorded at 420 nm. The assay was performed in a 3.0 mL cuvette at 25°C with an uv/vis kinetics spectrometer (model UNICAM 8630). The reaction mixture contained 50 mM Tris-HCl (pH 8.2); EDTA 1 mM and pirogallol 0.12 mM. One unit of superoxide dismutase was defined as the amount of enzyme which inhibited 50 % the rate of pirogallol autooxidation at 25°C and pH 8.2. All enzymatic measurements were performed in duplicates and statistically analyzed.

Peroxidase assay. Peroxidase activity was based on the method of Willstatter (7) with certain modifications. The assay measurement contained pirogallol 33 mM; H_2O_2 40 mM and Na_2HPO_4/NaH_2PO_4 100 mM (pH 6.8). The reaction was initiated at 25°C after addition of 0.1 mL enzymatic extract. One unit of peroxidase activity was defined as the amount of enzyme that caused an increase of 0.1 in the absorbance per minute at 25°C. All enzymatic measurements were performed in duplicates and statistically analyzed.

Protein assay. The protein content of the extract was determined by Bradford's method (8) with bovine serum albumin as the standard protein.

RESULTS

SOD activity depended on the dosis applied and the type of analogue. Therefore, 0.05 ppm of MH5 analogue enhanced SOD activity three days after sprayed. Following this abrupt increase, SOD activity declined over three subsequent days; however, its levels remained higher than the control (Figure 1A). By contrast, the SOD activity significantly decreased three days after treated with MH5 0.01 ppm following an increase attaining a similar level to 0.05 ppm at six days. SOD activity of control seedlings slightly diminished at six days. On the other hand, SOD activity remained relatively stable three days after sprayed with either BB6 concentration. Nevertheless, six days after sprayed, only the concentration of 0.01 ppm showed a significant increase (Figure 1B).

The changes on peroxidase activity depended also on the type of analogue and dosis applied. MH5-treated seedlings markedly increased peroxidase activity levels three days after treatment using both MH5 doses. However, six days after treatment, the concentration of 0.05 ppm reduced peroxidase activity whereas the concentration of 0.01 ppm had similar levels to control seedlings. BB6-treated seedlings showed a significant increase three days after applying 0.05 ppm. However, the concentration of 0.01 ppm decreased peroxidase activity at that time. At six days, peroxidase activity was unaffected by BB6. Peroxidase activity of controls changed significantly during the experiment (Figure 2 A and B).

Protein content depended less on the concentration of either analogue. Protein levels were unchanged by both MH5 concentrations three days after sprayed. Protein content was only increased by 0.05 ppm of MH5 at six days and no differences were found between MH5 (0.05 ppm) and MH5 (0.01 ppm) at that time (Figure 3A). Surprisingly, protein content in BB6-treated seedlings was slightly lower than control at three days using both BB6 concentrations. However, only 0.01 ppm of BB6 enhanced protein content six days after application. No variations on protein content of controls were shown over the experiment except a slight increase at three days (Figure 3B).

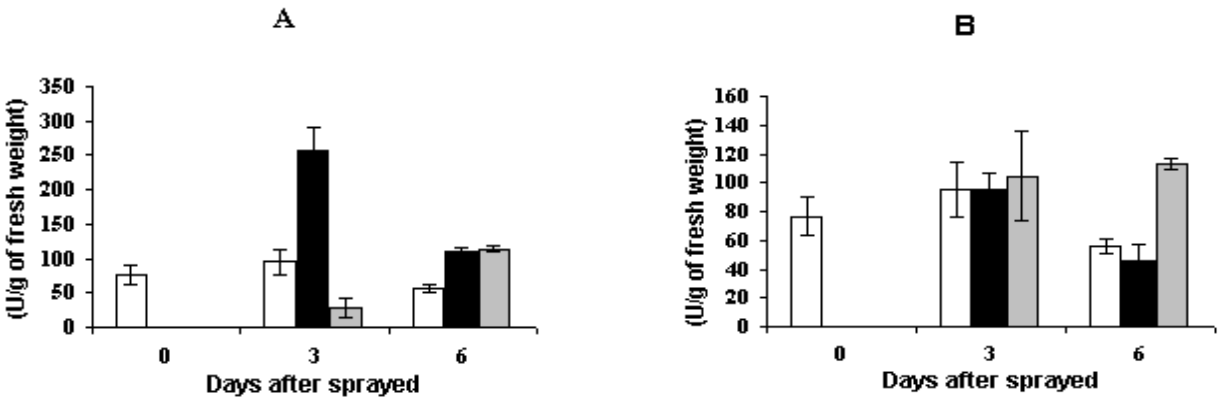

Figure 1 A. Superoxide dismutase activity of leaves from tomato seedlings sprayed either with MH5 0.01 ppm (gray bars) or with MH5 0.05 ppm (black bars). Controls (white bars). Lines above bars represent the SE (n=6). B. Superoxide dismutase activity of leaves from tomato seedlings sprayed either with BB6 0.01 ppm (gray bars) or with BB6 0.05 ppm (black bars). Line above bars represent the SE (n=6)

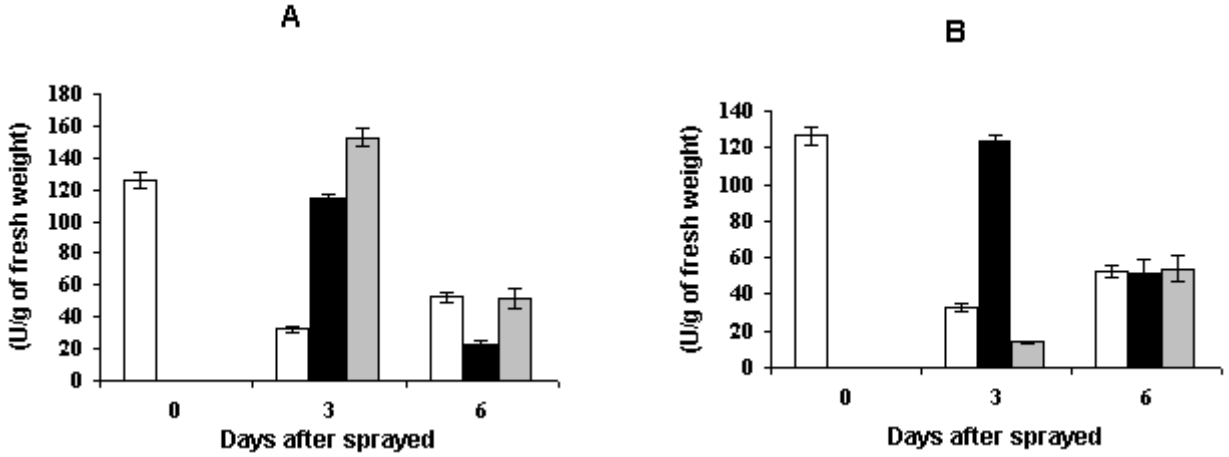

Figure 2A. Peroxidase activity of leaves from tomato seedlings sprayed either with MH5 0.01 ppm (gray bars) or with MH5 0.05 ppm (black bars). Controls (white bars). Lines above bars represent the SE (n=6). B. Peroxidase activity of leaves from tomato seedlings sprayed either with BB6 0.01 ppm (gray bars) or with BB6 0.05 ppm (black bars). Lines above bars represent the SE (n=6)

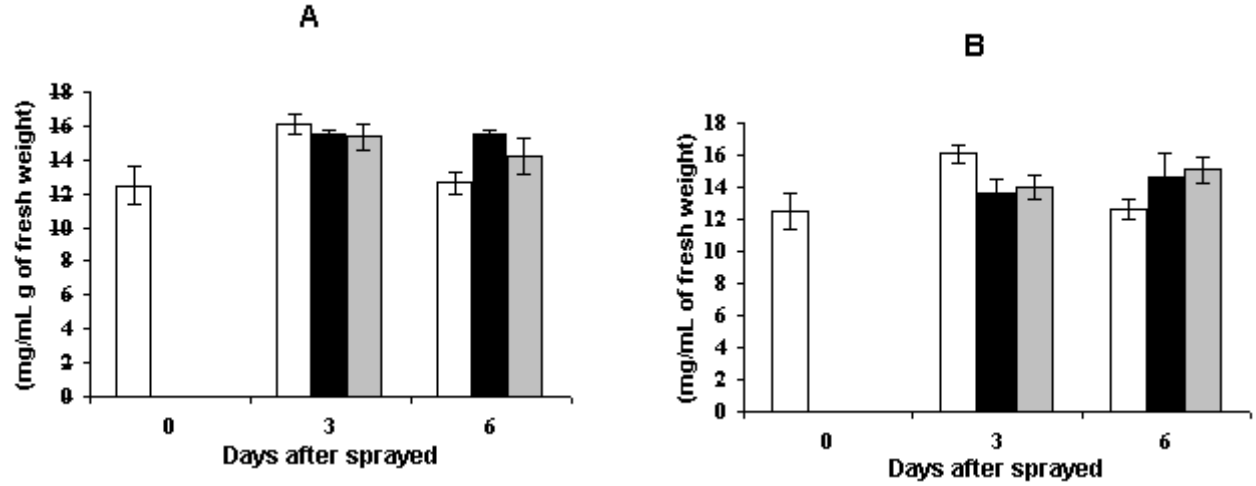

Figure 3A. Protein content of leaves from tomato seedlings sprayed either with MH5 0.01 ppm (gray bars) or with MH5 0.05 ppm (black bars). Controls (white bars). Lines above represent the SE (n=6). B. Protein content of leaves from tomato seedlings sprayed either with BB6 0.01 ppm (gray bars)or with BB6 0.05 ppm (black bars). Lines above represent the SE (n=6)

DISCUSSION

This is the first report, as far as we know, on BR analogues promoting changes on superoxide dismutase and peroxidase activity. This paper will be more concerned with discussing the importance of the effects of these BR analogues on these enzymes of the antioxidant system than on the influence of structural variations to each other for affecting that system. Several studies have shown that brassinosteroids alter the antioxidant capacity in plants mainly under stress conditions. Some authors (9) suggested that treatments with homobrasinolide increase the scavenging ability of activated oxygen in rice seedlings and strengthen the cold resistance of the varieties to some extent. In addition, others (10) concluded that the increased tolerance to drought stress induced by brassinolide in the resistant cultivar appears to be due to that maintenance of increased antioxidant enzyme activity (superoxide dismutase, catalase, ascorbate peroxidase) and antioxidant substance levels. It was demonstrated that under our conditions several treatments show an enhanced activity of each enzyme similar to that observed by the authors mentioned above; nevertheless, only tomato seedlings treated with MH5 (0.05 ppm) were able to increase both superoxide dismutase and peroxidase activity at three days after spraying, it may be suggesting a possible enhanced tolerance of this treatment to oxidative stress.

It was observed that epibrassinolide had no effect on superoxide dismutase activity at 22°C, but decreased the activity of this enzyme at 48°C. Epibrassinolide had no significant effects on peroxidase and catalase at 22°C (11). Similarly, BB6 analogue did not enhance significantly SOD and POX activity except at a concentration of 0.01 ppm (at six days) and 0.05 ppm (at three days) respectively. It may suggest that in spite of preliminary controlled laboratory experiments have indicated that the tomato leaf ultrastructure was less affected in BB 6-treated leaves under high temperature stress conditions (12), and that its promoting activity was higher under drought stress conditions (13), this BR analogue does not likely lead a putative anti-stress mechanism via modification of the SOD and POX activities.

Although it would be relevant to point out that neither superoxide dismutase activity nor peroxidase activity was simultaneously reduced at any treatment combination, it suggests that an enhanced oxidative injury, in terms of decreased peroxidase and superoxide dismutase activities, would not be likely taking place.

It has been reported that the superoxide dismutase (SOD), an enzyme catalyzing the reaction of superoxide radical (O_{2},) with itself to yield H_2O_2 and O_2, is the cell primary defense against damage by O_2, and that the peroxidase enzyme catalyzes the dehydrogenation of a large number of organic compounds, which consists of the transfer of hydrogen from a donor to H_2O_2. Since the enzymatic activities of both enzymes were differentially modified after MH5 and BB6 application, it is suggested that these BR analogues might regulate the levels of toxic oxygen species such as superoxide and peroxide radicals. It would be interesting to evaluate if the BR analogue-promoted changes on these enzymes lead to modifications of the amounts of reactive oxygen. Besides, those altered activities may be provoked as a consequence of qualitative and/or quantitative variations of isoenzymes. Therefore, it would also be interesting to test if the BR analogue-induced changes on these enzymes are mediated by changes on the levels of specific isoforms.

The fact that the effects of these analogues depended on dosis applied and that temporal changes of these enzymatic activities were also related to concentrations might indicate different mechanisms of brassinosteroid analogue-promoted antioxidant changes. The implications of this observation may be that selection of all experimental conditions at a useful model for studying the antioxidant metabolism regulated by brassinosteroids and their analogues should be well defined and controlled.

Although these results failed to show the extent to which a change to structure was more important than a change in the concentration itself, it is speculated that the structural modifications between both analogues strongly led to important changes in the activities of antioxidant enzymes. If it is assumed that both analogues (or their active metabolic forms) target the reception site at the same time, then the evidence that seedlings treated with the same concentrations of them provoked different enzymatic responses could explain our hypothesis.

BR-induced increase in protein content has been reported earlier (14, 15). Homobrasinolide application increased total soluble protein content under irrigated, stressed and recovered conditions. In spite of references cited have shown that these compounds increase protein content, under these experimental conditions, significant increases have only been found in the protein content at six days with 0.05 ppm of MH5 and 0.01 ppm of BB6. This finding suggests that these analogues may be involved in molecular process leading to protein content increase, but in a dose-dependent manner.

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