BIOLOGIA PLANTARUM 46 (3): 451-453, 2003

BRIEF COMMUNICATION

Differential expression of peroxidase isoenzymes in soybean roots treated with the benzothiadiazole

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Abstract

The protection compound benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) was applied to soybean roots or leaves in a dose of 20 cm^3 of a solution containing 25 $\mu g\ g^{-1}$ of the active ingredient. Electrophoretic profiles of chitinase and superoxide dismutase were not altered by the product. Increased activity of two root anionic peroxidases and three differential isoforms of these enzymes were observed in plants with roots treated by BTH, which can be used as biochemical markers of the BTH effect.

Additional key words: *Glycine max*, systemic acquired resistance.

Contact with pathogens can stimulate plants to activate mechanisms that make them more resistant to new infections. Induced resistance shown not only in the contact area but throughout the whole plant is named systemic acquired resistance (SAR). BTH is a chemical SAR inducer (Friederich *et al.* 1996, Görlach *et al.* 1996, Lawton *et al.* 1996) being developed for the purpose of disease control in commercial crops. SAR leads to gene transcription and protein synthesis that may vary among species. Increased activity of chitinase, β-(1,3)-glucanase, and peroxidase were detected in treated green bean plants (Siegrist *et al.* 1997). The objective of this work was to study the effect of BTH in soybean plants.

Soybean [*Glycine max* (L.) Merril] cv. IAC 8.2 seeds were pre-germinated and transplanted to pots containing 1.6 kg of an autoclaved soil and sand mixture (3:1; v/v). The temperature of the greenhouse varied from 18 to 30 °C and relative humidity from 50 to 90 %. The photon flux density averaged 900 $\mu mol.m^{-2}s^{-1}$ at noon on sunny days and 400 $\mu mol\ m^{-2}\ s^{-1}$ on cloudy days. The photoperiod was 12.1 h in the first assay and 13.2 h in the second assay. The plants were harvested 30 d after transplanting to pots and samples were stored at -20 °C.

Two assays were conducted, and the following treatments were randomly applied: *1*) control, *2*) BTH on leaves, manually sprayed, *3*) BTH on roots, placed in the soil next to the seedling, *4*) BTH on leaves and roots simultaneously. The product was applied on the 13th and 14th days after planting, using 20 cm^3 on leaves and 20 cm^3 on roots of a 25 $\mu g\ g^{-1}$ solution of the active ingredient of BTH (*Syngenta*, Basel, Schwitzerland), of molecular mass 210.3. Ten repetitions were used per treatment in the first assay and eight in the second assay.

The protein extraction was made by grinding roots or leaves with mortar and pestle in the presence of an buffer containing 34 mM K_2HPO_4, 0.2 M sucrose, 2.56 % polyvinylpyrrolidone (PVP-40); 3 mM dithiotreitol (DTT), 5.7 mM L-ascorbic acid, 5.8 mM diethyl-

Received 27 February 2001, *accepted* 4 December 2001.
Abbreviation: BTH - benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester.
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dithiocarbamic acid (DIECA), 2.6 mM $NaHSO_4.H_2O$, 2.5 mM $Na_2B_4O_7.10\ H_2O$, 0.2 % 2-mer-captoethanol, and 1 % polyethylene glycol (m/v). The suspensions were then transferred to tubes, centrifuged at 3 026 *g* for 30 min at 4 °C, and the supernatant was collected. Protein concentration in each sample was determined according to Bradford (1976), using bovine albumin as a standard. Native proteins were separated by polyacrylamide gel electrophoresis (PAGE) under alkaline conditions, according to the procedure described by Davis (1964).

The solutions used for visualisation of peroxidase activities were: *1*) acetic acid containing 2 mM *o*-dianizidine and 0.3 % H_2O_2; *2*) 2.7 mM benzidine, 1 % H_2O_2, 77 % ethanol, and 5 % acetic acid; *3*) phosphate buffer (pH 6.0) containing 0.1 % H_2O_2 and 0.5 M guaiacol; and *4*) 0.1 M sodium phosphate buffer (pH 7.2) containing 0.072 % (m/v) catechol, 0.072 % (m/v) N,N-dimethyl-phenylenediamine, and 0.072 % (m/v) thiourea. The amount of root protein applied was standardized at 30 μg per sample, except the case where catechol was the substrate, when 60 μg of protein per sample were applied. The gels were prepared with 7 % polyacrylamide, except when guaiacol was the substrate, in which case they contained 10 % polyacrylamide. For observation of superoxide dismutases, the gels were placed under illumination in a solution containing 0.004 % (m/v) riboflavin, 0.3 % (m/v) Na_2EDTA, and 2 % (v/v) dimethylthiazol(-diphenyl)tetrazolium bromide (MTT) in 0.05 M Tris-HCl pH 8.5. Polyacryl-amide concentration was 8.9 or 12 %. Chitinases isoforms were observed in gels prepared as described by Trudel and Asselin (1989), using 15 % polyacrylamide gels. For each sample, 25 μg of proteins were applied.

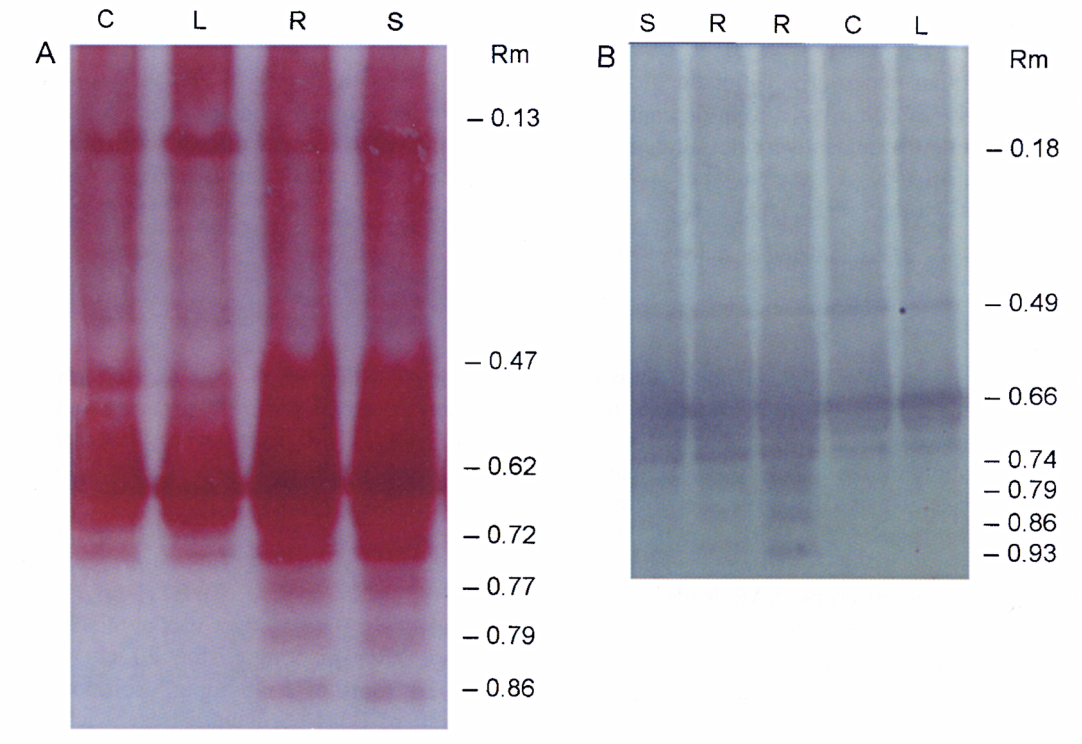

Fig. 1. Proteins of roots stained with guaiacol (*A*) or in solution containing catechol (*B*). Each well received soybean protein extract of one plant. Relative mobilities (Rm) are indicated in the right margins. C - control; L - BTH applied on leaves; R - BTH applied on roots; S - simultaneous application on leaves and roots.

At least four bands were observed in the profiles of root protein extracts of control plants on native activity-stained gels developed in the presence of the substrates guaiacol, *o*-dianizidine, or catechol. Two bands showed even greater activity in the protein extracts of plants in which BTH was applied to roots, when compared to the control plants; and three weaker, more mobile bands were observed. Application of BTH onto the leaves did not produce any detectable effect. Plants treated on leaves only or on roots and leaves simultaneously presented equal profiles (Fig. 1*A,B*). The relative mobilities were consistent for all substrates, suggesting that a single class of enzyme is functional. The peroxidase nature of the enzyme is indicated, because guaiacol (Rudolph and Stahmann 1964) is considered a specific substrate for peroxidases and catechol (Macko *et al.* 1967), and *o*-dianizidine (Brune and Alfenas 1998) are peroxidase substrates, although unspecific. The peroxidase activity of an enzyme with unspecific substrate is consistent with the properties of the enzyme EC 1.11.1.7. The electro-

phoretic profile was the same using plants from both assays, showing repeatability of the BTH effect. Thus the differential isoforms can be used as biochemical markers of the effect of the application of BTH on soybean roots, indicating how long the product has an effect on the field.

In the gel incubated in a solution containing benzidine, only one band was revealed, and this was of lesser intensity in the root protein extracts of plants not treated with BTH on roots, with or without simultaneous application on leaves. This band had equivalent relative mobility to the most intense band revealed by the other peroxidase substrates, which also presented higher activities when extracts were from plants treated on roots.

Differences between treatments were not observed in gels developed for chitinases or superoxide dismutases, neither for root nor for leaf protein extracts.

Peroxidase induction has been observed in host plants after pathogen infection. Peroxidase takes part in the last step of lignin biosynthesis, which is synthesised in plants after contact with pathogens (Vance *et al.* 1980). Peroxidases are capable of producing hydrogen peroxide, which has a direct function in the defense of plants against pathogens because it diffuses through the cellular membrane and can be toxic to microorganisms. It also can have an indirect function, serving the plant cell as an intermediary messenger in the signalling of induction of resistance (Hammond-Kosack and Jones 1996). Anionic peroxidase is considered a marker of SAR (Siegrist *et al.* 1997). The induction of new peroxidase isoforms in the present work is consistent with the hypothesis that BTH also induces SAR in soybeans.

In the plants that received BTH on the leaves only, induction of new isoforms or increase of intensity of bands was not observed, suggesting that BTH has greater efficiency when applied to roots.

A question associated to the usefulness of protection compounds in commercial cultures is whether or not their activation of defence mechanisms is significant in the field, since under these conditions they also may be activated by incompatible pathogens and micro-symbionts. Moving from greenhouse to the field should be possible with the commercial liberation of protection compounds such as BTH. Biochemical markers could become important tools in understanding the frequency and intensity with which the biotic incentive is enough to activate the defence mechanisms, and whether there is a synergistic effect between chemical inducer and biotic stimulus.

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