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Benzothiadiazole as an inducer of β-1,3-glucanase and chitinase isozymes in sugar beet

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Abstract

The effect of benzothiadiazole (BTH) on protein synthesis was studied in sugar beet plants. Extracellular proteins induced by 0.025 % BTH were examined and their pattern was compared with that induced by sodium salicylate, chitosan, paraquat, $AgNO_3$, and by tobacco necrosis virus. BTH induced synthesis of at least 9 acidic and 6 basic proteins; three of them appeared as acidic chitinase isozymes, three as acidic β-1,3-glucanase isozymes, three as basic chitinase isozymes, and one as a basic β-1,3-glucanase isozyme. One of the basic chitinase isozymes was found also in control plants. The most of the newly formed proteins was also induced by the other inducers under study regardless of the necrotic or symptomless reaction of plants. The benzothiadiazole proved to be an efficient inducer of proteins in sugar beet.

Additional key words: *Bion*, chitosan, paraquat, pathogenesis-related proteins, salicylic acid, tobacco necrosis virus.

Introduction

Inoculation of plants with pathogens or the treatment with some chemical compounds can result in the establishment of systemic induced (acquired) resistance (SAR) (Ryals *et al.* 1996). SAR is usually accompanied by synthesis of pathogenesis-related proteins (PR-proteins) (Linthorst 1991). It has been shown that some PR-proteins have β-1,3-glucanase (PR-2 group) or chitinase (PR-3 group) activity at least *in vitro* (Broekaert *et al.* 1988, Jacobsen *et al.* 1990). They are suggested to play a role in the

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Abbreviations: BTH - benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester, IF - intercellular fluid, INA - 2,6-dichloroisonicotinic acid, PQ - paraquat, PR-proteins - pathogenesis-related proteins, SAR - systemic acquired resistance, TNV - tobacco necrosis virus.

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defense ability of the plant, *e.g.*, they are capable of degrading fungal cell wall polysaccharides and so could inhibit fungal growth (Roulin and Buchala 1995), and could also cleave bacterial cell walls (Bernasconi *et al.* 1987). Low levels of these proteins are also present in uninfected plants where their concentrations change during maturation, caryopsis development and germination (Seetharaman *et al.* 1996, Repka *et al.* 1997).

A number of exogenously applied chemicals, salicylic acid, polyacrylic acid, inorganic salts, chitosan, a minor component of the fungal cell wall, free oxygen radicals producing compounds such as paraquat, ozone, and others have been shown to induce extracellular proteins and/or resistance to subsequent infection with viruses, fungi or bacteria (Van Loon 1985, Van Loon and Antoniw 1982, Hadwiger and Wagoner 1983, Tamás *et al.* 1997). Salicylic acid and its synthetic derivates 2,6-dichloroisonicotinic acid (INA) and benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Kunz *et al.* 1997) (Fig. 1) have been proved to be efficient inducers of local and systemic resistance to pathogens in a number of plants (Nielsen *et al.* 1994, Métraux *et al.* 1991, Ward *et al.* 1991, Uknes *et al.* 1992, Lawton *et al.* 1996, Malamy and Klessig 1992). BTH, the member of a new category of compounds for plant protection called 'plant defense activators', induced resistance to a number of fungal and viral pathogens in *Arabidopsis* (Lawton *et al.* 1996), in wheat (Görlach *et al.* 1996), and in tobacco (Friedrich *et al.* 1996).

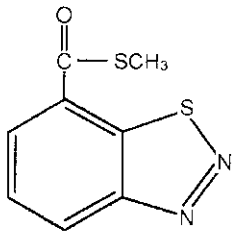

Fig. 1. Structure of BTH.

In sugar beet plants, the presence of newly synthesized proteins in extracellular fluid was reported by Fleming *et al.* (1991), who detected both acidic and basic proteins as a result of tobacco necrosis virus (TNV) infection and salicylic acid treatment. The induction of protein synthesis, their characterization, histological localization and expression of both β-1,3-glucanase and chitinase genes were intensively studied in sugar beet infected with *Cercospora beticola* (Nielsen *et al.* 1994, Nielsen *et al.* 1993, Gottschalk *et al.* 1998, Berglund *et al.* 1995).

As far as we know, there is no literature report on the effect of benzothiadiazole on sugar beet. The aim of the work presented in this paper, was to study the effect of BTH on the induction of PR-proteins in sugar beet plants and to compare their spectrum with the spectrum induced by some other known inducers. The results obtained will serve in future studies of induced resistance of sugar beet to pathogens.

Material and methods

Plants: Seeds of sugar beet (*Beta vulgaris*) cv. Rimini were sown into moisture sand. After one week, seedlings were transplanted to pots filled with *Perlit*, and regularly watered with a half strength Steiner (1984) nutrient solution. Plants were grown under a 16-h photoperiod (irradiance of 60 μmol m^{-2} s^{-1}) and temperature of 24 °C.

Inducer treatment: Leaves of 5-week-old sugar beet plants were sprayed with 0.025 % BTH (and/or 0.01 % and 0.05 % in experiments dealing with concentration dependence), 10 mM $AgNO_3$, 20 mM sodium salicylate (SA), and 0.75 % chitosan, respectively, or 5 mm^3 droplets of 0.1 mM paraquat solution were applied to the adaxial surface of three intact leaves. The optimum concentrations were assessed in previous experiments as well as the screening for the most convenient cultivar (data not shown). Chitosan solution was prepared from crab-shell chitosan (*Sigma Chemical Company*, St. Louis, USA) as described by El Ghaouth *et al.* (1992). Control plants were treated with distilled water. Four days after the treatment, leaves were used for intercellular fluid (IF) extraction.

TNV was inoculated on carborundum dusted leaves at the same time as chemical inducers. Inoculum was prepared from fresh TNV infected tobacco leaves displaying lesions. Leaves were harvested and IF extracted 8 - 10 d after inoculation.

Extraction of intercellular fluid: Freshly harvested leaves were used for the extraction of intercellular fluid (IF) using the method as described by Pierpoint *et al.* (1987), and centrifuged at 1 000 *g* for 10 min.

Protein content in IF was determined according to Bradford (1976) using bovine serum albumin as a standard.

Electrophoresis: Discontinuous nondenaturing polyacrylamide gel electrophoresis (PAGE) in 1 mm thick 12.5 % resolving gel and 4 % stacking gel was performed to analyse acidic (Laemmli system) and basic (Reisfeld system) proteins (Hames and Rickwood 1990), using the *Mighty Small II* apparatus (*Hoefer Scientific Instruments*, San Francisco, USA). All separations were performed at constant current of 25 mA. Gels were silver stained (Hames and Rickwood 1990). The amount of proteins loaded to each well corresponded to IF derived from 0.2 g (8 - 14 μg of proteins) and 0.4 g of fresh leaf tissue for silver staining and enzyme activities detection, respectively.

Detection of enzyme activities of protein bands: Chitinases were detected in overlaying gel containing 1 % glycolchitin (Trudel and Asselin 1989), bands with chitinase activity appeared as dark bands on the Calcofluor white stained gel. The β-1,3-glucanase activity was detected directly in the gel by the method of Shimoni (1994) using 2 % laminarine solution as a substrate and 2,3,5-triphenyltetrazolium chloride for visualization of the hydrolyzed products.

Chemicals: Benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester, CGA-245 704, was kindly supplied by Willy Ruess, *Novartis Crop Protection AG*, Basel,

Switzerland, as a water dispersible granule with 50 % active compound (trade name *Bion 50WG*). Other chemicals were purchased from *Sigma Chemical Company* (St. Louis, USA).

Gel analysis: Documentation of the gels was provided using the Laboratory Universal Computer Image Analysis (*LUCIA*) system (*Laboratory Imaging*, Prague, Czech Republic).

Results

Visual symptoms: Spraying of plants with all concentrations of BTH did not cause any visible symptoms on leaves. Slight yellowing of sodium salicylate treated leaves was recorded 10 d after the treatment; chitosan solution and $AgNO_3$ induced minute necrosis 2 - 4 d after the induction. After paraquat application, firstly translucent spots of 5 mm in diameter appeared, which became necrotic after next 2 d. Plants inoculated with tobacco necrosis virus started to form necrotic local lesions 5 d after inoculation. The maximum number of lesions was observed between day 7 and 10. In this time, leaves with the highest amount of lesions were used for IF preparation.

Induction of PR-proteins: The BTH, SA, chitosan, paraquat, $AgNO_3$ and tobacco necrosis virus were used for the induction of proteins. Compared to distilled water treated control, the 0.025 % BTH induced increased accumulation or *de novo* synthesis of 9 acidic (Fig. 2*A*) and 6 basic (Fig. 3*A*) extracellular proteins. Similarly to other inducers used in experiment, the bands of acidic proteins displaying chitinase activity appeared at R_f 0.53, 0.62 and 0.64 (Fig. 2*B*) and β-1,3-glucanase activity (Fig. 2*C*) at R_f 0.53, 0.56 and 0.42, respectively.

The protein of R_f 0.45 without glucanase or chitinase activity was found only in BTH treated, TNV infected and paraquat stressed plants (Fig. 2*A*). The intensity of the band corresponded with the concentration of BTH used for induction (Fig. 4*A*). The most pronounced difference in the pattern of acidic proteins between control and treated plants was found following the paraquat induction, where the band of R_f 0.30 was strongly enhanced and proteins of R_f 0.27, 0.47 and 0.49 markedly increased (Fig. 2*A*). The protein of R_f 0.27 was synthesized exclusively under oxidative stress caused by paraquat.

The pattern of basic proteins analyzed by Reisfeld buffer system in BTH sprayed plants was similar to that of the other inducers (Fig. 3*A*). Three basic isozymes of chitinases and one β-1,3-glucanase isozyme were detected (Fig. 3*B,C*). With the exception of TNV and $AgNO_3$, the bands showing chitinase activity of R_f 0.57 and glucanase activity of R_f 0.39 appeared, the chitinase isozyme of R_f 0.25 was induced by all inducers. On the other hand, the chitinase isozyme of R_f 0.37 was present both in treated and control plants.

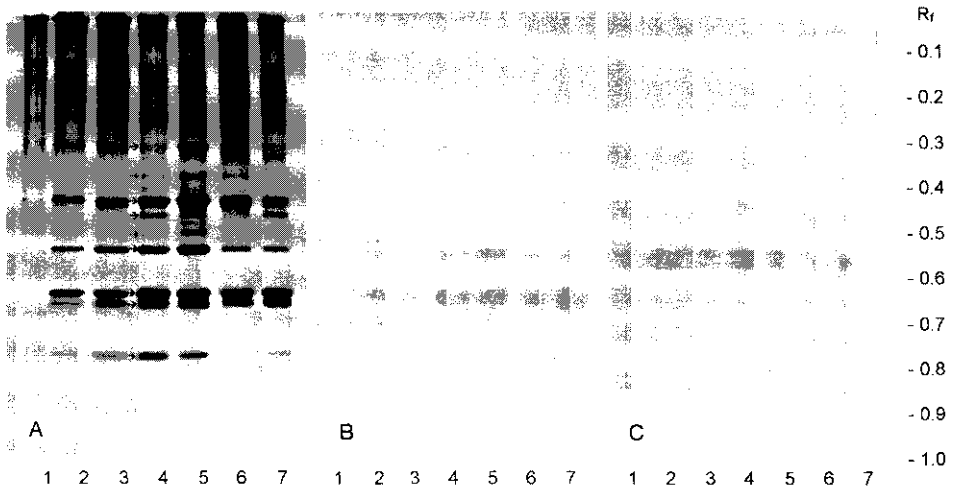

Fig. 2. Analysis of sugar beet acidic extracellular PR-proteins induced by chemical treatment or virus infection by electrophoresis in a 12.5 % polyacrylamide gel under native conditions (Laemmli system). *A* - silver stained proteins, *B* - chitinase activities in the gel, *C* - β-1,3-glucanase activity. Newly synthesized proteins following BTH treatment are marked by *arrows*. *Lanes: 1* - water treated control plants; *2* - 20 mM sodium salicylate; *3* - 0.75 % chitosan; *4* - 0.025 % BTH; *5* - 0.1 mM paraquat; *6* - 10 mM $AgNO_3$ and *7* - TNV infection.

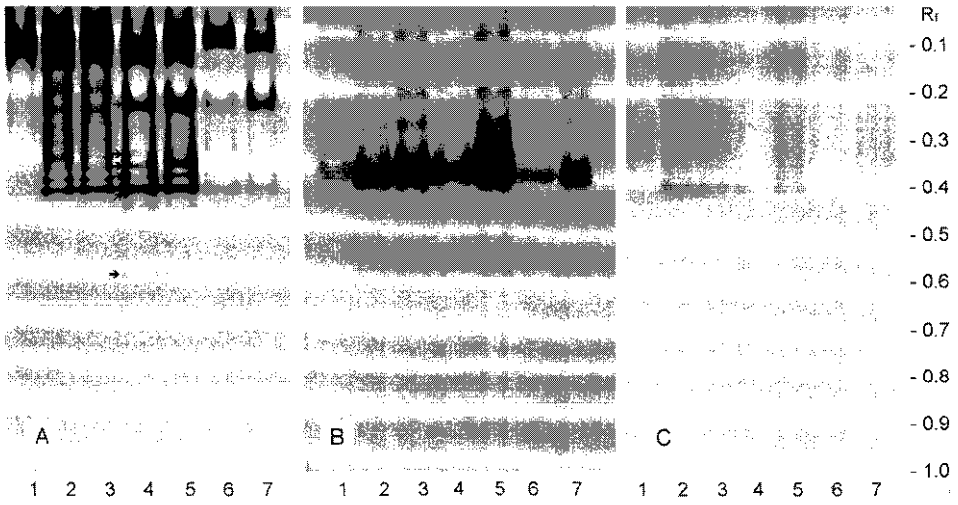

Fig. 3. Analysis of sugar beet basic extracellular PR-proteins induced by chemical treatment or virus infection by electrophoresis in a 12.5 % polyacrylamide gel under native conditions (Reisfeld system). See Fig. 2 for details.

Discussion

In the experiments, a benzothiadiazole efficiently induced a wide spectrum of PR-proteins including proteins displaying glucanase and/or chitinase activities. The detection of these hydrolases is not consistent with results of Nielsen *et al.* (1994), who used a similar compound INA for the induction of resistance of sugar beet to *Cercospora beticola*. Interestingly, they reported that the resistance against the pathogen had been established in spite of the accumulation of β-1,3-glucanase and chitinase gene transcripts had not been observed. Although INA and BTH are related

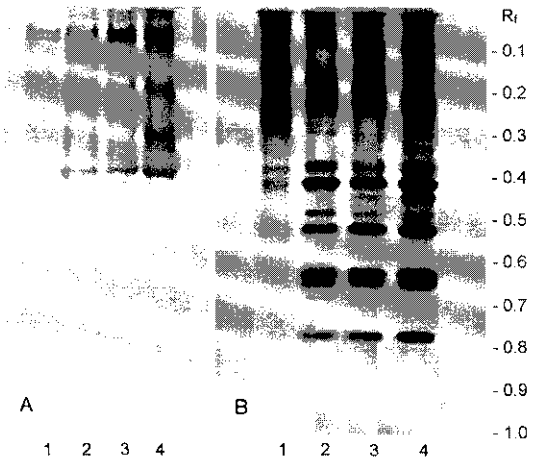

Fig. 4. Analysis of sugar beet extracellular PR-proteins induced by increasing concentrations of BTH by electrophoresis in a 12.5 % polyacrylamide gel under native conditions: *A* - basic proteins (Reisfeld system), *B* - acidic proteins (Laemmli system), *1* - control; *2* - 0.01 % BTH; *3* - 0.025 % BTH; *4* - 0.05 % BTH.

compounds, INA most likely primes sugar beet cells for fast activation of defence genes whereas BTH switches on these genes directly after the treatment. The induction of PR-proteins in BTH-treated sugar beet corresponds with findings of Lawton *et al.* (1996) and Friedrich *et al.* (1996), who found increased accumulation of mRNAs from β-1,3-glucanase and/or chitinase genes in *Arabidopsis* and tobacco, respectively, in addition to other SAR-associated genes. On the other hand, the response to BTH in monocot system may differ from these results. In wheat, the onset of the resistance to several fungal pathogens after BTH spraying was accompanied by the accumulation of five cDNAs which revealed no homology with previously described SAR genes (Görlach *et al.* 1996).

To compare the effect of BTH with various inducers of SAR, several compounds were used: SA as a key chemical inducer, chitosan as an elicitor, silver as metal, paraquat as an oxidative stress mediating compound and TNV as a pathogen. The most of the BTH-induced proteins was also induced by the other inducers under study regardless of the necrotic or symptomless reaction of plants. Accumulation of

defence-related proteins in connection with abiotic stress and elicitors was found previously in different plant species. Paraquat, similarly to ozone, mediates oxidative damage of plant tissues through the production of reactive oxygen species. Paraquat, used in our experiment, induced effectively sugar beet proteins including β-1,3-glucanase and chitinase isozymes, that was in accordance with Ernst *et al.* (1992), who reported markedly increased the mRNA level of basic β-1,3-glucanase, basic chitinase, as well as PR-1b, in tobacco treated with ozone. Similarly, chitosan, which elicits various defence responces, enhanced activity of both β-1,3-glucanase and chitinase isozymes in sugar beet in our experiment, as well as in pea (Mauch *et al.* 1984). On the contrary, the chitosan oligomer, chitoheptaose heptahydrochloride, did not induce neither β-1,3-glucanase nor chitinase gene expression in sugar beet (Nielsen *et al.* 1994). Induction of both basic and acidic proteins in sugar beet by SA and TNV in our experiment is in accordance with Fleming *et al.* (1991), who reported the localization of 12 acidic and 8 basic proteins in intercellular space of sugar beet leaves infected with TNV and 7 acidic and 8 basic proteins induced by salicylic acid. Comparison of the pattern of induced proteins slightly differed from that analyzed by the above-mentioned authors, probably because of the different cultivar was chosen on the base of its capability to induce a wide range of proteins by BTH. According to our previous experiments (data not shown), the pattern of induced proteins is highly dependent on cultivar used. Metals represent another possible inducer, which can cause leaf necrosis accompanied by protein synthesis similarly to pathogens. In sugar beet, proteins were induced by silver nitrate, in accordance with Edreva (1990), who found the same protein pattern both in Mn^{2+} treated and potato virus Y^N inoculated tobacco.

Among the inducers used, BTH together with paraquat manifested the broadest spectrum as well as quantitatively the most intense induction of PR-proteins. Only one acidic protein without glucanase or chitinase activity of R_f 0.45 showed to be dependent on the BTH concentration (Fig. 4). In contrast to acidic proteins, the content of basic proteins was increased with increased BTH concentration. One of the BTH-induced basic chitinase isozymes was found also in control plants. The presence of chitinase isozyme in non-induced plants is consistent with the results of Nielsen *et al.* (1994), who reported the expression of basic chitinase gene in control plants following mechanical shaking. Thus, the chitinase found in control plants might be caused by the ventilation in a growth chamber. Low concentration of this basic chitinase in control plants was not sufficient for silver staining, indicating a high sensitivity of the method used for the determination of enzyme activity of the bands. The same effect was observed for other basic chitinase isozyme and some acidic glucanases.

The benzothiadiazole proved to be an efficient inducer of new proteins in sugar beet, similarly to other inducers under study. Regarding that the effective concentration of BTH was not toxic to sugar beet and induced both β-1,3-glucanase and chitinase isozymes, BTH represents a useful tool for SAR studies in sugar beet.

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