Qualitative profiling of phenols and extracellular proteins induced in mustard (Brassica juncea) in response to benzothiadiazole treatment

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Abstract

Treatment of mustard [Brassica juncea (L.) Czern. & Coss.] cv. Varuna with benzothiadiazole (BTH) induced changes in the qualitative profile of total soluble phenols and acid soluble extra cellular proteins. There was temporal increase in the level of total soluble phenolics after BTH treatment and maximum content was observed 72 h after treatment. Thin layer chromatography of an aqueous methanol extract of BTH treated leaves of mustard revealed presence of new phenolic compounds which were not present in control. Twelve acid soluble proteins with apparent molecular masses ranging from 13.2 – 69.5 kDa accumulated in BTH treated leaves of mustard plants. Proteins P13.8, P33.7 and P34.5 were present in traces in control. The most prominent proteins 24 h after BTH treatment were with apparent molecular mass of 33.0 and 33.7 kDa indicating towards their early induction, whereas, P33.0 was the most prominent protein 48 h after treatment with BTH. It is suggested that changes in specific phenols and proteins as a result of BTH treatment might be the useful markers of induced resistance in mustard.

Key words: Brassica juncea, benzothiadiazole, extracellular proteins

Özet

Benzotiyadiazol uygulamasına cevap olarak hardal bitkisinde (Brassica juncea) teşvik edilen fenol ve hücre dışı proteinlerin kalitatif profili

Benzotiyadiazol (BTH) ile hardal [Brassica juncea (L.) Czern. & Coss.] cv Varuna’nın muamelesi toplam çözünürlü fenoller ve asidik çözün espan hücresel proteinlerinin kalitatif profillerinde değişiklikleri teşvik eder. BTH uygulamasından sonra toplam çözün espan fenollerin seviyesinde geçici bir artış vardı ve maksimum miktar uygulamadan 72 saat sonra gözlemdi. Hardal bitkisinin BTH muameleli yapraklarının sulu methanol ekstraktının ince tabaka kromotografisinde, kontrolde mevcut olmayan yeni fenolik birleşikler açığa çıktı. 13.2 - 69.5 kDa arasında değişen moleküler kütlesi 12 asidik çözün espan protein BTH ile muamele edilen hardal bitkilerinin yapraklarından toplandı. P13.8, P33.7 ve P34.5 proteinleri kontrolde çok az miktarda vardı. BTH uygulamasından 24 saat sonra en çok göz çarpan proteinler erken teşvik edilenlerin görülüğü 33.0 ve 33.7 kDa lik moleküler kütüde görüldü. Halbuki P33.0 BTH uygulamasından 48 saat sonra en çok görülen proteini. BTH uygulaması sonucunda spesifik fenoller ve proteinlerde değişiklikler hardal bitkisinde teşvik edilen dirençin faydali bir belirleyicisi olabileceği ortaya koymaktadır.

Anahtar kelimeler: Brassica juncea, Benzotiyadiazol, ekstra hücresel proteinler.
**Introduction**

Plants have several lines of defense against invading pathogens including preformed barriers and induced responses. The latter include a rapid production of reactive oxygen species, synthesis of phenolics and production of large amount of pathogenesis related (PR) proteins (Kombrink et al., 1993). Van Loon (1985) defined them as proteins which have low molecular weights, are extremely soluble in acids, have low isoelectric points (pI), are resistant to photolytic digestion, are secreted into the intercellular fluid and have no apparent enzymatic activities. PR-proteins have been shown to possess antimicrobial activity (Brunner et al., 1995). Their synthesis occurs after pathogen attack at a faster rate in incompatible than compatible interaction (Kombrink and Somssich, 1997). Apart from this, they also accumulate in response to treatment with abiotic elicitors such as salicylic acid (SA), benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) and 2,6-dichloroisonicotinic acid (INA) (Cohen, 1994; Vernooij et al., 1995; Gorlach et al., 1996; Spletzer and Enyedi, 1999). The expression of genes encoding basic PR-1 from barley and acidic PR-1 from maize has also been shown to be induced after plant treatment with INA or BTH respectively (Kogel et al., 1994; Morris et al., 1998). Two groups of PR-proteins, namely chitinases and β-1, 3-glucanases were found to have enzymatic functions (Legrand et al., 1987; Kauffmann et al., 1987). A role for these hydrolytic enzymes in plant defense response against fungal pathogens is suggested as they degrade chitin and β-1, 3-glucan, major structural polysaccharides in the cell walls of many fungi (Mauch et al., 1988).

Induced synthesis of phenolic compounds is associated with host-pathogen interaction and specific phenolics have been implicated in host resistance (Glazener, 1982; Hammerschmidt et al., 1982; Friend et al., 1973). Ferulic and p-coumaric acids in bound form have been found to be involved in the resistance of wheat leaves to *Puccinia recondita f. sp. tritici* (Southerton and Deverall, 1990). Benzo[1,2,3]thiadiazole has been developed by Novartis for the protection of crop plants from potential pathogens by inducing systemic acquired resistance (SAR) in the host plant. SAR refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens (Hunt and Ryals, 1996). Kaur and Kolte (2001) showed that foliar treatment of mustard plants by BTH protects them against staghead phase of white rust (*Albugo candida*) by activating plants own defense response.

In this paper we demonstrate that treatment of mustard plants cv. Varuna with BTH induce changes in the qualitative profile of phenols and extracellular proteins.

**Materials and methods**

Seeds of mustard cv. Varuna were surface sterilized with two changes of sterile distilled water before sowing in 800 ml (16 cm height) pots containing a medium of soil: sand:vermi compost (2:1:1 v/v/v). Plants were maintained under controlled conditions and irrigation was applied in the pots every third day. For studying the qualitative profile of phenols 4-week-old plants were sprayed with 1.428 mM aqueous BTH solution. The leaves sprayed with water alone served as control. Level of total soluble phenols was determined in second and third leaves harvested 24, 48, 72 and 96 h after elicitor treatment. Qualitative profile of phenols was determined by sampling the second and third leaf of 4-week-old mustard plants 72 h after elicitor treatment. For the determination of qualitative protein profile, second and third leaves of 4-week-old plants were treated with elicitor (BTH) solution. The elicitor was applied in sterile aqueous solution (0.095 mM) through the petioles for 12 h and then the leaves were placed in sterile distilled water in petri plates. The analogous treatment with water served as control. The intercellular fluid (IF) was isolated 24 and 48 h after treatment.

**Quantification of total soluble phenolics**

The method of Swain and Hillis (1959) was used for the extraction and quantification of total soluble phenolics. Fresh leaves (1 g) were extracted in 80% methanol for 90 min at 80 °C. The extract was centrifuged at 14,000 g for 15 min, and 100 µl of the extract was diluted to 1 ml with water and mixed with 0.5 ml of 2.0 M Folin-Ciocalteu’s reagent and 0.5 ml of 1 M Na₂CO₃. After 1h, absorbance of the sample solution was measured at 725 nm using T117 spectrophotometer (Systronics, India). Concentration of total soluble phenolics in the extract was calculated from a standard curve prepared with gallic acid. The data were analyzed using t-test (Gupta and Kapoor, 2001).
1993). Values in Fig.1 are the means and the bars indicate the standard deviations.

Thin layer chromatography

Thin layer chromatography was used for the qualitative profiling of phenolic compounds in the leaves of Brassica juncea cv. Varuna using the methods of Harborne (1973). Leaves were extracted in 80% methanol and the extracts concentrated to the aqueous phase using a vacuum rotary evaporator. Phenolics were then partitioned into ethyl acetate (2 x equal volume). A single spot of extract (25 µl) was applied onto silica gel TLC plates (250 µm) and the plates developed in n- butanol-acetic acid-water (BAW, 4:1:5, v/v/v). Response to ultra-violet light and diagnostic spray reagents was determined.

Extraction of intercellular fluids

The procedure adopted was a simplified form of the in vacuo infiltration method described by Parent and Asselin (1984). Leaves were submerged in a 32 mM/84 mM phosphate/citrate buffer pH 2.8 containing 1% β-mercaptoethanol (20 ml g⁻¹ fresh weight) and vacuum infiltrated for 10 min. The leaves were dried on the filter paper, rolled into cylindrical bundle and placed in a 5 ml syringe inside a centrifuge tube. The intercellular fluid was obtained by centrifuging the tissue at 1000 g for 10 min at room temperature. The protein content of the intercellular fluid was determined as µg BSA equivalents using Bradford (1976) method.

SDS-polyacrylamide gel electrophoresis

The proteins were separated on the gels according to Lämmli (1970) with the following concentrations used in the stacking /separation gels-5/12% acrylamide :N,N'-methylenebisacrylamide (30:0.8); 125/375 mM Tris-HCl pH 8.8; 0.1% (w/v) SDS; 0.05/0.01% (v/v) ammoniumpersulphate. The running buffer was 25 mM Tris, 190 mM glycine, and 0.1% (w/v) SDS, pH 8.3. Protein in the intercellular fluid was precipitated with nine volumes of ice cold acetone. After 16 h at 4 °C, the precipitates were collected by centrifugation at 5000 g for 20 min at 4°C. The pellets were suspended in sample buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue), heated to 95 °C for 3 min, spun rapidly, and loaded (max. 20 µg protein per slot) onto 80 x 70 x 0.1 mm slab gels. The current was maintained at 10 mA per gel until the samples had passed the stacking gel then increased to 20 mA and run for approximately 2 h. The gel was stained and fixed in 40% MeOH, 10% acetic acid and 0.1% Coomassie brilliant blue G-250. Overnight destaining was carried out in 10% MeOH and 7.5% acetic acid. Relative molecular weight of the proteins was determined using protein molecular weight marker (Fermentas, USA).

Results and discussion

The resistance-inducing activity of BTH probably relies on the activation of a natural defense pathway called systemic acquired resistance (Sticher et al., 1997; Lucas, 1999) Treatment of Brassica juncea cv. Varuna with BTH caused temporal changes in concentration of soluble phenolics and accumulation of acid soluble proteins. It is evident from the Fig.1 that total soluble phenolic content is significantly enhanced (P<0.01) in mustard leaves 48, 72 and 96 h after BTH treatment. Maximum total soluble phenolic content (1.15 mg gallic acid g⁻¹ fresh wt) was observed after 72 h of treatment.

When leaves of Brassica juncea cv. Varuna treated
with water (control) or BTH were run on the TLC plates which contained fluorescent indicator and plates observed under short wave UV (254 nm), separated compounds appear as dark spots. When plates were sprayed with Folin-Ciocalteu reagent, blue to blue gray bands indicated the presence of at least eight phenolic compounds in BTH treated and six in control (Table 1). Of these, two of \( R_F \) values 0.64 and 0.93 were yellow-brown and yellow in the visible. One major band of high \( R_F \) (\( R_F = 0.64 \)) changed to blue immediately after spraying with Folin-reagent, a characteristic of phenols which are derivatives of catechol. A major band of high \( R_F \) (\( R_F = 0.76 \)) which was observed only in BTH treated leaves, showed pink yellow colour after spraying with vanillin-HCl. Bands corresponding to \( R_F \) values 0.76 and 0.89 were only observed in the extract from BTH treated leaves and were absent in the control. Induced synthesis of phenolic compounds is a common feature of host pathogen interaction or treatment with certain chemicals (elicitors) and specific phenolics have been implicated in host resistance. Southerton and Deverall (1990) reported that ferulic and \( p \)-coumaric acids were involved in the resistance of wheat leaves to \textit{Puccinia recondite} f. sp. tritici. Similarly ferulic and \( p \)-coumaric acids in corn leaves infected with \textit{Colletotrichum graminicola} were inhibitory to spore germination (Nicholson et al.,1989). Differences in phenolic profile of \textit{Phytophthora cinnamomi} resistant and susceptible \textit{Eucalyptus} cultivars have been reported (Cahill and McComb, 1992). In our study of the types of phenolics found in BTH treated and control leaves of mustard differences in phenolics were evident. There was a change in number and type of phenolics after BTH treatment and accumulation of new compounds.

A typical separation of acid soluble proteins accumulated in the intercellular spaces of BTH treated mustard leaves obtained by SDS-PAGE is shown in Fig.2. Intercellular fluid isolated from control leaves contained 15 \( \mu \)g acid soluble proteins per 1g fresh

<table>
<thead>
<tr>
<th>( R_F ) values</th>
<th>Control</th>
<th>Treated</th>
<th>Visible</th>
<th>UV(^{a} )</th>
<th>Folin(^{b} )</th>
<th>Van-HCl(^{c} )</th>
</tr>
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<tbody>
<tr>
<td>0.09</td>
<td>0.09</td>
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<td>0.25</td>
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<tr>
<td>0.38</td>
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<td>db</td>
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<td>0.52</td>
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<tr>
<td>0.64</td>
<td>0.64</td>
<td>y-br</td>
<td>-</td>
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<td>-</td>
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<td>b</td>
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<tr>
<td>0.93</td>
<td>0.93</td>
<td>y</td>
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</table>

\(^{a}\)Colour reaction of the phenolic after treatment: y, yellow; br, brown; b, blue; db, dark blue; p, pink, \(^{b}\)Folin-Ciocalteu reagent (2 M), \(^{c}\)Vanillin-HCl (1 g Vanillin in 10 ml concentrated HCl).
weight infiltrated water treated leaves after 48 h. The amount of acid soluble protein in the IF extracted from BTH treated leaves was 50 µg and 75 µg per 1g fresh weight infiltrated BTH treated leaves respectively after 24 and 48 h after elicitor treatment. The numbers on right hand side of Fig.2 indicate the apparent molecular mass of each protein band. The induction of PR-proteins has been reported in several crop plants after treatment with biotic and abiotic elicitors (Kagale et al., 2004; Reiss and Bryngelsson, 1996; Silué et al., 2002). In total, 12 acid soluble proteins, with molecular masses ranging from 13.2-69.5 kDa accumulated in BTH treated leaves of mustard plants with P18.6, P26.8, P33.0 and P33.7 accumulating to the greatest extent. The protein P55.1 was present only in the control and its level reduced after BTH treatment. Proteins P13.8, P33.7 and P34.5 were present in traces in control. The most prominent proteins 24 h after BTH treatment were with apparent molecular mass of 33.0 and 33.7 kDa indicating towards their early induction, whereas, P33.0 was the most prominent protein 48 h after treatment with BTH. The expression of genes encoding acid soluble PR-protein (PR-1) from maize has also been shown to be induced after treatment with BTH (Morris et al., 1998). Similarly, Sauerborn et al. (2002) reported accumulation of PR-proteins in sunflower (Helianthus annuus) treated with BTH.

From above study it may be concluded that BTH induces changes in qualitative profile of total soluble phenols and extracellular proteins in mustard by activation of natural defense pathway and changes in specific phenols and proteins might be the useful markers of induced resistance in mustard.

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