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CHANGES IN THE ACCUMULATION OF SOLUBLE AND CELL WALL-BOUND PHENOLICS IN ELICITOR-TREATED CELL SUSPENSION CULTURES AND FUNGUS-INFECTED LEAVES OF *SOLANUM TUBEROSUM*

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Key Word Index—*Solanum tuberosum*; Solanaceae; cell culture; phenolics; phenylpropanoids; *Phytophthora infestans*; elicitation; late blight.

Abstract—Cell suspension cultures of potato (*Solanum tuberosum* cv. Datura) treated with an elicitor preparation from *Phytophthora infestans* and potato leaves infected with the same fungus were used to study changes in the accumulation patterns of soluble and cell wall-bound phenolics. The compounds were identified by chromatographic comparison with authentic substances and by spectroscopic methods (FAB mass spectrometry, ^{1}H and ^{13}C NMR). The soluble phenolics were 4-*O*-β-glucopyranosylhydroquinone (arbutin), 4-*O*-β-glucopyranosylbenzoate, 3-methoxy-4-*O*-β-glucopyranosylbenzoate (vanillate glucoside), *N*-(*E*)-caffeoylputrescine, 2-*O*-β-glucopyranosylbenzoate (salicylate glucoside), *N*-(*E*)-feruloylputrescine, and *N*-(*E*)-feruloylaspartate. The cell wall-bound phenolics were 4-hydroxybenzoate, 4-hydroxybenzaldehyde, 3-methoxy-4-hydroxybenzaldehyde (vanillin), 4-(*E*)-coumarate, (*E*)-ferulate, *N*-4-(*E*)-coumaroyltyramine, and *N*-(*E*)-feruloyltyramine. The most prominent phenolics showing elicitor- or fungus-induced increases in accumulation rates were the soluble putrescine amides and cell wall-bound 4-hydroxybenzaldehyde and tyramine amides. In addition, there was a secretion of large amounts of coumaroyltyramine into the cell culture medium.

INTRODUCTION

The most destructive fungal disease in potato (*Solanum tuberosum*) is late blight caused by infection with the oomycete *Phytophthora infestans* (*Pi*). Besides the accumulation of rishitin and several structurally related sesquiterpenoid phytoalexins in potato tubers [1, 2] in response to infection by *Pi*, a rapid tissue browning followed by hypersensitive cell death has been observed. This phenomenon is correlated with the induction of phenylalanine ammonia-lyase (PAL) and hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase leading to the formation of chlorogenic acid [3]. Stimulation of phenylpropanoid metabolism also results in the incorporation of phenolics into the cell wall [4]. Along with 4-coumaric and ferulic acids, their tyramine and octopamine amides were observed to be associated with the cell wall [5] as a result of the hypersensitive reaction of potato tubers.

In field-grown plants, however, it is the potato leaves and not the tubers that are the primary target of *Pi*. In contrast to infected tubers, the infected leaves do not accumulate the sesquiterpenoid phytoalexins [6], but there is a significant stimulation of phenylpropanoid metabolism, including transcriptionally activated PAL and 4-coumarate:CoA ligase (4CL) [7–9]. Treatment of the potato leaves with the PAL inhibitor L-2-aminooxy-3-phenylpropionic acid (AOPP) prior to infection results in a complete collapse of late blight resistance, emphasizing the importance of phenylpropanoid derivatives in a successful defence response [10].

As part of our studies on the induction of phenylpropanoid metabolism in potato by *Pi*, we report here the structural elucidation of soluble and cell wall-bound phenolics and elicitor- and fungus-induced changes in their accumulation patterns in cell suspension cultures and leaves of potato.

RESULTS AND DISCUSSION

Identification of phenolics

HPLC analyses of methanolic extracts from suspension-cultured potato cells and from alkaline treatments of the remaining cell debris showed complex patterns of constitutively present soluble and cell wall-bound phenolic compounds, respectively (controls in Fig. 1). The majority of soluble phenolics comprise arbutin (**1**), 4-hydroxybenzoate glucoside (**2**), vanillate glucoside (**3**), caffeoylputrescine (**4**), salicylate glucoside (**5**),

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feruloylputrescine (**6**), and feruloylaspartate (**7**). The cell wall-bound phenolics identified, are 4-hydroxybenzoate (**8**), 4-hydroxybenzaldehyde (**9**), vanillin (**10**), 4-coumarate (**11**), ferulate (**12**), 4-coumaroyltyramine (**13**), and feruloyltyramine (**14**); see structure scheme. Most of these structures are well known from other sources [11].

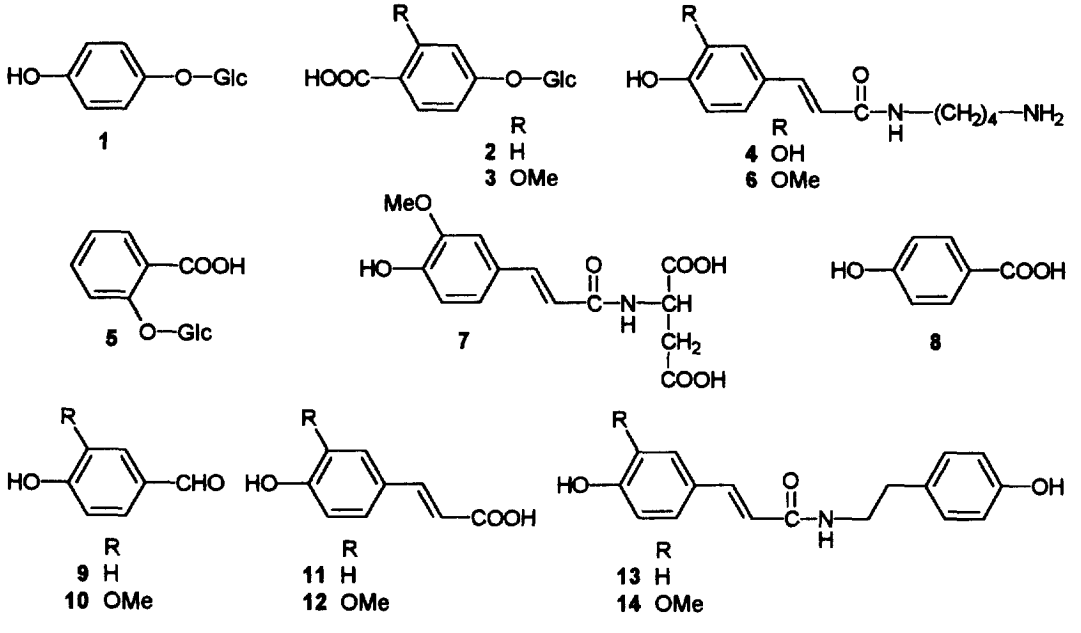
Compounds **1**–**7** and **9** were readily determined from the combination of NMR and MS data and chromatographic comparison with authentic compounds (HPLC, TLC). Compounds **8** and **10**–**14** were identified only from their chromatographic behaviour (TLC and HPLC) by comparison with authentic samples [**8**, **10**–**12** from commercial sources (Merck, Darmstadt); **13** and **14** from synthetic material]. In addition, the spectroscopic data of **13** and **14** extracted from cell walls were identical with those of the synthetic compounds (not documented).

Compound **7** has been previously identified from cell cultures of *Beta vulgaris* [12] and the related 4-coumaroylaspartate from *Arabidopsis thaliana* [13]. The hydroxycinnamoylputrescines (**4** and **6**) are of widespread occurrence in the plant kingdom [14] and their distribution in different organs of solanaceous species, including potato, has recently been described [15].

The pattern of cell wall-bound phenolics is comparable to that reported from some other plants, e.g. from tomato [16] or from parsley [17]. Besides hydroxybenzoates and hydroxycinnamates, which are most often found in cell walls [18], the hydroxycinnamate tyramine amides are rare, but have also repeatedly been detected as insoluble constituents of cell wall fractions [5]. The location within the cell wall and types of chemical bonds are unknown, except for the hydroxycinnamates which are probably ether- and ester-bound to cell wall fractions and cross-linked to the polysaccharides [19–21].

The spectroscopic data were compared with the respective literature data. The following characteristic data substantiated the structural elucidation. The nature of the sugar moieties was deduced from their ^{1}H (**2**, **3**, **5**) and ^{13}C (**5**) chemical shifts. Their β-configuration was apparent from the magnitude of the vicinal coupling constant of the anomeric protons (7.1–7.3 Hz). The number of protons in each aromatic and aliphatic system was determined from the integrated 1D ^{1}H spectra and the identity of each moiety was established from their characteristic ^{1}H shifts and coupling constants. The aromatic moiety of **1** showed the multiplet pattern of an AA'BB' spin system with $J(AB) + J(AB')$ of 9.0 Hz and high-field shifts denoting a substituted quinol system. A similar system was found for **2** and **9** with shifts indicative of a 4-hydroxybenzoate derivative and 4-hydroxybenzaldehyde, respectively. An alternative aromatic four-spin system for **5** and a three-spin system for **3**, **4**, **6** and **7** were identified from the *ca* 8 Hz three-bond and 2 Hz four-bond couplings. The configuration of the olefinic bond in the putrescine and aspartate derivatives was apparent from the magnitude of the three-bond coupling (15.7 Hz for *E*). For **2**, **3** and **5** the glucose moieties were bound to the aromatic ring system and not at the carboxyl group, as the ^{1}H shifts of the anomeric protons were between 4.9 and 5.1 ppm.

As reported previously for spermidine derivatives [22], monoacylation was detected in **4** and **6** by a 0.4 ppm low-field shift of the methylene protons (H-1) adjacent to the substituted nitrogen, compared to those adjacent to the unsubstituted one (H-4). Although only



three protons of the aspartate moiety of **7** are observable as an ABX spin system, in the ^1H spectrum in CD_3OD they have characteristic chemical shifts and couplings that allow ready identification [23]. This was confirmed by the observation of the correct molecular masses in the positive ion FAB mass spectra.

Elicitor-induced changes in the phenolic patterns

The putrescine conjugates **4** and **6** are among the major constituents of the soluble phenolics, reaching constitutive concentrations between 5 and 10 nmol (g fresh weight)$^{-1}$. The accumulation of both compounds is significantly increased after application of *Pi* elicitor. Figures 1 and 2 illustrate the induced accumulation of **4**, which reaches levels of about 50 nmol (g fresh weight)$^{-1}$ within 24 hr. At 48 hr after elicitation, however, **4** decreased to levels of the control cultures, whereas **6** increased 5–6-fold reaching levels between 25 and 30 nmol (Fig. 2). Among the minor soluble phenolics, the tyramine conjugates (**13**, **14**), hardly detectable in the control experiments, appeared upon elicitation at 4–5 nmol (**13**) and 0.5–0.6 nmol (g fresh weight)$^{-1}$ (**14**).

Alkaline treatments of the extensively washed insoluble residues, which remained after extraction of the soluble compounds, liberated more than 20 phenolics. Seven were identified (**8–14**). One of the major components, 4-hydroxybenzaldehyde (**9**), showed the most prominent elicitor-induced accumulation, reaching 50 to 60 nmol (g fresh weight)$^{-1}$ (Fig. 3). This level is about 5 to 6 times higher than that detected in control cultures. Among the minor constituents, the levels of the hydroxycinnamate tyramine amides (**13** and **14**) increased significantly. In the control cultures these cell wall-bound amides were present in trace amounts or could hardly be detected.

Surprisingly there was a specific secretion of large

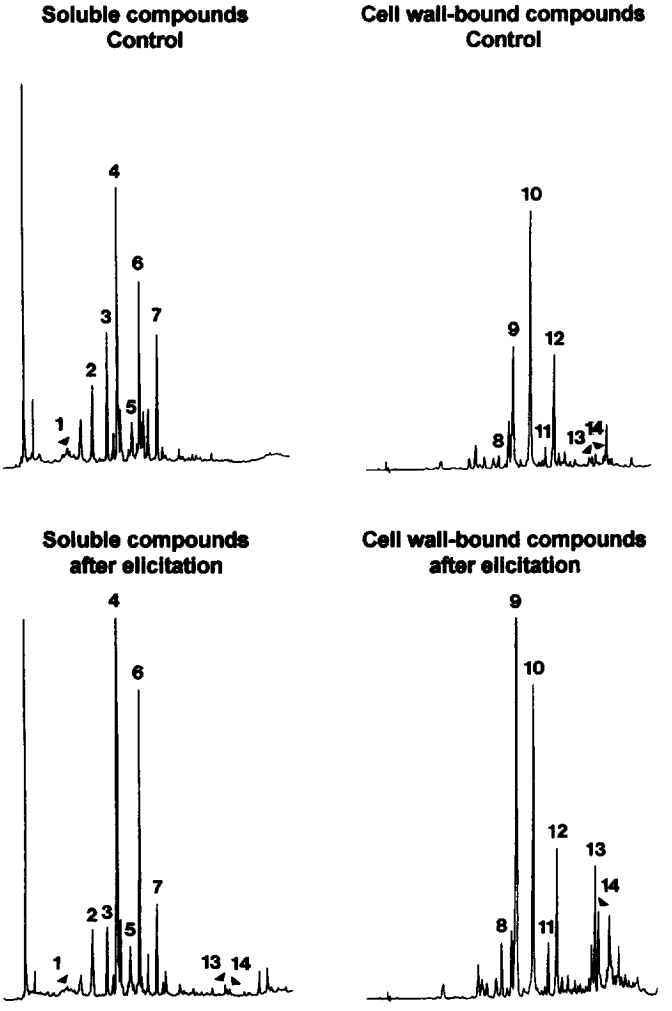


Fig. 1. Traces of HPLC analysis (35 min; maxplot detection between 260 and 340 nm) of soluble phenolics (**1–7**) and cell wall-bound phenolics (**8–14**) 24 hr after application of water (control) and of *Pi* culture filtrate [λ_{max}, nm: **1**, 283; **2**, 250; **3**, 255 (292); **4**, 319 (295); **5**, 287; **6**, 318 (296); **7**, 320 (299); **8**, 256; **9**, 285; **10**, 279 (309); **11**, 310; **12**, 324; **13**, 292 (315); **14**, 319 (294)]. For peak identification see structure scheme.

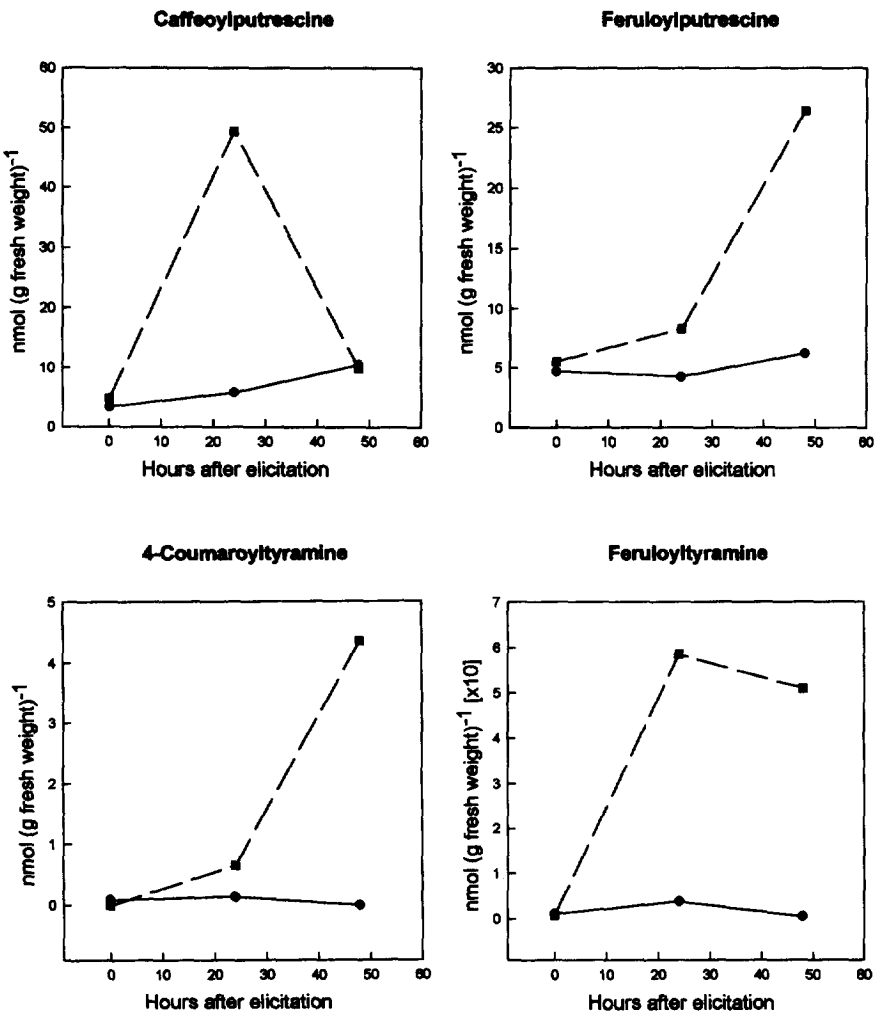


Fig. 2. Content of some soluble compounds of potato cell suspension cultures at the time of application of elicitor (day 5 = 0 hr) and after 24 and 48 hr after elicitation. Solid lines represent non-elicited cultures (application of water) and broken lines elicited cultures (application of *Pi* culture filtrate). Note the different scale reading in the *Y* axis for feruloyltyramine.

amounts of **13** into the cell culture medium (Fig. 3). Between 24 and 48 hr after elicitation, this compound accumulated to a level of about 200 nmol (g fresh weight)$^{-1}$. This is almost 50 times higher than the levels reached in the soluble or cell wall-bound pools. However, secretion of **13** depends on the amount of elicitor added to the cultures and is accompanied by browning of the cell cultures. When elicitor was applied at final concentrations below 10 μg ml^{-1} glucose equivalents, e.g. 5 μg, no browning and no secretion of **13** was observed. Accumulation of the soluble and cell wall-bound phenolics was significantly reduced upon elicitation with 5 μg ml^{-1}.

Potato leaves infected with virulent and avirulent *Pi* races accumulated the same phenolics and revealed the same pattern as elicitor-treated cells. This is shown in detail for the tyramine amides in cell walls of leaves after infection with the virulent *Pi* race 1 (Fig. 4). Leaves inoculated with race 4 accumulated the same compounds to slightly lower amount (not shown), indicating that the induction of this defence response may not be causally related to race-specific resistance of potato against *Pi*, but rather be a component of multigenic field resistance. Similar results have previously been reported for enzymes of general phenylpropanoid metabolism that are involved in the biosynthesis of compounds described here and the corresponding transcripts [7, 24].

The increase in cell wall-bound phenolics may enhance the effect of a chemical barrier against pathogens [16, 17, 25, 26] by decreasing the digestibility of cell walls [20]. But cell wall-bound phenolics may also directly inhibit hyphal growth of a potential fungal invader [27]. In this context it is interesting to note that several groups report on reduced activities of cell wall-degrading enzymes on cell walls with high phenolic content [16 and refs therein].

The elicitor-stimulated accumulation of the tyramine amides requires special attention. The hydroxycinnamate moiety of these conjugates originates from

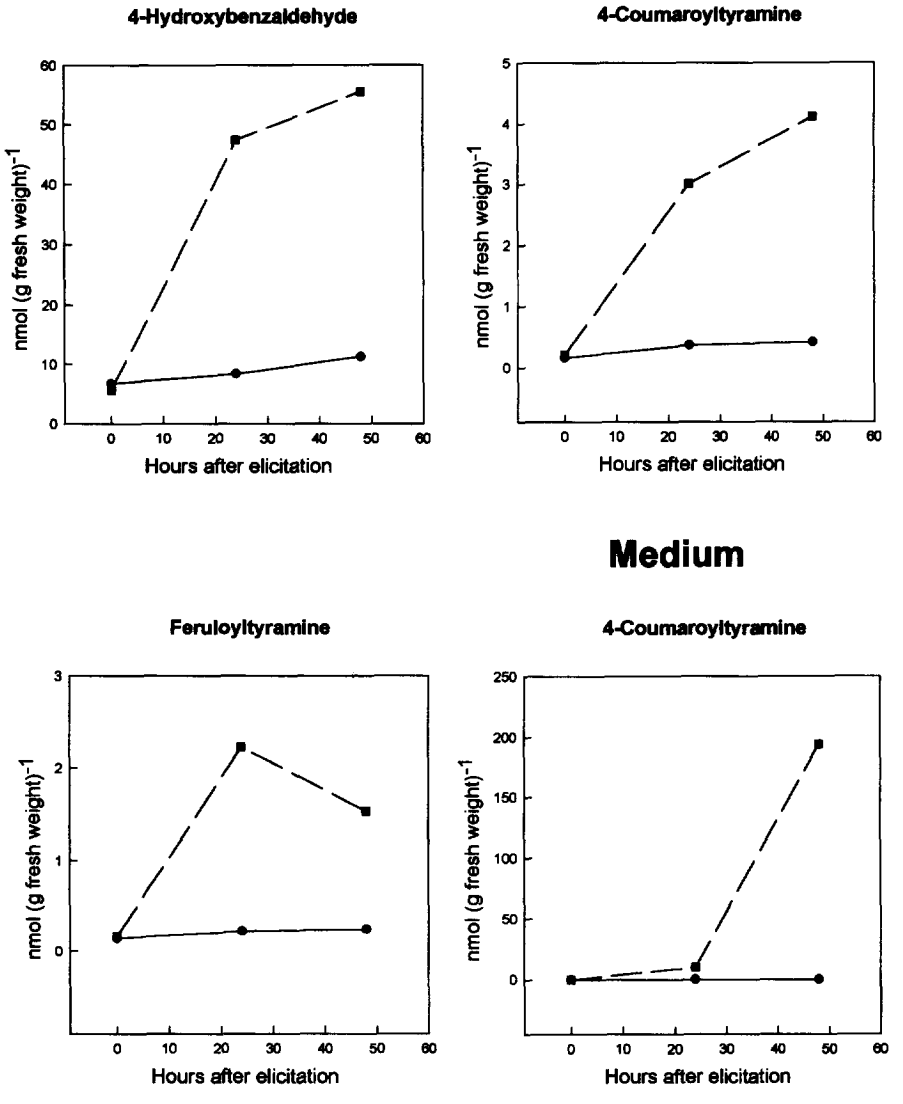


Fig. 3. Content of some cell wall-bound compounds and a secreted compound (medium) in potato cell suspension cultures at the time of application (day 5 = 0 hr) and after 24 and 48 hr after elicitation. Solid lines represent non-elicited cultures (application of water) and broken lines elicited cultures (application of *Pi* culture filtrate). Note the different scale reading of the *Y* axis in "Medium".

phenylalanine, whereas the tyramine group is generated by decarboxylation of tyrosine. PAL and 4CL were found to be transcriptionally activated by elicitor treatment of cultured potato cells [7–9]. Interestingly, one elicitor-responsive gene from parsley was identified as tyrosine decarboxylase [28]. The timing of changes in enzyme activities and accumulation of mRNA were similar for PAL and tyrosine decarboxylase. In the course of our studies on the elicitor-induced incorporation of the tyramine amides, we recently found that the activity of the enzyme that catalyses their formation from tyramine and hydroxycinnamate-CoA esters, the hydroxycinnamoyl-Coa:tyramine hydroxycinnamoyltransferase (THT, EC 2.3.1) [29], is also markedly stimulated by *Pi* elicitor (not shown). The biosynthesis of these compounds, therefore, appears to be regulated by coordinated, transient activation of two biosynthetic pathways, which both involve aromatic amino acids and merge at the conjugation step by THT. Our future work will focus on the investigation of the mechanism of this regulatory network.

EXPERIMENTAL

Plant material. Cell suspension cultures from potato (*Solanum tuberosum* L. cv. Datura), established from surface-sterilized leaves, were grown on MS medium [30] supplemented with 2 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg l^{-1} of nicotinic acid, 1 mg l^{-1} of pyridoxine (vitamin B6) and 10 mg l^{-1} of

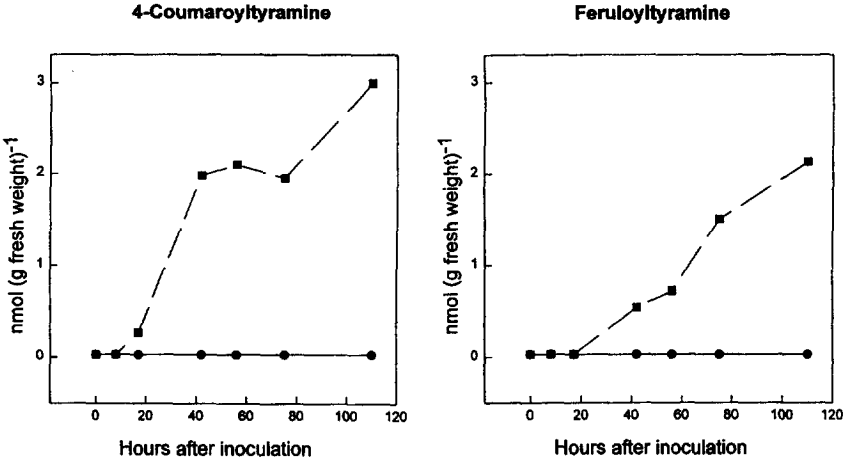


Fig. 4. Content of cell wall-bound tyramine amides in potato leaves during 120 hr after inoculation with *Pi* spores (broken lines) compared with non-inoculated leaves (application of water; solid lines).

thiamine (vitamin B1). Subcultivation was carried out after 7 days by 2 g of cells in 40 ml medium. The cultures were grown at 22° in the dark on a shaker operating at 100 rpm. Potato plants were grown from tubers in a phytochamber as described [6].

Fungi. Pathotypes 1 and 4 of *Pi* (Mont.) de Bary were grown as described [6]. Infections of detached leaves with spore suspensions were performed according to [6].

Elicitor preparation and elicitation. Concentrated culture filtrate was prepared from *Phytophthora infestans* race 4 grown for several weeks in liquid Henniger medium [31] as described elsewhere [6]. Filter sterilized elicitor solution was added to suspension cultures 5 days after transfer to give a final concentration in the medium of 10 μg ml^{-1} glucose equivalents as determined by the method of [32].

Extraction of soluble phenolics. Analytical. Freshly harvested cells were filtered and washed twice with H_2O. Cells were then transferred into 80% aq. MeOH (2 g fr. wt per 10 ml), treated with an Ultra Turrax homogenizer, allowed to stand with continuous stirring for 30 min and centrifuged. The supernatants were used for HPLC analysis. Potato leaves were extracted essentially according to this procedure. *Semipreparative.* Frozen cells (200 g fr. wt) were transferred into 200 ml MeOH, treated with an Ultra Turrax homogenizer, allowed to stand for 1 hr with continuous stirring and centrifuged. The pellet was re-extracted with MeOH (×2) and 50% aq. MeOH (×2). The combined extracts were reduced (*in vacuo*) to a few ml and kept at −20°.

Isolation of soluble phenolics. The methanolic extract was fractionated on a polyamide column (CC 6, 30 × 4 cm i.d.; Macherey-Nagel, Düren) with a stepwise gradient (750 ml each) of H_2O (elution of compounds **1**, **2**, **3**, **6**), 40% aq. MeOH, 80% aq. MeOH, and finally 0.01% NH_4OH in MeOH (elution of compound **7**). For purification of components **4** and **5** a reduced extract was directly fractionated on a Sephadex LH-20 column (see below). Compounds were purified by semipreparative HPLC.

Extraction of cell wall-bound phenolics. Analytical. Pellets from the analytical extractions of soluble phenolics (see above) were consecutively treated with the following solvents, ×2 each for 15–30 min stirring followed by centrifugation: (i) 50% aq. MeOH, (ii) H_2O, (iii) 0.5% aq. SDS, (iv) 1 N aq. NaCl, (v) MeOH, (vi) Me_2CO, and (vii) Et_2O. The residues were dried in an exsiccator. The white powders (30 mg aliquots) were suspended under N_2 in 2 ml 1 N NaOMe in 80% aq. MeOH, kept for 2 hr at 80°C and allowed to stand for 1 hr at room temp. with continuous stirring. The hydrolysates were centrifuged, 1 ml of the supernatant acidified by adding 100 μl 85% aq. H_3PO_4, centrifuged and 20 μl of the supernatants taken for HPLC. In order to enrich the liberated hydroxycinnamic tyramine amides (**13**, **14**), alternatively the supernatants were extracted prior to HPLC ×3 with Et_2O, which was taken to dryness and the residues redissolved in a small vol. of MeOH. *Semipreparative.* A pellet from 400 g fr. wt. of cells, which were treated according to the analytical procedure, was suspended under N_2 in 1 N aq. NaOH, kept for 1 hr at 80° and allowed to stand for 15 hr at room temp. with continuous stirring. The hydrolysate was centrifuged, the supernatant acidified with 85% aq. H_3PO_4 and extracted ×3 with Et_2O. The Et_2O extracts were combined, taken to dryness (*in vacuo*) and the residue redissolved in a small vol. of MeOH.

Isolation of cell wall-bound phenolics. The cell wall-bound phenolics liberated by the semipreparative extraction procedure were fractionated by polyamide column chromatography according to the procedure for isolation of the soluble phenolics. The 40% aq. MeOH

fraction contained compounds **9** and **10**, which were purified by semipreparative HPLC.

Synthesis of hydroxycinnamic acid tyramine amides. Syntheses of compounds **13** and **14** were carried out according to the procedure described in [33] via the *N*-hydroxysuccinimide ester [34]. Identity of the products was proven by NMR and MS analysis (data not shown).

HPLC. Analytical. The liquid chromatograph (Waters™ 600-MS System Controller; Waters GmbH, Eschborn) was equipped with a 5 μm-Nucleosil C_{18} column (250 × 4 mm i.d.; Macherey-Nagel, Düren) and the following two-step linear gradient elution system was applied with a flow rate of 1 ml min^{-1} within 20 min from solvent A (1.5% H_3PO_4 in H_2O) to 35% solvent B (80% aq. MeCN), then within 10 min to 70% solvent B in (A + B), followed by 5 min with 100% solvent B. Injections of 20 μl were carried out by an automatic sampler (Waters™ 717 Autosampler). Compounds were photometrically detected (maxplot between 260 and 340 nm) by a Waters™ 996 Photodiode Array Detector. Compounds were quantified by external standardization with authentic compounds using the Millenium software 2010 (Millipore, Eschborn). The quantitative data are the mean of two independent experiments that showed deviations up to 10% and 20% for the soluble and cell wall-bound compounds, respectively. *Semipreparative.* The column used was prepacked with 10 μm Silica C-18 (300 × 40 mm i.d.; Latek, Eppenheim) and the following linear gradient was applied with a flow rate of 20 ml min^{-1} within 200 min from solvent A (1% aq. HCO_2H) to solvent B (80% aq. MeOH).

TLC. Microcrystalline cellulose ('Avicel'; Macherey-Nagel, Düren) and silica gel (silica gel 60 with fluorescence indicator; Merck, Darmstadt) with $CHCl_3$–HOAc, 3:2, H_2O satd) and toluene–HOAc (2:1, H_2O satd); detection with UV light (345 nm) with and without ammonia vapour.

NMR and MS. ^1H and ^{13}C NMR spectra were recorded on a Bruker ARX 400 or a Bruker WM 400 NMR spectrometer at 300 K locked to the major deuterium resonance of the solvent, CD_3OD. Fast atom bombardment mass spectra were measured on either a Kratos MS 50 RF or Finnigan MAT 8430 mass spectrometer with 3-nitrobenzylalcohol (3NBA) or glycerol as matrix. Mass spectra of compounds **6** and **7** were obtained by ion spray MS as described previously [13].

Arbutin (4-O-β-*glucopyranosylhydroquinone*) (**1**). ^1H NMR (CD_3OD) = 7.00, 6.73 [*d* ×2, H-2/H-6, H-3/H-5, *J* (2–3) + *J* (2–5) 9.0], 4.77 [*d*, H-1′, *J* (1′–2′) 7.5], 3.92 [*dd*, H-6′A, *J* (6′A–6′B) 12.0, *J* (6′A-5′) 1.4], 3.73 [*dd*, H-6′B, *J* (6′B-5) 5.1], 3.50–3.30 [*m*, H-2′–H-5′]. (+)FAB-MS (glycerol) *m/z*: 295 $[M + Na]^+$.

4-O-β-*Glucopyranosylbenzoate* (**2**). ^1H NMR (CD_3OD) = 7.99 [*d*, H-2/H-6, *J* (2–3) + *J* (2–5) 8.7], 7.15 [*d*, H-3/H-5], 5.03 [*d*, H-1′, *J* (1′–2′) 7.1], 3.94 [*dd*, H-6′A, *J* (6′A-5) 2.0, *J* (6′A–6′B) 12.2], 3.74 [*dd*, H-6′B, *J* (6′B-5) 5.6], 3.53–3.35 [*m*, H-2′–H-5′].

Vanillate glucoside (3-*methoxy*-4-O-β-*glucopyranosylbenzoic acid*) (**3**). ^1H NMR (CD_3OD) = 7.67–7.65 [*m*, H-2, H-6], 7.23 [*d*, H-5, *J* (5–6) 8.9], 5.04 [*d*, H-1′, *J* (1′–2′) 7.3], 3.94 [*s*, 3-OMe], 3.92 [*dd*, H-6′A, *J* (6′A-5) 2.0, *J* (6′A–6′B) 12.2], 3.73 [*dd*, H-6′B, *J* (6′B-5) 5.1], 3.59–3.35 [*m*, H-2′–H-5′]. (−)FAB-MS (3NBA) *m/z*: 329 $[M - H]^-$.

N-(E)-*Caffeoylputrescine* (**4**). ^1H NMR (CD_3OD) = 7.44 [*d*, H-7′, *J* (7′–8′) 15.7], 7.05 [*d*, H-2′, *J* (2′–6′) 2.0], 6.94 [*dd*, H-6′, *J* (5′–6′) 8.2], [*d*, H-5′], 6.40 [*d*, H-8′], 3.38 [*t*, H-1, *J* (1–2) 6.7], 3.01 [*t*, H-4, *J* (3–4) 7.2], 1.78–1.65 [*m*, H-2, H-3]. ^{13}C NMR (CD_3OD) = 169.49 (*s*, C-9′), 148.86, 146.79 (*s* ×2, C-3′, C-4′), 142.44 (*d*, C-7′), 128.21 (*s*, C-1′), 122.11 (*d*, C-6′), 118.18 (*d*, C-5′), 116.46 (*d*, C-8′), 115.03 (*d*, C-2′), 40.36 (*t*, C-1), 39.52 (*t*, C-4), 27.62 (*t*, C-3), 25.90 (*t*, C-2). (−)FAB-MS (3NBA) *m/z*: 499 $[2M - H]^-$, 249 $[M - H]^-$.

Salicylate glucoside (2-O-β-*glucopyranosylbenzoic acid*) (**5**). ^1H NMR (CD_3OD) = 7.85 [*dd*, H-6, *J* (5–6) 7.7, *J* (4–6) 1.7], 7.56 [*ddd*, H-4, *J* (4–5) 7.5, *J* (3–4) 7.8], 7.45 [*dd*, H-3, *J* (3–5) 0.9], 7.18 [*ddd*, H-5], 4.92 [*d*, H-1′, *J* (1′–2′) 7.3], 3.96 [*dd*, H-6′A, *J* (6′A-5) 2.0, *J* (6′A–6′B) 12.0], 3.77 [*dd*, H-6′B, *J* (6′B-5) 5.7], 3.59–3.42 [*m*, H-2′–H-5′]. ^{13}C NMR (CD_3OD) = 158.77 (*s*, C-2), 134.86 (*d*, C-4), 132.35 (*d*, C-6), 123.87, 119.16 (*d* ×2, C-5, C-3), 104.59 (*d*, C-1′), 78.61, 77.53 (*d* ×2, C-3′, C-5′), 74.97 (*d*, C-2′), 71.30 (*d*, C-4′), 62.60 (*t*, C-6′). The signals of the quaternary carbons C-7 and C-1 could not be unambiguously identified. (−)FAB-MS (3NBA) *m/z*: 599 $[2M - H]^-$, 299 $[M - H]^-$.

N-(E)-*Feruloylputrescine* (**6**). ^1H NMR (CD_3OD) = 7.49 [*d*, H-7′, *J* (7′–8′) 15.7], 7.19 [*d*, H-2′, *J* (2′–6′) 2.0], 7.07 [*dd*, H-6′, *J* (6′–5′) 8.3], 6.85 [*d*, H-5′], 6.54 [*d*, H-8′], 3.94 [*s*, 3′-OCH_3], 3.39 [*t*, H-1, *J* (1–2) 6.9], 3.02 [*t*, H-4, *J* (4–3) 7.4], 1.79–1.68 [*m*, H-2, H-3]. (+)IS-MS (1% HCOOH in 50% aq. MeOH) *m/z*: 265 $[M + H]^+$, 287 $[M + Na]^+$.

N-(E)-*Feruloylaspartate* (**7**). ^1H NMR (CD_3OD) = 7.51 [*d*, H-7′, *J* (7′–8′) 15.7], 7.20 [*d*, H-2′, *J* (2′–6′) 1.9], 7.09 [*dd*, H-6′, *J* (6′–5′) 8.2], 6.84 [*d*, H-5′], 6.58 [*d*, H-8′], 3.93 [*s*, 3′-OCH_3], 2.94 [*dd*, H-3A, *J* (3A–3B) 16.7, *J* (3A-2) 5.3], 2.90 [*dd*, H-3B, *J* (3B-2) 6.7]. The signal of H-2 was under the residual water signal at 5.0–4.6 ppm. (+)IS-MS (1% HCOOH in 50% aq. MeOH) *m/z*: 310 $[M + H]^+$, 332 $[M + Na]^+$, 348 $[M + H]^+$, 641 $[2M + Na]^+$.

4-*Hydroxybenzaldehyde* (**9**). ^1H NMR (CD_3OD) (*d* ppm): 9.81 [*s*, 1-CHO], 7.82 ["*d*", H-2/6, *J* (2–3) + *J* (2–5) 8.7], 6.96 ["*d*", H-3/5]. EI-MS was identical with reference material.

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