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Benzothiadiazole-Mediated Induced Resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in Tomato[1]

Nicole Benhamou* and Richard R. Bélanger

Recherche en Sciences de la Vie et de la Santé, Pavillon Charles-Eugène Marchand (N.B.), and Département de Phytologie, Faculté des Sciences de l'Agriculture et de l'Alimentation (R.R.B.), Université Laval, Sainte-Foy, Québec, Canada G1K 7P4

Benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), a synthetic chemical, was applied as a foliar spray to tomato (*Lycopersicon esculentum*) plants and evaluated for its potential to confer increased resistance against the soil-borne pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL). In nontreated tomato plants all root tissues were massively colonized by FORL hyphae. Pathogen ingress toward the vascular stele was accompanied by severe host cell alterations, including cell wall breakdown. In BTH-treated plants striking differences in the rate and extent of fungal colonization were observed. Pathogen growth was restricted to the epidermis and the outer cortex, and fungal ingress was apparently halted by the formation of callose-enriched wall appositions at sites of fungal penetration. In addition, aggregated deposits, which frequently established close contact with the invading hyphae, accumulated in densely colonized epidermal cells and filled most intercellular spaces. Upon incubation of sections with gold-complexed laccase for localization of phenolic-like compounds, a slight deposition of gold particles was observed over both the host cell walls and the wall appositions. Labeling was also detected over the walls of fungal cells showing signs of obvious alteration ranging from cytoplasm disorganization to protoplasm retraction. We provide evidence that foliar applications of BTH sensitize susceptible tomato plants to react more rapidly and more efficiently to FORL attack through the formation of protective layers at sites of potential fungal entry.

Plant-pathogen interactions are mediated by a complex network of molecular and cytological events that ultimately determine outcomes ranging from susceptibility to resistance (Lamb et al., 1989). Recent advances in molecular biology have given rise to the notion that exogenous and/or endogenous factors could substantially affect host physiology, leading to rapid and coordinated defense-gene activation in plants normally expressing susceptibility to pathogen infection (Ward et al., 1991). Corroborating data relevant to this concept are now beginning to emerge from studies of SAR (Ryals et al., 1992), a phenomenon initially linked to plant "immunization" against a broad range of biotic agents by previous inoculation with a pathogen (Ross, 1961; Madamanchi and Kuc, 1991). A similar activation of the natural plant defense system has been shown to occur upon exogenous application of chitosan (Benhamou et al., 1994), salicylic acid (Malamy and Klessig, 1992), or certain chemicals such as 2,6-dichloroisonicotinic acid (Métraux et al., 1991) and β-aminobutyric acid (Cohen et al., 1994). In all cases, characterization of the biochemical changes associated with chemical-mediated induced resistance revealed a correlation between the establishment of resistance and the accumulation of defense molecules such as pathogenesis-related proteins (Cohen et al., 1994).

A new product, promoted as a safe, reliable, and nonphytotoxic plant protection agent, BTH, was recently identified by scientists at Novartis as a novel disease-control compound. Exogenous application of BTH to tobacco and Arabidopsis leaves has been shown to activate a number of SAR-associated genes, leading to enhanced plant protection against various pathogens (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996). These studies provided evidence that induction of SAR gene expression by BTH did not require the contribution of salicylic acid and/or jasmonate, suggesting that this compound could act as a secondary messenger analog capable of activating the SAR signal transduction pathway independently of the accumulation of other signal molecules (Lawton et al., 1996). In a recent ultrastructural investigation, we demonstrated that application of BTH to cucumber leaves before challenge with the root pathogen *Pythium ultimum* triggered a set of plant defense reactions that resulted in the creation of a fungitoxic environment, which protected the roots by restricting pathogen growth to the outermost tissues (Benhamou and Bélanger, 1998). Evidence was provided from these cytological studies that the beneficial effect exerted by BTH in cucumber was mainly associated with a massive accumulation of phenolic-enriched deposits at sites underlying fungal penetration. These observations raised the question of to what extent activation of the secondary metabolism was a general feature of BTH-mediated induced resistance in plants.

In an attempt to determine whether BTH, known to be an active inducer of systemic resistance against *P. ultimum*-incited disease (Benhamou and Bélanger, 1998), was operational against a vascular pathogen, we investigated the

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* Corresponding author; e-mail nben@rsvs.ulaval.ca; fax 1–418–656–7176.

Abbreviations: BTH, benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester; FORL, *Fusarium oxysporum* f. sp. *radicis-lycopersici*; SAR, systemic acquired resistance.

effectiveness of a pretreatment with BTH in inducing systemic resistance against crown and root rot caused by FORL in tomato (*Lycopersicon esculentum*) (Jarvis, 1988). The objectives of the present research were first to investigate ultrastructurally the outcome of the tomato-FORL interaction upon BTH treatment, and second to compare the nature and extent of the host reactions with those previously reported in BTH-treated cucumber plants (Benhamou and Bélanger, 1998).

MATERIALS AND METHODS

Fungal Culture and Growth Conditions

A tomato isolate of FORL (kindly provided by P.O. Thibodeau, Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, Canada) was grown on potato dextrose agar (Difco, Detroit, MI) at 24°C and subcultured every week. It was periodically inoculated and reisolated from ripe tomato fruits.

Plant Material

Tomato (*Lycopersicon esculentum* Mill. cv Bonny Best, susceptible to FORL) seeds were sterilized by immersion in 1% (v/v) sodium hypochlorite for 30 min and sown in a mixture of peat:perlite:vermiculite (2:1:1) at a density of four seeds per 6-cm pot. Plants were propagated in a greenhouse at 22°C to 24°C with 16 h of light supplemented by high-pressure sodium lamps (100 μE m^{-2} s^{-1}). They were fertilized twice a week with a nutrient solution containing, in milliequivalents, NO_3 (12.0), PO_4 (1.0), K (1.7), Mg (1.5), Ca (2.8), and S (0.5), and in microequivalents, Fe (70.0), Mn (18.0), Zn (7.7), Cu (1.5), B (27.5), and Mo (0.5). The pH of the solution was adjusted to 6.2, and the electrical conductivity to 2.4 millisiemens. Seedlings were grown on a greenhouse bench at 24°C to 26°C with a 16-h light regime supplemented by high-pressure sodium lamps (100 μE m^{-2} s^{-1}). Experiments were performed with 5-week-old tomato plants carrying five or six fully expanded leaves.

Chemical Application and Pathogen Inoculation

BTH was kindly supplied in powdered form by Dr. A. Schmitt (Federal Biological Research Center for Agriculture and Forestry, Darmstadt, Germany) as 25% active ingredient. For plant treatment, water or BTH, from which wettable powders were removed by filtration, was applied as a fine mist to tomato leaves (approximately 1.0 mL per plant). A fresh solution of BTH at a final concentration of 1.5 mM in distilled water was prepared on each day of application and maintained at room temperature. Plants were maintained in a greenhouse at 22°C to 24°C under the environmental conditions described above. Four days after treatment, tomato plants were challenge inoculated by introducing two plugs (5 mm in diameter) of actively growing mycelium of FORL as close as possible to the main root. Control plants were treated similarly but with sterile agar plugs. Ten plants were used for each treatment and the experiment was repeated twice. The roots were pulled out of the substrate and examined daily for fungal infection (visible necrotic lesions). For electron microscope investigations, samples from the main roots were collected 6 d after fungal inoculation.

Tissue Processing for Ultrastructural Investigations

Samples (2 mm^3), collected from the crown and the main root at potential sites of fungal entry, were fixed by immersion in a mixture of 3% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C overnight and postfixed with 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4°C. Root samples were dehydrated in a graded ethanol series and embedded in Epon 812 (JBEM Chemical, Pointe-Claire, Québec, Canada). Thin sections (0.7 μm), cut from the Epon-embedded material using glass knives, were mounted on glass slides and stained with 1% aqueous toluidine or methylene blue before examination with a microscope (Axioscope, Zeiss). Ultrathin sections (0.1 μm), collected on Formvar-coated nickel grids (JBEM Chemical) using a diamond knife, were either contrasted with uranyl acetate and lead citrate for immediate examination with a transmission electron microscope (model 1200 EX, Jeol) operating at 80 kV, or further processed for cytochemical labeling. For each treatment, an average of five samples from five different roots were investigated. For each sample, 10 to 15 ultrathin sections were examined.

Cytochemical Labeling of Ultrathin Sections

Colloidal gold with particles averaging 15 nm in diameter was prepared according to the method of Frens (1973). The pH of the colloidal gold solution was adjusted according to the pI of each enzyme used.

For localization of cellulosic compounds, a β-1,4-exoglucanase (β-1,4-glucan cellobiohydrolase), purified from a cellulase produced by the fungus *Trichoderma harzianum*, was directly complexed to colloidal gold at pH 9.0 (Benhamou et al., 1987). Localization of callose, a polymer of β-1,3-glucans, was performed using a β-1,3-glucanase extracted and purified from tobacco plants reacting hypersensitively to tobacco mosaic virus (Kauffmann et al., 1987). The enzyme was complexed to gold at pH 5.5 (Benhamou, 1992).

Localization of phenolic compounds was performed by using a laccase (*p*-diphenol:oxygen oxidoreductase; EC 1.10.3.2) purified from the white rot fungus *Rigidoporus lignosus* (Geiger et al., 1986). Fungal laccases are blue copper-containing glycoproteins that play a key role in lignin breakdown in addition to being involved in the oxidation and polymerization of endogenous plant phenols (Mayer, 1987). Because of their multifaceted function, laccases have a very broad substrate specificity, including monophenols, *o*-, *m*-, and *p*-diphenols, and a variety of substituted phenolics, as well as thioglycolic lignin. The enzyme was complexed to colloidal gold at pH 4.0, a pH value close to its pI, which is reported to be 3.83. For preparation of the complex, 100 μg of the purified laccase (50 μg/mL) was mixed with 10 mL of colloidal gold at pH

4.0 (Benhamou et al., 1994). The solution was further stabilized by adding 1 mL of 1% (v/v) PEG 20,000 and centrifuged at 27,000*g* for 60 min. The resulting pellet was carefully recovered and resuspended in 0.5 mL of PBS, pH 6.0, containing 0.2 mg/mL of PEG 20,000. All gold-conjugated probes were stored at 4°C.

Ultrathin sections of resin-embedded material were first floated for 5 min on a drop of 0.01 M sodium PBS containing 0.02% (w/v) PEG 20,000 at the pH corresponding to the optimal activity of the enzyme tested. Sections were thereafter transferred to a drop of each gold-complexed probe for 30 to 60 min at room temperature in a moist chamber. They were washed thoroughly with PBS, pH 7.4, and rinsed with distilled water and allowed to dry before staining with uranyl acetate and lead citrate.

Specificity of the different labelings was assessed by the following control tests: (a) addition of the corresponding substrate to each enzyme-gold complex for a competition experiment: β-1,4-glucans from barley (1 mg/mL) for the β-1,4-exoglucanase-gold complex; laminarin (1 mg/mL) for the β-1,3-glucanase; and *p*-coumaric acid, ferulic acid, or sinapinic acid (1 mg/mL) for the laccase; (b) substitution of the enzyme-gold complex under study with BSA-gold complex to assess the nonspecific adsorption of the protein-gold complex to the tissue sections; (c) incubation of the tissue sections with the enzyme-gold complexes under nonoptimal conditions for biological activity; and (d) incubation of the tissue sections with colloidal gold alone to assess the nonspecific adsorption of the gold particles to the tissue sections. In addition, the specificity of the labeling pattern obtained with the gold-complexed laccase was verified by incubating either sections from fungal hyphae grown in vitro or sections from FORL-infected roots of BTH-free plants with the enzyme-gold complex.

RESULTS

Effect of BTH on the Development of Root Lesions

By 6 d after inoculation with FORL, nontreated tomato seedlings showed a slight wilting of the upper leaves. When plants were removed from the substrate, typical symptoms of crown and root rot, mainly the formation of brown lesions along the primary and lateral roots, were readily detected. Treatment of the leaves with BTH 4 d before inoculation with the pathogen reduced the symptom severity of FORL wilt compared with controls, and also significantly reduced the number of root lesions (Table I). Although some tiny lesions could be seen on the lateral roots, their frequency and severity never reached levels similar to those observed in control plants. In addition, BTH treatment was associated with a delay in the appearance of the lesions. Noninoculated, BTH-treated tomato plants showed no symptoms and their root system appeared healthy (not shown).

Table I. *Effect of BTH on the number of root lesions induced by FORL*

Values are ±SE.

Days after Inoculation	BTH Concentration	
	0 mM (control)	1.5 mM
	no. of root lesions	
1	0	0
2	2[a] ± 0.50	0
3	5 ± 1.0	0
4	9 ± 1.5	2 ± 0.5
5	10 ± 1.0	2 ± 1.5
6	10 ± 1.5	3 ± 0.5

[a] The number of root lesions was determined from observations of 10 main roots per day after inoculation with FORL.

Effect of BTH on the Rate and Extent of Pathogen Colonization

Histology

In the absence of pathogen challenge, treatment of tomato plants with BTH failed to stimulate visible cellular changes, as judged by the absence of typical wall appositions or intercellular space occlusions (data not shown). All root tissues from nontreated plants were massively colonized by FORL hyphae 6 d after inoculation (Fig. 1A). Pathogen ingress toward the vascular stele was accompanied by severe host cell alterations, including cell wall breakdown, as shown by the reduced density of wall staining even at a distance from the sites of fungal penetration (Fig. 1A, arrows).

Pretreatment of tomato plants with BTH before inoculation with FORL did not halt pathogen penetration into the root tissues (Fig. 1, B–D). However, in these plants striking differences in the rate and extent of fungal colonization were observed compared with controls. Pathogen growth was usually restricted to the outermost root tissues, including the epidermis and the outer cortex (Fig. 1B). A close examination of the colonized, outer root area showed that restriction of fungal growth correlated with the establishment of discrete structural changes, mainly characterized by an increase in blue-staining density of the host cell wall (Fig. 1D, large arrow) and by the occlusion of most intercellular spaces with an amorphous material that stained blue-green with toluidine blue (Fig. 1, C and D, arrowheads). Such a staining pattern suggested the deposition of lignin and/or tannins in intercellular spaces and host cell walls (O'Brien and McCully, 1981). Other typical modifications concerned the formation of elongated wall thickenings at sites of potential pathogen penetration (see Fig. 3A).

These microscopic observations indicated that treatment of tomato plants with BTH reduced the rate and extent of FORL colonization and triggered the elaboration of structural barriers. Complementary information at the ultrastructural level were essential to provide further insight into the biological significance of the barriers thought to be involved in restricting pathogen growth and development.

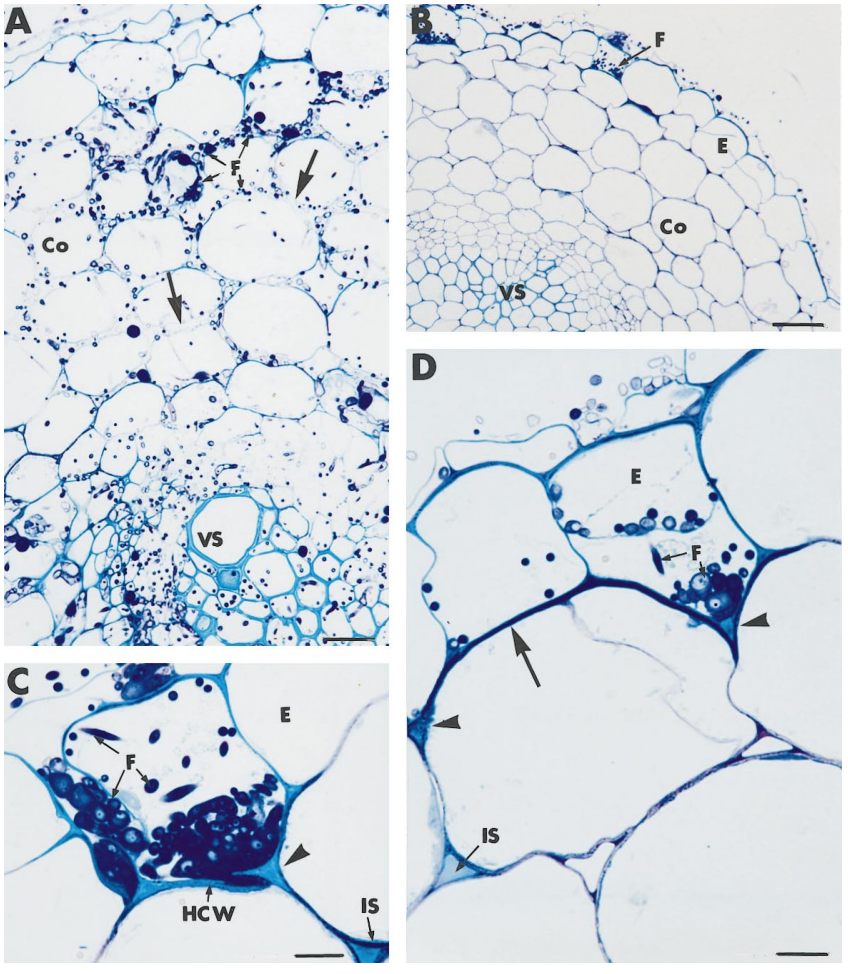

Figure 1. Light micrographs of samples from tomato roots. A, Sample from a nontreated (control) tomato root collected 6 d after inoculation with FORL. Hyphae of the pathogen abundantly colonize the epidermis and the cortex and reach the vascular stele. Fungal growth occurs both intracellularly and intercellularly. Pathogen ingress toward the vascular stele coincides with local cell wall alterations (arrows). Bar = 40 μm. B to D, Samples from BTH-treated tomato roots collected 6 d after inoculation with FORL. Fungal growth is mainly restricted to the epidermis and occasionally to the first outer cortical layers (B). Restriction of fungal growth correlates with the establishment of discrete structural changes, which are mainly characterized by an increase in staining density of the host cell wall (D, arrow) and by the occlusion of most intercellular spaces with an amorphous material that stains blue-green with toluidine blue (C and D, arrowheads). B, Bar = 80 μm; C, bar = 20 μm; D, bar = 10 μm. Co, Cortex; E, epidermis; F, FORL hyphae; HCW, host cell wall; IS, intercellular space; vs, vascular stele.

Ultrastructure and Cytochemistry

Observation of sections from FORL-inoculated tomato plants that were not sprayed with BTH confirmed the massive colonization of all root tissues (Fig. 2A). At this stage of infection, mycelial growth occurred both intercellularly and intracellularly (Fig. 2A). Fungal ingress toward the vascular stele paralleled marked cell wall damage involving loosening and/or splitting of the fibrillar layers (Fig. 2B) and, in some cases, complete wall breakdown leading to tissue maceration (Fig. 2A). Incubation with the gold-complexed β-1,4-exoglucanase revealed that gold particles were associated with the strands of disorganized wall fibrils (Fig. 2B). Host reactions such as wall appositions and intercellular space plugging could not be detected. This massive root-tissue colonization coincided with the presence of numerous dark-brown lesions on the root system and the expression of symptoms such as leaf chlorosis and wilting.

Examination of root samples from BTH-treated plants provided evidence that fungal growth occurred mainly in the epidermis and occasionally in the outer cortex (Fig. 2C). Examination of about 50 sections revealed that successful penetration of the root epidermis was achieved either through direct host wall penetration or, most often, through localized cell wall disruptions at the junctions between epidermal cells (Fig. 3A). When epidermal cells were colonized, hyphae of the pathogen multiplied so extensively that they completely filled the space originally occupied by the host cytoplasm (Fig. 2, D and E). In spite of such a massive colonization of some epidermal cells in BTH-treated plants, pathogen growth toward the cortical area was greatly impaired because fungal cells were seldom seen in the inner tissues. A close examination of this area revealed that the walls of all colonized epidermal cells were of higher electron opacity than normal (Fig. 2E) in addition to being frequently bordered by elongated wall appositions (Fig. 3, A and B). The wall appositions formed in the colonized areas were found to vary enormously in size, shape, and texture. They were usually composed of variously shaped zones containing numerous vesicles (Fig. 3B). Frequently, the wall appositions were delimited by a band of disorganized host cytoplasm (Fig. 3, B and C). Another striking feature of host reaction was the accumulation of amorphous deposits in densely colonized epidermal cells (Fig. 3C). This polymorphic material frequently established close contact with the invading hyphae (Fig. 3D, arrow). Most intercellular spaces were also filled with an aggregated material resembling that accumulating as intracellular deposits, although it appeared to be of higher electron density (Fig. 3E).

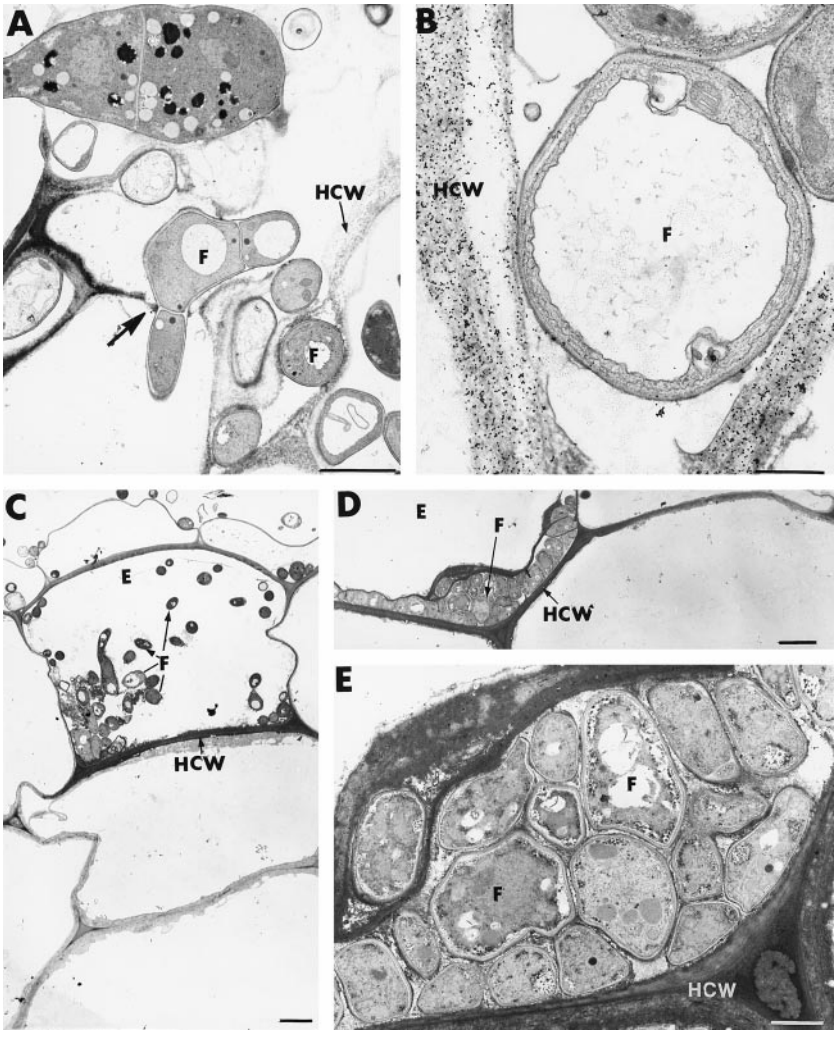

Figure 2. Transmission electron micrographs of control (A and B) and BTH-treated (C–E) tomato root tissues collected 6 d after challenge with FORL. A and B, In control roots from plants grown in the absence of BTH, FORL hyphae colonize the root tissues rapidly, causing extensive cell damage and host cell wall alterations. Cell invasion occurs through direct host cell wall penetration (A, arrow). Incubation with the gold-complexed exoglucanase for the localization of cellulosic compounds results in the deposition of gold particles over the strands of disorganized wall fibrils (B). A, Bar = 2 μm; B, bar = 0.5 μm. C to E, In roots from BTH-treated plants, fungal cells are restricted to the epidermis, where they multiply extensively. In spite of such a massive colonization of some epidermal cells, the host cell walls are of much higher density than normal (D and E). C and D, Bars = 3 μm; E, bar = 1 μm. E, Epidermis; F, FORL hyphae; HCW, host cell wall.

Application of the β-1,4-exoglucanase-gold complex to sections of infected root tissues from BTH-treated plants resulted in heavy and regular deposition of gold particles over the electron-dense host cell walls (Fig. 4, A and B). Labeling also occurred over the heterogeneous wall appositions, but it was less densely distributed (Fig. 4B). The aggregated material formed in the reacting host cells was unlabeled (not shown). Control tests, including preincubation of the enzyme-gold complex with β-1,4-glucans before section labeling, resulted in the absence of labeling over both the cell walls and the wall appositions (not shown).

Upon incubation of sections with the tobacco β-1,3-glucanase for localization of callose, a considerable number of gold particles were detected over all wall appositions, regardless of their size, shape, and texture (Fig. 4, D and E). A qualitative evaluation of labeling clearly showed that electron-lucent appositions, formed between the wall and the retracted plasma membrane (Fig. 4E), were more intensely labeled than electron-dense appositions (Fig. 4D). In electron-lucent appositions labeling appeared to be mainly associated with the underlying matrix, with a predominant accumulation over the area bordering the host cell wall (Fig. 4E). The fibrillogranular material formed in some intercellular spaces appeared substantially labeled (Fig. 4C), whereas the polymorphic, amorphous material was free of gold particles (Fig. 4E). A few scattered gold particles were occasionally detected over the host cell walls (Fig. 4E). Control tests, including incubation of the enzyme-gold complex with laminarin before section labeling, yielded negative results (not shown).

Incubation of sections from inoculated, BTH-treated plants with the gold-complexed laccase for localization of phenolic-like compounds resulted in a slight deposition of gold particles over both the host cell walls and the wall appositions (Fig. 5A). No labeling could be detected over the amorphous deposits of aggregated material formed in the invaded host cells, possibly because the structural organization of this material prevented access of the probe to its target receptors (Fig. 5B). Gold particles were detected over the walls of fungal cells surrounded by this aggregated material (Fig. 5, A and B). In all sections examined, these fungal cells showed signs of obvious alteration ranging from cytoplasm disorganization to protoplasm retraction. A few scattered gold particles were seen in the fungal cytoplasm (Fig. 5B). Preincubation of the laccase-gold complex with either ferulic acid or *p*-coumaric acid before

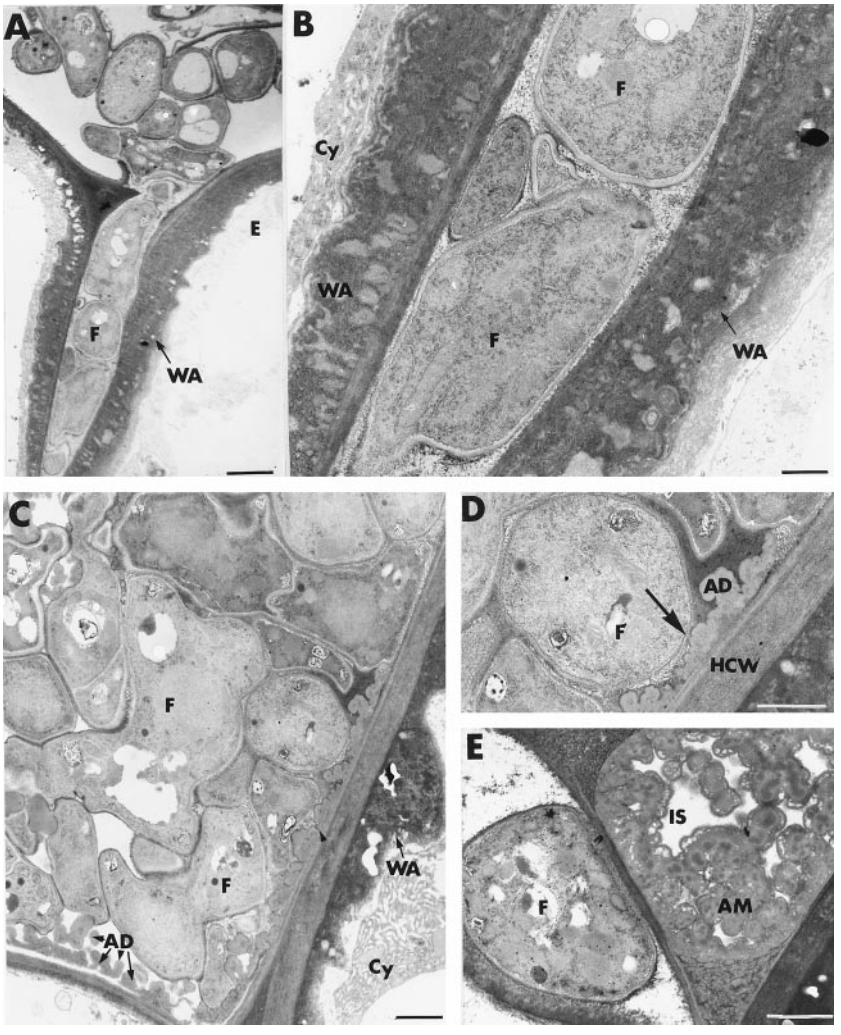

Figure 3. Transmission electron micrographs of BTH-treated tomato root tissues collected 6 d after challenge with FORL. A and B, Successful penetration of the root epidermis is achieved through localized cell wall disruptions at the junction between epidermal cells. Elongated wall appositions are formed along the pathway of fungus penetration. Such wall appositions are delimited by a band of cytoplasm. A, Bar = 2 μm; B, bar = 0.5 μm. C to E, Amorphous deposits accumulate in densely colonized epidermal cells (C and D). This material sometimes interacts with the wall of the invading hyphae (D, arrow). Electron-opaque aggregates fill an intercellular space (E). C, Bar = 2 μm; D and E, bars = 1 μm. AD, Amorphous deposits; AM, aggregated material; Cy, cytoplasm; E, epidermis; F, FORL hyphae; HCW, host cell wall; IS, intercellular space; WA, wall appositions.

section treatment abolished labeling over the fungal cell walls, the wall appositions, and the dense material (Fig. 5C). Similarly, labeling of sections from either the fungal mycelium grown in vitro (Fig. 5D) or the infected root tissues from nontreated tomato plants (Fig. 5E) resulted in a near absence of labeling over the fungal cell walls. A few scattered gold particles could be seen over the secondary walls in xylem vessels (Fig. 5E).

DISCUSSION

The results of the present study demonstrate that susceptible tomato plants develop a systemically induced resistance to FORL infection in response to BTH application and support the concept that this new chemical could become a potential disease-control agent in a wide array of cultivated crops. Although earlier observations have highlighted the potential of BTH in activating SAR in tobacco (Friedrich et al., 1996), wheat (Görlach et al., 1996), Arabidopsis (Lawton et al., 1996), and cucumber (Benhamou and Bélanger, 1998), the data reported here provide, for the first time to our knowledge, evidence that BTH induces SAR in tomato. This response against FORL attack, which normally correlates with genetically determined resistance (Brammall and Higgins, 1988), has been frequently obtained through preinoculation of tomato plants with avirulent or nonhost pathogen isolates (Lemanceau and Alabouvette, 1993; De Cal et al., 1997). The possibility of triggering the expression of this response in susceptible plants after treatment with BTH brings new insights into the concept of "nonspecific immunity," which was previously shown to be induced after treatment with chitosan (Benhamou et al., 1994). Evidence is provided that the beneficial effect of BTH in reducing the extent of fungal colonization in the root tissues is primarily associated with a massive accumulation of structural barriers (i.e. wall appositions), a reaction that was also amplified in chitosan-treated tomato plants (Benhamou, 1996).

It is interesting that this BTH-induced response differs from that observed in *P. ultimum*-infected cucumber plants, in which a direct inhibitory effect of fungal growth by phenolics was detected (Benhamou and Bélanger, 1998). Although phenolic-like compounds likely accumulated in the tomato root tissues as a result of elicitation, their levels never reached those monitored in cucumber roots. These observations suggest that the mechanisms by which BTH

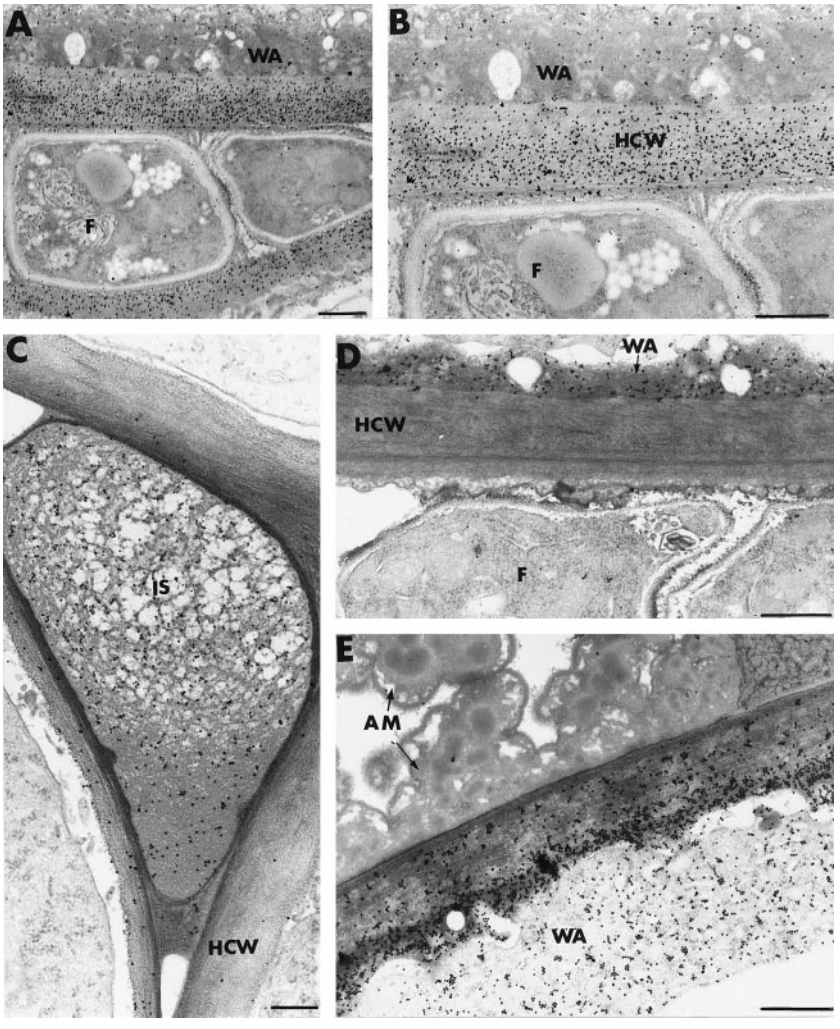

Figure 4. Transmission electron micrographs of BTH-treated tomato root tissues collected 6 d after challenge with FORL. A and B, Incubation with the β-1,4-exoglucanase-gold complex results in a heavy and regular deposition of gold particles over the electron-dense host cell walls. Randomly distributed gold particles also occur over the heterogeneous wall appositions. Bars = 1 μm. C to E, Upon incubation of sections with the tobacco β-1,3-glucanase for localization of callose, the fibrillogranular material formed in some intercellular spaces is labeled (C), whereas the aggregated material is free of gold particles (E). A substantial number of gold particles is seen over the wall appositions. C, Bar = 0.25 μm; D and E, bars = 0.5 μm. AM, Aggregated material; F, FORL hyphae; HCW, host cell wall; IS, intercellular space; WA, wall apposition.

may provide biological control against soil-borne pathogens are selective, probably because a strong heterogeneity in both the nature and the extent of defense reactions exists among plant species. This concept agrees with the observations of Görlach et al. (1996), who reported that the set of genes induced in tobacco, Arabidopsis, and wheat during the onset of BTH-mediated induced resistance was different. The authors pointed out that such a distinction was also reflected in the nature and spectrum of defense responses against various pathogens in the different plant species.

In line with earlier studies dealing with the use of resistance inducers (Chérif et al., 1992; Benhamou and Lafontaine, 1995), the present data confirmed that the expression of defense reactions in BTH-treated plants occurred with a much higher magnitude after fungal challenge. This feature of the general defense response suggests that contact with the pathogen is essential for the plant to mobilize its defense strategy. However, the absence of such reactions in FORL-infected plants that did not receive BTH treatment demonstrates that defense mechanisms cannot be triggered by the pathogen alone. In light of these observations, one may suggest that BTH has the potential to sensitize tomato plants to respond faster and to a greater extent to FORL attack.

Our results, based on the investigation of the cytologically visible consequences of the induced response, indicate that the increased resistance of tomato seedlings to FORL attack is directly associated with restricted fungal growth in the root tissues, which correlates with massive deposition of new structures and products in the host cells. Examination of the spatial distribution of these host reactions revealed that both the intensity and the magnitude of the response decreased at the cortical level, to become barely discernible in the endodermis and the vascular parenchyma. Conceivably, cell wall strengthening in the outermost root tissues is likely to provide strong protection against vascular invasion and diffusion of toxins and lytic enzymes. This reinforcement process, mediated by the early deposition of both callose and phenolics in the wall appositions formed in BTH-treated tomato plants, likely leads to drastic changes in both the rigidity and the vulnerability of cells and tissues. According to our cytochemical observations, the wall appositions formed in tomato root tissues upon BTH treatment and fungal challenge were found to contain small amounts of cellulosic com-

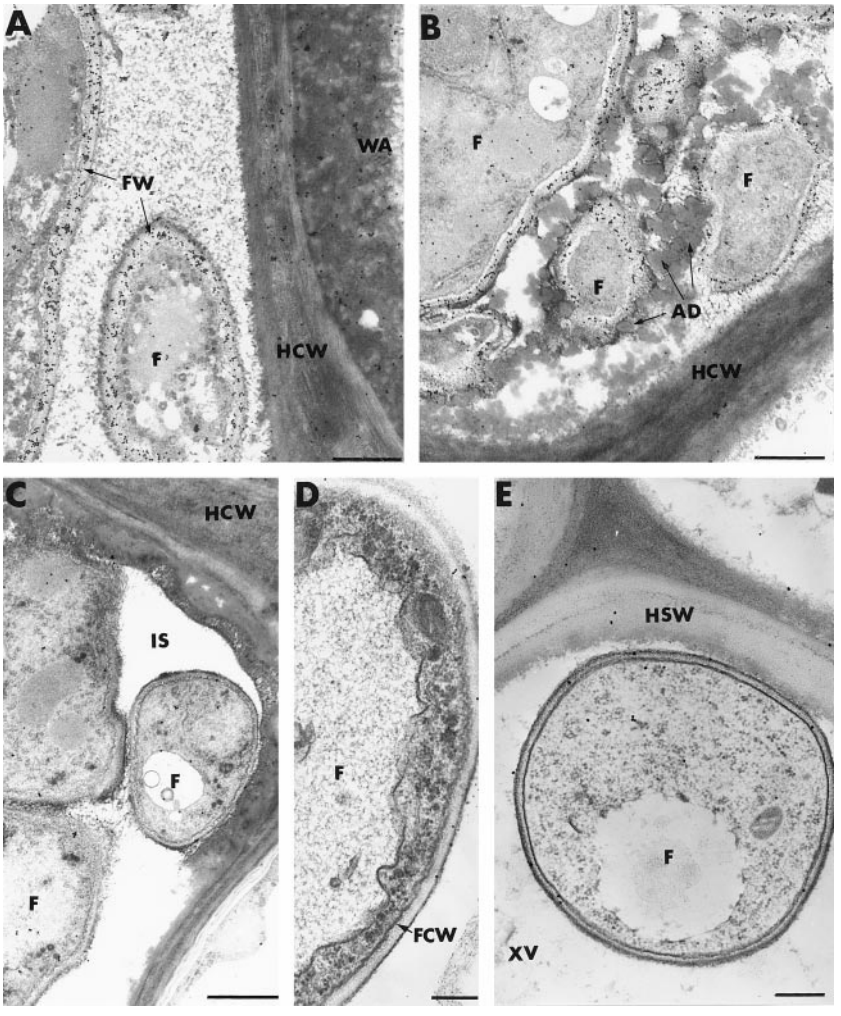

Figure 5. Transmission electron micrographs of BTH-treated tomato root tissues collected 6 d after challenge with FORL (A–C), FORL hyphae grown in vitro (D), and nontreated tomato root tissues (E). A and B, Incubation with the gold-complexed laccase for localization of phenolic-like compounds results in a slight deposition of gold particles over both the host cell walls and the wall appositions (A). Labeling is absent over the amorphous deposits (B). Gold particles are detected over the walls of all fungal cells showing signs of obvious alteration. Bars = 0.5 μm. C, Control test. Incubation of sections from infected roots of BTH-treated plants with the gold-complexed laccase, which was previously adsorbed with *p*-coumaric acid, results in an absence of labeling over the fungal cell walls as well as over the plant structures. Bar = 0.5 μm. D, The fungal cell wall of *Fusarium* hyphae grown in vitro is unlabeled after treatment with the laccase-gold complex. Bar = 0.25 μm. E, In control tomato root tissues from nontreated tomato plants, incubation with the laccase-gold complex results in an absence of labeling over the cell walls of invading hyphae. A few gold particles occur over the secondary wall of xylem vessels. Bar = 0.25 μm. AD, Amorphous deposits; F, FORL hyphae; FW, fungal cell wall; HCW, host cell wall; IS, intercellular space; WA, wall appositions; XV, xylem vessel.

pounds (see Fig. 4B). Although the origin of the accumulating cellulosic material is still uncertain, one may suggest that splitting of the host cell walls as an early event preceding the formation of papillae may have resulted in the release of cell wall fragments that accumulated in the paramural space and likely contributed, in association with callose, to the elaboration of a single unified material with reduced porosity and permeability. Subsequent infiltration of phenolics and related substances (i.e. lignin) (Blanchette, 1991) likely promoted compaction of this polysaccharidic matrix, leading to physical barriers preventing pathogen spread in the tissues (see Fig. 1, C and D).

In addition to the formation of wall appositions, another important feature of the host defense strategy was the heavy accumulation of densely stained deposits, frequently encircling pathogen hyphae in the colonized epidermal cells and also accumulating in some intercellular spaces. Although these deposits failed to be labeled by the laccase-gold complex, a probe known to bind to monophenols and diphenols (Benhamou et al., 1994), the structure and electron density of the accumulating material suggest that it may be enriched with phenolic compounds containing *O*-dihydroxy groups (Scalet et al., 1989). The absence of labeling with the gold-complexed laccase may be explained by an inaccessibility of the probe to its target substrate molecules because of the structural organization of this material. A growing body of evidence from a number of studies supports the concept that active secretory processes associated with increased synthesis and activity of enzymes involved in the phenylpropanoid pathway (Niemann et al., 1991) account for the formation of protective layers at sites of potential fungal entry (Benhamou et al., 1994). Although the role played by this material in preventing FORL invasion is difficult to assess from the present ultrastructural data, it seems reasonable to assume that it may enhance the mechanical strength of these first defensive barriers in addition to causing inhibition of fungal growth, as indicated by the often-distorted aspect of the fungal hyphae that were trapped or coated by this opaque material (see Fig. 3C). Based on the present results, the defense strategy occurring at the onset of BTH-mediated induced resistance in tomato plants appears to follow a specific scheme of events, including (a) the rapid formation of callose-enriched wall appositions at or beyond the infection sites to slow the growth of the pathogen, and (b) the activation of secondary responses with antimicrobial activity. Secondary responses would include the polymerization of preexisting phenols and/or the synthesis of new phenolic compounds

followed by their deposition and/or their infiltration at strategic sites, such as the wall appositions and the intercellular spaces.

An interesting aspect of the BTH-mediated induced resistance in tomato was the occurrence of gold particles over the fungal cell wall upon incubation with the laccase-gold complex. Whether such a phenomenon reflects the infiltration of phenolic compounds produced by the plant in response to infection warrants further investigation. However, the observation that cell walls of hyphae either grown in vitro or colonizing root tissues from nontreated plants were unlabeled with the probe favors the concept of a BTH-mediated induced reaction, leading to the synthesis and accumulation of phenolics in both the host cells and the fungal cell walls. Another argument that reinforces the hypothesis of a specific deposition of phenolic compounds comes from the correlation established between accumulation of wall-bound phenolics and fungitoxic activity, as indicated by the finding that labeled fungal hyphae were morphologically and structurally altered (see Fig. 5, A and B). Several studies have shown that phenolics disturb fungal metabolism by promoting internal osmotic imbalances, leading to plasmalemma retraction and cytoplasm aggregation (Southerton and Deverall, 1990). A similar phenomenon was reported by Ride (1986), who suggested that lignification of fungal hyphae could be a mechanism of resistance elaborated by wheat plants to fend off invasion.

In a recent study, Bennett et al. (1996) suggested that accumulation of bright autofluorescing material within the fungus cell wall in the lettuce-*Bremia lactucae* interaction correlated with strong leakage of phenolics from the host vacuole, leading to changes in ionic balance and formation of compounds with fungitoxic activity. The authors concluded that irreversible membrane damage in lettuce was a key signaling event leading to widespread activation of defense responses in surrounding cells. In tomato, we still have little understanding of the mechanisms underlying the transfer of phenolics to the plant and the fungal cell walls.

In summary, evidence is provided in this study that BTH treatment confers increased protection of tomato plants against infection by FORL by stimulating a number of defense reactions that culminate in both the deposition of structural compounds and the infusion of phenolics into the infested root tissues. As the mechanisms underlying the biological functions of chemical elicitors are revealed, the possibility of sensitizing a plant to respond more rapidly to pathogen attack by previous inoculation with selected products such as BTH can be considered a promising option for effective management of plant diseases in the near future.

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LITERATURE CITED

Benhamou N (1992) Ultrastructural detection of β-1,3-glucans in tobacco root tissues infected by *Phytophthora parasitica* var. *nicotianae* using a gold-complexed tobacco β-1,3-glucanase. Physiol Mol Plant Pathol **41:** 351–370

Benhamou N (1996) Elicitor-induced plant defense pathways. Trends Plant Sci **1:** 233–240

Benhamou N, Bélanger RR (1998) Induction of systemic resistance to *Pythium* damping-off in cucumber plants by benzothiadiazole: ultrastructure and cytochemistry of the host response. Plant J **14:** 13–21

Benhamou N, Chamberland H, Ouellette GB, Pauzé FJ (1987) Ultrastructural localization of β-1,4-D-glucans in two pathogenic fungi and in their host tissues by means of an exoglucanase-gold complex. Can J Microbiol **33:** 405–417

Benhamou N, Lafontaine PJ (1995) Ultrastructural and cytochemical characterization of elicitor-induced responses in tomato root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Planta **197:** 89–102

Benhamou N, Lafontaine PJ, Nicole M (1994) Seed treatment with chitosan induces systemic resistance to *Fusarium* crown and root rot in tomato plants. Phytopathology **84:** 1432–1444

Bennett M, Gallagher M, Fagg J, Bestwick C, Paul T, Beale M, Mansfield J (1996) The hypersensitive reaction, membrane damage and accumulation of autofluorescent phenolics in lettuce cells challenged by *Bremia lactucae*. Plant J **9:** 851–865

Blanchette RA (1991) Delignification of wood-decay fungi. Annu Rev Phytopathol **29:** 381–398

Brammall RA, Higgins VJ (1988) A histological comparison of fungal colonization in tomato seedlings susceptible and resistant to *Fusarium* crown and root rot disease. Can J Bot **66:** 915–925

Chérif M, Benhamou N, Menzies JG, Bélanger RR (1992) Silicon induced resistance in cucumber plants against *Pythium ultimum*. Physiol Mol Plant Pathol **41:** 411–425

Cohen Y, Niderman T, Mösinger E, Fluhr R (1994) β-Aminobutyric acid induces the accumulation of pathogenesis-related proteins in tomato (*Lycopersicon esculentum* L.) plants and resistance to late blight infection caused by *Phytophthora infestans*. Plant Physiol **104:** 59–66

De Cal A, Pascual S, Melgarejo P (1997) Involvement of resistance induction by *Penicillium oxalicum* in the biocontrol of tomato wilt. Plant Pathol **46:** 72–79

Frens G (1973) Controlled nucleation for regulation of the particle size in monodisperse gold solutions. Nature Phys Sci **24:** 20–22

Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Gut Rella M, Meier B, Dincher S, Staub T, Uknes S, and others (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. Plant J **10:** 61–70

Geiger JP, Rio B, Nandris D, Nicole M (1986) Laccases of *Rigidoporus lignosus* and *Phellinus noxius*. 1. Purification and some physicochemical properties. Appl Biochem Biotechnol **12:** 121–133

Görlach J, Volrath S, Knauff-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T, Ward E, Kessmann H, and others (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell **8:** 629–643

Jarvis WR (1988) *Fusarium* crown and root rot of tomatoes. Phytoprotection **69:** 49–64

Kauffmann S, Legrand M, Geoffroy P, Fritig B (1987) Biological function of "pathogenesis-related" proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J **6:** 3209–3212

Lamb CJ, Lawton MA, Dron M, Dixon RA (1989) Signals and transduction mechanisms for activation of plant defense against microbial attack. Cell **56:** 215–224

Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T, Ryals J (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J **10:** 71–82

Lemanceau P, Alabouvette C (1993) Suppression of *Fusarium* wilts by fluorescent *Pseudomonads*: mechanisms and applications. Biocontrol Sci Technol **3:** 219–234

Madamanchi NR, Kuc J (1991) Induced systemic resistance in plants. *In* GT Cole, TA Hoch, eds, Fungal Spores and Disease Initiation in Plants and Animals. Plenum Publishers, New York, pp 347–362

Malamy J, Klessig DF (1992) Salicylic acid and plant disease resistance. Plant J **2:** 643–654

Mayer AM (1987) Polyphenol oxidases in plants: recent progress. Phytochemistry **26:** 11–20

Métraux JP, Ahl Goy P, Staub T, Speich J, Steinemann A, Ryals J, Ward E (1991) Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. *In* H Hennecke, DPS Verma, eds, Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 1. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 432–439

Niemann GJ, Van der Kerk A, Niessen WMA, Versluis K (1991) Free and cell wall-bound phenolics and other constituents from healthy and fungus-infected carnation (*Dianthus caryophyllus* L.) stems. Physiol Mol Plant Pathol **38:** 417–432

O'Brien TP, McCully ME (1981) The Study of Plant Structure: Principles and Selected Methods. Termarcarphi, Melbourne, Australia

Ride JP (1986) Induced structural defense in plants. *In* GW Gould, RM Coopre, RG Board, eds, Natural Antimicrobial Systems in Plants and Animals. University Press, Bath, UK, pp 159–165

Ross AF (1961) Systemic acquired resistance induced by localized virus infections in plants. Virology **14:** 340–358

Ryals J, Ward E, Métraux JP (1992) Systemic acquired resistance: an inducible defense mechanism in plants. *In* JL Wray, ed, Inducible Plant Proteins: Their Biochemistry and Molecular Biology. Cambridge University Press, Cambridge, UK, pp 205–229

Scalet M, Crivaletto E, Mallardi F (1989) Demonstration of phenolic compounds in plant tissues by an osmium-iodide post-fixation procedure. Stain Technol **64:** 273–290

Southerton SG, Deverall BJ (1990) Changes in phenolic acid levels in wheat leaves expressing resistance to *Puccinia recondita* f. sp. *tritici*. Physiol Mol Plant Pathol **37:** 437–450

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Metraux JP, Ryals JA (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3:** 1085–1094